Productivity and Trophic Role of Bacteria in Aquatic Food Webs

by

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A thesis submitted to the Faculty of Science, University of Adelaide for the Doctor of Science Degree.

December, 1989.

Preface

I was introduced to aquatic ecology 20 years ago through research into the productivity of Lake George, Uganda, organised by the Royal Society, London as part of the British contribution to the International Biological Programme. When I started working in the field of aquatic microbial ecology there were no convenient methods for quantifying the total numbers of bacteria in water or sediment. There were no methods for determining the growth rates and productivity of the bulk of the heterotrophic bacterial populations, i.e. most of the bacteria. Without this information, it was difficult to quantify the roles of bacteria in food webs and biogeochemical cycles of the major elements, especially carbon and nitrogen. Many marine biologists dismissed bacteria as having an insignificant role.

There was a widely-held notion that cyanobacteria (blue-green algae) were poorly digested by animals and poor contributors to the productivity of food chains. In particular, a paper was published in Nature (London) in 1952, reporting that tilapia, herbivorous African fishes, did not digest cyanobacteria. That report led me to question why tilapia ate cyanobacteria if they could not digest them. From studies on the physiology of digestion and feeding biology of tilapia in Lake George, I found that cyanobacteria were indeed the principal food of tilapia and could be almost completely digested. There was, however, a diel cycle in digestion, linked to a diurnal feeding period, so that for a short period, some algal cells passed through the gut undigested. Colleagues working with me on the Royal Society team studying Lake George then showed that the other important herbivores in the lake, the fish *Haplochromis nigripinnis* and the copepod *Thermocyclops hyalinus* also digested and assimilated cyanobacteria. These studies provided the essential links needed for integrating the ecological studies on Lake George into a description of food web dynamics. By quantifying the major portions of the carbon cycle that concerned the transfer of organic matter from the primary to secondary producers, we were able to compare grazing rates on phytoplankton with net primary production. Furthermore, from a knowledge of the average daily feeding rates on phytoplankton and assimilation efficiencies, the rate of transfer of primary production to the detrital system via faeces could be quantified.

We could not proceed further with studies on detritus dynamics at that stage. Overall rates of mineralisation could be quantified in terms of oxygen uptake or ammonium and phosphate ion release, but there were no methods for accurately quantifying the total numbers and biomass of bacteria and their growth rates or productivity in the detritus. Thus the role of heterotrophic bacteria in the carbon and nitrogen cycles could not be quantified (the cyanobacteria are autotrophs). With the exception of the deep ocean and deep lakes, the sediments are the main sites for bacterial regeneration of the nutrients required to support primary production, and therefore the sediments are important sites for food webs dependent on bacteria and the detritus that they decompose. While at Lake George, therefore, I was stimulated to consider procedures for quantifying heterotrophic bacterial productivity and decided that probably the best way to do

this would be to measure the rate of DNA synthesis in bacteria with radioactive precursors with tritiated thymidine and ^{32}P-phosphate. I discussed these ideas with some microbial ecologists at an international meeting of limnologists at Reading, UK in 1972 and was told that these methods had already been tried and didn't work in natural systems. I was not entirely discouraged and decided on my return to Australia to develop a method for quantifying bacterial biomass in sediments before tackling the more difficult topic of quantifying growth rates, both of which are necessary for a comprehensive understanding of microbial food webs in sediments.

I developed a technique for determining bacterial biomass in sediment, based on the quantity of muramic acid, which is a compound unique to the cell walls of bacteria. Initially, the method involved a biochemical determination of D-lactic acid, but there were problems due to interference with the enzymatic determination. These problems were partially overcome by further improvements, and ultimately eliminated by determination of muramic acid directly with high pressure liquid chromatography (HPLC). Provided one has access to HPLC with automatic sample loading, determination of muramic acid is not a complicated procedure and is very useful for sediments where bacteria are difficult to see directly with a microscope and are difficult to separate from the sediment for microscopical counting. At the same time that I was developing this technique, the direct counting of bacteria in natural waters with epifluorescence microscopy and acridine orange staining was developed into a simple technique by research workers in Germany and the USA. These methods now provide a simple alternative to muramic acid determination, but, because it is difficult to separate bacteria from sediments, their accuracy remains questionable. The direct counting method gives total numbers of bacteria, but it is more difficult to measure the sizes of the bacteria and thus convert numbers to biomass. Its great advantage, however, is that it is a simple technique and gives results very quickly. It can be improved by combining it with the muramic acid method. I have been able to improve separation of bacteria from sediment using muramic acid to monitor the release of bacteria. This works because muramic acid is a measure of the total bacterial biomass and thus one can determine whether bacteria that cannot be seen by the microscope are left behind on the sediment.

In 1977 I returned to a consideration of the problem of measuring bacterial growth rates from measurements of the rate of incorporation of tritiated thymidine into DNA. Although this technique had been used by biochemists for many years with pure cultures of bacteria, there were a number of problems that needed to be solved before the technique could be used in a complex community of bacteria in water or sediment. One problem was specificity, in other words, in knowing which specific components of the microbial community would take up thymidine and incorporate it into DNA. By 1975 it was clear that the technique could be used specifically for the general heterotrophic bacterial populations in water and sediments; microalgae and fungi could not compete with bacteria at low concentrations of thymidine and many of them lacked a necessary enzyme for incorporation of thymidine into DNA. The other problem was to determine the specific radioactivity of tritiated thymidine in the bacterial cell at the site where it was incorporated into DNA. This is essential for calculating the rate of DNA synthesis. Sediments presented particular problems because much of the tritiated thymidine that was mixed with the sediment was adsorbed to the sediment, and thus the concentration around

growing bacterial cells was perhaps several orders of magnitude lower than might be expected from the amount added to the interstitial water in the sediment. This problem was solved by the application of isotope dilution theory to estimate the contribution of *de novo* synthesis of nucleotides for DNA within the bacterial cells and the relative contribution of added thymidine. Another problem was that the radioactive labelling of DNA had to be distinguished from that of other macromolecules in bacteria as well as other microorganisms in the sediment and the labelled DNA had to be efficiently extracted from the sediment. Clays and organic compounds such as polymers enveloping bacterial cells and sediment particles strongly adsorb DNA or thymidine. Thus some time elapsed before the technique was developed sufficiently for publication. By this time others working with bacteria in the water column, which is a much simpler system, had developed a technique along similar lines. The tritiated thymidine method for determining bacterial growth rates in water and sediments is now a well established technique for microbial ecologists. Its successful application does require some knowledge of the biochemistry of DNA synthesis and methods of extraction that do not result in degradation of DNA.

Aquatic scientists can now quantify more accurately than 10 years ago, the pathways of carbon and nitrogen flux in benthic and pelagic food webs and biogeochemical cycles. Most research workers have studied the water column of lakes and the sea, probably because these systems are easier than sediments to study quantitatively. Most of my work, has been the application of these techniques to the study of benthic food webs and biogeochemical cycling in seagrass beds, coral reefs and, more recently, aquaculture ponds. In benthic coastal ecosystems with vascular plants, bacteria are the dominant consumers of primary production by the macrophytes, because most animals cannot digest the fibrous plant material, which also has a low protein content. Thus where seagrasses are major primary producers, they may be important in marine food chains, but only indirectly. Most animals that utilise seagrass production are dependent on decomposition by bacteria and a detrital food chain, or on epiphytic and benthic microalgae in the seagrass beds. Quantitative studies on the cycling of carbon from the primary producers through bacteria to animals are now under way. Carbon cycling through bacteria and the potential bacterial biomass available each day as food for deposit-feeding animals can be determined from estimates of bacterial productivity, which are calculated from rates of bacterial growth and the biomass of bacteria.

Bacterial productivity is controlled by seagrass productivity, on both a diel and seasonal basis. Bacterial growth rates increase markedly during the day when organic matter that is synthesised during photosynthesis by the seagrasses is excreted from the roots. In the tropical seagrass beds of northern Australia, the productivity of many seagrass species varies throughout the year, in some cases being five times greater in summer than in winter. Similarly, bacterial productivity is five times greater in summer than in winter in the rhizosphere of those seagrasses. The rhizosphere is a zone of intense bacterial activity. The productivity of bacteria in the rhizosphere in those tropical seagrass beds is very high in summer, in some cases up to 5 grams of carbon/m^2/day, which is a very high value, and indicates that bacteria are, potentially at least, an important food source for deposit feeding animals. The question of what controls bacterial

biomass and production in sediments is a difficult one. It seems, from my work, that in the surface sediments in summer, grazing by animals is the most important factor. But below the surface, i.e. below 1 cm, lysis of bacterial cells followed by recycling of organic matter within the microbial community is more important than animal grazing. The intense bacterial activity in the rhizosphere has important implications for seagrass productivity. The bacterial activity results in deoxygenation and so the sediment is strongly reducing and this means that nitrogen fixation and release of phosphate from insoluble complexes is facilitated. In collaboration with a post-graduate student, I have found that nitrogen fixation supplies up to 50% of the daily needs of the seagrass. As seagrasses are so important in stabilising coastal environments and as nursery grounds for many important fish, crustacean and mollusc species, this has an important bearing on the management of impacts to the coastal environment.

In coral reefs, bacterial biomass is an important constitutent of organic matter in reef sediments and provides an important food source, together with microalgae, for the many deposit-feeding animals. The activity of bacteria is very intense in coral reef sediments, particularly the sands of the reef flats and lagoons and is important in the degradation of coral mucus and detritus and the regeneration of nutrients for primary production in the reef system. As in tropical seagrass systems, on coral reefs there is a marked seasonal variation in bacterial productivity with summer being a season of much greater bacterial activity than winter. The studies on the productivity of bacteria with the thymidine method have led to questions of a more general ecological nature, for example: Do the many deposit-feeding animals lower their rate of food consumption in winter to match the lower productivity of bacteria, or are the population densities of the animals much lower in winter also? This question has yet to be answered.

The application of biological principles to aquaculture in the disciplines of nutrition, physiology, reproduction and pathology has allowed for rapid expansion and intensification of production from aquacultural systems. Little effort has been devoted to the controlled use of nutrients from decomposing organic matter, particularly in tropical systems. Some extremely successful culture systems are based in large part on microbial-detrital production, notably pond fertilization with livestock manure. The potential for utilizing such detrital systems to reduce or eliminate the need for costly supplementary feed in pond aquaculture is excellent. Full development of this potential is dependent upon more complete knowledge of the systems and assessment of methods for their manipulation.

Detritus develops when organic material is decomposed, particularly in soils and aquatic ecosystems. Studies of aquatic detrital food chains have been largely limited to fundamental research focused on natural waters; little effort has been directed to their importance and manipulation in aquaculture systems. Microbial production in detritus affords a rich source of nutrients for fish and a mechanism for rapid recycling of nutrients released upon death of plants or breakdown of waste products. However, the interrelationships between such heterotrophic food chains and autotrophic food chains (fuelled by light and dissolved nutrients and based on phytoplankton) are poorly understood. Because detritus is a complex mixture of different types of organic matter, its composition is very variable, depending upon its derivation.

Thus we have to be cautious when discussing the role of detritus in food chains, not to generalize from particular situations without adequate knowledge of whether the processes occurring in those situations are generally applicable. The wastefed fishpond is commonly treated as a black box in experimental aquaculture. The constraints to production, such as water quality, dissolved oxygen and feed availability, are partially understood but the prospects for channeling more nutrients through detrital food chains into fish flesh have scarcely been considered.

The major advances in the methodology available for the study of detritus and its associated biota can now be applied to aquaculture ponds. The prospect of applying new techniques to aquaculture systems is exciting because in these systems the environment, which is semi-natural, can be modified, e.g., by supplying detritus of known composition at known rates, varying nutrient supply, and altering grazing pressures, fish species composition, circulation patterns and flushing rates. Manipulating detrital food chains has far-reaching implications for the utilization of wastes in aquaculture. The vast quantities of agricultural wastes and low value byproducts (straws, sugarcane bagasse, coffee residues, banana wastes, rice hulls, other food processing wastes, trash vegetation and aquatic weeds generated annually in the tropics) may be useable as supplementary detritus, added to culture ponds as composts and microbial substances either alone or mixed with fertilizers such as livestock manure. This could greatly reduce the need for inputs of high quality feeds for aquaculture: an important innovation particularly where feedstuffs are expensive or scarce. The development of such techniques appears feasible and would constitute a major advance in aquaculture, but firstly, additional information must be collected and research gaps must be filled. This is one direction that my work will take in the future.

Acknowledgements

This thesis is dedicated with thanks to Christine, my wife, for her love, support, encouragement and scientific collaboration throughout my research career. She noticed that tilapia slept in Lake George at night and proceeded from this observation to quantify the amount of phytoplankton eaten by them each day. Without this, we could not have quantified the carbon cycle in the lake.

I am grateful to Dr. George G. Ganf and my other colleagues on the Royal Society African Freshwater Biology Team for teaching me the concepts of aquatic ecology.

I wish to record my thanks to Dr. William Dall, at the CSIRO Marine Laboratories at Cleveland, who appointed me to my present position in CSIRO. Under his guidance and direction, the Cleveland Laboratory has become an excellent environment in which to conduct research.

I thank Peter C. Pollard, D. Grant Roberts, Margaret C. Jackson (neé Barclay), Bill G. Hunt and Ted J. Wassenberg, without whose assistance much of the experimental work reported in this thesis could not have been done. In particular, I wish to record my appreciation to Peter Pollard, who has enthusiastically assisted me in these studies since 1979. He has helped develop the thymidine method into a convenient technique, especially with his suggestion that dialysis be used to decontaminate labelled DNA.

Many scientific advances result from teamwork, or at least from the stimulation of discussions with colleagues or the published writings of others. It is difficult to single only some out, but I would like to thank Dr. John F. Jackson, Waite Agricultural Research Institute, Adelaide, for many helpful discussions on measuring rates of DNA synthesis. I am also grateful to Dr. David C. White, University of Knoxville, Tennessee, USA, who introduced me to lipid chemistry and its application to microbial ecology.

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A. Trophic Role of Cyanobacteria and Bacteria in Fish and Prawns

Reprinted from *J. Zool., Lond.* (1973) **171**, 25–39

The physiology of digestion of blue-green algae in the cichlid fish, *Tilapia nilotica*

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(*Accepted 10 April 1973*)

(With 6 figures in the text)

The cells of blue-green algae are lysed by high concentrations of acid (pH 1·9–1·4) in the stomach of *Tilapia nilotica*. After lysis, cell contents are digested in the intestine. Acid secretion follows a diurnal cycle related to feeding, and thus there is a cycle from zero to maximum digestion each day. Some of the digestive enzymes have been studied.

Contents

Introduction

Digestive enzymes in *Tilapia* species have been investigated by a number of workers. Fish (1960) searched unsuccessfully for enzymes which might lyse blue-green algae in *T. mossambica* Peters. He reported the presence of amylase and acidic and alkaline proteases in the wall of all parts of the alimentary canal. Nagase (1964) examined the effect of diet on the distribution of digestive enzymes in the wall of the alimentary canal of *T. mossambica*. He reported that amylase and lipase were present throughout the gut. His results demonstrate the presence of an esterase, but not necessarily pancreatic lipase, because he used tributyrin as an assay for lipase. Similar results were obtained by

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Al-Hussaini & Kholy (1953) who reported that amylase and lipase were present in the wall of the alimentary tract of *T. nilotica* (L.). They also used a general esterase substrate (glycerol triacetate) for lipase assay. Because these authors used the tissues of the alimentary tract, rather than the secreted fluid, there is the possibility that pancreatic tissue was present or that intracellular tissue enzymes (e.g. the phosphorylases and cathepsins) were assayed. Keddis (1957) studied enzymes in the intestinal juice of *T. nilotica*. In order to collect juice free from particulate matter, he was forced to starve fish for a few days after capture. However, he was unable to demonstrate pancreatic lipase or esterase activity in the intestinal juice or pancreas.

In order to clarify the role of enzymes in the digestion of phytoplankton in *T. nilotica*, I have made a brief examination of enzymes in the alimentary tract fluid from fish actively feeding in their natural environment.

Gastric glands are present in the stomach of *T. esculenta* Graham which feeds on phytoplankton, and Greenwood (1953) points out that the initial stages of digestion may occur there. Gastric glands have been reported in the anterior part of the stomach of *T. nilotica* (Al-Hussaini & Kholy, 1953). Acid production in the stomach of *T. mossambica* was studied by Fish (1960), who commented on some factors controlling the secretion of acid. In particular, he found that treatment and handling of the fish caused a reduction in acid concentration. Acid production is shown (see below) to have an important role in the digestion of blue-green algae.

Fish (1952, 1955) observing undigested cells of blue-green and green algae in the rectum, concluded that these algae could not be digested by *Tilapia*. Quantitative experiments have shown that *T. nilotica* can assimilate a maximum of 70–80 % of the carbon in the blue-green algae *Microcystis* and *Anabaena* (Moriarty & Moriarty, 1973*a*). The proportion of carbon assimilated was not always maximal. The main trend was low assimilation of *Microcystis* consumed in the morning and maximum assimilation after the end of the day's feeding period. Thus undigested cells could often be observed in the faeces. An explanation for this variability in digestion is given in this paper.

Methods

Tilapia nilotica in Lake George (Uganda) were caught by trawling. They were brought to the laboratory, then killed and dissected within half an hour of capture.

Values of pH in the stomach or intestine were measured with indicator papers at 03.00 and 06.00 hrs and with a glass electrode at later times (e.g. 10.00 and 16.00 hrs) when there were more contents.

Chlorophyll and phaeophytin were determined by a spectrophotometric method (Golterman, 1969: 114). A Unicam Sp 600 spectrophotometer was used. Aggregates of faeces and gut contents were not concentrated on filters, but analysed directly after neutralizing, if necessary, with solid $MgCO_3$. Sufficient acetone at 5°C was added to give a final concentration of 90% acetone. Pigments were extracted for chromatography at 4°C in dim light with 90% acetone, then transferred to petroleum ether (60–80% fraction) and washed with 5 volumes of 10% NaCl. Chromatograms were run in a two-way solvent system (Jeffrey, 1961) on thin layer cellulose.

Algae from the lake were harvested in a tow net (80 µm pore size) immediately before use. For *in vitro* digestion studies, they were exposed to light for 5 h in sealed flasks containing ^{14}C-$NaHCO_3$. The labelled phytoplankton was collected with a net of 40 µm pore size and washed well with filtered lake water. Hydrochloric acid (1 M) was added to 5 ml of the concentrated labelled algae to give the pH values of 2·0 or less as shown in Figs 3 and 4. After 2 h,

0·2 ml of K_2HPO_4 (1 M) was added and the algae were titrated to pH 7·0 with sodium hydroxide (0·05 M). 1 ml aliquots were removed and incubated with 1 ml of intestine juice (7·5 mg protein/ml). 1 ml of boiled intestine juice or 1 ml water were added to other aliquots as controls. After incubation with intestine juice and controls for the times shown in the figures, 10 µl aliquots of the soluble fraction were removed for chromatography.

Chromatography of sugars formed during digestion was carried out routinely with 80% (v/v) isopropanol in water on Whatman No. 1 paper. Other systems used in the identification of glucose were butanol—acetic acid—water, (3 : 1 : 1) and butanol—pyridine—water, (2 : 2 : 1). Areas of ^{14}C-glucose were cut out and counted by liquid scintillation.

Stomach and intestinal juice were prepared by slitting longitudinally the walls of the stomach and anterior half of the intestine from fish captured around 16.00 hrs. The contents were collected in centrifuge tubes in an ice bath, and then centrifuged at 5° to 10°C for 15 min at 25,000 × **g**. The supernatants were filtered through Millipore filters (0·45 µm) and stored at about −10°C. The method of Bramhall, Noack *et al.* (1969), was found to be satisfactory for protein estimation in these fluids. Methods based on the reactions of copper with peptide bonds or amino acids were unreliable, possibly because of interference by mucilaginous compounds.

Stomach walls were washed free of contents in phosphate buffer (pH 7·0, 0·01 M) at 2°C, then homogenized in a blender with 1 volume of deionised water for 2 min at 2°C. Pancreatic tissue was dissected out of the liver, from which it may be distinguished by its lighter colour, and homogenized as above. Acetone powders (Morton, 1955) were prepared at −10°C, and were dried with ethyl ether. When required, they were extracted for 2 h at 2°C with distilled water (200 mg powder per 10 ml H_2O).

For the enzyme assays, protein was estimated by the method of Warburg & Christian (1941). All assays were conducted at 30°C.

α-Amylase was assayed by the method of Rutter & Brosemer (1961) except that the buffers were tris-succinate (0·1 M). For some experiments NaCl was omitted or replaced by $CaCl_2$, or tris-HCl was used in place of NaCl and tris-succinate.

Electrophoresis of proteins in intestine juice was carried out in Difco Ionagar (1% w/v) made up with sodium barbiturate-HCl (0·05 M pH 8·0). It was run at 15 V/cm for 3 h at 10°C. Amylase was detected by placing a strip of Ionagar containing soluble starch (each 1% w/v) over the sample for 10 min. After the starch-agar strip was removed, exposure to iodine vapour revealed the location of amylase.

Pepsinogen was assayed as described by Ryle (1970) with the exception that casein was used as the substrate.

Trypsin-like activity was assayed with TAME (p-tosyl-L-arginine methyl ester hydrochloride) and chymotrypsin-like activity with BTEE (benzoyl-L-tyrosine ethyl ester) by the methods of Hummel (1959). Carboxypeptidase A activity was assayed with hippuryl-L-phenylalanine using the method of Folk & Schirmer (1963). Carboxypeptidase B activity was assayed with hippuryl-L-arginine using the method of Folk, Piez *et al.* (1960).

The lipase assay was that of Marchis-Mouren, Sarda *et al.* (1959) using olive oil.

Esterase was assayed with tributyrin in phosphate buffer (0·001 M, pH 7·0). Using a pH meter (Radiometer), the pH was kept constant by titration with 0·01 M NaOH.

TAME, BTEE, hippuryl-L-phenylalanine and hippuryl-L-arginine were obtained from Sigma Chemical Company.

Results

Observations on fish in the laboratory

When the fish start feeding in the morning, the faeces produced are brown in colour, if the fish are adjusted to laboratory conditions. Phaeophytin, but no chlorophyll, was

detected in these faeces (Table I). After about four to six hours, the faeces become green in colour, with a clear demarcation between the brown and green material. There is usually a high proportion of chlorophyll to phaeophytin in the green faeces (Table I). Throughout the day, the colour of the faeces changes gradually from green to brown and the proportion of chlorophyll to phaeophytin decreases. Chromatography of the pigments in the faeces confirms that chlorophyll *a* is present in the green faeces, with variable amounts of phaeophytin *a*. Likewise, phaeophytin *a*, but no chlorophyll *a*, is present in brown faeces. There are also differences between other pigments in green and brown faeces, but these were not examined in detail.

TABLE I

Proportions of chlorophyll to phaeophytin in faeces of ***T. nilotica***

Colour of faeces	% Chlorophyll : % Phaeophytin
Green	80 : 20
Green/Brown	20 : 80
Brown	0 : 100

The cyclic colour change in the faeces is repeated every day, unless the fish are stressed, in which case, if they continue feeding, their faeces remain green.

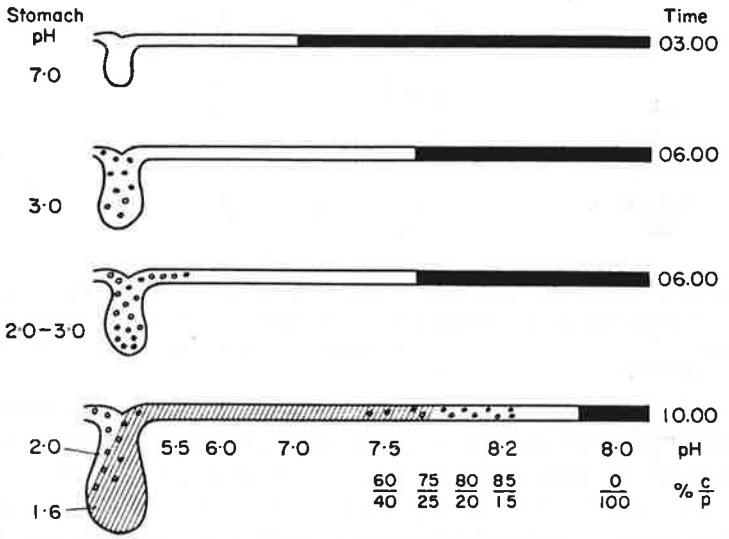

FIG. 1. Diagram of pH change in stomach at start of feeding, and typical values for pH and proportions of chlorophyll to phaeophytin $\left(\frac{\%C}{\%P}\right)$ in the intestine at about 10.00 hrs. The intestine is drawn to a smaller scale than the stomach. □, Empty; ▒, green material; ///, greenish-brown material; ■, brown material.

Observations on fish from the lake

Stomach

In most fishes the stomach is empty from about 02.00 to 04.00 hrs, except for a small amount of mucus. Stomach pH values range from 5·0 to 7·0 (Fig. 1). The stomachs are empty long enough for the intestines to empty partially. Fish start feeding between 04.00

and 06.00 hrs. During this period a small amount of algae of natural appearance and colour (green) is found in the stomach, and the pH of the stomach fluid is about 2·5 to 3·0. The recently ingested, fresh algae soon pass into the intestine, where they are distinguished from the previous day's food by their colour and the intervening empty region of the intestine (see Fig. 1). The stomach pH is between 2·0 and 3·0 at this stage. Later in the morning, when more phytoplankton has accumulated in the stomach, values of pH 2·0 and lower are recorded. It is evident that the stomach does not secrete acid when it is empty. Acid secretion starts when ingestion begins. Later in the day, lower pH values were measured in the stomach (Fig. 1).

When the stomach is full or nearly full, the pH and colour of the ingested algae vary according to position within the stomach. Figure 2 depicts the situation in the majority

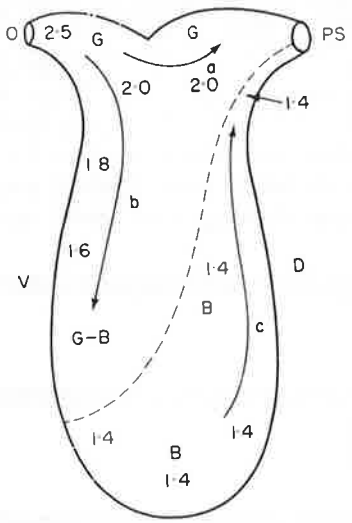

FIG. 2. Diagram of typical pH values and colouration of algae in stomach of *T. nilotica* captured at 16.00 hrs. An interpretation of the movement of algae through the stomach is shown by arrows a, b and c. B, Brown; D, dorsal; G, green; G-B, greenish-brown; O, oesophagus; PS, pyloric sphincter; V, ventral.

of fish. The whole area to the right of the dotted line represents material of pH 1·4 and brown colour. Phaeophytin, but no chlorophyll, was present. Near the oesophagus, the algae are natural in appearance (green colour) and the proportion of chlorophyll to phaeophytin is variable, but usually around 50% : 50%. On the ventral side, the pH becomes progressively lower and there is an antero-posterior colour change, from green to brown. Along the dorsal wall, a band of brown material, pH 1·4, extends to the pyloric sphincter. In the pyloris, near the sphincter, the brown material is in contact with green contents, pH 1·8 to 2·0.

In vitro observations with concentrated lake algae treated with hydrochloric acid, show that at values of pH 1·8 and higher, most of the algae retain their natural colour. A few small colonies turn brown after three hours. At pH 1·65, most algae are still green after one hour, but there is a noticeable brown colouration after two hours, giving the "brownish-green" colour described in the *in vivo* samples. At pH 1·5, brown colouration is first observed after ten minutes and all the algae have turned brown after 30 minutes.

Intestine

The proportions of chlorophyll to phaeophytin in food material in the intestine have been measured during the day. In the morning, the brown material from food ingested on the previous day has 0 % chlorophyll : 100 % phaeophytin. Green material, ingested on the morning of capture, has high proportions of chlorophyll to phaeophytin near the posterior end of the intestine (Fig. 1). The proportions of chlorophyll to phaeophytin in the intestine become lower as the day progresses and eventually reaches 0 % chlorophyll : 100 % phaeophytin around midnight.

These ratios were measured in the posterior half of the intestine only. Other pigments, possibly from the bile, interfered in the assay in the anterior half. Chromatographic separation of the pigments confirmed that the quantitative determination of chlorophyll to phaeophytin proportions is satisfactory for material from the faeces and the posterior half of the intestine.

Near the pyloric sphincter the pH is 5·5 to 6·0 (measured with a glass electrode). Further along the intestine the pH increases, reaching values around pH 8·0 near the anus (Fig. 1). This distribution of pH is similar throughout the day.

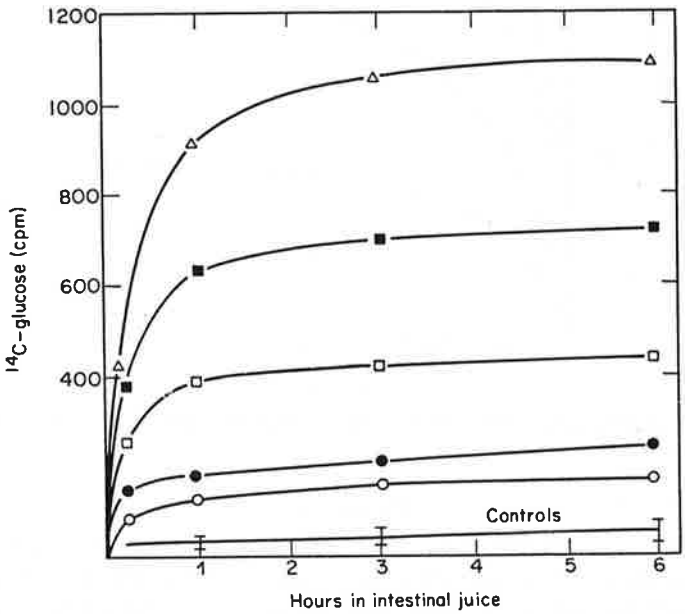

FIG. 3. Digestion of blue-green algae after treatment with acid at various pH values. This is measured by the release of glucose from storage polysaccharides in lake algae (mainly *Microcystis* spp.), labelled with ^{14}C. ○, pH 7·0; ●, pH 2·0; □, pH 1·9; ■, pH 1·75; △, pH 1·4.

Lysis of blue-green algae

The digestion of carbohydrate in lake algae by intestinal juices occurs after treatment of the algae with acid at pH values less than 2·0 (Fig. 3). Values of pH most often recorded in the stomachs of *T. nilotica* are 2·0, 1·75, and 1·4. Digestion is greatest after treatment at pH 1·4 (Fig. 3). The amount of polymeric carbohydrate made available by acid for subsequent digestion depends also on the time spent by the algae at a low pH. At pH 1·5,

a significant effect occurs within about 30 minutes (Fig. 4), although food would remain at this pH in the stomach for about two hours or longer. At higher pH values the acid acts more slowly on the algae. For example, at pH 1·75, two hours is insufficient for maximum effect (Fig. 3).

It can be seen that two hours' incubation with intestinal juice is sufficient to digest most of the available carbohydrate. The controls, of water or boiled intestine juice, show that the formation of glucose is an enzymic reaction in the intestine juice.

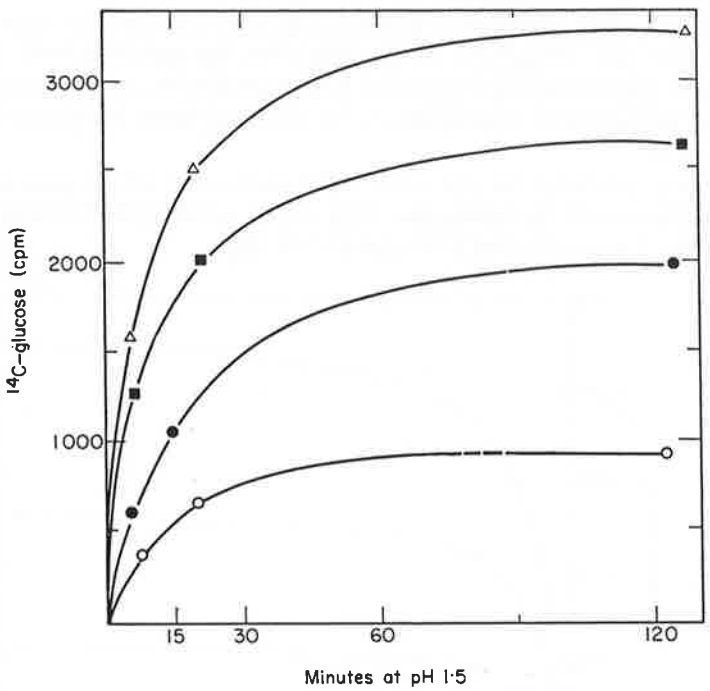

FIG. 4. Digestion of blue-green algae after treatment with acid at pH 1·5 for various times. Time in intestinal juice: ○, 10 min; ●, 1·5 h; ■, 3 h; △, 8 h.

Gastric enzymes

In the late afternoon the pH of the prepared stomach juice is usually about 1·8. Protein concentration is about 65 μg/ml when measured by the method of Warburg & Christian (1941): E_{260}=0·250, and E_{280}=0·165. When measured by the method of Bramhall, Noack *et al.* (1969) protein is not detectable, i.e. less than 25 μg/ml. No proteolytic activity was detected at any time in the secretion prepared by centrifuging down the stomach contents. There was no amylase activity in this fluid, when it was adjusted to pH 7·0.

Pepsinogen is present, however, in the acetone powder prepared from the stomach wall. Its specific activity at various pH values is shown in Fig. 5. The optimum pH is around 2·1.

Intestinal enzymes

In the late afternoon, the protein concentration of juice collected from the anterior half of the intestine is about 7·5 mg/ml (measured by the method of Bramhall, Noack *et al.*

(1969)). Its pH is usually between 6·8 and 7·0. As noted above, however, the pH varies along the intestine.

Carbohydrase activity.

The main end-product of digestion of photosynthetic storage compounds in the phytoplankton is glucose (Figs 3 and 4). It has been identified as such because the radioactivity is coincident with a spot which reacted readily with alkaline silver oxide and aniline-diphenylamine reagents (Smith, 1969: 316, 317) after separation in three different solvent systems. It did not separate from authentic glucose when co-chromatographed in these solvents. Glucose is often present in the intestinal juice, but not in the stomach juice, of fish caught in the lake in the afternoon.

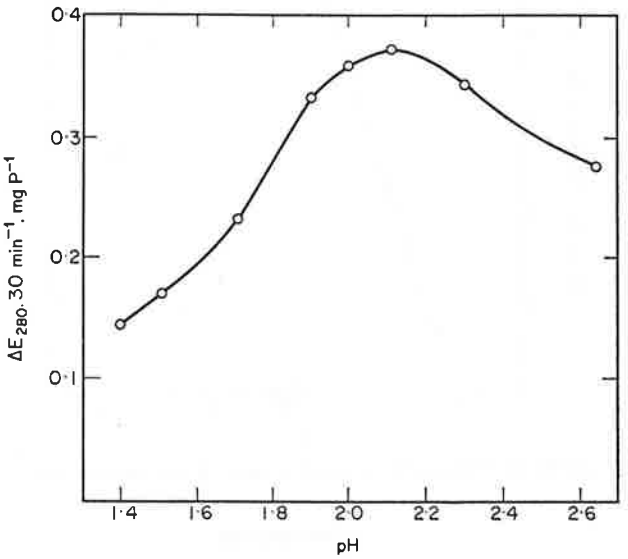

FIG. 5. Specific activity at various pH values of Pepsin(ogen) from *T. nilotica*.

Glucose is the major product of digestion of starch by intestinal juice. When the proteins in the intestinal juice were separated by electrophoresis, the fraction which hydrolysed starch yielded maltose as the main end-product. It is likely therefore, that this fraction contains α-amylase.

In the acetone powder made from intestinal juice, the specific activity of the α-amylase at pH 7·0 was 8·8 mg starch hydrolysed/mg protein. min. The pH optimum occurs between pH 7·0 and pH 8·0 (Fig. 6). There was no change in α-amylase activity when NaCl in the assay mixture was replaced by $CaCl_2$ or HCl, but if none of these compounds was present, starch was not hydrolysed. Thus the chloride is necessary for activation of the enzyme in the acetone powder.

α-Amylase was present in the acetone powder made from pancreatic tissue, with a specific activity of 2·1 mg starch hydrolysed/mg protein. min at pH 7·0.

Proteolytic activity

Trypsin-like activity is present in the intestinal juice. Its specific activity was 0·26

absorbance units/mg protein. ml. min. Chymotrypsin-like activity is also present, specific activity 0·58 absorbance units/mg protein. ml. min. There was a very slight hydrolysis of hippuryl-L-phenylalanine and no hydrolysis of hippury-L-arginine by the intestinal juice. Thus carboxypeptidase-like activity is absent (or virtually absent).

Lipase and esterase

No lipase activity was detected in the intestinal fluid or pancreatic tissue. A strong esterase activity was detected, however. It was not characterized further.

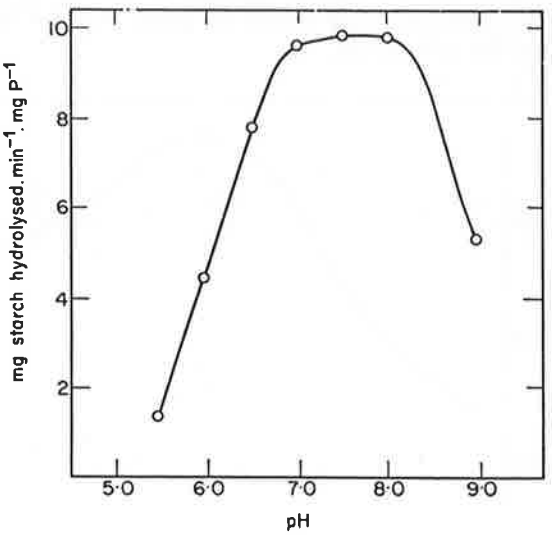

FIG. 6. Specific activity at various pH values of α-amylase in intestinal juice from *T. nilotica*.

Discussion

Acid secretion

The observations reported here show that there is a diurnal cycle of acid secretion in *T. nilotica* which closely follows its feeding pattern. A similar cycle occurs in *Haplochromis nigripinnis* Regan. Acid affects the pigments in the algae, converting chlorophyll to phaeophytin, and at pH values less than about 1·6, causes an overall colour change from green to brown. This correlation of pigment alteration with acidity is important. In the laboratory an examination of the colour of faeces and the proportion of chlorophyll to phaeophytin shows whether acid secretion has occurred, and gives an approximate indication of the concentration of acid in the stomach, without disturbing the animal in any way. As has been pointed out elsewhere (Moriarty & Moriarty, 1973*a*) these fish are very susceptible to disturbance. It was noticed on some occasions that if fish, whose faeces had started to turn from green to brown, were stressed, they might continue feeding but their faeces changed back to the green after some hours. Thus the conditions of stress inhibited acid secretion in the stomach (see also Fish, 1960). The high proportions of chlorophyll to phaeophytin in the green coloured material passing down the intestine in the morning (see Fig. 1), confirm that this material has not been affected by acid. No lysis

of the blue-green algae would have occurred and thus very little of this material would be digested or assimilated.

Movement of food through the stomach

The stomach and about half of the intestine of fish in the lake empty completely overnight. When feeding commences in the morning, the stomach is contracted. The distance from oesophagus to pylorus is therefore small, so much of the algae consumed initially passes rapidly into the intestine and is not affected by acid. While *T. nilotica* are feeding in the lake, algae pass into the intestine at about three to four times the rate at which they accumulate in the stomach (Moriarty & Moriarty, 1973*b*). This must mean that a large proportion (up to 70%) of the ingested algae passes rapidly through the stomach and a small proportion (minimum about 30%) remains for a longer time. The pattern of colour and pH in the stomach support this conclusion. In Fig. 2, there is a large amount of green material, pH 2·0 and some brown material, pH 1·4 in the pyloric region. An interpretation of this pattern is that some algae pass across the anterior end of the stomach from oesophagus to pylorus and are affected only a little by acid (pH 2·0). Part of the ingested algae, however, move down the ventral side of the stomach and accumulate in the posterior end. The concentration of acid increases during the day, with concomitant change in colour of the algae.

Some of the accumulated algae are moved along the dorsal side of the stomach while the fish is feeding, and pass into the intestine together with freshly ingested algae. This is clearly demonstrated in Fig. 2. The line of pH 1·4 material on the dorsal side can only have come from the posterior end, because acid-secreting tissue occurs only on the ventral side of the stomach (unpubl. obs.). Peristaltic waves have been observed progressing along the ventral side from anterior to posterior (path b, Fig. 2) and in the reverse direction along the dorsal side (path c, Fig. 2) of stomachs from freshly-killed specimens. The consequent mixing of algae in the centre of the stomach and the apparently large proportion which is moved directly across the anterior end of the stomach to the intestine indicates that the treatment of algae by acid over the whole feeding period is not efficient. Figure 2 summarizes the movements of algae through the stomach while the fish is feeding. A large proportion of the algae follows path a, and a smaller proportion follows paths b and c. Because some material is moved out of the stomach along path c during feeding, a greater proportion of algae will be affected by low pH values than that derived from the relation between rate of stomach filling and intestine filling.

Lysis of blue-green algae

The experiments reported here show that lysis of the cells of blue-green algae occurs in an acid medium, permitting subsequent access to the cell contents by intestinal enzymes. The lysis of cells and the denaturation of their proteins is pH dependent. The Lake George algae, used in these experiments, were collected in a net which retained mostly the colonial blue-green algae. Diatoms possibly constituted about 20% of the algae. The formation of an amylopectin type of carbohydrate during photosynthesis is typical of the blue-green algae (Lang, 1968), and not diatoms (Meeuse, 1962). Thus the assay of digestion in terms of ^{14}C-glucose from a photosynthetic storage compound is a method for studying the effect of acid on the blue-green algae, mainly *Microcystis* species, ingested by *T. nilotica*

in Lake George. Conclusions from the *in vitro* experiments are therefore directly applicable to fish in the lake.

Blue-green algae which are retained in the stomach during the day and are subjected to pH values of around 1·5–1·4 will be more fully digested in the intestine than the material which passes through more quickly. As Fig. 2 shows, many of the algae pass through the anterior part of the stomach only; here the pH does not drop much below 2·0 while the fish is feeding. The blue-green algae will therefore be only partially digested. The high proportion of chlorophyll to phaeophytin in the intestine at this time demonstrates the incomplete effect of acid. Because there is also a movement of acid-lysed algae (Fig. 2, path c) into the intestine, some blue-green algae will be more completely digested at the same time.

It is likely, however, that most of the diatoms will be digested throughout the day, because pH 2·0 should be sufficient to denature their cell membranes. This may explain the observations of digested diatoms and undigested blue-green algae found together in the rectum of *Tilapia* (Fish, 1952, 1955).

When the fish stop feeding, acid secretion must continue for some time, because the pH drops below 1·6 in the anterior part of the stomach. Thus all the algae present in a full stomach at the end of feeding will be more fully digested. Some material in or near the pylorus will pass into the intestine before the acid concentration rises sufficiently to cause maximum lysis. Laboratory experiments with uniformly labelled ^{14}C-*Microcystis* eaten at the end of a feeding period support this conclusion (Moriarty & Moriarty, 1973*a*). It was found that maximum digestion occurred at this time but the small variations in the percentage carbon assimilated suggest that cell lysis was not complete. The faeces from this last phase of ingestion and digestion are all brown in most fish. In some fish, both in the laboratory and in the lake, the faeces, or the algae in the intestine after feeding stopped, retained a greenish colour. This colour indicates that less gastric acid was secreted by these fish, and that subsequent digestion would not be complete. Variability in digestion and assimilation of phytoplankton is therefore to be expected (Moriarty & Moriarty, 1973*a*).

In general terms, the structure of the wall of an algal cell will affect its susceptibility to lysis by acid and the penetration of digestive enzymes, or the release of its components into the intestinal juice. Since blue-green algae are closely related to bacteria, especially in the composition of their walls (Echlin & Morris, 1965) it is likely that all bacteria can be digested by these herbivorous fish. This means that where detritus contains a high proportion of bacteria and blue-green algae (as it does in Lake George), its digestion will be dependent upon acid secretion in the stomach. Thus the physiology of digestion in *T. leucosticta* Trewavas, which feeds mainly on the bottom deposits in Lake George, will be similar to that of *T. nilotica*.

The fact that 50 % of the carbon was assimilated from the green alga *Chlorella* sp. by *T. nilotica* (Moriarty & Moriarty, 1973*a*), does indicate that acid will lyse these algae in the stomach. As this value is lower than that obtained for *Microcystis* under similar conditions, the cellulose cell wall of green algae may be more resistant to lysis.

Stomach fluid

Pepsinogen is present in the stomach wall of *T. nilotica*, and has also been reported in *T. mossambica* (Fish, 1960). Strong binding of pepsin to the algal material and its removal by centrifuging might explain the lack of proteolytic activity in the stomach fluid. It is also

possible that the level of secretion is low. Mucus in the fluid interfered with methods for determining protein, but the ultra-violet spectrum confirmed that if pepsin or any protein were present, its concentration would be very low. Phycocyanin leached from the blue-green algae, and mucus from the fish, might also account for the U.V. absorbance.

The low U.V. absorbance also shows that there could be few or no peptides present in the stomach juice. This suggests that peptic activity in the stomach is negligible. Even if pepsin were present, it could not play a significant part in the digestion of blue-green or green algae because the cells must first by lysed. Maximum lysis occurs around pH 1·6–1·4, but the lysed algae remain at this pH until they pass through the pyloric sphincter. This pH is well below the optimum pH for activity of the pepsin (assuming that casein is a typical substrate).

It is apparent that in *T. nilotica* and *H. nigripinnis* the high concentration of acid in the stomach is serving a function not only unrelated to peptic digestion, but one which is not readily compatible with it. The lack of evidence for peptic activity in the stomach juice of *T. nilotica* suggests that the stomach may be losing the ability to synthesize and secrete pepsin, while the fish is changing from an omnivorous to a herbivorous diet. The dominance of blue-green algae in the phytoplankton might encourage such an evolutionary step. They are easily lysed by low pH values and the subsequent digestion of their cytoplasm is readily accomplished by the usual proteases and carbohydrases synthesized by vertebrates. It would be interesting to compare the concentrations of acid, of pepsin and of other digestive enzymes in the alimentary secretions of *T. nilotica* taken from a variety of habitats in which it occurs naturally.

Another point to note in this context is that *H. nigripinnis* assimilates *Microcystis* more efficiently over a diurnal feeding cycle (Moriarty & Moriarty, 1973*a*). The low efficiency of *T. nilotica* is a result of much of the *Microcystis* passing through the stomach too rapidly for lysis to occur. This implies that *H. nigripinnis* has evolved a more effective mechanism for the treatment of the ingested *Microcystis* with acid. Further work is needed to test this hypothesis.

Pancreas and intestinal fluid

The digestion of an amylopectin with glucose as the main end product indicates that an α-amylase, an α-1,6-glucosidase and a maltase are present in *T. nilotica*. This result is in agreement with that of Keddis (1957), but differs however, in showing that an inorganic anion (e.g. chloride) is essential for activation of the α-amylase, as is the case in higher vertebrates (see Cole, 1904). The methods used by Keddis (1957) may not have removed all anions initially. He concluded that the α-amylase originated in the pancreas.

The pancreas in *T. nilotica* is diffuse, occurring within the connective tissue surrounding the walls of the stomach and intestine, and around blood vessels in this mesentery. Pancreatic cells are also embedded in the liver. A duct system collects into one pancreatic duct which with the bile duct enters the intestine at the pyloric sphincter (Naguib, 1958). Because of the diffuse nature of the pancreas, α-amylase can be detected in all alimentary tissues in the abdominal cavity. It could easily be transferred into the buccal cavity and other areas by dissecting instruments. Thus workers (Fish, 1960; Nagase, 1964; Cockson & Bourne, 1972) studying the distribution of amylase and other enzymes in different regions of the alimentary canal of *Tilapia* are likely to have been measuring enzymes in the pancreas. There need be no correlation between enzyme activities in the walls and those

of the digestive fluid enclosed by them. Barrington (1957) has stressed the need for stringent precautions to avoid contamination of extracts by pancreatic tissue in teleosts.

The inability of fluid from the intestine in *T. nilotica* to hydrolyse olive oil can be interpreted as an absence of a pancreatic lipase. Keddis (1957) also found no lipase in the intestinal juice and pancreatic tissue of *T. nilotica*. Although Keddis could not demonstrate esterase activity (ethyl acetate hydrolysis) an esterase is present, as I have shown (p. 33). Enzymes with similar substrate specificities to mammalian trypsin and chymotrypsin are present, but no activity attributable to a carboxypeptidase was detected. More detailed investigations of the proteases in *T. nilotica* are needed to confirm the absence of carboxypeptidases A and B.

General conclusions

The digestion of blue-green algae and bacteria occurs in the intestine, but before this takes place the cells must be lysed by acid. Acid has been recorded in the stomach of the herbivorous *T. esculenta* (Greenwood, 1953) and *T. mossambica* (Fish, 1960). These and any other *Tilapia* or *Haplochromis* species in which algae or bacteria are retained in the stomach long enough for the pH to reach values around 1·6 or even lower, will be able to digest such material. Grey mullets (Mugilidae) and the milkfish *Chanos chanos* (Forsk.) which feed on algae and detritus, have an anterior thin-walled stomach (Hickling, 1970). Gastric glands have been reported (Pillay, 1953) in this part of the stomach of *Mugil capito* (=*Liza ramada* (Risso)) and *M. tade* (=*Liza planiceps* (C and V)). A similar mechanism for lysis and digestion of blue-green algae may occur in these herbivores. The grey mullets have a gizzard which may improve the efficiency of acid lysis on the algal cells. Weatherly (1972: 130), discussing the relation of food to the growth and ecology of fish, suggests that it ". . . may be relatively simple to infer the general nature of a fish's food from a knowledge of the functional morphology . . ." of its alimentary tract. One of the characteristics of herbivorous fish that he cites is the lack of a true stomach, i.e. a dilatation in the anterior part of the alimentary tract in which acid is secreted. I would disagree with this generalization; it is because *T. nilotica* and *H. nigripinnis* possess a true stomach that blue-green and green algae can be digested. The general nature of a fish's food can often be told from field studies, but only from a detailed knowledge of its digestive physiology can we determine the extent to which the various elements are utilized.

Summary

Blue-green algae are lysed by a high concentration of acid in the stomach. pH 1·4 is the lowest value recorded in the stomach of *T. nilotica*; lysis is most effective at this pH.

Enzymatic digestion occurs in the intestine, after the algae are lysed.

Acid secretion in the stomachs of *T. nilotica* and *H. nigripinnis* in Lake George, Uganda, follows a diurnal cycle associated with feeding. Secretion starts when feeding starts in the morning. In *T. nilotica*, low pH values are not obtained throughout the stomach until feeding ceases in the evening. Thus there is a gradation through the day, from zero to maximum in number of blue-green algal cells lysed, and therefore digested and assimilated.

Colour changes in the ingested phytoplankton can be used as an indicator of gastric acid secretion by fish in the laboratory. Acid is not secreted by stressed fish.

Pepsinogen was detected in the stomach wall of *T. nilotica*. Evidence for the absence of peptic digestion in the stomach of *T. nilotica* is presented.

A pancreatic α-amylase, pH optimum 7·0 to 8·0, and requiring chloride, is present in the intestinal juice of *T. nilotica*.

Trypsin and chymotrypsin are also present, but carboxypeptidases A and B could not be detected.

Esterase activity, but no lipase, was detected in the intestine juice.

This project forms part of a joint contribution by the United Kingdom and Uganda to the International Biological Programme. It was financed by the Royal Society whose support, with that of the Freshwater Biological Association, is gratefully acknowledged. I wish to thank my colleagues at Lake George for their assistance during this work. I am grateful to Professor E. J. W. Barrington for providing facilities in his department, where some of this work was carried out. Dr P. H. Greenwood has kindly read and criticized the manuscript.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

Reprinted from *J. Zool., Lond.* (1973) **171**, 41–55

The assimilation of carbon from phytoplankton by two herbivorous fishes: *Tilapia nilotica* and *Haplochromis nigripinnis*

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(*Accepted 10 April 1973*)

(With 2 figures in the text)

With the exception of their very young stages, *Tilapia nilotica* and *Haplochromis nigripinnis* in Lake George ingest phytoplankton, especially *Microcystis* species. *Tilapia nilotica* can assimilate a maximum of 70–80% of ingested carbon from *Microcystis* sp., *Anabaena* sp., and *Nitzschia* sp. *Haplochromis nigripinnis* also assimilates a similar proportion of carbon from *Microcystis*. Assimilation is lower at the start than at the end of a feeding period. *T. nilotica* assimilates a maximum of about 50% of ingested carbon from *Chlorella* sp.

An average of about 43% of ingested carbon is assimilated by *T. nilotica* from the Lake George phytoplankton during a daily feeding cycle. An average of about 66% is assimilated by *H. nigripinnis*.

Contents

Introduction

The herbivorous fish, *Tilapia nilotica* (L.) and *Haplochromis nigripinnis* Regan dominate the fauna of Lake George, Uganda (Dunn, 1972). Fish (1955) stated that the *T. nilotica* feed on the soft bottom deposits in this lake, whereas Lowe-McConnell (1958) reported

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that they feed on phytoplankton as well as the bottom deposits. There are no reports concerning the food of the fry of *T. nilotica*, but Dunn (1972) found zooplankton in the guts of young *Haplochromis* species (probably including *H. nigripinnis*).

Before embarking on a quantitative study of the assimilation of phytoplankton by these fishes, it is necessary to clarify the nature of their food. It is shown in this paper that adult *H. nigripinnis* feed mainly on the colonies of *Microcystis* and their associated flora (mainly *Nitzschia* sp.). *Tilapia nilotica* feed on most of the phytoplankton species, of which about 70% are blue-green algae (especially *Microcystis aeruginosa*, *M. flos-aquae* and *Anabaenopsis* spp., Ganf, 1972).

In many inland and estuarine water bodies in the tropics and warm temperate regions, where blue-green algae dominate the phytoplankton, there are herbivorous fish which can directly utilize the primary production (e.g. Hickling, 1961, 1970). *Tilapia* are among the commercially exploited herbivores, especially in the lakes of Africa (Hickling, 1961). Where these fish are not endemic, they have often been introduced and farmed in ponds (e.g. Hickling, 1971).

Fish (1952) reported that *T. esculenta* Graham which ingests phytoplankton, cannot digest blue-green algae. Although Fish (1955) found some evidence for digestion of blue-green algae by *T. nilotica* in Lake Rudolf, which he regarded as an unusual situation, it is generally accepted that *Tilapia* species cannot digest blue-green algae (e.g. Hickling, 1961, 1970; Fryer & Iles, 1972).

During a study of productivity in Lake George as part of the International Biological Programme (Dunn, Burgis *et al.*, 1969), it was found difficult to reconcile the presence of a high biomass of herbivorous fish with the idea that these algae could not be digested.

Quantitative studies with ^{14}C-labelled algae were undertaken. The methods employed were based partly on those of Sorokin (1968), who commented on the inaccuracy of the widespread view that blue-green algae are trophically unimportant.

These experiments were designed to show whether certain species of algae, representative of three major algal groups occurring in Lake George, could be digested by *T. nilotica*. Two different types of blue-green algae were chosen: *Microcystis* sp., in which colonies of cells are embedded in mucilage, and *Anabaena* sp. a filamentous form containing three different types of cells. A species of *Nitzschia* was used as a representative of the diatoms, which make up about 20–30% of the phytoplankton. A *Chlorella* sp. was studied as a representative of the green algae. The assimilation of *Microcystis* sp. by *H. nigripinnis* was also measured.

In vitro studies indicate that digestion of blue-green algae occurs in the intestine of the fish, if the cells have been subjected to a high concentration of acid in the stomach. Since acid secretion follows a diurnal cycle, a trend is expected from minimum digestion of algae ingested at the start of a daily feeding period to maximum digestion at the end (Moriarty, D., 1973). This hypothesis is supported by *in vivo* experiments reported in this paper.

Experiments were also designed to estimate the average proportion of carbon assimilated daily by *T. nilotica* and *H. nigripinnis* from the phytoplankton ingested in the lake. As a direct measurement was not possible, their natural feeding rhythm (Moriarty & Moriarty, 1973) has been simulated in the laboratory. It is important to maintain this schedule, because digestion is variable, being affected particularly by the fullness of the stomach and the time spent by algae in it (Moriarty, D., 1973).

Assimilation is measured as the difference between the amount of ^{14}C or total carbon ingested and the amount defaecated.

Methods

Gut contents

Qualitative examinations were made of the gut contents of a size range of *T. nilotica* caught in most areas of the lake by a variety of fishing methods. Counts were made of the zooplankton and algae in the guts from a size range of *H. nigripinnis* from seine catches made in the mid-lake area.

Algae

For culture of *Chlorella* and *Nitzschia* the ASM medium of McLachlan & Gorham (1961) was used. It was made up in filtered lake water (Whatman GF/C). These algae were uniformly labelled with ^{14}C in sealed flasks. After autoclaving, 1·7 g $NaHCO_3$ (solid) and from 10 to 50 μc $^{14}NaHCO_3$ were added to each 400 ml of medium.

In the ASM medium for *Microcystis*, $NaNO_3$ was replaced by NH_4Cl at the same molar concentration. It was then diluted 5 times. It was made up in deionised, distilled water. Colony formation was better in this dilute medium and the growth of *M. flos-aquae* was favoured over other species during the purification procedure.

Anabaena was grown in the diluted ASM medium, but nitrogenous salts were omitted.

These blue-green algae were labelled with 350 mg $NaHCO_3$ and from 10 to 50 μc $^{14}NaHCO_3$ per 400 ml medium.

All media were autoclaved at 15 psi for 15 min.

Cultured algae

Microcystis flos-aquae colonies were picked out of Lake George water and placed in medium with actidione (cycloheximide) at 50 μg/ml to inhibit growth of eukaryotic algae. After repeated sub-culturing during the exponential phase of growth, unialgal cultures were obtained.

During a bloom of *A. flos-aquae*, enrichment cultures were set up with actidione in the *Anabaena* medium. A species of *Anabaena* was subsequently obtained in unialgal culture.

Under lake conditions, Ganf (1972) identified the *Microcystis* and *Anabaena* species as *M. flos-aquae* and *A. flos-aquae*. The morphology of the organisms in culture however, was variable. They were tentatively identified as *Microcystis c.f. M. flos-aquae* and *Anabaena c.f. A. torulosa* (Thienemann, 1962) and are referred to in this paper as *Microcystis* and *Anabaena*.

Nitzschia sp. and *Chlorella* sp. were isolated by streaking Lake George water on ASM medium solidified with 1 % Ionagar (Difco). Colonies were picked off and grown in liquid culture.

Cells in all labelled cultures were kept in suspension with magnetic stirrers. They were harvested, and washed twice in filtered lake water by centrifuging, immediately before use.

Lake algae

A tow net (about 100 μm pore size) was used to collect plankton from the lake for feeding fish maintained in the laboratory. This material is referred to as lake algae in the text.

Fish

Juveniles, which fed on organisms other than phytoplankton, were not studied. Fish for the assimilation experiments were caught by trawling for periods of ½ to 1 min duration. They were transferred to 20 l tanks, 20 cm deep. Fresh lake water was supplied at about 3 day intervals, and lake algae were provided each day. After the fish commenced feeding in these tanks, they were transferred to laboratory aquaria. Fish up to 13 cm length were kept individually in small

tanks holding 3 l of lake water, and larger fish in 20 l tanks. The water was changed every two days.

Lengths of the fish were measured as total lengths, i.e. the length from the tip of the snout to the most posterior point of the caudal fin.

Fish were used for experiments only when they could be transferred between aquaria without their showing resistance or alarm reactions, such as raising the dorsal fin. Also, fish were not used unless their faeces showed a diurnal change in colour from green to brown, which indicates that acid secretion has occurred in their stomachs (Moriarty, D., 1973). If these conditions were not met, ingestion and assimilation were low and unpredictable (see discussion on stress).

Determination of ^{14}C

Particulate material (microorganisms and faeces) was concentrated onto Millipore (0·45 μm) or Whatman GF/C filters of 24 mm diameter, and dried in a desiccator.

Respired $^{14}CO_2$ was collected as $BaCO_3$ and counted on Millipore filters (0·45 μm) as described by Sorokin (1968).

Gut contents were separated from the gut wall and pipetted onto 25 mm planchets. Fish tissues were blended at high speed for ten minutes with sufficient water to give a liquid, homogeneous suspension. A few drops of amyl alcohol were included to reduce froth. Aliquots were transferred with a wide mouthed pipette into planchets. Planchets were dried at 70°C in an oven. Self-absorption was corrected for by extrapolation to zero thickness. A Tracerlab manual gas-flow system using proportional gas was used to assay ^{14}C.

Radioactivity in the faeces was measured by suspending them in a known volume of water and filtering replicate aliquots. No soluble ^{14}C was detected in the filtrates.

Assimilation is expressed as a percentage of the total ^{14}C ingested. Standard errors were calculated for all replicate ^{14}C determinations.

Assimilation of ^{14}C-algae

For the *ad lib.* feeding method, *Microcystis* and *Anabaena* were resuspended as colonies in a small volume of filtered lake water. *Nitzschia* and *Chlorella* were resuspended with a small volume of concentrated lake algae. The labelled suspensions were stirred into a known volume of water in an aquarium to give an algal concentration approximately equal to that in the lake (*c.* 30 mg C/l). A sample of about 10% of the volume was removed and shaken vigorously to disperse colonies before being subsampled for ^{14}C determinations (8–10 replicates). The fish was then placed in the aquarium with an aerator to keep the cells in suspension. As these fish feed by filtration, a suspension of cells is necessary. The tank was partially covered with a dark cloth to give a light intensity a little above the compensation point of the algae, in order to reduce changes in the amount of particulate ^{14}C due to algal respiration or photosynthesis. Small samples were removed occasionally with a wide-mouthed pipette to check the progress of feeding. The fish was removed as soon as a significant consumption had occurred, usually within 2 h. The remaining algae were stirred to disperse colonies and again sampled for ^{14}C. The decrease in ^{14}C in the aquarium was a measure of the amount ingested.

For the pipette feeding method, *Microcystis* was resuspended homogeneously in a known volume of filtered lake water and then sub-sampled for determination of total ^{14}C present. It was then centrifuged again and taken up in a small volume of water as a concentrated suspension in the feeding pipette. The fish was trained to take the algae directly from the pipette. After feeding, the fish was removed and the amount of ^{14}C remaining in the water, pipette and centrifuge tube was measured. The amount ingested was the difference between this and the original total.

The faeces of these fish, being enclosed in mucus, are easy to collect quantitatively with a wide-mouthed pipette. They did not break up in the aquarium when collected at 1 or 2 h intervals during the day. Overnight, however, some faeces did break up, and these were collected by

filtration the next morning. On the day following a ^{14}C experiment, faeces were collected until none contained ^{14}C.

Assimilation of lake algae

Lake algae were collected immediately prior to each experiment. They were evenly suspended in filtered lake water at a concentration of about 0·5 mg chlorophyll *a*/l. For determination of particulate carbon, 10 replicate samples were removed with a wide-mouthed pipette. They were placed in a thick-walled glass bottle, where the gas vacuoles were ruptured by pressure shock. Particulate carbon was collected by centrifuging at 20,000 × g for 5 min. The concentrated material was resuspended in a small amount of distilled water, transferred to combustion boats and dried at 70°C.

Carbon was determined by the method of Ganf & Milburn (1971). Carbon dioxide, derived from the sample by dry combustion, was quantitatively absorbed by a solution of NaOH and the change in conductivity was monitored.

After removing the initial samples for carbon measurement, the fish was placed in the tank for 12 h. A dark cloth was placed over the tank, giving a light intensity close to the compensation point of the phytoplankton. Controls were conducted to check that changes in particulate carbon due to respiration or photosynthesis were negligible.

Particulate carbon was measured again after removal of the fish. The difference between initial and final values represented the amount ingested.

Faeces resulting from phytoplankton ingested during the experimental feeding period were collected and aliquots were placed in combustion boats for carbon determination. It is necessary to distinguish between those faeces resulting from the experimental feeding period and those from previous or subsequent periods. Provided the stomach is fully evacuated between feeding periods, as occurs naturally in the lake, mixing of ingested material from two periods will not occur and the diurnal colour change in faeces permits distinction between two different periods (see discussion, Moriarty & Moriarty, 1973; Moriarty, D., 1973). This was checked by feeding the fish on phytoplankton stained with alcian blue on the day before and the day following each experiment. The faeces from the stained phytoplankton contained alcian blue, but the faeces from the intervening period did not.

All experiments were carried out at 25 ± 1°C.

Results

Gut contents

The guts of *T. nilotica* fry contain a large variety of plant and animal material collected from the swampy and littoral areas they inhabit. Among the items eaten are algae, Aufwuchs, macrophytic detritus, rotifers and other zooplankton, insect larvae, and water mites.

Small *T. nilotica* between 3 and 6 cm in length are caught in water less than 1 m in depth, and between 1 and 10 m from the shoreline. Their guts contain a higher proportion of phytoplankton compared to the fry, and not as much zooplankton.

Fish of larger sizes are found further offshore. They consume mainly phytoplankton, and occasionally some detritus, but only a very low number of planktonic animals (mainly small rotifers). The percentage of *T. nilotica* with some detritus mixed with the phytoplankton in the gut varies between 0 and 24% of the fish in trawl samples, the maximum numbers being found in samples taken between 08.00 and 12.00 hrs.

About 70% of the food items ingested by *H. nigripinnis* between 2 and 3 cm in length are from the zooplankton, and about 30% are algae. In successively larger *H. nigripinnis*

there is a steady increase in the percentage of algae eaten, reaching over 95% in the 4 to 5 cm length group and levelling off at this value in the larger size groups.

The zooplankton eaten by the small *H. nigripinnis* includes copepods (mainly *Thermocyclops hyalinus*), *Daphnia*, *Ceriodaphnia*, *Moina* and some chironomid larvae. The algae eaten are mainly the large colonial species *Microcystis aeruginosa* and *M. flos-aquae*, plus the diatoms bound in the mucilaginous sheaths of these colonial algae.

A more extensive account of the food items of these fishes in Lake George is given by Moriarty, C. (1973).

TABLE I

Assimilation by T. nilotica of carbon from algae ingested at the end of a feeding period. For the first 8 to 10 h of the day, the fish were fed lake algae. For a subsequent period of 1 to 3 h they were fed by the ad lib. method on the experimental algal species. This was followed by a period of 8 h in filtered lake water. Finally, the next day, they were again fed lake algae. Fish length: 9·5 to 12·5 cm

Algae	Assimilation (% ^{14}C) Average	Range	Number of experiments
Microcystis	70	67–73	6
Anabaena	75	68–82	4
Nitzschia	79	78–81	2
Chlorella	49	45–52	3

Assimilation of ^{14}C-algae

At the end of a feeding period, high proportions of carbon are assimilated by *T. nilotica* from *Microcystis*, *Anabaena*, *Nitzschia*, and rather less from *Chlorella* (Table I). As these microorganisms were uniformly labelled, ^{14}C determinations are directly proportional to total carbon in their cells. A high proportion of carbon is assimilated by *H. nigripinnis* from *Microcystis*, when ingested towards the end of a feeding period (Table II).

TABLE II

Assimilation of Microcystis by H. nigripinnis. The procedure used is described in Table I, except that the fish were fed for 4 h on lake algae after the ^{14}C algae and before being placed in filtered lake water. Fish length: 6·3 to 7·8 cm

Alga	Assimilation (% ^{14}C) Average	Range	Number of experiments
Microcystis	71	56–80	7

The maximum values for assimilation are especially noteworthy. For example, 73% of *Microcystis* carbon was assimilated by *T. nilotica* in one experiment, and 80% by *H. nigripinnis*. Experimental errors contribute to an uncertainty range of *c.* ± 2 to ± 6% in each value for percentage assimilation. The variability shown by the range of values for assimilation is discussed below.

TABLE III

Assimilation of carbon from Microcystis by T. nilotica when fed at the start of a feeding period.

Fish were kept in filtered lake water overnight prior to the experiment. Fish 1 was fed ^{14}C-Microcystis from a pipette for 15 minutes (see Fig. 1). The other fish fed ad lib. for 2 h in a suspension of Microcystis. After feeding on the labelled cells, all fish were transferred to tanks containing lake algae

Fish number	Assimilation % ^{14}C (and experimental error)	Ratio $\frac{\text{cpm ingested}}{\text{cpm offered}}$
1	33±2	0·65
2	0±20	0·16
3	43±2	0·38
4	60±5	0·25

When *T. nilotica* are fed ^{14}C-*Microcystis* at the beginning of a day's feeding period, less carbon is assimilated and the results are more variable. Some examples are given in Table III. Fish 1 ingested more labelled *Microcystis* in a shorter time than the other fish,

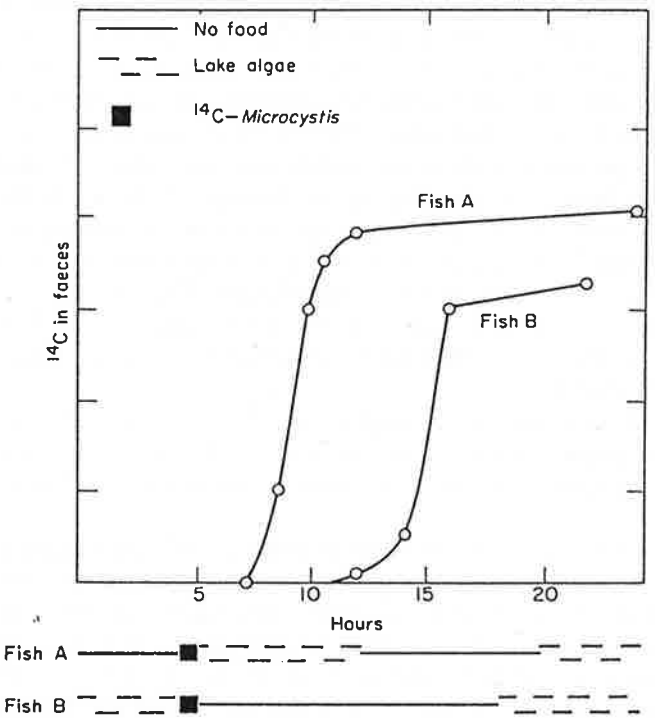

FIG. 1. Time for recovery of ^{14}C in faeces of *T. nilotica*. Fish A was fed ^{14}C-*Microcystis* after being kept in filtered water for 10 h. Fish B was fed ^{14}C-*Microcystis* after feeding on lake algae for 8 h. Cumulative totals of ^{14}C in the faeces are plotted. Fish lengths 10 cm each.

because it was fed by pipette. Fish 2 did not start feeding until shortly before it was removed from the tank of labelled cells. As it consumed a small proportion of the ^{14}C offered, experimental errors were large in comparison to the amount assimilated.

Algae ingested at the end of a feeding period take longer to pass through the gut than do algae eaten at the beginning of a feeding period (Fig. 1). The majority of undigested ^{14}C from fish A was detected as green faeces, four to seven hours after feeding started. At the time the first labelled faeces appeared, (four hours), the colour of the faeces changed abruptly from brown to green. Throughout the day, the faeces changed colour gradually from green to brown again. Some ^{14}C was present in all faeces from that day's food. In this experiment, 33 % of the ingested ^{14}C was assimilated.

The first of the undigested ^{14}C from fish B (Fig. 1), which received ^{14}C-*Microcystis* at the end of a feeding period, was found in the faeces about seven hours after feeding on the labelled cells. It assimilated 70 % of the ingested ^{14}C-*Microcystis*. The faeces showed the usual colour change, the first from that day's food being green and later faeces becoming brown. The ^{14}C was recovered in the brown faeces only.

Both these fish were fed lake algae on the day after an overnight period in filtered lake water. The brown faeces produced during the morning, originating from the previous day's labelled food, contained ^{14}C. After the abrupt colour change from brown to green occurred, no further ^{14}C was found in the faeces. The overnight period without food was long enough for the stomach and part of the intestine to empty, as is the case with fish in the lake. The colour change in faeces confirms that the stomach has emptied, because acid secretion stops (Moriarty, D., 1973). The presence of ^{14}C in the brown faeces next morning, but not in the subsequent green faeces, shows that although there is mixing of food material during a feeding period, there is no mixing between feeding periods. This suggests that mixing occurs in the stomach when the fish is feeding. Provided the stomach is emptied between feeding periods, food from two different periods will not be mixed.

The difference in proportions of carbon assimilated from *Microcystis* at the beginning and at the end of a feeding period was demonstrated in one individual *T. nilotica* during one period (Fig. 2). In the faeces, it is necessary to distinguish between the ^{14}C from feed A and from feed B. The total ^{14}C in the faeces from feed A was found by extrapolating the curve from hour 11 to hour 24, giving point A in Fig. 2. Experiments such as that with Fish A in Fig. 1 demonstrate the validity of this extrapolation. The ^{14}C in the faeces from feed B was given by the difference between the total ^{14}C in the faeces (point T, Fig. 2), and the ^{14}C from feed A (point A, Fig. 2). It was found that about 43 % carbon was assimilated from feed A and 68 % from feed B.

In all experiments referred to above, it has been assumed that the decrease in ^{14}C in the feeding tank represented the amount ingested by the fish. This has been verified (Table IV). Experimental errors or small losses due to $^{14}CO_2$ may account for the slight differences recorded.

The assumption that the difference between the amount of ^{14}C-algae ingested and the amount defaecated is due to assimilation has also been confirmed. Fish have been left undisturbed in a closed container for some hours after ingesting ^{14}C *Microcystis*, and then killed. The ^{14}C in their faeces, respired CO_2 and bodies agrees well with the amount ingested. An example is given in Table V. In this case, where the fish was left for only four hours, a considerable amount of ^{14}C was still in the gut. When the fish was allowed to digest the labelled food for a longer period (nine hours) proportionally more ^{14}C was

found in the respired CO_2, body tissue and soluble fractions in the intestine. Thus more assimilation had occurred.

Assimilation of lake algae

The average proportion of carbon assimilated from Lake George phytoplankton by *T. nilotica* during a 24 hour day in the laboratory was about 43 % of the total ingested

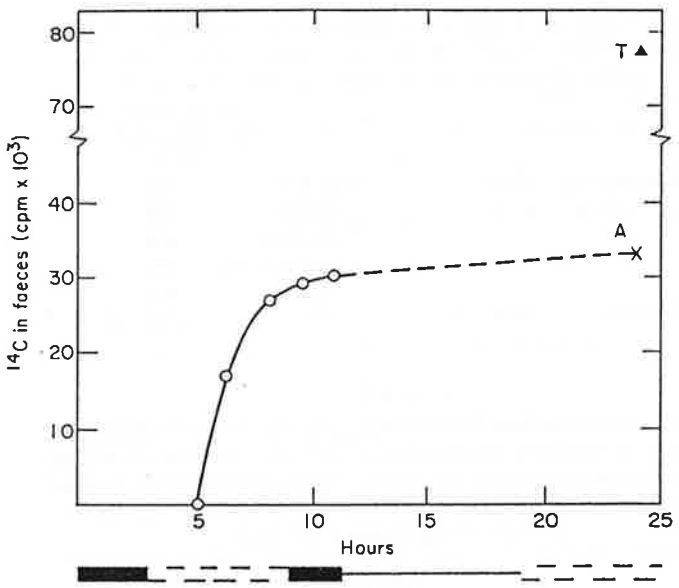

FIG. 2. Assimilation by *T. nilotica* of ^{14}C-*Microcystis* ingested at beginning and end of feeding period. After being kept in filtered lake water for 10 h, the fish was fed ^{14}C-*Microcystis* (feed A, 60,000 cpm ingested), then lake algae followed by ^{14}C-*Microcystis* again (feed B, 136,500 cpm ingested). Point T is the total ^{14}C in all faeces collected from the experimental period. Fish length: 9·5 cm.

TABLE IV

Correlation of ^{14}C loss in tank with ingestion. A T. nilotica was fed lake algae for 5 h before being placed in a tank containing ^{14}C-Microcystis for 2 h. It was then killed by immersion in crushed ice and dissected immediately

^{14}C in tank (cpm) × 10^3		^{14}C in fish (cpm) × 10^3	
Before feeding	459	Stomach	40
After feeding	367	Intestine	40
		Fish tissues	3
Difference	92±2	Total	88±5

(Table VI). Most values were between 40 and 60 %. The standard error of the sampling procedure for determining carbon was ±2 % or less in all cases, and so these methods were not the cause of the considerable variation in results noted between experiments.

Variations in the amount of phytoplankton ingested by one particular fish do not seem to have a predictable effect on the proportion of carbon assimilated. For example, a fish

of 15·8 cm length ingested 72 mg C on one occasion and 252 mg C on another, but there was very little difference in the proportion assimilated (Table VII).

TABLE V

Correlation of ^{14}C loss in tank with ingestion and assimilation. A T. nilotica was fed as in Table IV. After feeding on ^{14}C-Microcystis it was transferred to a solution of NaCl in distilled water (2·5 meq/l) in a sealed tank for 4 h. It was then removed and killed. Respired $^{14}CO_2$ was collected from the water

^{14}C in tank (cpm) $\times 10^3$		^{14}C in fish (cpm) $\times 10^3$	
Before feeding	4310	Stomach	700
After feeding	1350	Intestine	1080
		Fish tissues	630
		Respired CO_2	270
		Faeces	200
Difference	2960±70	Total	2880±50

TABLE VI

Assimilation of carbon from Lake George phytoplankton by T. nilotica and H. nigripinnis Fish were fed plankton for 12 h and were kept in filtered water for 12 h before and after the feeding. The total carbon of the ingested phytoplankton, and in the faeces was measured. Fish size: T. nilotica 9·4–20·3 cm; H. nigripinnis 6·3–7·8 cm

Fish		Assimilation % C Mean	Range	Number of experiments
T. nilotica		43±4	30–60	15
H. nigripinnis	Group 1	66	60–71	6
	Group 2	33	28–40	4

TABLE VII

Assimilation of carbon from Lake George phytoplankton by T. nilotica. One individual (length 15·8 cm) was used on four separate occasions. See Table VI for procedure

Amount ingested (mg C)	Assimilation (% C)
72	45
111	45
163	60
252	48

These results for assimilation are applicable to the phytoplankton only. Zooplankton, although present in the water, are not ingested by these *T. nilotica*; being positively rheotactic, the copepods swim away from the mouths of feeding fish.

In one group of experiments with *H. nigripinnis*, a high proportion of carbon (66 % average) was assimilated from the Lake George phytoplankton (Table VI). The faeces of fish in Group 1 showed the usual colour change from green to brown during the experiments. These fish consumed more than 50 % of their natural daily intake in the lake (see Moriarty & Moriarty, 1973). A lower proportion of carbon (average 33 %) was assimilated by other *H. nigripinnis* (Group 2, Table VI). In two of these experiments, it was noted that the faeces did not turn brown overnight, indicating that acid secretion was reduced. These results are mainly applicable to the assimilation of *Microcystis* colonies and their associated microflora, as very little zooplankton was eaten by fish in the size range used in these experiments.

Discussion

Assimilation of ^{14}C-algae

An important feature of the methods used here is that the labelled algae were grown over several generations in a closed system with ^{14}C-bicarbonate. This ensured that all carbon compounds, including cell walls and mucilage (where present) were uniformly labelled. It was possible therefore, to study quantitatively the digestion and assimilation of given species of microorganisms at different times of the day, while the fish were feeding on their natural food and following their natural feeding rhythm. The maximum proportion of carbon is assimilated from *Microcystis*, for example, when it is ingested at the end of a feeding period. The lowest figures for assimilation were obtained when the ^{14}C-*Microcystis* was eaten at the start of a feeding period. This supports the work reported previously on the physiology of digestion of blue-green algae in *T. nilotica* (Moriarty, D., 1973).

Not all algal cells of a given species are digested equally. In fact some are not digested at all. The digestibility is influenced by a number of factors which are discussed below. The differences in digestion between algal species have been discussed elsewhere (Moriarty, D., 1973).

Experimental error

Experimental errors were due primarily to the problems of sampling a heterogeneous suspension for the determination of ^{14}C. These errors were large with colonial algae. In experiments where the fish ingested less than 15 % of the total ^{14}C offered, the experimental error was large in proportion to the amount ingested. These experiments were discontinued. Fish 2 (Table III) ingested only 16 % of the total offered, and this contributed to a large uncertainty in the calculation of ^{14}C assimilated. The conclusion drawn from this experiment however, is still valid, *viz.* little or no carbon was assimilated from the ^{14}C-*Microcystis* eaten at the start of that feeding period. In most other experiments with *Microcystis*, the fish ingested about 30–40 % of the total offered if feeding *ad lib.*, or 55–65 % if feeding from a pipette.

When *Nitzschia* and *Chlorella* were offered to *T. nilotica*, the fish usually ingested less than 25 %. The inclusion of colonial blue-green algae in the water usually enhanced ingestion of *Nitzschia* and *Chlorella*. Feeding was stimulated by the presence of colonial blue-green algae, but many cells of *Nitzschia* and *Chlorella* passed out of the oro-branchial cavity in the respiratory current.

Occasionally some individual *T. nilotica* altered their feeding mechanism to ingest single cells, including very small species of Chroococcales. The reasons for this change were not elucidated, nor could an individual fish be trained to ingest small cells when required. This was the main difficulty encountered in attempting to replicate the experiments with *Chlorella* and *Nitzschia*. Although more replicates are required, it is likely that the maximum digestibility of *Nitzschia* is about the same as that of the blue-green algae, but that the green alga, *Chlorella* is significantly less digestible.

Loss of ^{14}C into the atmosphere or into solution was negligible in the time period of the experiments.

Bacteria were not excluded from the cultures of algae used in these experiments. As they did not constitute a large proportion of the biomass, however, their presence would contribute only a small error to the estimates of algal assimilation.

Stress

Fish were stressed during capture. Many small individuals of *T. nilotica* (less than 13 cm in length) recovered within about two weeks. *Tilapia nilotica* larger than 13 cm length rarely adapted to laboratory conditions. *Haplochromis nigripinnis* were even more susceptible to stress; many did not survive in the laboratory. One indication of stress is that the faeces remain green in colour, provided the fish continues to feed while stressed. Stress inhibits acid secretion in the stomach (Moriarty, D., 1973). It is important therefore, that the fish be well adapted to laboratory conditions and handled carefully during studies on digestion and assimilation.

Physiology of digestion

The physiology of digestion has the most pronounced effect in determining the percentage of carbon assimilated. This has been studied in detail in *T. nilotica* (Moriarty, D., 1973), but in general, the conclusions should also be applicable to *H. nigripinnis*.

Maximum values can be expected for assimilation of *Microcystis* consumed at the end of a feeding period because there is already a high concentration of acid in the stomach, and the retention time increases when feeding ceases (Fig. 1 and Moriarty & Moriarty, 1973). The retention times for algae ingested during a feeding period are complex (Moriarty, D., 1973). Some cells pass rapidly from oesophagus to pylorus and are affected only slightly by acid. Others are retained in the fundus, where acid has the most effect. Thus, values for assimilation of a given batch of *Microcystis* cells will vary with the relative proportions retained in the fundus or passing rapidly into the intestine. It was observed that ^{14}C was present in all faeces from the food ingested on the day fish A (Fig. 1) was fed ^{14}C-*Microcystis*. This indicates that some of the ^{14}C-*Microcystis* cells were retained in the stomach until it emptied, and were continually released during the day. The figure of 33 % carbon assimilation is thus an average figure for all the labelled cells eaten at the start of that feeding period. *Microcystis* cells retained in the stomach would have been more fully digested than those passing rapidly into the intestine. In fact, many of the latter would not have been digested at all.

A similar explanation applies to the results in Table III. In fish 2, all the ingested ^{14}C-*Microcystis* cells would have passed rapidly through the stomach before secreted acid could cause lysis. Some of the ^{14}C-*Microcystis* cells may have been retained in the stomachs

of fish numbers 1, 3, and 4. These cells, being subjected to a low pH, would be readily digested and thus the overall assimilation is an average figure.

The experiments with *H. nigripinnis* were carried out before it was realized that assimilation reached a maximum at the end of a feeding period. In two experiments with *H. nigripinnis* assimilation values of 80 % were obtained. These two fish may not have continued feeding after ingesting the ^{14}C-*Microcystis* because 80 % is likely to be close to the maximum possible assimilation. Some of the cell carbon of *Microcystis* occurs in the cell wall and mucilage, and it is unlikely that the fish have enzymes capable of hydrolysing such materials (see Fish, 1960). Similarly, the maximum possible assimilation of carbon from *Microcystis* may also be about 80 % in *T. nilotica*.

Assimilation of lake algae

The proportions of carbon assimilated from phytoplankton by these fish during a complete diurnal cycle show considerable variation. The amount of phytoplankton ingested by fish in the lake has been estimated (Moriarty & Moriarty, 1973). On this basis, it was observed that *T. nilotica* in the laboratory were consuming about 20–50 % of the amount consumed by fish of equivalent sizes in the lake. If this reduced rate of feeding influences the relative proportions of algae being retained in the stomach or passing rapidly into the intestine, then it will alter the proportion of cells which are lysed in the stomach and subsequently digested in the intestine (Moriarty, D., 1973).

Although results such as those in Table VII show that variations in the actual amount consumed by individual fish may not affect assimilation percentages, some doubt will remain until fish in the laboratory can be made to consume as much as they would in the lake. Some of the *H. nigripinnis* (in Group 1, Table VI) did consume as much as fish of an equivalent size in the lake.

The species composition of phytoplankton in Lake George does not vary greatly (Ganf, pers. comm.) and thus would have little effect of the differences in assimilation values between experiments on one species of fish. As *H. nigripinnis* tended to select colonies of *Microcystis*, some of the interspecific differences in assimilation may be due to the slightly different food material ingested. Some variation in assimilation may be due to differences in the physiological state of the phytoplankton.

Perhaps the most important factors causing variation in assimilation within a species of fish and between the two species, are those concerned with the physiology of digestion. These and the effects of stress have been discussed above. If it is assumed that variation in the laboratory due to these factors is random, then the figure of 43 % carbon assimilated by *T. nilotica* and 66 % by *H. nigripinnis* should be applicable to fish in the lake.

It has been pointed out that much of the phytoplankton consumed by *T. nilotica* in the lake passes through the stomach too rapidly for much lysis to occur (Moriarty, D., 1973). On this basis, the figure of 43 % is probably not unreasonable as an average for a full 24 hour feeding cycle. Since the acidity of the stomach is the most important factor influencing assimilation, it is possible that ingested phytoplankton is treated more efficiently with acid in *H. nigripinnis*.

Our results show that blue-green algae in Lake George are digested by at least two species of herbivorous fish. This work with the natural population of phytoplankton, especially *Microcystis*, supports the experimentally determined values for assimilation of laboratory cultures. The morphology of the colonies of *Microcystis* grown in the laboratory

differed from that in the natural environment. Colonies in laboratory cultures were more easily dispersed into units of a few or even single cells by agitation than were natural colonies. This means that variations in the amount or structure of mucilage in *Microcystis* may not have much effect on the digestion of these microorganisms. This is in contrast to the conclusions of Fish (1952, 1955).

As not all the ingested phytoplankton is digested, undigested algae can be observed in the rectum and faeces of fish in the lake, especially in the late morning or in the afternoon. Thus a visual examination of gut contents may lead to false conclusions about the value of food items to fish. It is necessary to study patterns of feeding and the physiology of digestion over a 24 hour period at least.

The brown colour of algae affected by acid is similar to that of the detritus in Lake George, which may explain why Fish (1955) states that *T. nilotica* ingests the soft bottom deposits and not the phytoplankton. Detritus in the gut tends to be brown/black in colour, but under the microscope, is significantly different in appearance from algae treated with acid.

Summary

Tilapia nilotica more than 6 cm in total length ingest mainly phytoplankton. *Haplochromis nigripinnis* more than 5 cm in length eat mainly the large colonial blue-green algae (*Microcystis* species). Smaller fish of both species are omnivorous.

Using unialgal cultures uniformly labelled with ^{14}C, it is shown that *T. nilotica* can assimilate a maximum of 70–80 % of carbon from *Microcystis* sp., *Anabaena* sp. and *Nitzschia* sp., and about 50 % from *Chlorella* sp. *Haplochromis nigripinnis* can assimilate a maximum of about 80 % of carbon from *Microcystis* sp.

Maximum assimilation in *T. nilotica* occurs when the algae are ingested at the end of the daily feeding period. When *Microcystis* is eaten at the start of a feeding period, assimilation values are obtained which vary from 0 to 60 % of the ingested carbon.

An explanation for these results is discussed in relation to the physiology of digestion. Differences in the concentration of acid in the stomach during the day, and differences in the passage of food through the stomach, affect lysis of the blue-green algae.

These and other factors, such as the species of algae and their physiological state, affect the proportion of ingested carbon assimilated. Estimates have been made of the proportion of carbon assimilated from that part of the natural phytoplankton population ingested by these fishes in Lake George. *Tilapia nilotica* assimilate an average of about 43 % per day and *H. nigripinnis* an average of about 66 %.

This project forms part of a joint contribution by the United Kingdom and Uganda to the International Biological Programme. It was financed by the Royal Society whose support, with that of the Freshwater Biological Association is gratefully acknowledged. We wish to thank our colleagues at Lake George for their assistance during this work. We are grateful to Professor E. J. W. Barrington for his help and the provision of facilities in his department. Dr P. H. Greenwood has kindly read and criticized the manuscript. We are grateful to Dr G. G. Ganf for assistance in the identification of the algae and for his comments on the manuscript.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

Proc. R. Soc. Lond. B. 184, 299–319 (1973)
Printed in Great Britain

Feeding and grazing in Lake George, Uganda

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The fauna of Lake George is dominated by herbivores, in particular the fish *Tilapia nilotica* and *Haplochromis nigripinnis*, and the cyclopoid copepod *Thermocyclops hyalinus*. Digestion, and the assimilation of carbon from algae, by these herbivores were studied in detail. It was found that, contrary to previous observations reported in the literature, both fish and zooplankton were able to digest and assimilate blue-green algae. The diurnal feeding patterns of the herbivores were examined, and methods devised for assessing, in terms of carbon, the daily ingestion of algae. Hence, using biomass data, the total amount grazed by the herbivores was calculated. Estimates of carbon requirements at other trophic levels were made, as was an assessment of the required level of net production by the algae for comparison with figures for standing crop and net algal production measured by other means.

Food selection by secondary and tertiary producers is discussed, and in several species age correlated changes in selectivity were examined. Both the major herbivorous species of fish adopt phytoplankton feeding after a period of carnivorous or omnivorous feeding as fry. *Thermocyclops hyalinus* is herbivorous all its life, but the size of particle taken changes with age. There are more species of carnivorous than herbivorous fish and these exploit a wider variety of food sources; the few species studied also show changes in food preference with age.

Introduction

The Royal Society–I.B.P. team was established to investigate productivity at all trophic levels in Lake George. Previous workers (Fish 1952, 1955) have concluded that blue-green algae, the dominant elements of the phytoplankton, are not digested by herbivores and therefore do not enter into the food chains. Blue-green algae are known to be ingested by the dominant herbivores in this lake, and thus the question of whether or not these species can digest and assimilate blue-green algae is critical to any interpretation of the relationship between primary and secondary production in the lake.

Digestion and assimilation of algae

The digestion of algae by the herbivorous fish in Lake George was studied by D. J. W. Moriarty (1973). From this investigation it appeared that enzymic digestion occurred in the intestine, but only after acid lysis of the algal cells had already taken place. The lowest pH value recorded in the stomach of *Tilapia nilotica* was 1.4. Experiments *in vitro* indicated, by the degree of subsequent digestion by intestinal enzymes, that lysis was more effective at this pH than at any higher value (figure 1). Very little digestion occurred after algae had been subjected to acid concentrations at or above pH 2.0. Experiments in which ^{14}C labelled monospecific cultures of algae were fed to fish in the laboratory showed

that *T. nilotica* could assimilate a maximum of 70 to 80% of ingested carbon from the blue-green algae *Microcystis* sp. and *Anabaena* sp., and the diatom *Nitzschia* sp. *Haplochromis nigripinnis* assimilated a similar proportion of carbon from *Microcystis*. *Tilapia nilotica* assimilated a maximum of about 50% of ingested carbon from the green alga *Chlorella* sp. (table 1), (Moriarty, D. J. W. & Moriarty, C. M. 1973). The proportion of carbon assimilated was, however, not always maximal.

In the stomach of *Tilapia nilotica* and of *Haplochromis nigripinnis* in the lake, acid secretion follows a diurnal cycle associated with feeding (figure 2). Secretion starts when feeding begins, at or before dawn, and in *T. nilotica* low pH values are

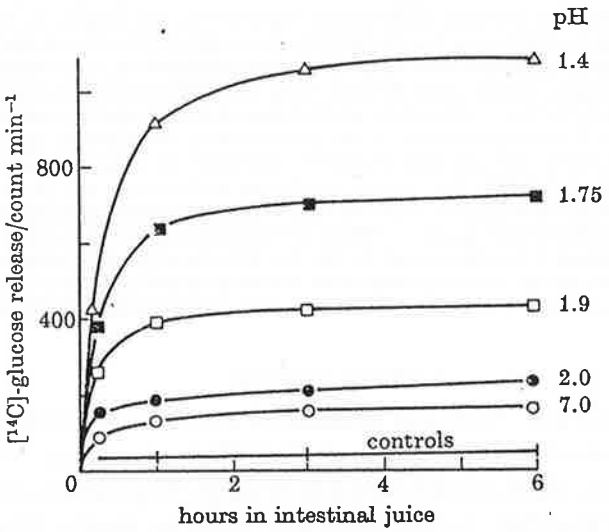

FIGURE 1. Digestion of blue-green algae after treatment with acid at various pH values. This is measured by the release of glucose from storage polysaccharides in lake algae (mainly *Microcystis* spp.), labelled with ^{14}C (Moriarty, D. J. W. 1973).

TABLE 1. PERCENTAGE ASSIMILATION OF CARBON FROM DIFFERENT SPECIES OF ALGAE BY *TILAPIA NILOTICA* AND *HAPLOCHROMIS NIGRIPINNIS* (MORIARTY, D. J. W. & MORIARTY, C. M. 1973)

	assimilation as percentage of carbon ingested		
	average	range	no. of experiments
by *Tilapia nilotica*,			
from *Microcystis* spp.	70	67–73	6
Anabaena sp.	75	68–82	4
Nitzschia sp.	79	78–81	2
Chlorella sp.	49	45–52	3
by *Haplochromis nigripinnis*,			
from *Microcystis* spp.	71	56–80	7

not obtained throughout the stomach until feeding ceases at sunset. When feeding begins the stomach is contracted and most of the algae pass straight through the stomach into the intestine. These algae will remain undigested. As feeding continues the stomach expands, but nevertheless a large proportion of the ingested algae passes through the anterior end of the stomach only. Consequently, this material is not subjected to pH values of less than about 2.0, and its subsequent digestion and assimilation will, therefore, be low. A smaller proportion of

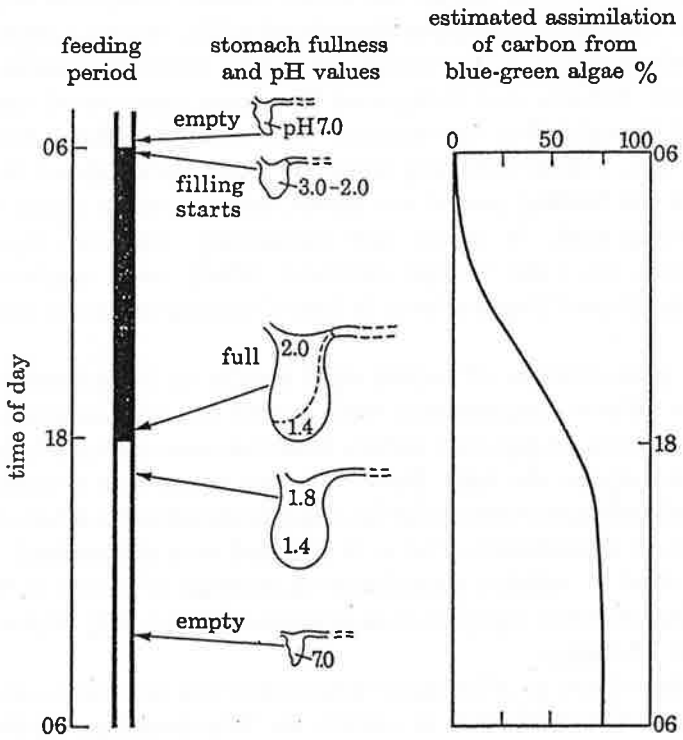

FIGURE 2. Diurnal cycle of feeding in *Tilapia nilotica*, stomach acidity, and digestion of algae *in the intestine*. The graph of carbon assimilation from blue-green algae which have been eaten during the feeding period shown, and which are present in the intestine, is a tentative estimate based upon data from Moriarty, D. J. W. (1973), and Moriarty, C. M. & Moriarty, D. J. W. (1973).

the ingested algae passes into the fundus of the stomach, where it accumulates as feeding proceeds, and is subjected to pH values of less than 2.0. There is a certain amount of mixing of the algae from the fundus with algae from the anterior part of the stomach, that is, with algae which would not otherwise be subjected to pH values of less than 2.0. As a result of this mixing, a certain proportion of algae that do not pass through the main body of the stomach before entering the intestine nevertheless will be digested and ultimately assimilated. Acid secretion continues for some time after feeding has ceased and algae accumulated in the stomach, except for a small proportion near the pylorus, are subjected to pH values of less

than 2.0. These algae are completely lysed and their subsequent digestion and assimilation is almost complete. The effect of this varying degree of exposure to acid is to cause a gradation through the day from zero to maximum in the proportion of ingested blue-green algal cells lysed, and therefore digested and assimilated.

The first faecal material derived from any feeding period is green, because the first algae to enter the intestine have not been affected by acid. Faeces resulting from the mixture of algae from the anterior and posterior parts of the stomach are voided next, and are green-brown in colour, the brown fraction being due to lysed and digested algae. Any diatoms appearing in these faeces will have been completely digested because pH values of 2.0 are low enough to destroy their cell membranes. This mixture of digested diatoms and undigested blue-green algae could explain why Fish (1952, 1955) thought that the diatoms were utilized as food but the blue-green algae were not. Faeces resulting from the algae accumulated in the stomach at the end of the feeding period are brown, because these algae have been subjected to strong acid. In colour and consistency, this well-digested faecal material resembles the Lake George sediment, which could explain the observations of Fish (1955) that *Tilapia nilotica* in Lake George feeds on the bottom sediment.

Having shown that pure cultures of certain algal species in Lake George are assimilated by *Tilapia nilotica*, experiments were carried out to determine the average degree of assimilation of ingested carbon from the community of phytoplankton occurring naturally in the lake. Because it was impossible to measure this directly, the natural feeding rhythm of the fish was simulated in the laboratory, and the average degree of assimilation over a 24 h period was determined. The results obtained show that *T. nilotica* assimilated an average of about 43 % of ingested carbon, and *Haplochromis nigripinnis* an average of about 66 % (Moriarty, D. J. W. & Moriarty, C. M. 1973).

The same monospecific culture of *Microcystis* used to feed the fish was used also to determine the degree of assimilation of carbon by *Thermocyclops hyalinus*. Copepodids and adults assimilated about 35 % of ingested carbon, and the nauplii about 58 % (M. P. Tevlin unpublished). Although these results were obtained with a laboratory culture of *Microcystis*, they should be similar for *T. hyalinus* in the lake, since it feeds mainly on *Microcystis* throughout its life cycle.

Utilization of primary production

That all three herbivores have a diurnal cycle of ingestion was established by observation throughout 24 h. With this approach it was possible to quantify the total daily ingestion, and by applying this figure to the population biomass, to indicate the total grazing of phytoplankton by herbivores in the lake. Estimates of the amount of algae necessary to sustain organisms at higher trophic levels have been made, and hence a rough assessment of the total daily loss from the phytoplankton community could be made. This estimate can be compared with the net primary production as measured by standard techniques.

Moriarty, C. M. & Moriarty, D. J. W. (1973) showed by measuring dry weight of stomach and intestinal contents every 2 h for 24 h, that *Tilapia nilotica* and *Haplochromis nigripinnis* have a diurnal cycle of feeding.

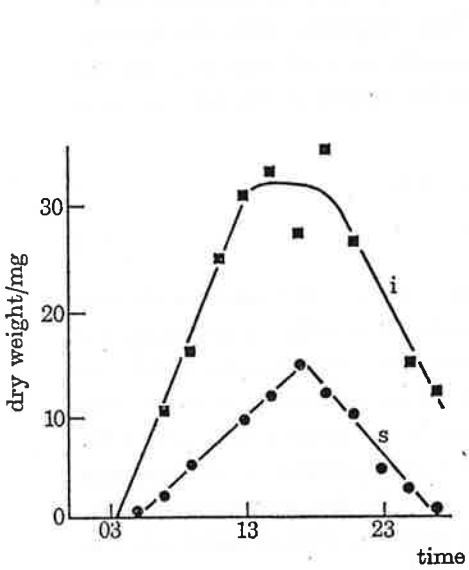

FIGURE 3

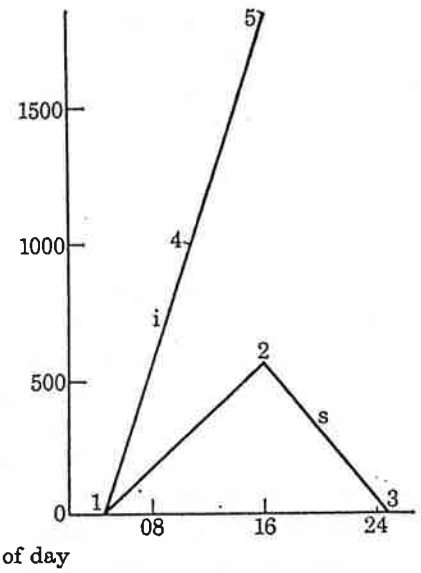

FIGURE 4

FIGURE 3. The diurnal changes in dry weight of stomach (s) and intestine (i) contents of *Haplochromis nigripinnis* (average total length 7.3 cm). Samples of fish were taken from the lake at 2 h intervals, and the gut contents removed and dried to constant weight (Moriarty, C. M. 1973).

FIGURE 4. Weighted regression lines of change in dry weight of stomach (lines 1–2, 2–3), and intestine (line 1–4) contents during the day, of *Tilapia nilotica*, average total length 19.0 cm. Line 4–5 is an extrapolation of line 1–4 (from Moriarty, C. M. & Moriarty, D. J. W. 1973).

In both species feeding begins at or before dawn, and the dry weight of stomach contents increases steadily until feeding stops. This generally occurs just before sunset (about 19.00 h), and is followed by a gradual decrease in the dry weight of stomach contents. Emptying of the stomach into the intestine is generally completed by about 01.00 to 02.00 h, and the intestinal contents decrease as defaecation proceeds. A typical feeding pattern is shown in figure 3.

A method for quantifying daily ingestion by *Tilapia nilotica* and *Haplochromis nigripinnis*, based on this diurnal feeding pattern, is illustrated in figure 4. The weighted regression lines 1 to 2 and 2 to 3 show the increase and decrease respectively in the dry weight of stomach contents with time. As feeding proceeds, in this case from 05.00 to 16.00 h, food passes from the stomach to the intestine. Initially, the passage rate of food from stomach to intestine is given by the slope of the weighted regression line 1–4. Point 4 represents the time at which defaecation of new food begins. Between this time and that at which feeding ceases (2) it was impossible to measure the rate at which stomach contents are emptied into the

intestine. It was assumed, therefore, that the rate represented by the slope of 1–4 would be constant throughout feeding, so that this line could be extrapolated to point 5, at which time feeding ceased. Point 5 gives, therefore, an estimate of the total food entering the intestine during feeding. This, together with the amount accumulated in the stomach (point 2), gives an estimate of total ingestion for the day. The quantities ingested are linearly related to the weight of the fish, as given by the following regression equations:

$$\text{for } T.\ nilotica \qquad y = 271 + 13.3x, \tag{1}$$

$$\text{for } H.\ nigripinnis \qquad y = -29.6 + 21.9x, \tag{2}$$

where y is the dry weight of phytoplankton ingested in mg per day and x is the wet weight of fish in grams. The equation for *H. nigripinnis* is only an approximate guide to ingestion by this species because insufficient data were available for one of the parameters on the rate of stomach emptying. These equations were derived from studies with *T. nilotica* in the size range 15 to 23 cm total length and with *H. nigripinnis* from 5 to 8 cm total length. They are not applicable to the juvenile and omnivorous stages of either species, and they may be inaccurate for individuals whose sizes lie outside the range studied. In order to obtain estimates for ingestion by the whole population of these species it was necessary to assume, however, that the equations do apply to all phytophagous members of the population. Since much of the biomass of both species is made up of individuals within the size ranges studied, there are probably no serious errors introduced by this assumption. Thus, for any population of fish equations (1) and (2) become:

$$\text{for } T.\ nilotica \qquad I = n271 + 13.3B, \tag{3}$$

$$\text{for } H.\ nigripinnis \qquad I = -n29.6 + 21.9B, \tag{4}$$

where I is the total ingestion in mg dry weight per day in the area occupied by the fish, n is the number of fish, and B is their biomass in grams fresh weight.

The biomass of fish is greatest around the shorelines of the lake, and least at its centre (Burgis *et al.*, this volume). This unequal distribution pattern of herbivorous fish must result in more intense grazing on the phytoplankton of inshore regions. To quantify this regional difference, grazing rates were calculated for inshore and midlake areas (table 2), inshore areas being defined as lying within 100 m of any shore, the remaining area being considered mid-lake. For calculation of overall grazing rates, a mean figure for fish biomass over the whole lake was used (J. J. Gwahaba, personal communication) from which a mean daily grazing rate of 34 mg C m^{-2} was calculated.

The raptorial stages of *Thermocyclops hyalinus* also showed a diurnal cycle of feeding. Ingestion rates were determined at 3 h intervals for 24 h by feeding freshly collected animals with ^{14}C labelled lake algae. The uptake of radioactive material was measured after a feeding period short enough to preclude defaecation of ^{14}C labelled pellets. The radioactivity of the feeding medium (expressed as

counts min^{-1} ml^{-1}), and its carbon content (as μg C ml^{-1}, determined by the method of Ganf & Milburn 1971), were measured. Thus, ingestion was expressed directly in terms of carbon. Corresponding defaecation rates (at intervals over 3 h) were measured, on some occasions, by counting the number of faecal pellets voided by

TABLE 2. CALCULATED TOTAL DAILY INGESTION OF PHYTOPLANKTON BY *TILAPIA NILOTICA* AND *HAPLOCHROMIS NIGRIPINNIS* IN INSHORE AND MIDLAKE REGIONS OF LAKE GEORGE

	mg (dry weight) m^{-2} day^{-1}		mg C m^{-2} day^{-1}	
	mid-lake	inshore	mid-lake	inshore
Tilapia nilotica	34	360	11	120
Haplochromis nigripinnis	57	110	19	36
total	91	470	30	156

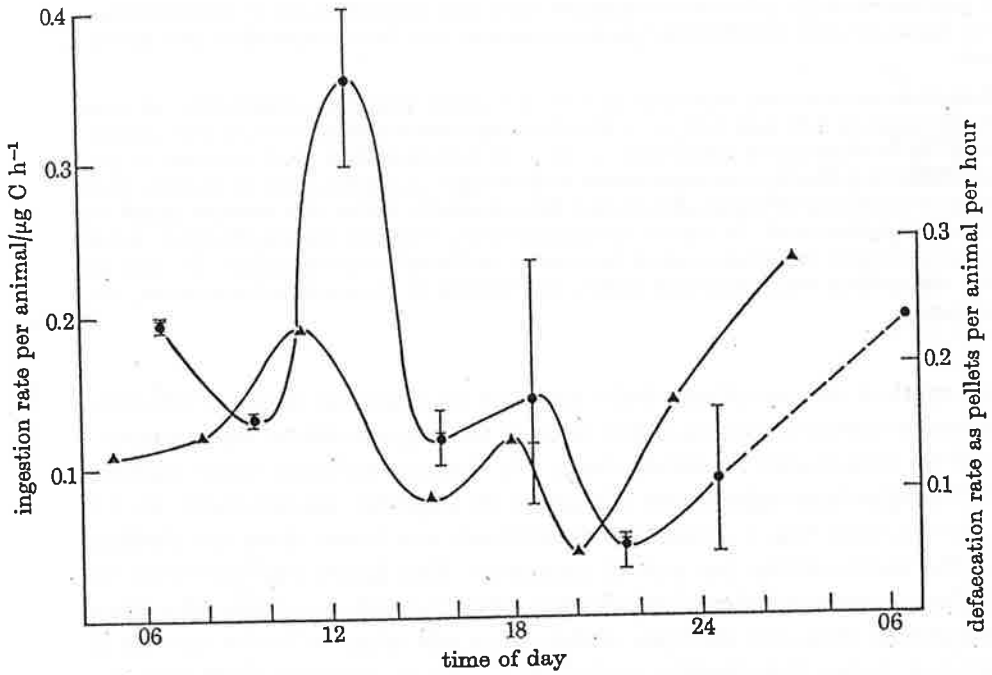

FIGURE 5. Diurnal variation in ingestion (•) and defaecation (▲) rates of *Thermocyclops hyalinus*. The area beneath the ingestion curve gives the total ingestion for that day, in this case 0.34 μg C per adult per day. Lines above the points show the range of values obtained. (M. P. Tevlin unpublished.)

freshly collected animals feeding on lake algae. The mean ingestion and defaecation rates per individual generally showed two or three peaks of feeding activity in each 24 h period (figure 5). The only period during which no feeding activity was recorded was between about 12.00 and 18.00 h, corresponding to the period when thermal stratification commonly occurs in the lake.

Feeding by the nauplii of *Thermocyclops hyalinus* was not investigated in detail, but the available data show that feeding activity was greatest at night. Of the other herbivorous zooplankton occurring in the lake, only *Daphnia barbata* was studied. Analysis of gut fullness indicated that the level of feeding activity did not vary appreciably during a 24 h period (A. Duncan, personal communication).

Results of the type illustrated in figure 5 were used to calculate the total daily ingestion of carbon by planimetric integration of the area beneath the ingestion curve. Mean figures obtained by this method are detailed in table 3*a*.

TABLE 3. DAILY INGESTION DATA FOR *THERMOCYCLOPS HYALINUS*

method	developmental stage	μg C ingested per animal per day	±s.d.	no. of experiments	corrected to lake concentrations
(*a*)	adult	0.33	± < 1%	2	0.86
	late copep.	0.18	—	1	0.47 †
	nauplius	0.38	—	1	0.38
(*b*)	adult	0.28–0.43	—	1	0.73–1.12
(*c*)	adult	0.31	±52%	8	0.81
	late copep.	0.12	±11%	4	0.31

Methods used (see text): (*a*) Planimetric integration of area beneath curve of diurnal ingestion rate. (*b*) Quantification of individual peaks of ingestion and their summation. (*c*) Laboratory method.

† In subsequent calculations, this value of 0.47 μg C ingested by late copepodids was used rather than the mean of 0.47 and 0.31 μg C. This is because the ingestion rate for late copepodids obtained by method (*a*) is considered to be more accurate than that obtained in (*c*). Greater experimental difficulty was experienced with method (*c*), particularly in manipulating sufficiently large numbers of copepodids in the time available before the animals began to defaecate the ^{14}C labelled food. As a result consistent results were not always obtained. Adult animals, because of their larger size, could be selected and handled more rapidly. Techniques for handling copepodids were improved before the method of planimetric integration, (*a*), was carried out.

A second method of quantifying daily ingestion by raptorial stages of *Thermocyclops* depended upon recurrence of the peaks of feeding activity at certain times, which could be anticipated to within about 2 h. Freshly collected adult animals were fed ^{14}C labelled lake algae about 3 h before the expected feeding peak. At 1 h intervals for the next 6 h, a number of individuals was taken from the feeding vessel and the radioactivity per animal measured. This figure was converted to ingested carbon per animal as described above (p. 305). The carbon uptake per animal was plotted against time, and the apex of this curve was taken to be the amount of carbon ingested during that feeding period (M. P. Tevlin personal observations). The apices of the first two expected feeding peaks were well-defined and ingestion values readily obtained. The third, however, was of longer duration, and a distinct apex was not observed since defaecation and ingestion of ^{14}C labelled material was occurring simultaneously. Nevertheless, from the data available, it was possible

to estimate the probable upper and lower limits of ingestion during that period. Calculated values for the three feeding periods are shown below:

first peak 0.092 μg C per adult *T. hyalinus*,
second peak 0.14 μg C per adult *T. hyalinus*,
third peak 0.05 to 0.20 μg C per adult *T. hyalinus*.

The total daily ingestion was taken to be the sum of these amounts (table 3*b*).

A laboratory method for measuring ingestion involved supplying a known number of animals with lake algae for 24 h, and counting the total number of faecal pellets produced as a result of ingestion during this period. In separate experiments, animals (of the same developmental stage) with full guts were deprived of food and allowed to defaecate completely. Thus, the mean number of faecal pellets produced from a full gut was obtained. By dividing this figure into the daily total, the number of times the gut filled per day was calculated. The mean amount of carbon in one full gut was determined by feeding previously starved animals on ^{14}C labelled algae for 40 min (i.e. long enough to fill the gut, but not to start defaecating) and measuring the uptake of radioactive material; this was converted into μg C as described above. Hence the total carbon uptake per individual per day was calculated as the product of the mean carbon content per full gut, and number of times the gut was filled per day (Tevlin 1973). The results are given in table 3*c*.

Table 3 shows that the results obtained by these three methods of measuring total ingestion are consistent. However, the figures could not be applied directly to animals in the lake, because the concentrations of zooplankton and phytoplankton used in the experiments were five to ten times higher than in the lake (except for those experiments on nauplii in which lake concentrations were used). Experiments to assess the effect of concentration showed that ingestion rates at lake concentrations were 2.6 times greater than those at the experimental concentrations. Thus, the measured total daily ingestions (except of the nauplii) were multiplied by 2.6 to make them applicable to animals feeding at lake concentrations (table 3). Using biomass data from Burgis (1973) the total ingestion by the whole *Thermocyclops hyalinus* population in the lake was calculated to be 504 mg C m^{-2} day^{-1} (table 4).

Total grazing and other losses from the phytoplankton

The total daily intake of phytoplankton by the herbivores has been estimated at 34 mg C m^{-2} for the fish, and 504 mg C m^{-2} for *T. hyalinus*, over the whole lake (table 4). Zooplankton grazing by species other than *T. hyalinus* was not measured, but was considered unlikely to alter the total grazing figure significantly because of their relatively small biomass (see Burgis, 1974). The total daily loss by grazing from the phytoplankton (538 mg C m^{-2}) is very much less than the mean standing crop of algae in the lake, which is about 30 g C m^{-2} and shows little variation (G. G. Ganf, personal communication).

Grazing by the plankton and fish does not, however, account for all the loss from the phytoplankton population. Export of algae from the lake occurs with outflow via the Kazinga Channel, but no quantitative data are available. It is thought that losses by this means are relatively small since the rate of flow down the Channel is slow. Terminal sedimentation of algae within the lake occurs, but again, no quantitative data are available. In addition to these losses, the benthic animals feed on sedimented algae, both live and moribund, and presumably, on the decomposers associated with them. A tentative quantitative estimate of benthic ingestion was

TABLE 4. TOTAL DAILY INGESTION OF PHYTOPLANKTON BY THE MAJOR HERBIVORES IN LAKE GEORGE

	mg (dry weight) m^{-2} day^{-1}	mg C m^{-2} day^{-1}
Tilapia nilotica	53	17.5
Haplochromis nigripinnis	50	16.5
Thermocyclops hyalinus	—	504
total		538

TABLE 5. RATE OF SEDIMENTATION OF PHYTOPLANKTON MATERIAL AS FAECES OF THE MAJOR HERBIVORES IN LAKE GEORGE

	ingestion mg C m^{-2} day^{-1}	assimilation %	faeces mg C m^{-2} day^{-1}
Tilapia nilotica	17.5	43	6.5
Haplochromis nigripinnis	16.5	66	6.5
Thermocyclops hyalinus (raptorial stages)	333	35	216
Thermocyclops hyalinus (nauplii)	171	58	72
		total faeces	about 300

made by using calculated population growth and respiration values in terms of carbon (J. P. E. C. Darlington, unpublished). Total daily assimilation of carbon by the benthos was obtained by summing population growth and respiration, assimilation of 15 % being assumed. The total daily ingestion by the benthos was estimated by this means at 1 g C m^{-2}.

Of this 1 g C m^{-2} day^{-1}, using assimilation efficiencies of 43 % for *Tilapia nilotica*, 66 % for *Haplochromis nigripinnis*, 58 % for *Thermocyclops* nauplii and 35 % for the raptorial stages of *T. hyalinus* (see p. 302), about 300 mg C m^{-2} day^{-1} could be supplied by the faeces of planktonic herbivores (table 5). The remaining 700 mg C m^{-2} day^{-1} required to maintain the benthic fauna must be derived from the phytoplankton, either directly or through the decomposers. Thus, in addition to a daily planktonic grazing of about 540 mg C m^{-2} the phytoplankton must supply 700 mg C m^{-2} day^{-1} for the benthic grazers. This total of 1240 mg C m^{-2} day^{-1} is a minimum estimate of the net primary production required to maintain both planktonic and benthic organisms at higher trophic levels. Net primary production must exceed this minimum estimate to account for the outflow and sedimentation losses mentioned above.

Net primary production has been estimated by standard limnological techniques, as 600 ± 800 mg C m^{-2} day^{-1} (Ganf 1972). Although there is considerable variation resulting from such factors as differences in light intensity, the mean rate is somewhat less than the estimated daily average of 1240 mg C m^{-2} taken by the 'grazers'. The discrepancy increases if attempts are made to include outflow and sedimentation losses. With the standard techniques for direct measurement of primary production it is difficult to make allowances for the complex interactions between supply and demand of oxygen and carbon dioxide for photosynthesis, photorespiration and dark respiration (Ganf & Viner, this volume). It is possible that estimates of total grazing could provide a useful independent assessment of minimum net primary production.

Food selection by herbivores

It has been shown above that the important herbivores in Lake George not only ingest, but can readily digest and assimilate blue-green algae, including *Microcystis* spp. Like many other specialized feeders, the young of the two dominant herbivorous fish species develop their characteristic adult feeding habits as they grow. The fry occupy a different niche or trophic level from the adults of their species.

As individuals of *Tilapia nilotica* increase in size they show changes in diet which are related to their changes in habitat. The fry inhabit the littoral and swampy regions of the lake, and ingest a wide variety of plant and animal material including aufwuchs, detritus, rotifers, copepods, hydracarines and various insects. At this stage their feeding behaviour involves active pursuit and 'pecking' at particles in the water. As the juvenile *T. nilotica* grow larger and move further offshore, they ingest an increasing proportion of phytoplankton until, when they are more than 6 cm long, they feed almost entirely on phytoplankton. Aquarium observations show that from this stage onwards the fish feed by a 'gulping' action resembling exaggerated respiratory movements, and that some zooplankters are able to avoid capture by swimming away from the feeding current. When live zooplankton was added to tanks of lake water in which the fish were feeding, there was no increase in the number of zooplankton remains found in the faeces after a fixed feeding period. However, after heat-killed zooplankton was added to the water, a sevenfold increase was observed (Moriarty, C. M. 1973).

The species composition of the phytoplankton ingested by large *T. nilotica* in the lake was examined in detail. Counts of several algal species in the stomachs of fourteen fish were compared with counts from an integrated column water sample collected at the same time and place, and their frequency distributions were found to be different. For each algal species Ivlev's coefficient of electivity was calculated; it is defined (Ivlev 1961) as:

$$\frac{(r-p)}{(r+p)},$$

where r is the percentage abundance of the species in the gut contents, and p is the

percentage abundance of that species in the environment. Results for five algal species (figure 6) show that there was a marked positive selection for the colonial blue-green alga *Microcystis*, the filamentous blue-green alga *Lyngbya*, and the colonial diatom *Melosira*, and negative selection for the small blue-green alga *Anabaenopsis* and the diatom *Synedra*.

This suggests that selection is based upon the size, and perhaps the shape, of the food particles. Collection of particles from the incurrent water involves a mucous-filtration mechanism, the effectiveness of which may well depend upon the particle size (Greenwood 1953).

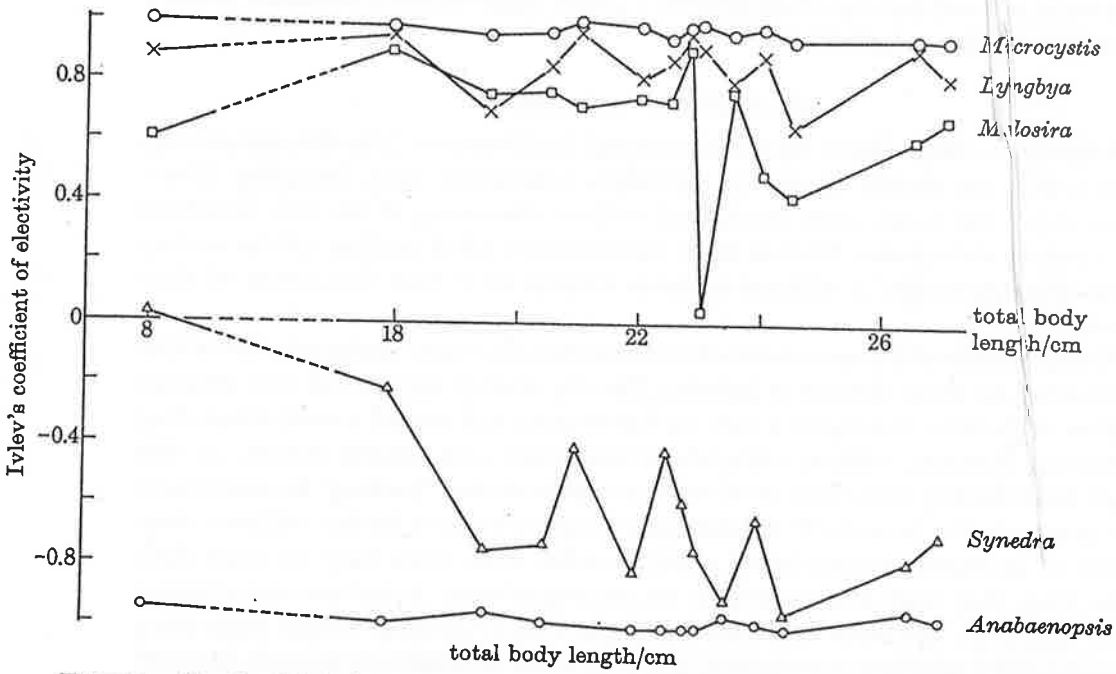

FIGURE 6. Graph of Ivlev's coefficient of electivity for five genera of planktonic algae ingested by *Tilapia nilotica*, against total length of the fish (after Moriarty, C. M. 1973).

All growth stages of *Haplochromis nigripinnis*, including the fry, are distributed throughout the lake. As much as 70% of the food particles taken in by fry are zooplankters, and Ivlev's coefficient suggests that there is some selectivity towards different components of the zooplankton (figure 7). There is positive selection for the larger cladoceran species *Daphnia* and *Moina*, which is greatest in the larger fish. For the smaller crustaceans, *Ceriodaphnia* and the copepods, there appears to be no selection by small fish (less than 5 cm long) and only weak selection, positive and negative respectively (for the *Ceriodaphnia* and copepods), by larger fish. The proportion of zooplankton in the total intake decreases as the fish approach maturity, when 97% of the particles ingested are phytoplankton (figure 8). Only the large colonial species of algae are found in the guts, including *Microcystis* spp.

and *Aphanocapsa*, together with diatoms bound in the mucilaginous sheaths of the colonies (Moriarty, C. M. 1973).

Only one other phytoplankton-eating fish, *Tilapia leucosticta* Trewavas, occurs in Lake George. The gut contents of individuals of this species caught in mid-lake areas show a preponderance of blue-green algae over other possible food items,

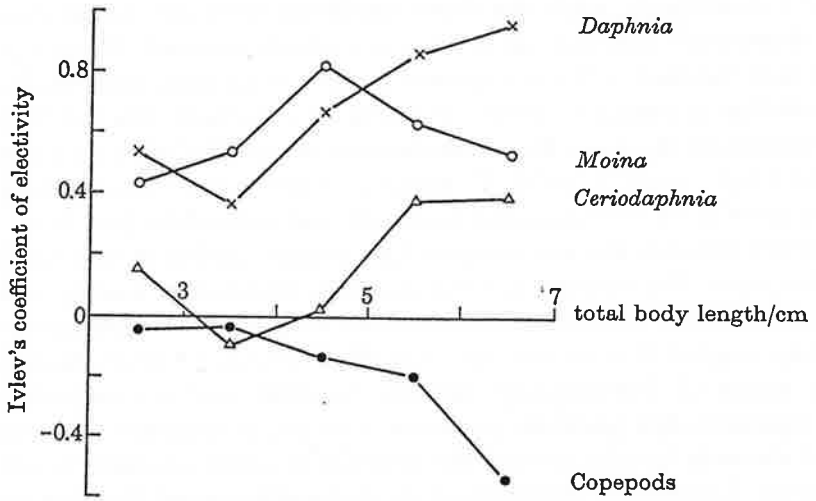

FIGURE 7. Graph of Ivlev's coefficient of electivity for four groups of planktonic crustaceans ingested by *Haplochromis nigripinnis* females and juveniles. Each point represents the gut contents of five individual fish whose total lengths are within the 1 cm size range indicated (Moriarty, C. M. 1973).

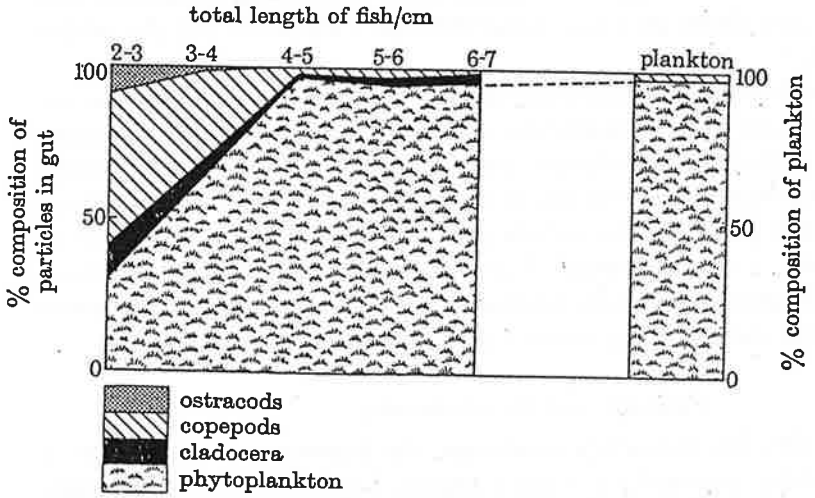

FIGURE 8. Numerical percentage composition of the total particles present in the guts of *Haplochromis nigripinnis* females and juveniles. The total gut contents of five individual fish were counted for each 1 cm size range of fish (after Moriarty, C. M. 1973). For comparison, the numerical percentage composition of the plankton is shown, as counted in vertical haul residues sieved through a plankton net with 4 meshes/mm (100 meshes/in).

whereas fish caught in inshore regions had ingested detrital material (Dunn 1971). Since this species is more abundant inshore than offshore (Burgis *et al.*, this volume), it is essentially a detritivore.

Thermocyclops hyalinus is a phytoplankton feeder throughout its development, but the mechanism of feeding and the size of particles taken differ in the different developmental stages. Adults and copepodids are raptorial feeders upon the large colonies of *Microcystis* spp., which consist of numerous cells bound together with mucilage. The animals can be seen to grasp a colony with their many-pointed maxillae and maxillipeds, while the sharp mandibles break off smaller pieces for ingestion. Microscopic examination of the gut contents revealed *Microcystis* cells which had been ingested without apparent rupture, suggesting that the function of the mandibles is simply to break off pieces of a suitable size for ingestion, and not to masticate the algae. Such mouthparts are typical of carnivorous species of cyclopoid (Fryer 1957) to which *T. hyalinus* is very closely related. Raptorial *T. hyalinus* individuals were observed to attack, and apparently feed from, moribund Cladocera, but this was not common. Laboratory cultures of raptorial stages were maintained on *Microcystis* taken from the lake. Subsequent feeding, assimilation and growth measurements showed that their daily carbon requirements could be adequately supplied from this source alone (M. P. Tevlin, personal observation).

Naupliar stages of *Thermocyclops* are not raptorial, and are unlikely to be capable of ingesting algal particles greater than *ca.* 10 μm diameter. Examination of their gut contents usually showed the presence of single *Microcystis* cells (*ca.* 8 μm diameter). Laboratory observations on the nauplii suggest that the animals simply ingest suitably sized particles that they encounter in their movements through the water.

The role of bacteria in naupliar nutrition has not been determined. Growth studies on *in vitro* cultures of nauplii show normal growth when the nauplii are kept in that fraction of lake water that has passed through a 400-mesh net (i.e. with a pore size of 40 μm).

The remaining herbivorous zooplankton species are small filter feeders that are unable to ingest the large algal colonies. The scarcity of algal particles of a suitable size may partially explain why Cladocera are not more abundant in Lake George (Burgis *et al.*, this volume). Adult *Daphnia barbata* can ingest particles about 60 μm in diameter, and their gut contents include green algal cells and brown material (A. Duncan, personal communication). Laboratory observations of behaviour, and the fact that *Daphnia* tends to be most abundant at the bottom of the water column, suggests that most feeding activity occurs close to the mud surface.

Food selection by carnivores

By comparison with the secondary producers, the tertiary producers show a greater species diversity, especially in inshore regions. They are exploiting a wider variety of food sources than the herbivores, and though some species are very selective in their choice of food, others, especially some of the fish, are opportunists.

Predation on fish

Predation on the fish population has not been quantified, nor are its effects clear, but some qualitative observations can be made.

Of the piscivorous species of fish in Lake George, the most abundant is *Haplochromis squamipinnis* Regan. It shows a change in food selection with age, the young fish being opportunistic feeders taking benthic, midwater and emergent

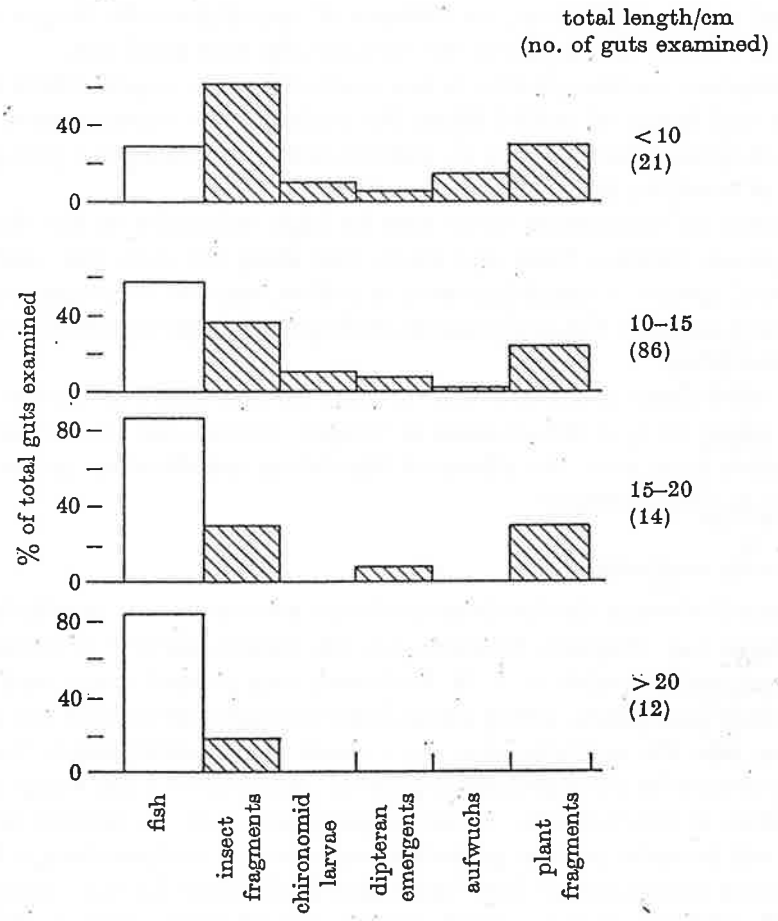

FIGURE 9. Histograms of the percentage of the total guts examined (excluding empty guts) in which six categories of food item occurred, in four 5 cm size ranges of *Haplochromis squamipinnis* (after Dunn 1972).

insects, as well as aufwuchs and plant fragments. As it grows, the young *H. squamipinnis* specializes increasingly on fish as a source of food (figure 9). Although it is the largest species of *Haplochromis* found in the lake, it is of moderate size, mature females being 15 to 25 cm long and the males rather less. Thus predation by this species will fall most heavily on the young stages of *Tilapia* spp. and on the *Haplochromis* spp. Young *H. squamipinnis* are most abundant close to shore where

they feed on fish fry. Only limited shelter is available for the prey in this region, which may contribute to the success of this predator. (Dunn 1972, and personal observations).

The other important piscivorous fish, *Bagrus docmac* (Forsk.), *Clarias lazera* Val., and *Protopterus aethiopicus* Heckel, are much larger fish than *Haplochromis squamipinnis* and are therefore able to take larger prey. *Protopterus* also eats molluscs in some areas. Other fish species also take fish fry, although their principal food source is different; for instance *H. angustifrons* Boulenger which feeds largely on benthic invertebrates will occasionally take small fish.

Haplochromis taurinus Trewavas is a localized inshore species which feeds on the embryos and larvae of cichlid fishes. By analogy with related species from Lake Victoria it seems probable that *H. taurinus* feeds by sucking the young out of the mouths of brooding females (Greenwood 1973).

Predation by piscivorous birds may be high, especially in the shallow water close to shore. Herons, ibises and storks feed along the shore line, and fish eagles and several species of kingfisher hunt in inshore regions. Cormorants and darters feed inshore and over the sandy shoals, while pelicans and terns feed over the entire area of the lake.

Up to 5000 tonnes of fish are removed from the lake each year by the commercial fishery. About 80 % of this consists of *Tilapia nilotica*, and the catching methods are selective as to size. The effects of this fishery are discussed in some detail in Burgis *et al.* (this volume).

Predation on zooplankton

In terms of biomass the dominant predators on zooplankton are the larval stages of *Chaoborus* spp. (Diptera, Nematocera). The food preference of the larvae varies in different instars (table 6; L. M. McGowan, unpublished data), and the instars are unevenly distributed, young larvae being concentrated inshore and older larvae extending into the mid-lake area. As a result of this distribution, the predation pressure exerted by the populations of larval *Chaoborus* on other components of the zooplankton is not uniform. Small zooplankters, such as rotifers and copepod nauplii, will be under heavier predation pressure close to shore than in the midlake region, since these animals form the major food source for the young *Chaoborus* larvae. In addition to this overall pattern, the *Chaoborus* larvae often occur in dense swarms in the plankton, so that the intensity of predation by the larval population varies very markedly. Most fourth and late third-instar *Chaoborus* larvae show a diurnal cycle of activity, burying themselves in the bottom sediments during the day and emerging into the water column at night to feed. It is possible that this cycle is linked with the diurnal cycle of feeding activity of *Thermocyclops* (M. P. Tevlin, personal observation). The copepod *Mesocyclops leuckarti* Kurz is also a planktonic predator taking cyclopoids as well as cladocerans.

By comparison with the *Chaoborus* species, predation by fish in the midlake area is relatively slight. *Haplochromis pappenheimi* (Boulenger) is the only fish

species in the lake which selectively feeds on zooplankton throughout its life, though the mechanism by which it selects animals from the total plankton is not known. Predation by *H. nigripinnis* fry on the zooplankton has already been discussed (p. 310). The surface-feeding, generalized carnivores *Aplocheilichthys eduardianus* David & Poll, and *A. pumilus* Boulenger take a proportion of zooplankton in their diet. Experiments in which a mixture of equal numbers

TABLE 6. DISTRIBUTION OF, AND FOOD SELECTION BY, DIFFERENT INSTARS OF *CHAOBORUS* SPP. LARVAE (L. M. MCGOWAN, UNPUBLISHED)

instar	distribution	prey organisms observed in the pharynx
1	predominantly inshore	ROTIFERS
2	predominantly inshore	ROTIFERS, nauplii, copepods
3	ubiquitous	rotifers, nauplii, COPEPODS, COPEPODIDS
4	ubiquitous	rotifers, *Daphnia*, ostracods, COPEPODS

Capitals, main components; lower case, other components.

Thermocyclops, *Daphnia* and *Ceriodaphnia* were offered to *A. eduardianus* in the laboratory, showed that the fish selected the Cladocera (M. P. Tevlin, unpublished observations).

Inshore, predation on the zooplankton by fish fry may be important. The paucity of large cladocerans in the inshore areas could well be correlated with this predation pressure (Burgis *et al.*, this volume).

Where dense swarms of *Chaoborus* larvae occur they are eaten by a great variety of fish. For instance, adult *Haplochromis nigripinnis* are sometimes found with their stomachs full of *Chaoborus* larvae instead of the usual phytoplankton. Similarly, *Chaoborus* and chironomid pupae ascending through the water column to emerge, or in the process of emerging, are frequently ingested by fish which are not otherwise carnivorous.

Predation on the benthos

Haplochromis angustifrons is the most important benthic predator throughout most of the lake; over mid-lake mud areas it is the only benthic predator except for the occasional young *H. squamipinnis*. At one mid-lake site, where the standing crop of benthos was 0.77 g (d.w.) m^{-2}, a minimum estimate for the daily take-off by the *H. angustifrons* population was 0.03 g (d.w.) m^{-2}, or 4 % of the total standing crop.

Individuals of *H. angustifrons* feed on benthic invertebrates throughout life, but show a change in feeding selectivity with growth (figures 10, 11). Gut content analyses show small fish select ostracods and copepods, while larger individuals select dipteran larvae. This may indicate size discrimination, since the crustaceans are about 1 mm in linear dimensions while the size range of dipteran larvae is from 1 to 18 mm in length. The distribution of the different instars of chironomid larvae in the gut contents (figure 12) shows that young instars are taken mainly by small fish and older instars by larger fish. This suggests that the upper limit to the size

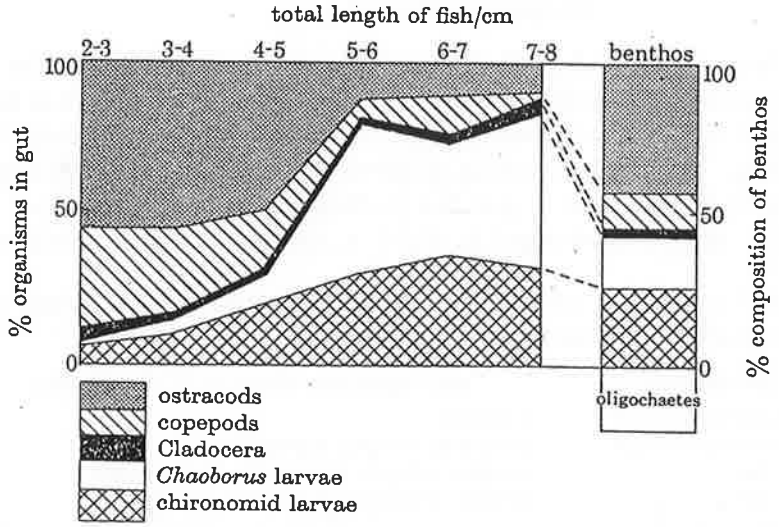

FIGURE 10. Numerical percentage composition of the total organisms present in the guts of *Haplochromis angustifrons* females and juveniles. The total gut contents of five individual fish were counted for each 1 cm size range of fish. For comparison, the numerical percentage composition of the benthos is shown, as counted in the residues of six Jenkins cores (enclosing the population occurring from 10 cm above the mud surface to 40 cm below it), retained in a wire sieve with 1.6 meshes/mm (40 meshes/in) (J. P. E. C. Darlington, unpublished). *N.B.* Oligochaetes are graphed as being in excess of 100 % of the benthos. This is because oligochaetes were not found in the guts of *H. angustifrons*. but if they were ingested it is probable that their remains would not have been recognized. They are, therefore, to be excluded from the comparison.

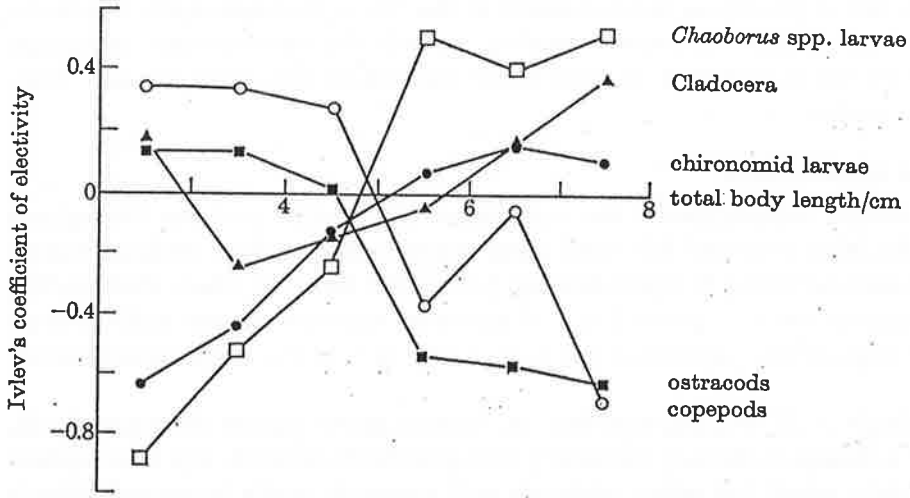

FIGURE 11. Graph of Ivlev's coefficient of electivity for five groups of prey organisms ingested by *Haplochromis angustifrons* females and juveniles. Each point represents the gut contents of five individuals whose total lengths are within the 1 cm size range indicated (J. P. E. C. Darlington, unpublished).

of prey selected is set by what can be swallowed, and the lower limit depends on a sieving mechanism whose 'mesh' coarsens as the fish grows. Laboratory observations indicate that the fish feed by sucking mud into the mouth, passing it through the buccal chamber, and expelling waste material through the opercular opening. The gill arch morphology shows peculiarities which may represent an adaptation to this mode of feeding (P. H. Greenwood, personal communication).

Food selection by different sized individuals of *H. angustifrons* may, however, be influenced by the accessibility of the prey. The *Chaoborus* and chironomid larvae burrow into the substrate where they may be beyond the reach of small fish. Furthermore, early instars of chironomids live closer to the mud surface than do later instars, and so might be more readily accessible to small fish. Ostracods and *Daphnia* occur on or near the mud surface, and the copepods in the water column, with the result that these species would be available to fish at all sizes (J. P. E. C. Darlington, personal observation).

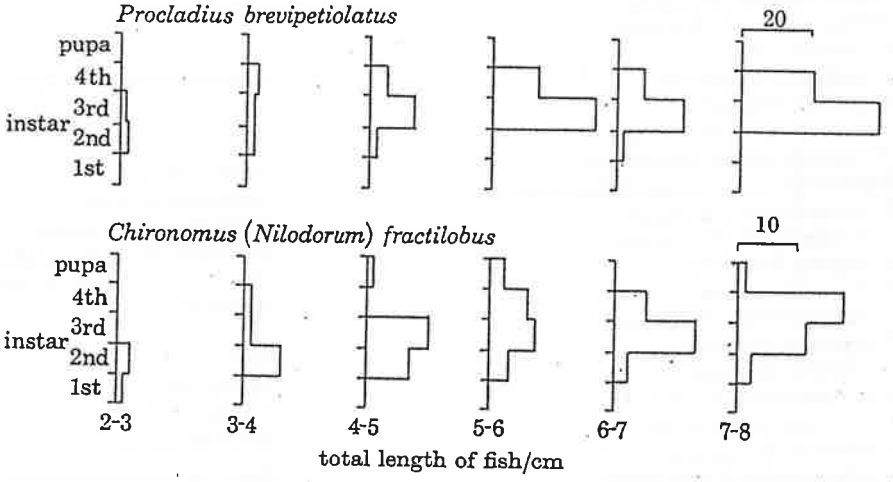

FIGURE 12. Histograms of the total numbers of each instar of two species of chironomid occurring in the guts of *Haplochromis angustifrons* females and juveniles. The total gut contents of five individual fish were counted for each 1 cm size range of fish (J. P. E. C. Darlington, unpublished).

Inshore and over sandy shoals many other species of benthic predators occur. *Haplochromis elegans* Trewavas and *Barbus neglectus* Boulenger are the most abundant, but a number of other species are also present (table 7). All feed mainly on chironomid larvae which are more abundant here than in the mid-lake mud. *Haplochromis mylodon* Greenwood has an enlarged pharyngeal mill which enables it to crush the shells of molluscs, especially *Melanoides tuberculata*, a benthic gastropod which occurs close to shore. Other inshore species of fish, whose overall food intake is more generalized, also include a proportion of benthic invertebrates in their diet; for instance, *H. aeneocolor* Greenwood takes food both from the water column and the substrate, thereby including plant fragments, dipteran larvae and adult aquatic insects in its diet.

Detritus and other feeders

The main detritus feeders are the benthic invertebrates, but very little is known about their feeding habits. The role of *Tilapia leucosticta* as a detritus feeder has been mentioned already (p. 311). Several inshore species of fish ingest detritus, including plant fragments which may be derived from hippopotamus dung. *Haplochromis limax* Trewavas has a dentition adapted for scraping detritus and aufwuchs off solid surfaces (Greenwood 1973).

TABLE 7. SPECIES LIST OF THE FISH IN LAKE GEORGE, WITH NOTES ON THEIR MAIN FOOD (DUNN, UNPUBLISHED OBSERVATIONS)

species	*main food*
Aplocheilichthys eduardianus David and Poll	dipteran larvae and emergents
A. pumilus (Boulenger)	
Astatoreochromis alluaudi Pellegrin	molluscs
Bagrus docmac (Fôrskal)	fish
Barbus altianalis Boulenger	chironomid larvae (benthos)
B. kerstenii Peters	
B. neglectus Boulenger	
B. perince Rüppell	
Clarias lazera Valenciennes	fish
Ctenopoma muriei (Boulenger)	insect larvae
Haplochromis aeneocolor Greenwood	opportunist omnivore
H. angustifrons Boulenger	benthic invertebrates
H. eduardianus (Boulenger)	phytoplankton and plant material
H. elegans Trewavas	chironomid larvae (benthos)
H. limax Trewavas	aufwuchs
H. macropsoides Greenwood	?
H. mylodon Greenwood	molluscs
H. nigripinnis Regan	phytoplankton
H. pappenheimi (Boulenger)	zooplankton
H. petronius Greenwood	dipteran larvae
H. schubotzi Boulenger	plant material and insect larvae
H. schubotziellus Greenwood	
H. squamipinnis Regan	fish (insect larvae when young)
H. taurinus Trewavas	embryos and fry of cichlid fish
Marcusenius nigricans Boulenger	dipteran larvae
Mormyrus kannume Fôrskal	insect larvae
Protopterus aethiopicus Heckel	fish (and molluscs)
Tilapia leucosticta Trewavas	detritus and phytoplankton
T. nilotica (L.)	phytoplankton

Comments on food selection

The dominant fish species in Lake George are herbivores, but their fry are omnivorous or zooplankton eaters. In turn these species are preyed upon by a variety of fish and other predators, especially when young. Individuals of both *Tilapia* species above a certain size are selectively preyed upon by man.

The zooplankton as a whole shows a paucity of the large forms, such as Cladocera, a characteristic of a 'predated' population (Burgis *et al.*, this volume) and

presumably the result of predation by fish. Smaller zooplankton, especially the dominant *Thermocyclops hyalinus*, are preyed on chiefly by *Chaoborus* larvae. These in turn, especially when they congregate in swarms, are subject to predation by fish in the plankton.

The benthos is preyed upon by one fish species in the midlake, but by many fish species inshore where its biomass is greater (Burgis *et al.*, this volume). Some of these species are fairly specialized in their choice of food, but many are opportunists and take a wide variety of material, including aufwuchs, detritus and natatorial insects.

The calculated grazing rates of the major planktonic herbivores show that only a small proportion of the phytoplankton standing crop is eaten daily (p. 307). The net primary production seems adequate to maintain this cropping rate, and to stand grazing and losses from other sources as well. It is suggested, therefore, that food is not a limiting factor for at least the adult stages of the various vertebrate and invertebrate herbivores of Lake George (see also Viner & Smith, this volume).

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Reprinted from:

NORTH HOLLAND

AMSTERDAM

J. exp. mar. Biol. Ecol., 1976, Vol. 22, pp. 131–143; © North-Holland Publishing Company

QUANTITATIVE STUDIES ON BACTERIA AND ALGAE IN THE FOOD OF THE MULLET *MUGIL CEPHALUS* L. AND THE PRAWN *METAPENAEUS BENNETTAE* (Racek & Dall).

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Abstract: The biomass of bacteria ingested by two deposit-feeding animals has been estimated by measuring the muramic acid in their gut contents. Bacteria comprised about 15 to 30 % of the organic carbon in the stomach of the mullet *Mugil cephalus* L. feeding on sea-grass flats, and about 20 to 35 % of organic carbon in the proventriculus of the prawn *Metapenaeus bennettae* (Racek & Dall) feeding on muddy estuarine sediments. Diatoms made up about 20 to 30 % of the carbon in the mullet stomachs, but algae were unimportant in the food of most of the prawns examined. In the mullet stomachs, total organic carbon was about 2 to 3 % of ash weight, an increase of 10 to 20 fold over that in the *in situ* sediments. The prawns were much more selective in their feeding since the organic carbon was about 30 to 45 % of ash weight in the proventriculus.

In laboratory experiments, five species of bacteria and one blue-green alga were digested and assimilated by the prawns. The highest percentage assimilation was > 90 % and this is interpreted to indicate that micro-organisms passing into the digestive gland were nearly completely digested whereas those which passed directly from the proventriculus to the mid-gut were poorly digested.

Evidence is presented which suggests that the mullet feed only during the day and not at night on the sea-grass flats. A regression equation is given for the dry weight of sediment in the full gut on fresh weight of fish. A calculation of the amount of sediment (and hence bacteria and algae) eaten per day by a given fish, exemplifies the application of the available tentative data.

INTRODUCTION

The mullet *Mugil cephalus* L. feeds on detritus, including mud and sand, and micro-algae (Thomson, 1954, 1963). The nature of the components of diet vary with the substratum; on sea-grass flats for example, epiphytic and epontic diatoms are important (Thomson, 1959; Wood, 1959). Thomson (1954) has suggested that much of the unrecognizable 'detritus' could be bacteria and protozoa. More recent quantitative work on the food of mullet is that of Odum (1970), who in addition to the usual qualitative and percentage abundance descriptions, measured organic matter, calorific value and total plant pigment.

The prawn *Metapenaeus bennettae* (Racek & Dall) is a detritus feeder, and it has been suggested that, in common with other penaeid prawns, micro-organisms are important dietary components (Dall, 1968). In general, micro-organisms are probably the main source of food for deposit-feeding animals (Newell, 1970; Fenchel, 1971). A convenient method has been proposed for estimating the biomass of bacteria, in

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terms of carbon, in the sediments ingested by deposit feeders (Moriarty, 1975). In this paper the method has been applied to a quantitative analysis of the food of *Mugil cephalus* and *Metapenaeus bennettae*. Bacterial carbon is compared with total organic and algal carbon. Since no published information was available, experiments to measure the digestibility of bacteria by the prawn were made and an attempt was made to estimate the daily food consumption of the mullet in its natural habitat.

Methods

CULTURES

One bacterium was isolated from estuarine mud on agar plates containing a mineral salts medium with 1 % (w/v) NaCl and 1 % glucose. The other bacteria, obtained from the Culture Collection in the Department of Microbiology, University of Queensland, were also grown in the same liquid medium with glucose as the sole carbon source. The bacteria were labelled uniformly with ^{14}C by growing them with ^{14}C(U)-glucose obtained from the Radiochemical Centre, Amersham, U.K. They were harvested by centrifuging at 10,000 *g* at 20 °C and washed twice in 1 % NaCl immediately before use.

The blue-green alga (probably *Oscillatoria* sp.) was isolated from a sea-grass flat on ASP2 medium solidified with agar (Provasoli, McLaughlin & Droop, 1957). The algae were labelled uniformly with ^{14}C by growing them in sealed flasks with 300 mg $NaHCO_3$ and about 50 μCi ^{14}C-$NaHCO_3$ added to the liquid medium after autoclaving.

ANIMALS

For the assimilation experiments, *Metapenaeus bennettae* (carapace length $\approx$ 20 mm) were caught in a seine net. They were kept in the laboratory at 25 °C and a salinity of 20 $^0/_{00}$ and maintained on cooked oatmeal. For ATP extraction, prawns were also caught in a seine net in shallow water and immediately placed in dry-ice (solid CO_2). Prawns for muramic acid analysis were caught by trawling near the mouth of the Brisbane River and further out in Moreton Bay, Queensland. About 40 prawns, carapace length 20–30 mm, were sampled on each occasion and their gut contents pooled. Contents were taken from the anterior chamber of the digestive gland and the mid-gut and freeze-dried.

Mullet were caught with a seine net over sea-grass (*Zostera capricorni* Aschers) flats on the western side of North Stradbroke Island, Moreton Bay. For muramic acid analysis the contents of the posterior and anterior stomachs were combined, and contents of the rectum were taken from the last 8 cm of intestine. This material was then treated as described elsewhere (Moriarty, 1975). The mullet used varied in length from 25 to 35 cm. For the dry weight of full gut contents, mullet were caught during the day, length to the caudal fork measured, and the complete gut placed in

previously weighed bottles. The bottles were weighed after drying at 70 °C for 4 days. To obtain the weight of the gut tissues without contents, mullet were caught at night (when the guts were empty) and treated similarly. Fresh weights of fish were calculated according to the formula: $W = 0.00808\ L^{3.025}$ (Chow, 1958, cited by Thomson, 1963). Regression equations were calculated for the dry weight of empty guts and full guts against fresh weights of fish. The values used for the regression equation for gut contents were obtained by difference.

ASSIMILATION

Filaments of the labelled algae were broken into small lengths in a blender. The bacteria and algae were suspended in centrifuge tubes with 1 % NaCl solution and aliquots were removed for counting ^{14}C. The organisms were then centrifuged. The pellets were mixed into an equal volume of gelatine (about 20 %) made up with 1 % NaCl solution and set in a freezer for 2–3 min. The pellet was broken with a spatula into small pieces which were offered to prawns in individual tanks. Prawns were left to feed for about 20 min and then removed to clean tanks. One prawn was immediately dissected and digested in Soluene (from Packard Corporation) to check the method for measuring ingestion. The water in which they were fed was filtered through glass-fibre (Whatman GF/C) and Millipore (0.45 μm) filters. Washings from centrifuge tubes and supernatants were combined and filtered. Faeces were collected by filtration through GF/C and Millipore filters at intervals over the following 30 h. Prawns were fed oatmeal from about 8 h after feeding on labelled micro-organisms. Materials on the GF/C filters were digested in Soluene.

Filters and aliquots of filtrates were counted on a Nuclear Chicago scintillation counter Mark II, using the sample channels ratio method to correct for quenching. Aqueous samples were counted with Triton X-100 in the scintillant fluid (Turner, 1971).

The amount of ^{14}C ingested was estimated as the difference between the ^{14}C in the original pellet suspended in gelatine and the amount recovered from the feeding water, washings *etc.* Assimilation was measured as the difference between ^{14}C ingested and ^{14}C in the faeces and expressed as a percentage of the total ingested.

CELL WALL DIGESTION

Bacillus subtilis cells were labelled uniformly with ^{14}C. The culture was boiled for 5 min, then harvested and washed twice in 1 % NaCl. Cell walls were prepared by the method of Salton & Horne (1951). The digestive glands from 10 prawns were dissected out, homogenized, and centrifuged at 30,000 *g* for 15 min at 4 °C. The supernatant and cell wall preparations were mixed and 5 μl subsamples removed for chromatography on Whatman No. 1 paper in butanol : acetic : water (3:1:1). A duplicate reaction was carried out with boiled supernatant. After chromatography, the paper was dried and cut into 2 cm squares for ^{14}C counting.

ANALYSES

Procedures for analysis of carbon, muramic acid, chlorophyll *a* and adenosine triphosphate (ATP) have been described elsewhere (Moriarty, 1975). Blue-green algae were counted in ≈ 1 mg samples of freeze-dried sediment, weighed on microscope slides and wetted with distilled water. Only cells easily recognizable by their colour and morphology were counted; each cell was taken to be 6 μm diameter. To measure ATP in the proventriculus contents, prawns were dissected while frozen and the contents of the proventriculus from about 40 prawns transferred to a container kept in dry-ice. The material was mixed, thawed to 0 °C, then 2 ml of potassium phosphate buffer (0.01 M, pH 7.5) at 0 °C was added and rapidly mixed. Two 0.5 ml aliquots were extracted immediately as described elsewhere (Moriarty, 1975). Chlorophyll *a*, total carbon, and dry weight were estimated using the remainder.

RESULTS

DIGESTION OF BACTERIA

A high proportion of bacterial carbon was digested and assimilated by the prawns (Table I). This indicates, especially in the cases where assimilation was almost complete, that, in addition to the cell contents, cell walls were also digested. If prawns were starved for 24 h prior to a feeding experiment, they ate rapidly when offered labelled bacteria, but overall assimilation was low because about 20 to 40 % of the ^{14}C was defaecated within about 2 h. If these apparently undigested bacteria in the faeces were left in the tanks, the prawns subsequently ate them. Assimilation was

TABLE I

Assimilation of bacteria and a blue-green alga by *Metapenaeus bennettae*.

Bacteria	Assimilation (% ^{14}C) Mean	Range	Number of experiments
Isolate No. 1	91	87–97	5
Escherichia coli	85	84–86	4
Pseudomonas fluorescens	93	90–95	4
Enterobacter aerogenes	96	95–98	5
Bacillus subtilis	84	80–88	2
Blue-green alga	63	48–87	6

then very high, *i.e.*, about the maximum shown in Table I. To check that the procedure for quantifying ingestion was adequate, a prawn was killed immediately after feeding on labelled bacteria. The amount of ^{14}C in the prawn was within 10 % of the estimated amount. A blue-green alga (probably *Oscillatoria* sp.) was also digested and assimilated by the prawns, but the results were more variable. When assimilation was low, it was noticed that many, apparently undigested, algal cells were defaecated within 1 to 5 h after ingestion.

About half the ^{14}C in boiled *Bacillus subtilis* cell walls was hydrolysed to many small molecular weight compounds by an extract of the digestive gland from *Metapenaeus bennettae* (Fig. 1). No identification was made of the many products of hydrolysis. There was no hydrolysis when the extract was first boiled.

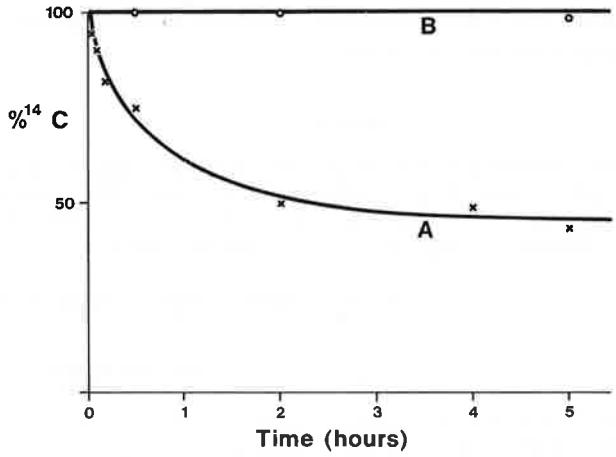

Fig. 1. *In vitro* digestion of *Bacillus subtilis* cell walls by an extract of prawn digestive gland: % ^{14}C remaining at origin is shown: A with untreated extract; B with boiled extract.

BACTERIA IN THE NATURAL DIET

Gut contents of prawns caught in Moreton Bay contain a considerable quantity of muramic acid (Table II). The ratio of muramic acid to ash in the proventriculus

TABLE II

Muramic acid in *Metapenaeus bennettae* gut contents. Prawns were caught in Morton Bay: range of variation in muramic acid, ±5 %; duplicates measured for each sample.

Date	Source	Muramic acid (mg/g ash weight)
28.x.74	Proventriculus	1.65
28.x.74	Mid-gut	0.99
3.ii.75	Proventriculus	2.63
3.ii.75	Mid-gut	1.70

was noticeably greater than the ratio in the mid-gut. The biomass of bacteria in the proventriculus was estimated from the muramic acid content, as described elsewhere (Moriarty, 1975) and assuming that the composition of the bacterial population in the area where the prawns were caught was similar to that in the Brisbane River. From the data of Table III, it may be seen that this biomass constitutes ≈ 20–30 %

of the organic carbon ingested by these prawns. One group of prawns from a shallow pond connected to the North Pine River, were found to feed on algae. The muramic acid method had not been developed at the time these prawns were examined but an estimate of total microbial biomass (from ATP measurements) showed that micro-organisms other than algae were important (Table III). By comparison with the analysis of the Brisbane River mud, it is evident that the prawns are selecting organic matter rich in bacteria from the sediments.

TABLE III

Analysis of *Metapenaeus bennettae* gut contents in terms of carbon: algal C, chlorophyll $a \times 30$; bacterial C, muramic acid/33; total living microbial biomass = ATP $\times 250$; range of variation in organic C, ± 1 %; in chlorophyll *a*, ± 5 %; in muramic acid, ± 5 %; in ATP, ± 5 %; duplicates measured for each sample; n.d. = not determined.

Material	Source	Total C	Organic C	Algal C	Bacterial C	Total microbial C
		mg/g ash weight				
Proventriculus contents	North Pine R.	376	n.d.	4	n.d.	35
Proventriculus contents	Moreton Bay	374	310	0	55	n.d.
Proventriculus contents	Moreton Bay	434	321	0	88	n.d.
Proventriculus contents	Brisbane River	530	456	0	147	n.d.
Mud	Brisbane River	n.d.	19.4	1	2.6	n.d.

Mullet were found to have a large amount of muramic acid in their gut contents (Table IV). In three out of four groups of fish, the ratio of muramic acid to ash was greater in the stomach than in the rectum. Blue-green algae were most abundant in their gut contents in spring (October–November), but at all times diatoms were the dominant algae ingested. The sea-grass sediments were sandy, with particles up to

TABLE IV

Muramic acid and blue-green algae in mullet gut contents: range of variation in muramic acid, ± 10 %; duplicates measured.

Date	Source	No. fish/sample	Muramic acid (μg/g ash)	Blue-green algae		
				cells/g ash		No. counts
				Mean	$\pm$S.E.	
28.viii.74	Stomach	8	525	2×10^4	6×10^3	6
28.viii.74	Rectum		318			
26.xi.74	Stomach	3	540	1.1×10^6	1.8×10^5	7
26.xi.74	Rectum		375			
5.ii.75	Stomach	10	214	2.2×10^5	9×10^4	7
5.ii.75	Rectum		125			
24.ii.75	Stomach	10	170	$< 1 \times 10^4$	—	6
24.ii.75	Rectum		200			

about 400 μm diameter. In the mullet gut contents, however, particles were rarely > 200 μm and mostly < 100 μm; these fish were, therefore, selecting small particles, which were richer in organic matter, especially algae and bacteria, as indicated by the higher ratio of these components to ash in the stomachs as compared with the sediments (Table V). Mullet caught on 28th August, 1974 had a high ratio of total carbon and bacterial carbon to ash weight. Microscopic examination showed that the stomach contents of these fish contained a much higher proportion of small sand particles (10–50 μm) than the other fish. With the exception of 3 fish caught on 26th November, 1974 bacterial carbon was estimated to be $\approx$ 20–30 % of the total carbon ingested by the mullet. Algae made up a similar proportion of their diet (Table V). Using narrow-range indicator papers, acidity in the stomach was found to vary from about pH 3.5 to pH 7.0.

TABLE V

Analysis of sediment from mullet gut and sea-grass flat in terms of carbon: algal C, chlorophyll $a \times 30$; bacterial C, muramic acid/44: inorganic C was less then 1 % of total C: range of variation in organic C, ± 1 %; in chlorophyll a, ± 5 %; in muramic acid: ± 10 % (gut contents) or ± 50 % (sea grass flat); duplicates measured.

Date	Sediment source	No. fish/sample	Total organic carbon	Algal carbon	Bacterial carbon
			mg/g ash		
1.v.74	Stomach	11	24.0	8.0	5.3
28.viii.74	Stomach	8	37.4	6.4	12
26.xi.74	Stomach	3	17.7	2.4	12.2
26.xi.74	Sea-grass flat		1.9	0.2	0.5
5.ii.75	Stomach	10	28.0	3.9	4.8
5.ii.75	Sea-grass flat		4.5	0.2	0.8
24.ii.75	Stomach	10	16.4	6.3	3.9
24.ii.75	Sea-grass flat		2.4	0.2	0.4

FEEDING CYCLE

The type of fishing gear required to catch mullet at all stages of the tides over a 24 h period was not available. From the data obtained, however, it seems that the mullet on these sea-grass flats have a diurnal feeding cycle which is not influenced by the tides (Table VI). No mullet caught at night were feeding; indeed, all had empty guts. Most mullet caught during the day on flood or ebb tides had full guts; occasionally one or two were found with empty or half empty guts at midday. Those caught in the morning had sediment in the stomach and anterior part of the intestine, indicating that they had begun feeding a little while before capture but had not fed overnight. Fish caught in the evening had sediment in the posterior part of the intestine but not in the stomach or anterior part, indicating that they had stopped feeding sometime before capture.

TABLE VI

Feeding cycle in mullet on sea-grass flats: filling, sediment present in the stomach and anterior part of the intestine only; emptying, sediment present in the posterior part of the intestine only: tide is shown as high water (H), low water (L), or hours after flood (F) or ebb (E).

Time	Gut	Tide	Number of fish	Date
08.00	Empty	F +2	7	19.xi.74
08.30	Filling	F +2.5	17	10.iv.74
09.30	Filling	F +3.5	2	19.xi.74
11.00	Full	E +5.5	19	5.ii.75
11.30	Full	L	10	1.v.74
12.45	Full	F +1	28	5.ii.75
13.30	Full	E +2	12	10.iv.74
13.30	Full	E +4	20	22.iv.74
14.40	Full	L	10	24.ii.75
16.00	Full	E +5	18	9.iv.74
19.00	Emptying	F +2	14	9.iv.74
00.00	Empty	H	8	10.iv.74
00.00	Empty	L	34	7.iii.75
03.00	Empty	L	9	25.ii.75
05.00	Empty	F +1	7	23.iv.74

The dry weight of sediment in the full gut was measured for 27 mullet, ranging in fresh weight from 130 to 300 g. The following regression equation was calculated:

$$\hat{Y} = 0.157X - 15.5.$$

where $\hat{Y}$ = dry weight of gut contents (g), X = fresh weight of fish (g). The standard error of the slope was ± 0.021.

DISCUSSION

DIGESTION OF BACTERIA

From the results it is clear that *M. bennettae* can readily digest and assimilate bacteria. The percentage assimilation is probably accurate to within 10 % for the bacteria. Precise values cannot be given because of uncertainty in the relative counting efficiencies of the heterogeneous materials containing ^{14}C. The values for assimilation of carbon from the blue-green alga are less accurate than those for bacteria, because the larger size of the filaments increased sampling and self-absorption errors.

The higher values in Table I probably indicate the maximum possible digestibility of the respective micro-organisms. Lower values were obtained, as previously described (p. 134), usually when large pieces of gelatine were eaten rapidly. It is likely that the organisms in these gelatine pieces did not pass into the digestive gland but went directly from proventriculus to mid-gut. The complex setae in the proventriculus allow only very fine particles to pass into the digestive gland (Dall, 1967).

The high assimilation values suggest that cell walls are not only lysed, but digested and assimilated. Enzymic digestion of cell walls is demonstrated by the experiments with *Bacillus subtilis*. The array of products from the cell wall digestion suggests that more than one enzyme was involved, but further characterization was not undertaken. Since the cell walls of blue-green algae and bacteria have a similar composition (Rogers & Perkins 1968), enzymic lysis and digestion of blue-green algae would be expected. Other crustaceans presumably have enzymes which lyse bacterial and blue-green algal cells. Tevlin (in Moriarty *et al.*, 1973) showed that adults of the copepod *Thermocyclops hayalinus* assimilated about 35 % of carbon from *Microcystis* sp. and the nauplii even more, about 58 %. The amphipod *Hyalella azteca* assimilated high proportions of carbon from bacteria, but the efficiency of assimilation of blue-green algae (*Anabaena* sp. and *Anacystis* sp.) was low (Hargrave, 1970). Reports in the literature indicate that the digestibility of blue-green algae is variable (see *e.g.*, Sorokin, 1968), which suggests that the physiology of digestion of these micro-organisms is complex.

It is probable that autolysis as well as enzyme activity in the digestive gland of the prawn would bring about almost complete digestion of bacterial. Hoogenraad & Hird (1970) have shown that in the sheep, autolytic as well as digestive enzymes are involved in the digestion of bacteria; $\approx$ 40 % of the cell wall mass of rumen bacteria was released by autolysis.

BACTERIA IN THE NATURAL DIET

The total living microbial biomass in the proventriculus of the first group of prawns in Table III, is probably an undere-stimate. Dall (1967) has suggested that digestive enzymes are transferred to the proventriculus. This, together with the environmental change, should cause hydrolysis of ATP in the ingested micro-organisms. Much of the microbial biomass which cannot be accounted for as algae is probably bacterial, as shown by the large amounts of muramic acid in the remaining groups of prawns. During the course of this work it was not possible to examine the bacterial populations in the areas of Moreton Bay where the prawns were caught and it was assumed, therefore, that the composition of the population was similar to that at the Brisbane River location. Gram negative rods comprised about 85 % of the total and, as discussed elsewhere, moderate variations do not greatly affect the estimates of biomass (Moriarty, 1975). The larger amount of bacteria eaten by the prawns in the Brisbane River compared with those in the Bay, may be due to a greater supply of allochthonous organic matter.

The quantitative data on bacterial and algal biomass confirms Dall's (1968) suggestion that the prawns select the epiflora and epifauna (bacteria, micro-algae and protozoa) of the mud substratum. The relative importance of protozoa, however, is still unknown. Flagellates and ciliates are common in similar areas elsewhere and are eaten by deposit-feeding animals (see, *e.g.*, Fenchel, 1971).

It is difficult to measure the digestibility of the food of these prawns. As indicated by Dall (1968), particles small enough to pass into the digestive gland will be more readily digested. The lower ratio of muramic acid to ash in the mid-gut compared with the proventriculus (Table II) suggests that about 30 % of the muramic acid in the bacterial cell walls is digested. A higher proportion of bacterial cell contents may be digested. The ^{14}C studies imply that there would be almost complete digestion of bacteria which pass into the digestive gland.

With the prawns it is clear that the muramic acid is bacterial in origin, because no blue-green algae were present. Blue-green algae did, however, occur in varying abundance throughout the year, in the sediments of the sea-grass flats where the mullet were feeding. A tentative estimate of the muramic acid content of blue-green algal cells suggested that 1×10^7 cells would contain about 2 μg (Moriarty, 1975). All the stomach contents contained much less than 10^7 cells, so that it is likely that the muramic acid is mainly bacterial. In most of the fish stomachs, bacteria comprise about 15 to 30 % of the carbon. The three fish caught on 26th November, 1974 have an unusually high ratio of muramic acid to ash and it is possible that some of the muramic acid was derived from blue-green algae. These fish had the highest number of cells/g of sediment and a bloom of blue-green algae had occurred earlier; decaying cells may have been present in sufficient quantity to affect the results. The possible occurrence of filamentous sulphur bacteria (*e.g. Beggiatoa* sp.) in large quantities in some regions of the sediment may contribute to such high muramic acid values. Bacterial cell wall fragments are unlikely to occur in large quantities in the surface sediment (Moriarty, 1975).

The methods for estimating algal and bacterial biomass in the sediments give approximate results because of the assumptions made to arrive at factors used to convert the primary data. Biomass to the nearest 0.1 mg in Table V for example, is not accurate to this level; it is given to allow calculation of the primary data by others. It is clear, however, that bacteria are as important as diatoms (the dominant algae) in the food of the mullet. Chlorophyll *a* has been calculated by a method which distinguishes it from phaeophytin *a*, but not chlorophyllide *a*. Since diatoms are the main algal component, the estimate of biomass may well be inaccurate (Jeffrey, 1974), but better methods are not yet available. Protozoan biomass is not measured by these methods, but could be important. Odum (1970) found that the fine particles eaten by *Mugil cephalus* were covered with bacteria and protozoa. Total living microbial biomass, estimated by the ATP method, is only a little lower than the combined algal and bacterial biomass in the sea-grass sediments referred to in Table V (Moriarty, 1975). Since this work was done, Karl & LaRock (1975) have shown that the extraction of ATP in boiling Tris (used here) is not complete and the control does not compensate for this. The total living biomass is, therefore, probably higher than the algal and bacterial biomass, which would be expected if protozoa were prominant.

The proportion of living biomass to detritus is rather high in the sea-grass sediments

studied here. It should be noted, however, that the samples are not representative of the sea-grass flats as a whole. They were taken close to *Zostera* plants, but passed through a 0.5 mm sieve to remove large debris, giving samples which should be similar to the sediment filtered by the mullet. The results suggest that the mullet filter small particles from the sediment and this is in agreement with Odum (1968) who demonstrated experimentally the selection of small particles by mullet. Both Odum (1970) and Wood (1953) observed that the small particles were rich in micro-organisms.

Odum (1970) observed that the number of micro-organisms on particles of detritus decreased along the digestive tract. In three of the groups of fish examined here, there was significantly less muramic acid/g ash in the rectum than in the stomach, suggesting that bacterial cell walls have been digested. Mucus in the rectum prevented comparisons of total organic carbon. It is difficult to draw firm conclusions from these comparisons, because during the time taken by food to pass from stomach to rectum, the fish could have been feeding on substrata with quite different ratios of carbon and micro-organisms to ash weight. The lowest stomach pH of 3.5 to 4.0 recorded here is probably insufficient to act as the primary mechanism of bacterial cell lysis in mullet, as suggested earlier (Moriarty, 1973). The process is more likely to be enzymatic.

FEEDING PERIODS IN MULLET

The mullet, like the herbivorous cichlids *Tilapia nilotica* and *Haplochromis nigripinnis* (Moriarty & Moriarty, 1973), apparently feed only during the day. Odum (1970) reported that the mullet he studied fed with greater intensity on the rising tide and less on the falling tide and implied that the time of day was irrelevant. He did not, however, state at what time of day he caught his fish, nor whether he looked at more than one tidal cycle. It is possible that feeding activity varies with locality, but there are no published data.

The average retention time for sediment in the mullet was found by Odum (1970) to be 4 to 5 h. I have observed a similar period for mullet in the laboratory. From the regression equation given above, a mullet of 200 g fresh weight may contain 16 g dry weight of sediment in its gut. For a feeding period of 12 h and a retention time of $\approx$ 4 h, this size of fish would eat about 50 g dry sediment/day, which corresponds to 1.5 g C/day for a sediment in the stomach containing 3 % of C. The amount of bacterial and algal carbon removed per day from a given area could be extrapolated from these results if the number and size distribution of fish were estimated. It is interesting to note that a 200 g *Tilapia nilotica* also eats about 1.5 g C/day and, with 45 % assimilation, this would be sufficient to supply its energy needs (Moriarty & Moriarty, 1973). The micro-organisms in the diet of these two fish are not dissimilar (diatoms, bacteria and blue-green algae).

Evidence that bacteria and other micro-organisms associated with detritus rather than the detritus itself, are the source of food for deposit-feeding animals has been reviewed by Fenchel (1971). Much of the previous work has been based on counting

micro-organisms, or nitrogen measurements. The work reported here analyses the diet of two deposit-feeding animals in terms of biomass (as carbon) of micro-organisms. Although further work is necessary, it is clear that bacteria are probably the main living component in the food of *Metapenaeus bennettae* and are of equal importance with diatoms in the food of the mullet on sea-grass flats. Mullet feeding in more turbid, muddy areas probably rely mainly on bacteria, and perhaps also on protozoa. The quantitative data on the amount eaten per day by the mullet, although tentative, suggests that the biomass of micro-organisms is probably sufficient for the energy needs of the fish. Further work is needed to assess the digestibility of bacteria in mullet.

The method for measuring the biomass of bacteria in the sediments ingested by deposit-feeding animals (Moriarty, 1975) requires further development, but it should simplify quantitative studies on the trophic rôle of bacteria and the flow of carbon through benthic ecosystems.

Acknowledgements

This work was carried out during tenure of a Queen's Fellowship in Marine Science awarded by the Australian Government. Professor J. M. Thomson, Department of Zoology and Professor V. B. D. Skerman, Department of Microbiology, kindly provided facilities.

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Aust. J. Mar. Freshwater Res., 1977, **28**, 113–18

Quantification of Carbon, Nitrogen and Bacterial Biomass in the Food of Some Penaeid Prawns

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Abstract

The proventriculus contents have been analysed in five species of penaeid prawns, *Penaeus plebejus*, *P. esculentus*, *P. merguiensis*, *Metapenaeus bennettae* and *Trachypenaeus fulvus*. Measurements of organic and inorganic carbon, protein and bacterial biomass show that these prawns are omnivores, feeding in an opportunistic manner. Most proventriculus contents contained about 100–150 mg organic C/g dry weight, of which 10–20% was bacterial C. The muddy sediments, over which the prawns were feeding, contained from 6 to 30 mg C/g dry weight with 6–16% bacterial C. A sandy seagrass flat contained 2 mg C/g dry weight with 30% bacterial C. Detritus was sometimes the main food item, bacteria constituting up to 30% of organic C in the proventriculus. Foraminifera, bivalves and crustaceans were important food items in some prawns. Low bacterial biomass, coupled with high $CaCO_3$ and high protein content (about 40% of organic matter) indicate that the fragments of animal skeletons are from live prey eaten by the prawns. Little difference was noted between species of prawns in their food preferences.

Introduction

The nature of the food of penaeid prawns has not been clarified. Hall (1962) analysed the proventriculus contents of many species and concluded that in general they were omnivores, although some were herbivores, and some were carnivores feeding on various groups of animals. Williams (1955) noted that animal remains and sand were present, and that the major component of the proventriculus contents was soft matter, probably made up of animal tissue and organic detritus. Racek (1959) observed remains of fish in the gut contents of *Penaeus* species, but he concluded that annelids, nudibranchs, small crustaceans and similar small organisms comprised the principal diet of penaeid prawns. Dall (1968) suggested that microorganisms from the bottom deposits were important constituents of the soft unrecognizable material. Bacteria are eaten and digested by at least one species of penaeid prawn (Moriarty 1976*a*).

This paper describes the analysis of proventriculus contents of five commercial species of penaeid prawns, *Penaeus plebejus* Hess, *P. esculentus* Haswell, *P. merguiensis* de Man, *Metapenaeus bennettae* Racek & Dall, and *Trachypenaeus fulvus* Dall. Measurements of carbon, nitrogen and bacterial biomass have been used to show that both microorganisms and small animals are important items in the food of these prawns.

Materials and Methods

Some prawns were taken from sandy banks of seagrass (mainly *Zostera capricorni* Aschers) in southern Moreton Bay, Queensland. A small beam trawl was used during high tide at night. In the deeper areas of Moreton Bay, prawns were caught with an otter trawl at night. They were transported to the laboratory on dry ice. Juvenile *P. merguiensis* were taken from the Norman River, North Queensland, by sampling with a small beam trawl about 3 m from the edge of the water at low tide. Adult *P. merguiensis* were caught north of Maria Island, Gulf of Carpentaria, by a commercial trawler. They had been caught at about midday, during a 5-min trawl through a school, and frozen to about −20°C within 20 min of capture. The proventriculus contents were dissected out of all prawns while frozen, and then freeze-dried. Samples of about 20–40 prawns were pooled by species for analysis.

Sediments from the seagrass flat and Norman River were collected in a polythene tube (2 cm diameter). The deeper sediments were collected in a Petersen-type grab. The top 1 cm was removed and freeze-dried before analysis.

Carbon and muramic acid were measured as described by Moriarty (1975, 1976*b*). Bacterial biomass was estimated from muramic acid content, using the conversion: C = muramic acid × 66 (Moriarty 1976*b*). Nitrogen was measured after Kjeldahl digestion using ninhydrin, as described by Fels and Veatch (1959). To distinguish between nitrogen in chitin and that in protein of ingested crustacea, gut contents were extracted with 1 M NaOH which does not hydrolyse chitin.

A microscope fitted with an epifluorescence attachment was used to examine gut contents. Bacteria were stained with magnesium 1-anilino-8-naphthalene sulphonate (Mayfield 1975).

Results

Two species of prawns taken from the seagrass flat had a similar diet. Fragments of small crustacea and molluscs were observed in the gut contents, and the proventriculus contained $CaCO_3$ which suggests that crustacea and molluscs were important items of food. Bacteria associated with detritus were eaten, especially by *M. bennettae* (Table 1).

Crustacea were an important part of the diet of *P. plebejus* from the 'main channel', a deeper, muddy area of Moreton Bay (Table 2). In prawns from another similar area, however, foraminifera were the predominant items in the proventriculus contents, their skeletons constituting up to 75% of the dry weight. The remainder of the dry weight was made up of silt, clay and detritus. Bacteria were an appreciable part of the organic matter in some cases (Table 2). In about 10% of the *P. plebejus* individuals the proventriculus was filled with polychaetes. These individuals were excluded from the analyses.

Juvenile *P. merguiensis* were sampled from two locations — an open shore with grass above a 4 m high bank, and a shore fringed by mangroves. The biomass of bacteria on the mangrove shore was twice that on the other shore (Table 3). The prawns caught on 30 April 1976 were feeding mainly on detritus containing fragments of what seemed to be crustacean skeletons. Under the microscope, a dense covering of bacteria was observed on the fragments. This is consistent with the large biomass of bacteria measured by the muramic acid procedure (Table 3). Prawns caught in the same locality 1 week later were feeding mainly on small bivalves. The proventriculus contents from a group of adult *P. merguiensis* contained fragments of molluscs and small crustacea (Table 3). Sand grains constituted about half of the dry weight, indicating that the prawns in this school had been feeding at the sediment surface.

Estimates were made of the amount of protein in the proventriculus contents of some of the groups of prawns analysed above. Protein constitutes about 30–40% of the organic matter, whereas bacteria constitute no more than 10% in these prawns (Table 4).

Discussion

Bacterial carbon constitutes more than about 20% of the total organic carbon in the proventriculus contents of many groups of prawns. In these, detritus and the microorganisms associated with it are the major food items. As the proportion of bacterial carbon is generally less than 20% in the sediments, a high value in the proventriculus demonstrates that the prawns are selecting particles rich in bacteria.

Table 1. Analysis of the proventriculus contents of prawns and the sediment from the seagrass flat on which they were feeding

The top 1 cm of sediment was passed through a 0·5-mm seive before analysis. Bacterial C was measured as muramic acid × 66. Mean values for duplicate measurements are shown. The accuracy of measurement for organic C is ±1%; for $CaCO_3$ ±2%, and for bacterial C about ±20% (see discussion). CL, Carapace length

Material	Microscopic observations	Organic C (mg/g dry wt)	Bacterial C (mg/g dry wt)	$CaCO_3$ (mg/g dry wt)
Sediment	Sand with bacteria and algae	2·0	0·6	<10
P. plebejus CL 10–20 mm; 8.x.75	Sand, detritus, crustacea, molluscs	160	11	250
M. bennettae CL 15–20 mm	Sand, detritus, crustacea, molluscs	140	22	290

Table 2. Analysis of sediment and proventriculus contents of prawns from muddy substrates in Moreton Bay

Sediment was collected with a grab and the top 1 cm (approximately) was analysed. Mean values for duplicate determinations are shown. Accuracy of measurements as in Table 1

Material and locality	Microscopic observations	Organic C (mg/g dry wt)	Bacterial C (mg/g dry wt)	$CaCO_3$ (mg/g dry wt)
St Helena, Mud Is., channel sediment 19.iii.76		13	2·2	75
P. plebejus				
24.ii.76	Mainly forams	100	9	460
19.iii.76		75	20	750
27.xi.75		76	9	750
P. esculentus				
24.ii.76	Mainly forams	100	9	400
19.iii.76		73	17	720
T. fulvus				
24.ii.76	Mainly forams, some detritus	160	26	370
19.iii.76		125	19	650
27.xi.75		120	18	650
M. bennettae				
24.ii.76	Mainly forams, some detritus	110	26	430
19.iii.76		100	25	650
27.xi.75		185	30	450
Main channel *P. plebejus* 10.xi.75	Mainly crustacea, some molluscs, forams	140	14	250

Table 3. Components of the proventriculus contents of *P. merguiensis*

Sediment cores, 1 cm deep, were taken near the low water mark for comparison with juvenile prawns caught at the same locality. Mean values for duplicate measurements are shown. Accuracy of measurements as in Table 1

Material	Microscopic observations	Organic C (mg/g dry wt)	Bacterial C (mg/g dry wt)	$CaCO_3$ (mg/g dry wt)
Sediment, exposed shore		6·8	0·4	30
Juveniles, 5.v.76, exposed shore	Detritus, bivalves	120	9	300
Juveniles, 5.v.76, exposed shore	Detritus, bivalves	170	19	370
Sediment, mangrove edge		7·4	1·0	46
Juveniles, 5.v.76, mangrove edge	Detritus, bivalves	110	16	420
Juveniles, 5.v.76, mangrove edge	Mainly bivalves	70	5	600
Juveniles, 30.iv.76, mangrove edge	Detritus, bacteria	240	70	0
Adults	Sand, molluscs crustacea	86	7	370

Table 4. Organic carbon and nitrogen in proventriculus contents

Nitrogen was measured on material extracted in 1 M NaOH at 100°C for 24 h. Organic C measurements as in Tables 1, 2, and 3. Nitrogen values are means of duplicate measurements, accuracy ± 5%

Species	Microscopic observations	Organic C (mg/g dry wt)	Protein	
			N × 6·25 (mg/g dry wt)	Percentage of organic matter
P. plebejus, 27.xii.75	Mainly foraminifera	76	62	40
P. plebejus, 10.xii.75	Foraminifera, crustacea, detritus	140	100	35
P. plebejus, 8.x.75	Crustacea, molluscs, detritus	160	75	45
P. merguiensis adults	Molluscs, crustacea	86	50	30

In one group of juvenile *P. merguiensis* the actual amount of bacterial carbon is the same as the total amount of organic carbon in another group of juveniles feeding mainly on bivalves (Table 3). Dall (1968) had suggested that a large proportion of the unrecognizable material in the proventriculus might be colonies of microorganisms selected from detritus. The results discussed above show that this is true at times. In many prawns, however, the proportion of bacterial carbon was less than 10%. The remainder of the organic carbon in these cases cannot be mainly detritus, because about 40% of it is present as readily extractable protein (Table 4). Algae were not common in any of the proventriculus contents examined here. Carbon to nitrogen ratios of small crustacea show that protein comprises about 20–50% of their dry weight (Mullin and Brooks 1970; Omori 1970). Thus, the source of the protein in the proventriculus is most likely to be the tissues of animals eaten by the prawns.

This supports the argument that the fragments of foraminifera, crustacea and molluscs were derived mainly from live prey.

The estimate of the content of bacterial carbon in the proventriculus is based on the assumption that the composition of the ingested bacterial population is the same as that in the sediment (Moriarty 1976*b*). Further work is necessary to check whether the proportion of gram-negative bacteria is correct. Hood and Meyers (1973) have shown that penaeid prawns have an indigenous bacterial flora in the digestive tract. They give a value of about 3×10^7 cells/g, and imply, but do not specify, that this applies to the proventriculus and is in terms of wet weight of contents. Prawns which contain 100 mg organic C/g dry weight of proventriculus contents and 10% bacterial carbon (Table 2) would contain about 1×10^{11} bacterial cells/g dry weight. This high value suggests that most of the bacteria have been ingested, although more experimental work is necessary to establish this.

The seagrass flat has a large bacterial biomass, although the total organic carbon content is low (Table 1). This implies that the system is very productive (see Marshall 1970). The actual amount of carbon on a weight basis is low, because it is present mainly as a film on heavy mineral sand grains. The presence of sand grains in the proventriculus indicates that the prawns are utilizing this source of food. In *M. bennettae* sand grains constitute about half of the dry weight of the gut contents (Table 1). This is equivalent to about 1 mg organic C/g or 0·3 mg bacterial C/g. Since the prawns contain 22 mg bacterial C/g, it may be concluded that they are removing organic matter from the sand grains before ingestion, or that they are selecting particles of organic detritus (with bacteria) which is not attached to sand grains. Comparison of the food value of the different sediments on a weight basis is not possible because the sediments vary in particle size and density. The results reported here suggest that prawns are better able to feed on detritus and micro-organisms in muddy rather than sandy sediments.

The penaeid prawns studied here cannot be placed in any one trophic category, such as carnivores or detrivores. At times, however, some of these prawns are very selective. Some examples are those feeding mainly on foraminifera (Table 2), or bivalves (Table 3). Generally, they are opportunistic omnivores, feeding mainly on microorganisms and on other animals which feed on microorganisms. Knowledge of bacterial productivity in sediments would be useful, therefore, in studies on growth, distribution and population density of prawns.

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Manuscript received 4 October 1976

Aust. J. Mar. Freshwater Res., 1981, **32**, 245–51

Carbon and Nitrogen Content of Food and the Assimilation Efficiencies of Penaeid Prawns in the Gulf of Carpentaria

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Abstract

The food of seven species of penaeid prawns from the Gulf of Carpentaria consists predominantly of Foraminifera, small molluscs, crustaceans and polychaetes. Measurements of organic and inorganic carbon, organic nitrogen and bacterial biomass were made. Foregut contents of adult prawns contained between 72 and 223 mg organic carbon/g dry wt. Protein constituted between 43 and 64% of the organic matter. Approximate assimilation efficiencies of food in prawns caught in the gulf, determined for four species, varied from 48 to 77% of organic carbon and from 42 to 77% of organic nitrogen. The food of juvenile *Penaeus merguiensis* was examined for two growing seasons. In the 1976–1977 season the foregut contents contained a mean of 41 mg organic nitrogen/g dry wt and 181 mg organic carbon/g dry wt. In the 1977–1978 season, significantly lower proportions of organic nitrogen and carbon were eaten, *viz*, 21 mg organic nitrogen/g dry wt and 101 mg organic carbon/g dry wt. Improved assay procedures for muramic acid have shown that bacteria are less important in the food of prawns than previously reported. Bacteria constituted less than 2% of the organic matter in the adults of all species, but in many juvenile *P. merguiensis* bacteria were more important, constituting up to 14% of organic matter.

Introduction

The Gulf of Carpentaria supports an important commercial fishery of penaeid prawns. A study is being made of the biology and ecology of the major species, particularly *Penaeus merguiensis* de Man, and this paper describes the food components and assimilation efficiencies of these prawns.

Preliminary work indicated that penaeid prawns are omnivores feeding mainly on live prey, but also eating some micro-organisms including bacteria (Moriarty 1977). Bacteria were thought to be an important part of their diet, based on estimates of biomass made by measurement of muramic acid. Recent improvements in the assay technique for muramic acid (Moriarty 1980) have shown that many of the early results overestimated bacterial biomass, particularly in samples containing $CaCO_3$. Revised estimates are published here. It is difficult to determine the food of prawns either quantitatively or qualitatively because they break much of it up with their mandibles or gastric mill. Other workers (e.g. Williams 1955; Hall 1962) noted that, in general, prawns are omnivores and that much of the unidentifiable material in the gut was probably animal remains. We have combined microscopic observations with a more objective analysis of carbon and nitrogen in the gut contents of the following species: *P. merguiensis*, *P. esculentus* Haswell, *P. semisulcatus* de Hann, *P. latisulcatus* Kishinouye, *P. monodon* Fabricius, *Metapenaeus endeavouri* (Haswell), and *M. ensis* (de Haan).

Condrey *et al.* (1972) have reported assimilation efficiencies of 55–87% of organic matter for two species of *Penaeus* that were fed in the laboratory on diatoms, bacteria and compounded diets. We have used the ratios of organic carbon and nitrogen to inorganic matter in the foregut, midgut and hindgut to estimate assimilation efficiencies of food of prawns caught in the field.

Materials and Methods

Adult prawns were collected by trawling in south-eastern, eastern and north-eastern areas of the Gulf of Carpentaria. Juvenile *P. merguiensis* were caught at low tide with a small beam trawl in the Norman River at Karumba, northern Queensland (cf. Staples and Vance 1979). All prawns were frozen within half an hour of capture and brought to the laboratory on dry ice. Foregut contents were dissected out while frozen and were then freeze-dried. Sample sizes ranged from 17 to 100 juveniles and from 10 to 30 adult prawns at each locality and time. They were pooled to provide sufficient material for analysis. To obtain midgut and hindgut contents, prawn tails were partly thawed and the intestine was removed and immediately dissected. The contents were frozen and freeze-dried.

A Perkin-Elmer CHN analyser was modified for measurement of organic carbon and nitrogen and carbonate. The main furnace was operated at 550 °C and the high-heat zone was extended slightly and packed with insulation to operate at 1000 °C. Samples were burnt for 20 min at 550 °C to give organic carbon and nitrogen and then combusted at 1000 °C to give carbonate (Moriarty and Barclay 1981). The uptake of water by CaO and MgO after combustion made it difficult to determine ash weights by weighing. We found it simpler to estimate ash weight by subtraction of the dry weight of organic matter from the original weight. Organic matter was calculated from the organic carbon values, assuming that these were 45% of organic matter. These calculations were checked by carefully weighing some ash samples; the results agreed with the estimated ash content. Organic carbon and nitrogen values are expressed in terms of dry weight and ash weight.

Assimilation efficiencies (U) in the digestive gland were calculated from the ratios of organic carbon and nitrogen to ash weight as follows:

$$U = [(C_1/A_1) - (C_2/A_2)]/(C_1/A_1) \times 100,$$

where C_1/A_1 is the ratio of organic carbon to ash in the foregut and C_2/A_2 is the ratio of organic carbon to ash in the midgut or hindgut. Assimilation efficiencies of organic nitrogen were calculated similarly. The results that were obtained were the same as those obtained by the more complicated equation for assimilation of a dietary component given by Condrey *et al.* (1972). As it is obviously impossible to obtain a representative sample of the food of prawns in their natural habitat before they eat, analysis of foregut contents is the only practicable method available. Animals selected were those that had full guts.

Chitin was estimated approximately by extracting samples with 1 M NaOH at 100 °C for 24 h and comparing organic nitrogen with untreated samples. Chitin obtained from B.D.H., Melbourne, Vic., was treated similarly as a control. Muramic acid was estimated and bacteria examined microscopically as described by Moriarty (1980). Foregut contents were examined under the microscope and the predominant food items were noted.

Results

Food Analyses in Adult Prawns

Foraminifera tests were the most common items observed in the gut contents of many adult prawns. Fragments of small crustaceans, molluscs and polychaetes were often present. Much of the $CaCO_3$ in the gut contents was due to Foraminifera tests, particularly in *P. esculentus* and *P. semisulcatus* which fed mainly on Foraminifera. In a few cases, molluscan shells predominated. Organic carbon constituted generally 10–20% of the diet and organic nitrogen about 2–4% (Table 1). The carbon to nitrogen ratios ranged from 4·4 to 6·7, which indicates that between 43 and 64% of the organic matter was protein (assuming that protein = N × 6·25). About 40% of the nitrogen was extracted by NaOH from control samples of chitin, whereas 93–97% was extracted by NaOH from prawn gut contents. The residual 3–7% of nitrogen in the gut contents,

when multiplied by the factor of 40% for nitrogen extracted from chitin, gives a value of about 5–10% for nitrogen present in chitin or other nitrogenous material insoluble in NaOH. No seasonal or geographical trends were apparent in the nature of the food within species. Under the microscope, more particles of sediment with attached bacteria and less Foraminifera and molluscan fragments were observed in the food of the *Metapenaeus* species than in the *Penaeus* species. Values for organic nitrogen and carbon were higher and for $CaCO_3$ lower in animals that had eaten few Foraminifera and molluscs.

Table 1. Analysis of food of adult penaeid prawns from the Gulf of Carpentaria
Each sample consisted of the pooled gut contents of 10–30 individuals. Food types are listed in order of abundance. Concentrations are given as mg/g dry wt

Species	Date	Locality[A]	Food[B]	Organic nitrogen concn	Organic carbon concn	$CaCO_3$ concn
P. merguiensis	20.x.77	S	C, F	36	164	406
	10.xi.77	M	C, F, P,M	25	117	554
P. esculentus	20.x.77	S	M, P	11	72	651
	20.xi.77	S	F, C, P, D	21	98	588
	6.xi.77	M	F, C, P, D	42	222	311
	4.xi.77	N	F, D, C, P	18	92	652
P. monodon	3.iv.78	M	P, M, S, C	37	182	304
	29.iii.78	N	P, C, F, M, D	25	122	556
	20.x.77	S	C, F	36	164	406
	10.xi.77	M	C, F, P, M	25	117	554
P. latisulcatus	17.x.77	S	C, P, M,F	23	104	440
	5.i.78	M	D, M, F, P, C	26	117	518
	2.xi.77	N	P, C, M, S	38	177	148
P. semisulcatus	6.xi.77	M	F, D, C, P	41	201	306
	11.xi.77	N	F, D, C, P, M	23	115	548
	17.x.77	S	C, P, M, F	23	103	440
M. endeavouri	15.xi.77	S	D, P, S, C	43	203	46
	6.xi.77	M	D, P, S, C, F	45	223	91
	8.x.77	N	C, P, D	43	205	124
M. ensis	6.xi.77	M	P, D, M, S, C, F	45	221	171
	12.x.77	N	C, F, P, D	48	220	158
	1.xi.77	N	S, P, C, D, F	39	194	199

[A] S, south-eastern gulf; M, midway along east coast; N, north-eastern gulf.
[B] F, Foraminifera; P, polychaetes; M, molluscs; C, crustaceans; S, sand; D, detritus.

Approximate assimilation efficiencies, calculated for four species, show that for most animals over 50% of the organic matter was assimilated (Table 2). Most assimilation occurred between the foregut and midgut. In the *P. merguiensis* sample there was no further nitrogen assimilation, but in the other species a further 8–14% of organic nitrogen and organic carbon was assimilated. The efficiency of assimilation in *P. esculentus* was lower than 50%. These animals had fed mainly on Foraminifera, many of which were not broken up. The other species had fed principally on crustaceans or polychaetes and molluscs. There was little alteration in the carbon to nitrogen ratio as food material moved through the gut, indicating that the efficiency of digestion and assimilation of protein was similar to that of other digestible organic material.

Food Analysis of Juvenile Prawns

Particles of sediment with bacteria attached were commonly observed in the foregut of juvenile *P. merguiensis*. Fragments of Foraminifera, crustaceans, molluscs and polychaetes were also observed. The food of juveniles in the 1976–77 season had twice

Table 2. Minimum assimilation efficiencies of food in penaeid prawns from the Gulf of Carpentaria
Samples of gut contents from 14–19 individuals with full guts were pooled. Values are means for duplicate or triplicate analyses. Values for assimilation efficiency between foregut and either midgut or hindgut are shown. Unit of concentration is mg/g dry wt

Species	Gut region	Organic nitrogen		Organic carbon		Ratio of carbon to nitrogen	$CaCO_3$ concn
		Concn	% assimilation	Concn	% assimilation		
P. latisulcatus	Fore	64	69	283	68	4·4	139
	Mid	20	77	89	77	4·6	367
	Hind	14	—	64	—	4·4	331
P. merguiensis	Fore	34	54	161	57	4·7	353
	Mid	16	54	69	52	4·5	327
	Hind	16	—	77	—	4·9	393
P. esculentus	Fore	22	34	114	44	5·2	520
	Mid	15	42	64	48	4·4	637
	Hind	12	—	59	—	4·8	629
M. endeavouri	Fore	56	56	264	60	4·7	120
	Mid	24	71	106	74	4·4	139
	Hind	16	—	68	—	4·2	210

the mean concentration of nitrogen as that of those sampled in the 1977–78 season (Table 3). The concentration of organic carbon was similarly greater. Detritus and Crustacea were predominant in their gut contents. Foraminifera were more prominent in the gut contents of the animals caught in the 1977–78 season. The carbon to nitrogen ratios indicate that protein constituted about 40–76% of the organic matter.

Table 3. Analysis of food of juvenile *P. merguiensis*
Samples of prawns were collected about once per month in 1976–77 and about once each fortnight in the 1977–78 season. Carapace length varied from 5 to 20 mm. Each sample consisted of 17–100 prawns. Unit of concentration is mg/g dry wt

Season		Organic nitrogen concn	Organic carbon concn	$CaCO_3$ concn	Ratio of carbon to nitrogen	No. of samples
Sept. 1976–	Mean	41[A]	181[A]	250	4·4	13
March 1977	Range	24–62	105–270	80–650	3·8–5·0	
Dec. 1977–	Mean	21[A]	101[A]	410	5·0	28
March 1978	Range	12–39	58–270	80–750	3·7–6·8	

[A] Significantly different $P = 0{\cdot}001$, rank test (Sokal and Rohlf 1969).

Of the juveniles dissected, only about 50% had a foregut that was more than half full. Many had empty guts, although they were collected at low tide which is probably the time of greatest activity (Staples and Vance 1979). There was no noticeable difference in the composition of the diet of juvenile prawns in the size range 5–20 mm carapace length.

Bacteria in the Food

Bacteria were observed microscopically in the foregut contents, usually in association with particles of sediment. Samples which obviously contained large amounts were analysed quantitatively and it was found that in juvenile prawns bacteria constituted 2–14% of the organic matter (Table 4). Juvenile prawns with large amounts of bacteria generally had lower values for organic carbon and nitrogen. In adult *Metapenaeus* spp., 1–3% of organic matter was derived from bacteria. Other samples of prawns contained less bacteria.

Table 4. Bacteria in the food of penaeid prawns

Mean values are given for duplicate analyses of foregut contents. *P. merguiensis* samples were juveniles from the Norman River; the other species were adults from the Gulf of Carpentaria

Species	Organic nitrogen (mg/g dry wt)	Organic carbon (mg/g dry wt)	Bacterial carbon (% organic carbon)	$CaCO_3$ (mg/g dry wt)
P. merguiensis	15	72	14	80
	17	100	10	120
	17	92	6	120
	18	97	6	120
	35	147	3	150
	35	130	4	100
	30	145	2	300
M. endeavouri	42	204	1	455
	44	220	1	490
	44	200	2	450
M. ensis	39	190	2	430
	45	220	2	490
	45	220	2	500

Discussion

Bacteria are less important in the food of most of the adult prawns than reported previously (Moriarty 1977). Measurement of trace amounts of muramic acid in sediment samples, particularly in those containing $CaCO_3$, has proved difficult, but these problems have been resolved and the method checked against another technique (Moriarty 1980). The *Metapenaeus* species did appear to be selecting sediment particles with bacteria, but the amounts involved (up to 3% of organic matter) are small. These species were caught in shallow water closer to shore than most adult *Penaeus* spp., so it is not clear whether the difference in diet between the two genera of prawns is due to differences in feeding selectivity. The juvenile *P. merguiensis* do ingest bacteria, although bacteria were not the predominant items in their diet.

The main components of the diet of all the prawns examined are meiofauna. As concluded previously, the low bacterial density and high protein content of their food indicates that most of these prawns are not feeding on detritus, but rather on living animals (Moriarty 1977). Bell and Coull (1978) have shown that the shrimp *Palaemonetes pugio* is an important controller of meiofauna abundance in salt-marsh environments. It is not unlikely that the penaeid prawns would have a similar effect on meiofauna. Few trends were noted in the types of food organisms eaten by the various species in different parts of the Gulf of Carpentaria, but *P. esculentus* consumed more

Foraminifera than did the other species. In Moreton Bay, *P. esculentus* also ate mainly Foraminifera (Moriarty 1977).

The content of protein and organic matter in the food varied over about a fivefold range. Without information on feeding rates, it is not possible to say whether those with small amounts were short of food. In the two seasons that were investigated, juvenile *P. merguiensis* contained significantly less nitrogen in the 1977–78 growing period. Staples (1980) has shown that the juveniles in the Norman River grow rapidly from the postlarval stage to a carapace length of about 10–20 mm over the period November–February. A point which could be investigated further, therefore, is whether food might be one of the factors limiting the growth rate of the juveniles and perhaps also the numbers entering the fishery. White (1978) has pointed out that the numbers of many insect populations are regulated by the supply of nitrogen to the juveniles, and as prawns select food with a high nitrogen content, a similar effect might occur.

It is obviously impossible to accurately measure assimilation efficiencies of prawns in their natural environment, but an approximation can be obtained from analyses of foregut and hindgut contents. The values recorded here are minimum estimates. These values were calculated on the assumption that mineral content remains constant. In three of the four groups of prawns analysed, $CaCO_3$ content increased from foregut to midgut, and in all groups it increased in the hindgut (Table 2). This suggests that little, if any, $CaCO_3$ was absorbed. Forster and Gabbott (1971) have discussed this problem in their study of assimilation in two species of carid prawns. They found that 32% of inorganic salts were apparently assimilated, although they could not rule out regurgitation as a possible explanation. In their experiments, however, inorganic matter was only a small percentage of the total and thus a small change would introduce a large error in calculations, whereas in this study, inorganic matter constituted 60–88% of the prawns' diet and it is most unlikely that a large proportion would be assimilated. Thus, errors due to ash assimilation would not be large. Variation in composition of the diet is another source of error, and we have assumed that the average composition over the period of time that food took to pass from foregut to hindgut was similar for a group of prawns caught in one area.

Another factor which would lead to underestimation of assimilation efficiency is the digestive process in the foregut. Much of the digestion in decapods probably occurs in the foregut. Digestive enzymes are secreted dorsally into the foregut and soluble products are filtered into ventral tubes and then pass into the digestive gland (Powell 1974; Dall and Moriarty 1981). We have minimized this problem by selecting prawns with full guts (i.e. those which had been feeding actively at the time of capture). Thus, although digestion would have been proceeding, much of the products would have remained in the frozen pellet of contents that was removed for analysis. The range of values recorded here is only a little less than the range of 52–87%, measured by analysis of food and faeces, for *P. setiferus* and *P. aztecus* that were fed on defined diets and an algal mat (Condrey *et al.* 1972). We may conclude therefore that the prawns are assimilating a high proportion of their diet in the field.

In their studies on the assimilation of various components of food by *Astacus* spp., Speck and Urich (1970) found that most assimilation occurred from the foregut, and only a small amount (about 2–5%) occurred from the midgut. The results that we have obtained with penaeid prawns support their observations (Table 2). In three of the samples, 10% of the assimilation occurred in the midgut, and in one (*P. merguiensis*) no

further food was assimilated in the midgut. These results also agree with Powell's (1974) model for digestive physiology in decapods, as discussed above.

Acknowledgments

We are grateful to J. A. Redfield, J. P. Salini and T. Wassenberg for supplying the adult prawns, and to D. J. Staples, D. J. Vance and D. S. Heales for the juvenile prawns. We thank Drs W. Dall and D. J. Staples for their helpful criticism of the manuscript.

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Manuscript received 18 August 1980, accepted 6 November 1980

B. Muramic Acid and Bacterial Biomass

Oecologia (Berl.) 20, 219—229 (1975)

A Method for Estimating the Biomass of Bacteria in Aquatic Sediments and Its Application to Trophic Studies

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Received May 17, 1975

Summary. A method is described for estimating the biomass of bacteria in aquatic sediments by an enzymic measurement of D-lactic acid derived from hydrolysis of muramic acid. A correlation is shown between muramic acid and biomass. The Gram-negative rod bacteria contain about 20 μg muramic acid/mg carbon whereas the Gram-negative or Gram-variable pleomorphic and Gram-positive bacteria contain about 100 μg muramic acid/mg carbon. Thus to measure biomass, the relative proportions of these bacteria in the population must be measured. The method is limited at present to sediments in which the biomass of blue-green algae is insignificant compared to bacteria. It is particularly suited to measuring the biomass of bacteria in sediments ingested by animals. This is illustrated by analysis of the gut contents of two deposit-feeders, a mullet (*Mugil cephalus*) and a prawn (*Metapenaeus bennettae*), in which it is shown that bacteria are an important component of their diet.

Introduction

Bacteria have an important trophic role in benthic ecosystems, especially in inshore areas. Much of the primary production from macrophytes such as mangroves and sea-grasses becomes available to higher trophic levels after it has been utilised by bacteria (Jorgensen, 1966; Newell, 1970; Fenchel, 1971; Mann, 1972). Studies such as these mostly suggest, rather than state explicitly, that bacteria are the major source of food for deposit feeders. Quantitative studies have been hampered by the difficulty of measuring the biomass of bacteria in sediments. The best available technique has been direct counting with fluorescence microscopy, but this suffers from some drawbacks and inaccuracies (Wood, 1953; Zhukova, 1963; Fenchel, 1971; Hobbie *et al.*, 1972; Dale, 1974; Jones. 1974). The ATP[1] method for measuring total microbial biomass (Holm-Hansen, 1973) is not applicable to the analysis of stomach contents because some ATP may be hydrolysed after ingestion. In the sediments, however, estimates of total microbial biomass may provide an independent check of bacterial biomass measured by the method described below.

While investigating feeding in the mullet *Mugil cephalus* (L.) and the prawn *Metapenaeus bennettae* (Racek and Dall) a method was required to measure the amount of bacteria in terms of carbon, in their food (Moriarty, 1975). Millar and Casida (1970) suggested that muramic acid in soils might be used as a measure

1 *Abbreviations.* ATP = adenosine triphosphate; LDH = lactic acid dehydrogenase; NAD = nicotinamide adenine dinucleotide; tris = tris(hydroxymethyl)aminomethane; C = carbon; M = muramic acid.

of the bacterial population. They showed that within each of the two major types of bacteria, the Gram-negative and the Gram-positive, the amount of muramic acid was closely related to bacterial biomass. This would be expected because muramic acid is an important component of peptidoglycan in the cell wall and occurs nowhere else in the cell. Muramic acid is present in the cell wall of all prokaryotes (bacteria and blue-green algae), except the halophilic bacteria (Rogers and Perkins, 1968).

This suggested a method for measuring bacterial biomass, and a technique has been developed which has given useful results in studying the estuarine sediments ingested by the prawns and mullet. The determination of muramic acid is adapted from Tipper (1968) and strictly speaking, it is D-lactic acid rather than muramic acid which is measured. Muramic acid, liberated from the cell wall by acid hydrolysis, is hydrolysed with alkali to release D-lactate. The lactate is oxidised to pyruvate by D-LDH with the concomitant reduction of NAD which is measured in a spectrophotometer. A disadvantage of the method is that it cannot be used in the presence of large numbers of blue-green algae (see "Discussion").

Methods

Bacteria. Bacteria, obtained from the culture collection in the Department of Microbiology, University of Queensland, were grown on a shaker at 28° or 37° (depending on their optimum temperature). The medium contained peptone 10 g, yeast extract 5 g, NaCl 5 g and distilled water 1 l; pH was 7.5. Bacteria, isolated on agar plates from sea-grass flats, were grown in Zobell's medium 2216E (Zobell, 1946: medium 2216 with 1% yeast extract) at 28°. Cultures were harvested in a centrifuge at 20°, washed twice in 0.5% (w/v) NaCl, or filtered sea-water if they were grown in sea-water medium, and then freeze-dried.

Sediments. Stomach contents were removed from 10 mullet soon after capture on a sea-grass (*Zostera capricorni*) flat in Moreton Bay, Queensland. Sediment was collected from the same area by coring to a depth of about 1.5 cm. About 50 prawns, caught in the Brisbane River estuary at night, were brought on dry ice to the laboratory where their proventriculus contents were removed and combined. A sample of the top 1 cm of mud just below the low water level was collected from the same area. All sediments and gut contents were kept on dry ice after collection and then freeze dried before analysis.

Muramic Acid. The procedure was modified from that of Tipper (1968) [see also Hohorst (1963) for further details]. Reagents: HCl: 3 M. NaOH: 5 M. Na_2HPO_4: 0.5 M. Buffer containing glycine 0.5 M and hydrazine 0.4 M, pH 9.0 (solid NaOH was used to adjust pH). NAD: 20 mg/ml in tris-HCl pH 7.5, 0.02 M. D-lithium lactate: 105.5 μg/ml (*i.e.* 100 μg/ml D-lactate). D-LDH: 1 mg/ml (diluted in tris buffer from stock 5 mg/ml) from Boehringer, Mannheim, W. Germany.

A weighed sample was hydrolysed with HCl (about 1 ml per 200 to 500 mg sediment or 10 to 20 mg bacteria) in a sealed tube at 100° for 6 hrs. After adding phosphate (0.2 ml per ml HCl) the sample was neutralised with hydroxide and centrifuged or filtered to remove particulate matter. At this stage portions of samples were put aside for assay of D-lactate from sources other than muramic acid. More hydroxide was added to raise the pH to 12.5 and the solution was incubated at 35° for 2 hrs. The pH was then reduced to about 8 to 8.5 with HCl and if necessary, it was centrifuged again. All samples were stored at −15° until assayed for lactate.

D-Lactate Assay. To small test tubes in crushed ice the following were added consecutively with mixing after each addition; glycine buffer (kept at 4°) 1 ml; sample 100 μl; NAD 100 μl; LDH 40 μl. Sample volumes were adjusted to contain about 1 to 12 μg D-lactate/100 μl. If they were more dilute than this, up to 300 μl was added to the reaction tube and buffer volume was correspondingly reduced. A duplicate set of sample reaction tubes was set up, but no LDH was added. To test for inhibition of D-LDH, a replicate assay should be carried out with a smaller volume of sample (*e.g.* half that used in the first assy tube). Standards

containing 0, 10, 20, 40, 60 and 120 µl of D-lactate solution were prepared, with complementary alterations to the buffer volume. Replicates were made of all reaction mixtures. After incubation, the reaction tubes were transferred to ice. Extinction at 340 nm was measured in a 1 cm × 1.5 ml cuvette, after allowing 1 minute for the solution to warm up.

The standard curve was almost linear over this range (0–12 µg D-lactate). For each sample, the E_{340} value without LDH was subtracted from that with LDH and the difference read off the standard curve to give µg D-lactate per sample. From this, muramic acid was calculated as shown in the following example for mud from mullet stomachs.

$\Delta E_{340} = 0.04 \pm 0.05 \equiv (0.9 \pm 0.1)$ µg D-lactate; 0.454 g sediment was hydrolysed; final volume 3.1 ml; 0.1 ml in each reaction tube. Molecular weight of muramic acid = 251, and of lactate = 89. $0.9 \times (3.1/0.1) \times (1/0.454) \times (251/89) = 170 \pm 20$ µg muramic acid/g sediment.

Carbon. Carbon was determined using Pregl's dry combustion method (Pregl, 1951; Steyermark, 1961). Magnesium perchlorate was used to absorb water and manganese dioxide for nitrogen oxides. Potassium persulphate was found not to be necessary to give maximum release of carbon (Newman and Tomlinson, 1964). A balance weighing to 0.01 mg was used. The furnace was operated at about 900°. Sediment samples were combusted with and without prior treatment with 1 M HCl to remove inorganic carbon.

Chlorophyll. Chlorophyll *a* was extracted with 90% (w/v) acetone at 4° for 6 hrs. It was estimated as described by Wetzel and Westlake (1969), using a value of 84 for the specific absorption coefficient.

ATP. The method for extraction of ATP was based on that of Holm-Hansen (1972) and measurement of ATP on that of Stanley and Williams (1969). Centrifuge tubes containing 9 ml tris buffer (0.02 M, pH 7.5) and 1 ml potassium phosphate buffer (0.01 M, pH 7.5) were heated in a boiling water bath. Freshly collected sediment was mixed in a small beaker and about 500 mg added to each of twenty tubes. To ten of these tubes, 0.5 ml of an overnight culture of bacteria (isolate no. 3, Table 1) was then added. A further ten replicate extractions were made of the bacteria only. After 5 min in the boiling water bath, tubes were cooled in tap water, centrifuged for 1 min and the supernatants frozen until assayed for ATP. The pellet of sediment was freeze-dried and weighed.

Assay solutions contained 1 ml potassium phosphate buffer (0.01 M, pH 7.5) with magnesium sulphate (4 mM) and 0.1 ml sample or standard ATP in tris buffer. A Nuclear Chicago Scintillation Counter Mark II was used out-of-coincidence.

Firefly extract (20 µl) was added as the print-out of the preceeding sample commenced. Counting was carried out for 0.4 min. Inhibition of the luciferase by material in the samples was monitored by assaying replicates of the sediment samples with an additional 20 ng ATP. Firefly extract and ATP were purchased from Sigma Chemical Co. Ltd., St. Louis, USA.

Composition of Bacterial Populations. Sediments were diluted in sterile sea-water, in ten-fold steps, with vortex mixing at each step. Aliquots of 0.1 ml from the 10^4 and 10^5 fold dilutions were plated on 2216E agar and incubated at 24° for 1 week. Smears from plates having about 25–50 colonies, were Gram stained and examined microscopically. The diluted sediment samples were placed in a boiling water bath for 5 min and further aliquots plated out to count the number of spores.

Blue-Green Algae. About 1 mg samples of freeze-dried sediment were weighed to the nearest 10 µg on microscope slides. Distilled water was added and all blue-green algal cells counted.

Results

Relationship between Muramic Acid Content and Biomass

According to the ratio of muramic acid to total cellular carbon, the bacteria occurring, or likely to occur in aquatic sediments, may be divided into three groups (Table 1). The Gram-negative rods analysed here all have about 20 µg M/mg C. The Gram-negative or Gram-variable, pleomorphic bacteria seem to have a more diverse relationship between muramic acid and carbon. On average however, they have about the same ratio as Gram-positive bacteria and so all are considered as one group with 100 µg M/mg C. As the actinomycetes were not

Table 1. Muramic content of various bacteria

Type of bacteria	μg muramic acid/mg carbon		Number of determinations
	Mean	Range	
Gram-negative rods			
Escherichia coli	20	17–22	4
Enterobacter aerogenes	20	17–22	2
Proteus vulgaris	20	19–20	2
Serratia marcescens	22	20–25	2
Pseudomonas aeruginosa	23	23–23	3
Isolate No. 5	17	—	1
Isolate No. 6	19	15–23	2
Average	20		
Gram-negative or variable, pleomorphic			
Arthrobacter globiformis	85	80–90	2
Isolate No. 1	110	approx. ± 20[a]	4
Isolate No. 2	110	approx. ± 20[a]	4
Isolate No. 3	110	approx. ± 20[a]	4
Isolate No. 4	60	approx. ± 20[a]	4
Gram-positive			
Bacillus subtilis	106	104–110	3
Micrococcus aurantiacus	115	107–123	2
Average	100		
Actinomycetes			
Streptomyces venezuelae	52	52–52	2

[a] Determined graphically: see text.

found in the sediments studied here, only one representative is included, which is shown to have a muramic acid to carbon ratio intermediate between the first two groups.

An apparent inhibition of the D-LDH was noticed in a few samples. When successively smaller volumes of sample were assayed, the amount of D-lactate per unit weight of sample increased to a maximum. This was observed with the Gram-negative pleomorphic bacteria isolated from the sea-grass flat (numbers 1–4, Table 1). A graph of μg M/mg C against volume assayed was plotted and extrapolated to zero volume, to give the mean figures quoted in Table 1. The amount of D-lactate present at low levels of dilution was near the lower limit for detection by this method, hence errors were large. An attempt to remove the interference with an ion exchange resin was unsuccessful.

A formula for calculating biomass from muramic acid derived from the data in Table 1 is:

$$C = \frac{M}{20n + 100p}.$$

Where C = carbon (mg), M = muramic acid (μg), n = proportion of Gram-negative rod bacteria and p = proportion of Gram-positive and Gram-negative or variable

pleomorphic bacteria ($n+p=1$). Muramic acid from other microorganisms and spores is neglected in this formula because they were uncommon in the sediments studied here (see "Discussion").

Effect of Hydrolysis on D-Lactate Recovery

Sediment samples from the stomachs of mullet have been hydrolysed with acid for periods of 4, 6 and 8 hrs. The variation in amount of D-lactate thus obtained was no greater than the variation between replicate samples hydrolysed for 6 hrs. The range of variation is about 10% for samples containing *ca.* 100 to 500 μg M/mg dry weight.

Extending the time of alkaline hydrolysis from 2 to 4 hrs did not alter the amount of D-lactate present.

The presence of sediment had no effect on the recovery of D-lactate (muramic acid) from *B. subtilis* cells added to it (Table 2).

Table 2. Recovery of muramic acid from *Bacillus subtilis* cells added to sediment from a sea-grass flat

Sediment and *B. subtilis* were mixed before acid hydrolysis. The range of variation for 3 determinations is shown.

Sample	Weight (mg)	Muramic acid (μg/g)	μg M in sample
Sediment	488	21 (±10)	10 (±5)
B. subtilis	2.29	41000 (±2000)	94 (±4)
Combined (expected recovery)			104 (±9)
Combined (actual recovery)			103 (±10)

Controls for Other Sources of D-Lactate

No D-lactate was found in samples which were hydrolysed in acid only. Sediments from sea-grass flats, mullet stomach and prawn proventriculus were tested.

Application to Trophic Studies

Substantial quantities of muramic acid were found in the sediments ingested by the mullet and prawns (Table 3). Muramic acid was also present in the sediments in the localities from which these animals were caught, but as the amounts were small and near the lower limit for detection, errors were proportionately large. The proportion of Gram-negative rods to Gram-negative pleomorphic and Gram-positive bacteria were determined from 4 plates with a total of 126 colonies, for the sea-grass flats and 2 plates (58 colonies) for the Brisbane River. Spores numbered about 1 per 6000 colonies in both localities.

The bacterial biomass thus derived is compared to algal biomass and total organic carbon in Table 4. In the sediments from both localities, bacterial biomass is greater than algal. It should be noted, however, that the algae (mainly diatoms) occur mostly on the surface, whereas these cores were taken to a depth of about 1.5 cm. Blue-green algae numbered less than 1×10^4 cells/g dry weight of the sediment in the stomachs of these mullet. The mullet have concentrated

Table 3. Muramic acid in sediment from various sources and equivalent bacterial biomass

The range of variation in assay of muramic acid is shown. The proportion of Gram-negative rods in the bacterial population ingested by the animals is assumed to be the same as that in the sediments of the locality of capture.

Sediment source	Muramic acid μg M/g ash	Proportion of gram-negative rods	Bacterial biomass mg C/g ash
Sea-grass flat	20±10	0.70	0.4
mullet stomach	170±20		3.8
Brisbane River	80±20	0.86	2.6
Prawn proventriculus	4415±200		142

Table 4. Examples of the application of the muramic acid method for measuring bacterial biomass

Bacterial biomass was measured as in Table 3. Algal carbon was measured as chlorophyll $a \times 30$.

Sediment source	Total organic	Algal	Bacterial	Bacterial C
	mg C/g ash			% of total C
Sea-grass flat	2.4	0.2	0.4	16
Mullet stomach	16.4	6.3	3.8	23
Brisbane River	19.4	1.0	2.6	14
Prawn proventriculus	456	0	142	31

Table 5. Comparison with ATP method for microbial biomass

Sediment, mainly sand, was from the top 1 cm of two different sea-grass flats. Total microbial C was calculated as ATP × 250; algal C as chlorophyll $a \times 30$; bacterial C as muramic acid/44. The range of variation due to experimental error is shown, except for ATP, where the standard error of 10 replicates is given.

Sample number	Total organic C	Algal C	Bacterial C	Total microbial C
	mg C/g ash			
1	2.7 (±0.1)	0.24 (±0.01)	0.47 (±0.25)	0.5 (±0.05)
2	2.1 (±0.1)	0.10 (±0.01)	1.3 (±0.3)	1.2 (±0.1)

the organic carbon, including bacteria by about 1 order of magnitude from the sediment. The prawns show much greater selectivity. They have selected not only organic from inorganic matter, but organic matter containing a high proportion of bacteria.

Comparison of ATP and Muramic Acid Methods

Two samples of sea-grass sediment, collected at different times from that in Table 4, have been analysed for ATP as well as the other components (Table 5).

The estimate of total microbial biomass thus obtained is only a little less than the combined algal and bacterial biomass. Addition of cultured bacteria containing a known amount of ATP to the sediments showed that ATP recovery was probably complete. Inhibition of the luciferase reaction by material in the extracts did not exceed 5%.

Discussion

Correlation of Muramic Acid with Biomass

The method reported here for determining bacterial biomass is dependent on a reasonably close correlation between muramic acid and total carbon in a cell. Since muramic acid has a key role in the cell wall and occurs nowhere else (Rogers and Perkins, 1968), its amount in the cell will be directly related to cell size and thus carbon. Some variation is expected of course, in the constituents of a cell during growth, but this would not be great. What does matter though, is whether the ratio of muramic acid to carbon varies greatly between different bacteria. From the data presented here, it seems that the bacteria can be divided into two main groups, those with 20 μgM/mgC and those with 100 μgM/mgC. The variability encountered with the Gram-negative or variable, pleomorphic bacteria may well be due to experimental problems rather than an actual variation in proportions of muramic acid. Some of these bacteria are characterised in young colonies, by the presence of rods and in older colonies by rods and cocci of variable size. This suggests they may be *Arthrobacter* spp.

A study of muramic acid levels with cell-cycle in the *Arthrobacter*-like organisms and methods for purifying D-lactate are under investigation. This present problem does not detract from the usefulness of the method for showing whether bacteria are an important component in the diet of deposit feeders. For example in the sediment from the mullet stomachs, the uncertainty in muramic acid content of the pleomorphic bacteria contributes at the most, a variation of ± 0.5 mgC/g ash.

Escherichia coli and the other Gram-negative bacteria were selected partly for comparison with the data of Millar and Casida (1970). In terms of dry weight, they found about half the amounts reported here of muramic acid in the Gram-negative bacteria and about one-third the amounts in Gram-positive bacteria. There are many differences in technique which might contribute to the different results.

Factors Affecting D-Lactate Measurement

Tipper (1968) has shown that losses during assay of D-lactate are less than 7% and racemisation less than 3%. He demonstrated complete recovery of muramic acid after acid hydrolysis for 18 hrs with other cell wall constituents. Although 4 hrs hydrolysis in acid seems to be sufficient, 6 hrs has been chosen here to ensure complete hydrolysis.

Alkaline hydrolysis at 37° for 2 hrs was shown by Tipper (1968) to be sufficient for maximum release of D-lactate from muramic acid.

Sediment does not adsorb or otherwise depress the recovery of muramic acid or D-lactate from bacteria as shown by the results reported here.

The possible inhibition of D-LDH by material in the extracts needs to be checked as described above.

Controls for Other Sources of D-Lactate

Experiments showing that no D-lactate was formed until extracts were hydrolysed in alkali, provide strong presumptive evidence that muramic acid is the only source of D-lactate. Tipper (1968) found that no D-lactate was released from other cell wall constituents. He showed also that the amounts of muramic acid measured in cell walls of various bacteria as D-lactate were similar to the amounts detected directly by amino acid analyser. As D-lactate has been reported in various organisms (*e.g.* Warburg *et al.*, 1957; Camien *et al.*, 1963; Long and Kaplan, 1968) the control described here should be used. In the examination of sediments or gut contents, it is unlikely that D-lactic acid from sources other than muramic acid would be present in concentrations of the same order of magnitude. Where actual amounts of D-lactate per unit weight of sample have been given (*e.g.* Camien *et al.*, 1963), they are several orders of magnitude lower than those derived from muramic acid in cell walls. Although Camien *et al.* (1963) argued to the contrary, it seems from later work (especially Tipper, 1968), that the D-lactate they found in bacterial cell walls was derived from muramic acid.

Composition of Bacterial Populations

The proportions of the main groups of bacteria shown in Table 1 need to be determined for the environment under study. For greatest accuracy in a heterogeneous population of bacteria, the main types should be isolated, characterised and their ratio of muramic acid to carbon measured. The simple counting procedure used here is probably sufficient for most purposes. It is assumed that the ratio of numbers of heterotrophs which form colonies on plates, is in fact equivalent to the ratio of total biomass of the different types of bacteria. The reasonably close correlation between total microbial biomass (ATP) and combined bacterial and algal biomass (Table 5) would suggest that this assumption may be justified. Further work is needed to check the validity of this assumption. Studies of aquatic and sediment bacterial populations in other localities (Wood, 1967; Hodgkiss and Shewan, 1968; Stevenson *et al.*, 1974) indicate that Gram-positive bacteria may be more numerous than found here. At this stage a detailed study of bacterial populations has not been undertaken and it is possible that estimates of Gram-positive bacteria in the present study were low and thus the biomass from muramic acid data has been overestimated. If Gram-positive bacteria made up 20% of the population in the sea-grass mud flat, for example, the bacterial biomass in the mullet stomachs would be 3.0, rather than 3.8 mgC/g ash. In other words, uncertainty of the exact composition of the bacterial population does not greatly influence the usefulness of this method.

Simple methods for characterising bacterial populations using selective media (Pratt and Reynolds, 1974) may be useful here. Crystal violet cannot be used for counting marine Gram-negative bacteria because, unlike soil bacteria, their growth is inhibited by it (Pratt and Reynolds, 1973).

The formula given here is applicable to environments were bacterial spores and blue-green algae are uncommon. If a locality is being studied in which it is found that spores are numerous, an allowance may be made for them in the formula since they contain about 3.5 times more muramic acid than Gram-positive bacteria (Millar and Casida, 1970). Obligate anaerobic bacteria and their spores are probably uncommon in marine muds (Wood, 1953).

Blue-Green Algae

Blue-green algae contain muramic acid in their cell walls (Rogers and Perkins, 1968). The cell size and thickness of their peptidoglycan layer is generally greater than that of the common Gram-negative bacteria in water (Carr and Whitton, 1973). As a rough estimate, blue-green algae may contain 500 times more muramic acid per cell. Calculation shows, therefore, that when blue-green algal cells number less than about 10^7/g sediment, their muramic acid contribution would be negligible (about 2 μg M/g).

Comparison with Other Methods

Direct counting of bacteria is probably the best alternative method with which to compare the muramic acid method. Wood (1953) counted about 10^9 bacteria/ml in estuarine mud. Dale (1974), using direct counts to study bacteria in intertidal sediments of Nova Scotia, has found from 1.17×10^8 to 9.97×10^9 bacteria/g dry sediment. Assuming the dry weight of each bacterial cell to be 2.2×10^{-13} g, he has transformed his data to biomass. The above figures become 2.6×10^{-2} mg to 2.2 mg/g dry sediment or about 1.5×10^{-2} to 1.0 mg C/g dry sediment, which is in good agreement with the data presented here. Dale comments that his estimates may be low because of losses in the technique and because only objects clearly distinguishable as bacteria were counted. In addition, he faced a considerable statistical problem in counting about 500 bacteria in a population of 10^8 to 10^{10}/g.

Bacteria can vary by more than one order of magnitude in size (Luria, 1960), so converting from numbers to biomass is not accurate, unless counting is made even more tedious by estimating size as well. The advantages of the muramic acid method over counting are that bacteria do not have to be removed from surfaces on which they might be adsorbed; they are readily distinguished from inert particles and micro-flagellates, and biomass is estimated directly.

The muramic acid, or more precisely D-lactic acid, is presumed to occur in living cells only (at least near the surface of the sediments). It is assumed that most bacteria either divide or are eaten, rather than die, or if they die, autolysis and decomposition by other bacteria rapidly degrade the cell walls. This assumption is difficult to test, but there are reports that the surface layers of the sediments are turned over constantly by the deposit-feeding animals (Odum, 1970; Whitlatch, 1974). Ciliates are presumed to be active in keeping bacteria in the growth phase (Lackey, 1967).

That the muramic acid method described here provides a reasonable estimate of bacterial biomass is substantiated by the comparison with total microbial and algal carbon (Table 5). If much muramic acid were derived from cell wall fragments, a large discrepancy would be expected between the results of these two methods. Biomass of protozoa is unknown, but is included by the ATP method for total microbial biomass. It is unlikely to be as high as the algal or bacterial biomass. The ATP method probably provides a minimum estimate of bacterial biomass, since losses during processing can occur easily. Cells in a dormant or resting state may contain lower amounts of ATP. The muramic acid method provides a maximum estimate, as some cell wall fragments may well be present. For studying gut contents, the ATP method cannot be used except to provide a minimum figure for microbial biomass. The muramic acid method

was developed for, and is particularly suited to, measuring bacterial biomass in gut contents. Losses are unlikely to occur during the initial stages of digestion and bacteria are present in large numbers in gut contents of deposit-feeders.

Application

The large amount of muramic in the gut contents of the animals investigated here shows that bacteria are important in their diet. There were less than 1×10^4 cells of blue-green algae per g sediment in the mullet gut and thus all the muramic acid is bacterial. In the sediments from the localities where the animals were caught, the concentration of muramic acid was near the lower limit of sensitivity of the assay. Errors, therefore, were large in proportion. Future improvements in the procedure may enable measurements of smaller quantities, thus making the method more widely applicable.

As mentioned above, a detailed study of the bacterial populations in these sediments was not undertaken. The conversion of muramic acid values to biomass is, therefore, an approximation (Table 3). In these prawns, bacteria and detritus were the only food. It has been found in prawns from a similar area, that muramic acid relative to ash in the intestine is about 60% of the amount in the proventriculus, which indicates that bacterial cell walls are digested (Moriarty, 1975). This conclusion is supported by laboratory studies with ^{14}C-labelled bacteria (Moriarty, 1975). Algae (mainly diatoms) as well as bacteria are the main food items in this group of mullet.

Acknowledgements. This work was carried out during tenure of a Queen's Fellowship in Marine Science awarded by the Australian Government. Professor V. B. D. Skerman, Department of Microbiology, and Professor J. M. Thomson, Department of Zoology, kindly provided facilities. I am also grateful to Mr. J. Kent, Department of Chemistry, for assistance with apparatus needed to measure carbon and to Dr. L. Sly, Curator, Culture Collection, Department of Microbiology.

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Oecologia (Berl.) 26, 317–323 (1977)

Oecologia

Improved Method Using Muramic Acid to Estimate Biomass of Bacteria in Sediments

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Summary. A method, which depends on the measurement of muramic acid content to estimate bacterial biomass, has been improved in sensitivity by two orders of magnitude. It is now applicable to any aquatic sediment, whereas previously it was mainly useful in the analysis of gut contents of deposit-feeding animals. Reduced NAD, a product of the oxidation of D-lactate derived from muramic acid, is assayed using bacterial luciferase. The amount of muramic acid in a number of terrestrial and marine bacteria was measured, and found to be lower than that obtained with the previous, less specific, assay procedure. The muramic acid content of a blue-green alga has been measured, thus allowing blue-green algae to be taken into account when estimating bacterial biomass. Experimental evidence is presented which shows that muramic acid in cell wall fragments of bacteria is rapidly degraded by microorganisms in a marine sediment.

Introduction

A correlation between muramic acid and carbon content of bacterial cells has been used to estimate biomass of bacteria (Moriarty, 1975). The method was developed to measure the importance of bacteria in the food of detritus-feeding animals, but was not very sensitive when applied directly to sediments. Another assay for D-lactate, which is more than 2 orders of magnitude more sensitive, is described below. The new assay has been tested with different types of bacteria (Gram negative and Gram positive) of marine and terrestrial origin and a blue-green alga. It was found that the content of muramic acid in the bacteria was less than that reported previously (Moriarty, 1975). Possible reasons for this discrepancy are discussed below. A new formula for estimating the amount of bacterial carbon in surface marine sediments is given in this paper.

One of the main assumptions made in applying this method, was that muramic acid in cell wall fragments of dead bacteria would not persist for long in sediments. To test this, cells walls of *Bacillus subtilis*, labelled with ^{14}C, were added to a marine sediment and the disappearance of ^{14}C-muramic acid was measured.

Materials and Methods

Microorganisms. The terrestrial bacteria were obtained from the Culture Collection, Department of Microbiology, University of Queensland. The marine bacteria were isolated from the top 1 cm of sediment on a sea-grass flat in Moreton Bay, Queensland. All bacteria were cultured as described previously (Moriarty, 1975). The blue-green alga, *Oscillatoria tenuis*, was obtained as an axenic culture from the Culture Collection of Algae, Indiana University, and grown on a solid medium (Allen, 1968). Filaments were carefully removed and freeze-dried.

Growth Phase Experiment. Two of the marine isolates (Nos. 6 and 11 in Table 1) were grown in 1 l of medium, aerated by bubbling, at 30°. Growth was monitored by optical density increase at 600 nm. Samples were withdrawn during the exponential phase and about 2 h after the start of the stationary phase of growth. Adenosine triphosphate was measured on 1 ml samples collected on a 0.45 μm filter as described by Hamilton and Holm-Hansen (1967). For muramic acid and carbon determinations, 200 ml samples were centrifuged at $10{,}000 \times g$ for 5 min, washed once in 100 ml of filtered sea-water and freeze-dried.

Carbon. Carbon was measured as described previously (Moriarty, 1975).

Muramic Acid. Hydrolysis was carried out as described previously (Moriarty, 1975).

D-*Lactate Assay.* The procedure is an adaptation of that described by Noll (1974) for L-lactate, in which the pyruvate is trapped as alanine by glutamate-pyruvate transaminase. The reduced NAD[1] is assayed with bacterial luciferase (Stanley, 1971). These references should be consulted for full details on the preparation and stability of reagents and other procedural aspects, although a few modifications have been made. It has not been possible to couple the two reactions in one vessel.

A small volume (up to 100 μl) of sample solution was placed in a small test tube. Distilled water was added to bring the volume up to 100 μl where necessary. Each test tube contained about 10 ng to 200 ng of D-lactate. A set of standards covering this range was also prepared, using lithium D-lactate. Immediately before use, a volume of the following reagents, sufficient for the number of samples desired, was prepared.

Glutamate buffer: 0.3 M, pH 9.0	0.5 ml/assay
NAD: 33 mg/ml in H_2O	10 μl/assay
D-LDH: 5 mg/ml	1 μl/assay
GPT: 10 mg/ml (80 units/mg)	0.5 μl/assay.

From a dispensing bottle 0.5 ml were mixed rapidly into each sample tube. The tubes were placed in a water bath at 30° for 15 min and then transferred to crushed ice. A volume of the following reagents, sufficient for triplicate analyses of each of the above samples, was prepared and equilibrated at 29°.

Phosphate buffer: 0.1 M, pH 7.5	2 ml/assay
2-mercaptoethanol	14 μl/assay
FMN: 5 mg/10 ml H_2O	10 μl/assay
Dodecyl aldehyde: saturated in ethanol	10 μl/assay.

Dodecyl aldehyde was suspended in ethanol and centrifuged immediately before use. Bacterial luciferase was prepared in phosphate buffer (1.5 mg/ml) with an equivalent amount of bovine serum albumen and kept on ice. Any material not dissolved after 15 min was removed by centrifuging at $3000 \times g$ for 5 min at 0°. The sensitivity of the assay depends on the amount of luciferase used; thus if samples contained about 10 to 50 ng lactate, 100 μl of this enzyme solution were used. For samples

[1] *Abbreviations:* ATP = adenosine triphosphate; FMN = flavin mononucleotide; GPT = glutamate pyruvate transaminase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2); D-LDH = D(−)-lactic acid dehydrogenase (D-lactate: NAD oxidoeductase, EC 1.1.1.28); MA = muramic acid; NAD = nicotinamide adenine dinucleotide (oxidised form); NADH = nicotinamide adenine dinucleotide (reduced form)

with 50 to 200 ng lactate, 50 µl were sufficient. Mercaptoethanol and bovine serum albumen stabilise the bacterial luciferase (Hastings and Gibson, 1963).

Two ml of the phosphate mixture were dispensed into a small polypropylene vial and luciferase was added and mixed. Then noting the time, 100 µl of the lactate reaction mixture were added. The tube was capped, mixed and placed in a scintillation vial. The counting sequence was started precisely 10 s (or a similar, reproducible interval) later. The scintillation counter (Packard Model 2250) was set up as follows: time 0.2 min; gain 100%; window 110–150; coincidence switch off.

Decomposition of Cell Walls. Bacillus subtilis cell walls, labelled with ^{14}C, were prepared as described elsewhere (Moriarty, 1976). A slurry of surface sediment was obtained from a mangrove area on Moreton Bay, Queensland. About 2 mg dry weight with an activity of about 1×10^6 cpm, were vortex mixed with 10 ml of mud for 30 s. Three ml were removed immediately and frozen. A piece of filter paper, moistened with 20% w/v KOH, was placed in a cup inside the flask of sediment. The flask was shaken at 30°. The filter paper was replaced at intervals, in order to monitor $^{14}CO_2$ release. After 24 h and 48 h, further 3 ml aliquots were removed and frozen. These samples were freeze-dried and then hydrolysed to form D-lactate from any muramic acid present. Each of the hydrolysates was subdivided. In one half of each, the D-lactate was converted to pyruvate, using D-LDH and 3-acetylpyridine NAD (Maurer and Poppendiek, 1974). D-LDH was then denatured by the addition of 1 M HCl to pH 2. Sodium citrate (0.5 M) was added, raising the pH to 6.0, followed by 1 mg/5 ml of pyruvate decarboxylase (2-oxo-acid carboxylyase, EC 4.1.1.1). Filter paper, moistened with KOH, was placed in a cup in the flask in order to collect $^{14}CO_2$ released from pyruvate. The flask was then sealed and shaken at 30° for 1 h. The reaction was terminated by injecting 2 M H_2SO_4 into the flask, and after shaking for a further 1 h, the paper was removed. Filter papers were counted by liquid scintillation, with Triton X100 in the scintillant fluid (Turner, 1971). As a control to allow for $^{14}CO_2$ from sources other than ^{14}C-muramic acid, the other half of each hydrolysate was treated as above, but D-LDH was omitted.

Bovine serum albumen, bacterial luciferase and pyruvate decarboxylase were obtained from Sigma Chemical Co., St. Louis, USA; D-LDH and GPT from Boehringer, Mannheim, F.R.G.; and dodecylaldehyde from Koch Light Labs., Colnbrook, U.K.

Results

The Gram negative bacteria, both marine and terrestrial, differ by about two fold in the ratio of muramic acid to mgC (Table 1). Much of this difference is due to variation in the sizes of the cells. The marine organisms and *Pseudomonas fluorescens* and *Proteus vulgaris* all have about the same amount of muramic acid per unit surface area. *Escherichia coli, Enterobacter aerogenes* and *Serratia marcescens* have about twice as much, which suggests that their murein layer is twice as thick. These results should be interpreted with caution, however, because it was difficult to measure the cell diameters accurately and it was assumed that the cells were perfect cylinders. The Gram positive bacteria vary considerably in their content of muramic acid. Of the marine bacteria, organism No. 11 (a *Bacillus*) stained strongly Gram positive and has a high content of muramic acid, whereas organisms 9 and 10 were Gram negative except in the early stages of growth when they were Gram positive. Isolate No. 9 exhibited a change in morphology during the cell cycle, with rods present during the exponential phase and cocci during the stationary phase.

The ratio of muramic acid to mgC in the blue-green alga, *Oscillatoria tenuis*, is similar to that of the Gram negative bacteria. Its murein layer is much thicker, however, as it has about 10 times as much muramic acid per unit area (Table 1). The amount of muramic acid per cell was measured by counting the number

Table 1. Muramic acid content of some terrestrial and marine bacteria. Range of variation: ±10% for 3 determinations each. Bacteria were harvested during the exponential phase of growth, except the coccoid form of No. 9 which was obtained during stationary phase. The ratios of muramic acid to surface area are an approximate calculation from the values of MA/mgC, using cell diameters measured in a phase contrast microscope, and assuming specific gravity is 1.1; C is 45% of dry weight, and dry weight is 20% of wet weight

Terrestrial bacteria	μgMA/ mgC	fgMA/ μm²	Marine bacteria	μgMA/ mgC	fgMA/ μm²
Gram negative			Gram negative		
Escherichia coli	10	0.30	Isolate No. 1	10	0.15
Enterobacter aerogenes	10	0.30	Isolate No. 2	9	0.13
Serratia marcescens	10	0.27	Isolate No. 3	9	0.15
Proteus vulgaris	10	0.15	Isolate No. 4	7	0.15
Pseudomonas fluorescens	7.5	0.15	Isolate No. 5	7	0.13
			Isolate No. 6	6	0.13
			Isolate No. 7	5	0.13
			Isolate No. 8	5	0.13
Gram positive, pleomorphic			Weak Gram positive or variable		
Arthrobacter globiformis	27	0.42	Isolate No. 9 (rod form)	13	0.20
			Isolate No. 9 (cocci)	16	0.20
			Isolate No. 10	14	0.35
Gram positive			Gram positive		
Bacillus subtilis	40	0.90	Isolate No. 11 (*Bacillus* sp.)	44	1.10
Micrococcus aurantiacus	57	1.00			
Blue-green algae					
Oscillatoria tenuis	11	1.60			

Table 2. Comparison of biomass and numbers with muramic acid and ATP content of two marine bacteria during growth. Range of variation in C, MA and ATP: ±5%; pseudomonad viable counts: ±20%; total counts are given for the *Bacillus*, but these are approximate because the cells occurred in pairs or short chains, and clumped together

Growth phase	*Bacillus* sp.		Pseudomonad	
	Exponential	Stationary	Exponential	Stationary
Number/ml	3×10^8	1×10^9	8×10^8	2×10^9
Carbon mg/cell	3×10^{-10}	3×10^{-10}	1×10^{-10}	1×10^{-10}
Muramic acid				
μg/mgC	44	39	6.5	4.7
μg/cell	1.4×10^{-8}	1.1×10^{-8}	6×10^{-10}	5×10^{-10}
ATP				
μg/mgC	3	2.8	5	5
μg/cell	3×10^{-9}	3×10^{-9}	0.5×10^{-9}	0.5×10^{-9}

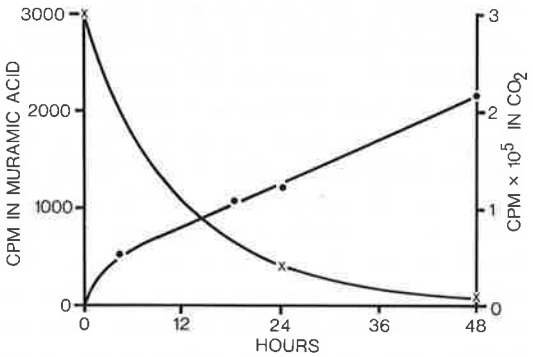

Fig. 1. Decomposition of ^{14}C labelled *Bacillus subtilis* cell walls in a marine sediment, monitored by the release of $^{14}CO_2$ (●) and the decrease in ^{14}C-muramic acid (×). ^{14}C-muramic acid was measured by enzymic release of $^{14}CO_2$ from the carboxyl group on its D-lactate moeity (see "Materials and Methods")

of cells in a portion of a sample that was subsequently assayed for muramic acid. A value of 1×10^{-7} µg MA cell was obtained (standard error: $\pm 0.2 \times 10^{-7}$, 10 counts).

The relationship between muramic acid and ATP content with biomass of two different marine bacteria was measured at two stages in the growth cycle (Table 2). It can be seen that the muramic acid content falls after the exponential phase, but the small change would have little effect on biomass estimation. The values calculated from the number of cells present, are given for comparison with enumeration methods for estimating biomass, but because it was difficult to obtain accurate numbers, these values are only approximate.

The cell walls of *Bacillus subtilis*, when mixed with a sediment, are metabolised by the microorganisms present (Fig. 1). In particular, about 90% of the muramic acid in the labelled wall material was degraded within 24 h. The $^{14}CO_2$ from pyruvate in the controls was about 10% of that from muramic acid at zero time.

Discussion

Assay Procedure. The values for the ratio of muramic acid to carbon (Table 1) are in general about half of those reported previously for the same bacteria (Moriarty, 1975). The method described above for the assay of D-lactate is much more sensitive than that used previously. It is also specific for NADH, whereas previously any reaction leading to an absorbance change at 340 nm would interfere. The discrepancy probably is due to other reactions in the complex bacterial hydrolysate which cause a change at 340 nm. Such changes could be caused by impurities in the D-LDH preparation. The values reported above have been checked by the use of internal standards (NADH and D-lactate). They agree reasonably well with values for muramic acid per unit dry weight of cells given by Millar and Casida (1970).

Precision in pipetting and reaction timing are essential for reproducible results with bacterial luciferase. To avoid quenching of light output and inhibition of this enzyme, the amount of hydrolysed bacteria in the LDH-GPT reaction mixture should not exceed about 100 µg dry weight per 0.5 ml. This can be checked by using internal standards or two different volumes of bacterial hydrolysate.

Two different volumes (say 20 µl and 100 µl) of each hydrolysed sediment sample should also be assayed, to check whether the various enzymes are being inhibited. If inhibition is occurring, the samples should be diluted.

The ultimate sensitivity is limited to about 10 ng lactate per assay. It could be improved by purifying further the commercial enzyme preparations and by using a scintillation spectrometer which can integrate all or most of the light flash from the luciferase. At low levels of NADH, the light flash decays within a few seconds. Much larger amounts of lactate can be measured by the technique described here. I have measured from 200 to 1 µg of D-lactate per assay by using twice the concentration of D-LDH and GPT, incubating for 20 min at 30° and by using 20 µl of luciferase. With larger amounts of D-lactate and thus NADH, the light flash reaches a peak more slowly. It is necessary to check, therefore, that the time delay between adding the final solution and starting the counting sequence is longer than the time taken for the light flash to reach a peak in all samples. With this system, it is better to integrate a portion of the decay period of light emission, rather than the initial increase in light emission.

Biomass Estimation. As pointed out previously (Moriarty, 1975) only an approximate indication of the proportions of the main groups of bacteria present in a natural environment is needed to give a reasonable estimate of biomass. The formula given previously (Moriarty, 1975) must be modified according to the values in Table 1. In marine sediments, if most of the bacteria are Gram negative or weak Gram positive (with low muramic acid content), a combined value of about 12 µg MA/mgC for these organisms would be sufficiently accurate for estimating biomass. Thus the formula should be modified for this environment to:

$$C = 1000\ \mathrm{MA}/(12n + 40p)$$

where n is the proportion of Gram negative or weakly Gram positive bacteria and p is the proportion of strongly Gram positive bacteria.

For surface marine sediments, where strongly Gram positive organisms (with a high content of muramic acid) are probably not abundant, a value of 15, substituted for the denominator, would accommodate some variation in population composition and includes blue-green algae. Blue-green algae could be counted using a fluorescent microscope with epi-illumination, and a corresponding estimate of their muramic acid content deducted from the total before applying the above formula. For example, if coccoid cells 6 µm in diameter are present, and it is assumed their murein layer is about the same thickness as *Oscillatoria tenuis*, it can be calculated that each cell would contain about 2×10^{-7} µg muramic acid. There is a possibility that the electron microscope could be used to obtain a better indication of the proportion of Gram negative bacteria in a sediment population. Weibull (1975) examined thin sections of bacteria concentrated from lake water and found that the majority were Gram negative.

Bacterial biomass has been measured in the food of two deposit-feeding animals (Moriarty, 1976). Although the method used to measure muramic acid overestimated the amount present, the ratio of bacterial carbon to total organic carbon is still about the same.

Muramic Acid Decomposition. In using muramic acid to estimate biomass of bacteria in sediments, I assumed that muramic acid in cell wall fragments from dead bacteria did not persist for long (Moriarty, 1975). The experiment described above (Fig. 1) shows that microorganisms in the sediment rapidly degrade muramic acid. The actual amount of wall material added to the sediment was large in comparison to the size of the living population, and yet most of the muramic acid was utilised within 24 h. Autolytic enzymes, inactivated in the above experiment, would be expected to increase the rate of cell wall decomposition. Harrison and Mann (1975) in an experimental study on sea-grass decomposition, have shown that protozoa graze heavily on the bacterial population. As argued earlier (Moriarty, 1975), this should reduce the proportion of senescent bacteria and thus cell wall fragments. Thus it is unlikely that muramic acid in cell wall fragments is a large source of error in the estimation of biomass.

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Received September 13, 1976

Reprint from
Microbial Ecology
Edited by M. W. LOUTIT and J. A. R. MILES

Springer-Verlag
Berlin Heidelberg New York

Estimation of Bacterial Biomass in Water and Sediments Using Muramic Acid

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A. Introduction

In many aquatic sediments, and the gut contents of animals which feed on them, techniques for estimating bacterial biomass do not give accurate results. This led me to develop a method for estimating biomass by using the relationship between muramic acid and carbon in bacteria (1-4). I have experienced a number of problems with both the assay procedure and the principle of the method and modifications are described which overcome these difficulties.

B. Assay Procedure

I. Acid hydrolysis. After hydrolysis with 3M HCl in sealed tubes at 100°C for 6 hours neutralisation with NaOH is possible, but HCl is better removed by evaporation in vacuo, thus reducing the final salt concentration. Most sediments can be dried before adding HCl but carbonate sediments must be degraded with 6M HCl until all CO_2 is driven off. A pH meter which reads negative pH can be used to adjust the HCl concentration to about 3 or 4M. Bacteria in water can be assayed after trapping the cells on 0.2 µm.Nuclepore polycarbonate filters which do not degrade during hydrolysis. Filters made of cellulose esters cannot be used because they release a substance which reacts with D-LDH.

II. Removal of cations. Divalent cations, especially Ca^{++}, inhibit bacterial luciferase and prevent extraction of D-lactic acid into ether. In siliceous sediments these cations can be removed by adjusting the pH to about 7.5 - 8.0 with NaOH and Na_2HPO_4 as described (1). Precipitates are removed by centrifugation. For coral reef sediments, Na_2HPO_4 (0.5M) should be added until no further Ca^{++} is precipitated. This should be checked after centrifuging and adjusting the pH to 8.0 with NaOH. All samples are then freeze-dried.

III. Ether extraction. Dry samples are extracted with diethyl ether acidified with concentrated HCl (0.2% v/v) to remove D. lactate liberated by acid hydrolysis and other compounds such as glycollic acid. After drying to remove excess ether, samples are redissolved in water and pH adjusted to 7.5 - 8.0 (NaOH). Half of each sample is then frozen until required, for assay of residual D-lactate (or any compound which reacts with D-LDH (D-lactate dehyrogenase) to reduce (NAD).

IV. Alkaline hydrolysis. To liberate D-lactate from muramic acid the pH of the other half of the sample is adjusted to 12.5 and incubated at 35° for 2 hours and the pH reduced to 9.0. The presence of muramic acid can be confirmed by running a thin layer chromatogram after the ether extract and assaying the region known to contain muramic acid (5). This procedure is necessary in samples containing much carbohydrate, as some carbohydrates can be degraded by alkali to D-lactate. An assay technique which does not use alkaline hydrolysis is desirable; gas-liquid chromatography (6) is being investigated.

V. Enzymatic assays. These are carried out as described (3), with the following modified reaction mixtures:- D-Lactate assay: Glutamate buffer 0.1M, pH 9.4-

0.5ml/assay; NAD, 33mg/ml in H_2O - 10µl/assay; D-LDH, 5mg/ml - 2µl/assay; GPT, (glutamate-pyruvate transaminase) 10mg/ml (80 units/mg) - 1µl/assay. NADH assay: Phosphate buffer, 0.1M, pH 7.5 - 2ml/assay; FMN, 5mg/10ml H_2O - 10µl/assay; Tetradecyl aldehyde, saturated in ethanol - 10µl/assay; Bacterial luciferase and bovine serum albumen, 1mg each/ml - 20µl/assay; D-lactate assay mixture - 0.5ml/assay. Three tubes per sample are prepared, one after acid hydrolysis, one after alkali hydrolysis and one after alkali hydrolysis plus 50 ng D-lactate as an internal standard. Activity of D-LDH and/or GPT may be less than specified by the manufacturer, perhaps due to high temperature during shipping, so an increase in incubation time to 60 minutes is sometimes necessary. Incomplete D-LDH reaction can be detected as an increase in the slope of a regression line for the lactate standards, when these are measured at intervals of about 30 min during storage in an ice bath.

An increase in intercept of a standard curve of lactate has been observed with some batches of D-LDH and GPT after placing the reaction tubes in an ice bath caused by contaminating glutamate dehydrogenase (GlDH), and can be stopped by briefly freezing and thawing the reaction tubes after the D-LDH incubation. Preferably, batches of D-LDH having no GlDH activity, and GPT with less than 0.0001% of GlDH should be used so that blank values for lactate will be less, thus giving greater sensitivity. Boehringer Mannheim will supply details of activity for each batch of enzyme they manufacture. To obtain precise results with bacterial luciferase, it is necessary to centrifuge for about 15 min at 3,000 x g and $0^{o}C$, to remove any solid material. Precise time intervals between adding luciferase and D-lactate mixture and starting the counting sequence are necessary.

C. Principle of the Method

The method depends on measuring muramic acid (MA) and using an empirical formula to convert to biomass. It is assumed that all gram positive bacteria have a ratio of 44µg MA/mg C and all gram negative bacteria, 12µg MA/mg C (3). There is however, no sharp distinction in the ratio of muramic acid to carbon between these groups of bacteria, and these amounts have been determined for only a few bacteria. Further,if an arbitrary assumption is made about the proportions of gram negative and gram positive bacteria in natural populations, biomass estimates may be inaccurate, especially in anaerobic sediments when gram positive bacteria predominate. This problem has been overcome by combining muramic acid determinations with direct counts on portions of homogenised sediment. Using direct counts to give an independent estimate of biomass, muramic acid values can be used to calculate the proportion of gram positive bacteria (with thick cell walls) in a natural population. Some examples are given in Table 1, and more information will be published elsewhere. These estimates may not be accurate, because they are strongly dependent on the biomass of the cells. They show that a substantial proportion of the total population of bacteria in anaerobic zones of the sediment are gram positive and that there are none or few in open sea water. To obtain accurate estimates of bacterial biomass in sediments, it is necessary to use both direct counts and muramic acid values, as only about 10 to 30% of bacteria can be separated from the sediment by homogenising. In water which does not contain suspended sediment, direct counting is a much simpler procedure than muramic acid determination.

Table 1. Estimation of the proportion of gram positive bacteria with a thick peptidoglycan layer in marine populations from Moreton Bay, Qld. Direct counts were made using acridine orange. Bacteria were separated from sediments by homogenising. The sediment was strongly reducing at depths below 2 cm. Values for biomass per cell are only approximate, based on the diameters of the most common cells in each sample

Sample	No./µgMA x 10^8	Biomass/cell µg x 10^{-7}	Gram positives % total population
Sea water	15	0.5	0
Sediment:			
0 - 1 cm	5	1.0	25
2 - 3 cm	2.4	1.5	50
5 - 6 cm	1.9	1.5	70

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Journal of Microbiological Methods 1 (1983) 111–117
Elsevier

Measurement of muramic acid in marine sediments by high performance liquid chromatography

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(Received 3 November 1982) (Revised version received 2 February 1983) (Accepted 21 February 1983)

Summary

Bacterial biomass in marine sediments may be estimated from the amount of muramic acid present. A method for determining muramic acid by high performance liquid chromatography is described, which is simpler and faster than other methods. Muramic acid is released from sediment by acid hydrolysis, and assayed as an *o*-phthaldialdehyde derivative.

Key words: *High performance liquid chromatography – Muramic acid*

Introduction

Bacteria have an essential role in marine systems as food for many animals, and in the cycling of organic matter. Measurement of bacterial biomass is therefore important. Direct microscopy is difficult, particularly in sediments where bacteria are strongly bound to sand grains or aggregates of slime. I have used muramic acid measurements to estimate bacterial biomass, but the methods were not quick and simple [1, 2]. Various methods have been used to determine muramic acid after its release from peptidoglycan by acid hydrolysis. These have included chromatography and detection as ninhydrin-positive peaks on an amino acid analyzer [3]; and gas-liquid chromatography (GLC) after derivatization [4]. Some methods have depended on alkali-catalyzed release of D-lactic acid from muramic acid. The D-lactic acid has been measured by (1) colorimetric assay after degradation to aldehyde [5, 6]; (2) enzymatic oxidation and spectrophotometric determination of

NADH [7]; and (3) enzymatic oxidation and assay of NADH with bacterial luciferase [8]. None of these methods is simple to apply to environmental samples, such as marine sediments. Muramic acid is present in trace amounts compared to other sugars or amino compounds, and thus any chromatographic method requires it to be well separated from similar compounds, or preliminary purification, such as ion exchange chromatography, is needed [4, 5]. High performance liquid chromatography (HPLC) gives good resolution of compounds with similar structure in a short time. This paper describes an adaptation of a HPLC procedure for primary amines that separates muramic acid from other amines in marine sediments and thus allows its quantification. The method is based on that of Lindroth and Mopper [9], who used precolumn derivatization of amino compounds with *o*-phthaldialdehyde.

Materials and Methods

Extraction

The extraction of muramic acid from sediments was described earlier [2]. Coral reef sediments (2 g dry weight) were treated with 20 ml acetic acid (30% v/v) overnight at room temperature to remove carbonate. The sediments were then centrifuged (3000 × *g*, 5 min) and washed with distilled water. Any residual calcium carbonate was dissolved with cold (4°C) 1 M HCl and the sediment again centrifuged and washed. The sediment was suspended in 2–3 ml of 6 M HCl and transferred to prescored 5 ml ampoules, sealed and heated at 100°C for 5 h. Dried siliceous sediments from a seagrass bed were weighed (2 g) directly into ampoules and hydrolyzed at 100°C with 2–3 ml 6 M HCl. To test for losses of muramic acid, 10 μg of pure muramic acid were added to replicate ampoules of sediment, which was then hydrolyzed as described above.

The liquid portion of each sample was then transferred to test tubes. The sediment was washed twice with distilled water and the washings were added to the test tubes also. Acid was removed in vacuo on a vortex evaporator. The residue was dissolved in 0.5 ml water and transferred to a graduated centrifuge tube; two additional 0.5 ml portions of water were used to rinse the sample into the tube to give a final volume of 1.5 ml. Then 200 μl of 0.5 M Na_2HPO_4 were added and NaOH (5 M at first, then 1 M) was added to give a final pH of 8.0. The precipitate was removed by centrifuging and the final volume adjusted to a known value (usually 2 ml). This step removes heavy metals and humic compounds. The solutions were transferred to vials for loading into the automatic injector of the HPLC.

Two samples were adjusted to pH 12.5 with NaOH, heated at 40°C for 1h and then the pH was lowered to 8.5 with HCl. This treatment degrades muramic acid, and provides a confirmation of peak identity.

The recovery of muramic acid from *Pseudomonas fluorescens* mixed with sediment was measured. Samples of *P. fluorescens* (2 mg) were combined with sediment (2 g) and analyzed as described above.

Chromatography

Apparatus

The apparatus was obtained from Waters Associates, Milford, MA. It consisted of an automatic injector (WISP), two pumps (models M45 and 6000A), a model 720 system controller and model 420 fluorescence detector. The column used was either a Waters 5 μm C18 radial 'Resolve' column or a 5 μm C18 radial compression cartridge in a 'Z module'. A narrow band excitation filter of 340 nm and a cut-off emission filter of 410 nm were used in the fluorescence detector. A Hewlett-Packard model 3380A integrator was connected to the fluorescence detector.

Mobile phases

Two mobile phases were used. A stock buffer solution was prepared, consisting of 0.05 M sodium acetate, adjusted to pH 7.5 with acetic acid. Phase A contained the acetate buffer and tetrahydrofuran (2% v/v). Phase B was 80% (v/v) methanol and 20% acetate buffer.

Reagent

This was made up as described by Lindroth and Mopper [9]. It consisted of 270 mg of *o*-phthaldialdehyde dissolved in 5 ml ethanol, and mixed with 200 μl of 2-mercaptoethanol and 45 ml of 0.4 M sodium borate buffer, pH 9.8. The reagent was stored in the dark in a refrigerator; for use, about 4 ml were loaded into the WISP in position 1 of the sampler tray.

Standard solution

Muramic acid (Sigma Chemical Co., MI) was prepared at a concentration of 10 μg ml^{-1}.

Procedure

Waters Associates supplied a program for the system controller which carried out derivatization and injection onto the column automatically. A brief description is given here. Reagent was taken up into the sample holding loop followed by a sample or standard. The pumps were held at zero flow at this stage. This was held for 1 min at zero flow, and then phase A was pumped at a flow rate of 0.1 ml min^{-1} for 1 min. A guard column packed with 0.5 mm diameter glass beads, placed in the line immediately after the sample holding loop, ensured that adequate mixing of reagent and sample occurred. Usually 100 μl of reagent and 25 μl of sample were mixed. After 2 min from the time the sample was taken up, the flow rate of phase A was increased to 1.5 ml min^{-1} and held until 10 min had elapsed. From 10 to 20 min a linear gradient from 100% A and 0% B to 80% A and 20% B was applied. At this stage, after muramic acid had eluted, 100% phase B was pumped through the column for 15 min to remove remaining compounds. A total time of 40 min was required to assay each sample.

Where separation of all amino acids or glucosamine or diaminopimelic acid was wanted, a slow linear gradient over 50 min total time to 100% B was used. Two extra isocratic steps were included: At 16 min for 5 min, the program was held at 75% phase A, then at 26 min it was held at 50% A for 10 min.

Results

With the procedure described here, muramic acid was clearly separated from other primary amines (Fig. 1). It could thus be accurately quantified with an integrator. Muramic acid eluted between glutamic acid and serine. At least eight other primary amines eluted in this region, from which muramic acid was well separated. Recovery of the standard muramic acid carried through the hydrolysis procedure was 66% ± 4% (mean ± standard error, 7 replicates).

Recovery of muramic acid from *Pseudomonas fluorescens* was 87%. Part of the reason for depressed recovery is that the derivatization appears not to be complete with large sample volumes. The ratio of peak area to sample volume was not linear with large volumes (more than 50 µl of sediment extract). For quantitative results, it is best to check completeness of derivatization with an internal standard of muramic acid added immediately before chromatography.

The lower limit of sensitivity, with the fluorescence detector used here, was 5

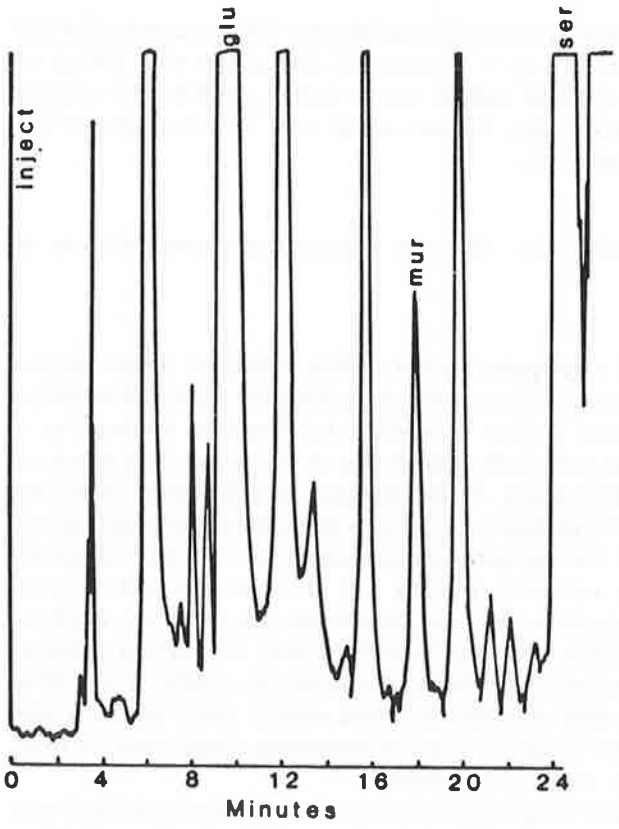

Fig. 1. Separation of muramic acid from seagrass bed sediment. The vertical axis is an arbitrary scale of fluorescence emission. Abbreviations as in Fig. 2.

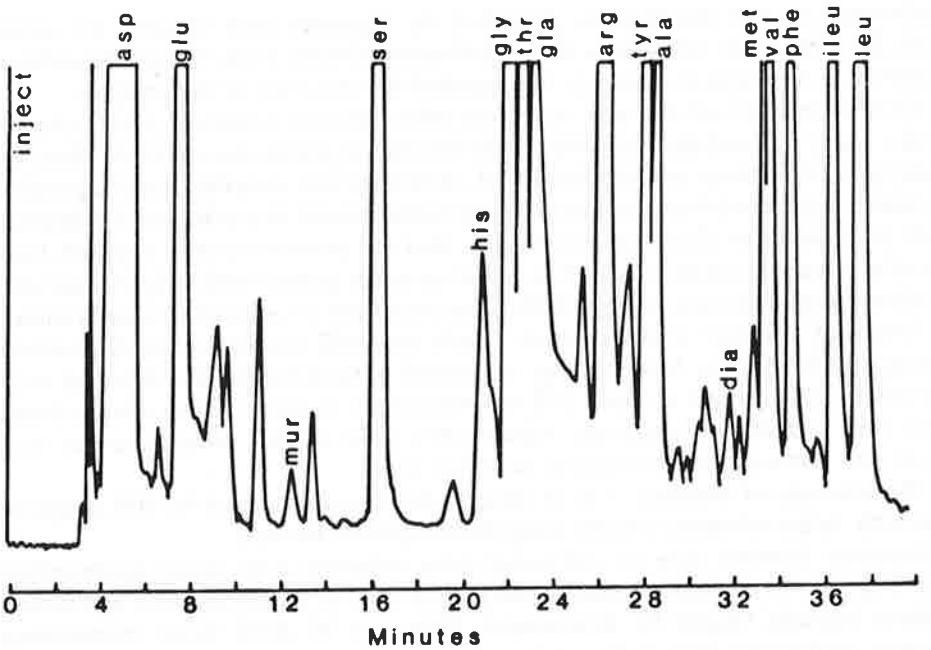

Fig. 2. Separation of amino compounds in hydrolysates of coral reef sediments. Abbreviations: asp, aspartic; glu, glutamic; mur, muramic; ser, serine; his, histidine; gly, glycine; thr, threonine; gla, glucosamine; arg, arginine; tyr, tyrosine; ala, alanine; dia, diaminopimelic; met, methionine; val, valine; phe, phenylalanine; ileu, isoleucine; leu, leucine.

ng of muramic acid, corresponding to a content of 0.2 μg ml^{-1} in the sediment extract when a 25 μl sample was injected. This was adequate for the marine sediments analyzed here. Diaminopimelic acid and glucosamine could be separated from other primary amines by an extended gradient elution (Fig. 2). The analysis depicted in Fig. 1 was of a siliceous sediment from a seagrass bed. It contained 1.6 μg muramic acid g^{-1} dry weight. A similar pattern of primary amines was found in chromatographs of coral reef sediment (Fig. 2). This example of reef sediment contained 1.5 μg of muramic acid g^{-1}. The attenuation in Fig. 2 is greater than that in Fig. 1 to show the positions of muramic acid and diaminopimelic acid in relation to common amino acids. In addition to establishing the identity of the muramic acid peak by retention time relative to a known standard, two other methods were used to confirm its identity. Firstly, a single peak of larger size was obtained when the sample was spiked with a standard. Secondly, the peak was not present in samples that were hydrolyzed in alkali. D-Lactic acid is cleaved from muramic acid in alkali.

Discussion

This procedure for determining muramic acid is much faster and simpler than either enzymatic [2] or GLC [4] methods. It may be carried out using manual

derivatization and injection as described by Lindroth and Mopper for amino acids [9]. The great advantage of the automatic system from Waters Associates, however, is that operator time is not required for this part of the analysis.

Good separation of muramic acid from other primary amines was not achieved until a 5 μm C18 column with 7000–9000 theoretical plates was used. As there are many primary amines in hydrolysates of environmental samples, good separation is essential for confidence in assigning particular peaks to particular compounds, such as muramic or diaminopimelic acids, that are present in trace amounts compared to common amino acids. The initial isocratic period with only 2% tetrahydrofuran in the aqueous acetate buffer was necessary to achieve this separation.

Losses of muramic acid did occur. These probably resulted from degradation during acid hydrolysis. Little change was noted in samples that stood for up to 12 h at pH 8. Dawson and Mopper [10] reported losses of amino sugars during drying after acid hydrolysis. Glycerine, which (they pointed out) would prevent such losses, did not improve recovery of muramic acid.

The amounts of muramic acid in these sediments determined by HPLC agreed well with values obtained earlier using the enzymatic method.

Bacterial biomass may be estimated from muramic acid values as described earlier [2, 8]. To do this accurately, the proportions of Gram-negative and Gram-positive bacteria should be determined. This may be done using transmission electron microscopy [11] or by relating direct counts to muramic acid values [2]. Fortunately, Gram-negative bacteria predominate in surface marine sediments (and probably freshwater sediments also) and thus as argued earlier [2], errors would not be large if a value of 15% Gram-positive bacteria was assumed.

Diaminopimelic acid, like muramic acid, occurs only in the cell walls of prokaryotes, but it is less useful as a measure of biomass because its distribution is more restricted than that of muramic acid. It occurs as a substitute for lysine, mainly in rod-shaped bacteria [12]. In some circumstances, however, it may be useful as a relative measure of biomass.

Acknowledgments

I am grateful for the helpful advice and suggestions for mobile phases that I received from Mr Brian Walker of Waters Associates, Sydney, Australia.

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CSIRO Marine Laboratories. Reprint No. 1151

Reprinted from Laboratory Practice, Vol.29 No.11 November, 1980

Determination of organic carbon and carbonate in the same sample with an elemental analyser

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Abstract

The determination of organic carbon and carbonate in the same sample often presents problems. Treatment of the sample for one determination may lead to the loss of the other component, and the instruments used in this method may not be capable of both determinations. This paper outlines modifications made by the authors to instruments and methods in an attempt to facilitate the determination of organic carbon and carbonate in the same sample.

Introduction

In our studies on the food of prawns, we wanted to determine organic carbon and carbonate in the same sample. We have also wanted to measure organic carbon in carbonate sediments from coral reefs. It is possible to treat the samples with acid and then dry them prior to combustion, but there are difficulties with this method. Volatile organic material may be lost, or not all carbonate may be decomposed with acid. In addition, acidic gases from the anionic part of the acid interfere with combustion tube catalysts in the Perkin-Elmer analyser. Giovannini *et al.* (1975), using a modified Carlo-Erba elemental analyser, showed that organic carbon and carbonate in soils could be measured by combusting at 500°C and 1100°C. They combusted separate samples at these two temperatures, obtaining values for organic carbon and total carbon respectively. Telek and Marshall (1974), measured organic carbon in the presence of carbonate with a Hewlett Packard elemental analyser by combusting replicate samples for different time periods. Carbonates decomposed more slowly than organic materials and by extrapolating to zero time, they could distinguish organic carbon from inorganic.

We have a Perkin-Elmer model 240 elemental analyser, which has a different mode of operation from the two instruments mentioned above. We attempted to use the procedure of Telek and Marshall (1974) but found that the combustion of carbonate was variable with time, and often was nearly complete at temperatures high enough to give complete combustion of organic material in the normal machine cycle (about 700°C). Samples in the model 240 are combusted in an atmosphere of pure O_2, usually at 950°C and the gases are passed firstly through oxidising catalysts and then into a second furnace in which nitrogenous gases are reduced to nitrogen (N_2). Modifications to this system are described below which enable organic carbon and nitrogen and carbonate to be determined on the same sample (Figure 1).

Modifications to the instrument

Soon after purchase, we discovered that the combustion furnace temperature could not be regulated below about 700°C, which was contrary to the specifications. Perkin-Elmer modified the temperature control circuitry so that the furnace could be used at temperatures between 500°C and 600°C. At these temperatures not all carbon compounds were fully oxidised to carbon dixoide (CO_2), and carbon (C) was deposited in valve D (after the reduction furnace). A small furnace, manufactured by Pyroco Products, Redcliffe, Queensland, was interposed between the combustion and reduction furnaces to overcome this problem. It carried a tube 110 mm long containing copper oxide (wire form) in a heated zone of 60 mm and was operated at 950°C. Power was taken to its controller from the outlet provided for an accessory furnace on the main instrument. The furnace was mounted on top of the main furnace block and gases were led into and from it by short lengths of copper tube (3 mm outside diameter) held close to the furnace to prevent cooling of the gases. New plugs with the copper tubing attached were made in our workshop for the ends of the combustion tubes.

The high heat coil was replaced with a longer one (15 turns of 1.5 mm diameter nichrome wire) and covered with 10 mm of insulation (Kaowool), which increased the length of the heated zone to a little over the full length of a combustion boat (35 mm), at 1000°C. The raised portion of the cam operating the microswitch (S804) for the high heat coil was lengthened with epoxy resin to keep the coil heated until immediately after the mixing volume had

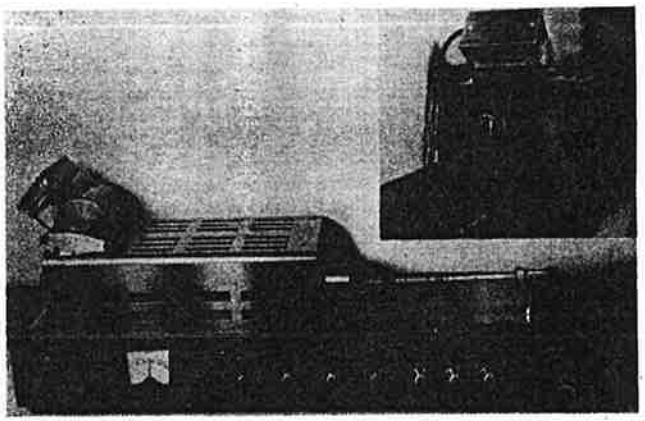

Figure 1. Elemental analyser showing modifications. The ladle system can be seen in place. Side view (inset) shows the secondary furnace and its controller mounted underneath.

filled, ie, it turned the heat off at position 5.1/4 on the programmer wheel. The heating period was thus increased to just under 3 min. A timer was inserted in the circuitry which holds the combustion cycle (at position 2.3/4 in the program) for any set period up to 30 min. This timer could be bypassed by a switch on the front panel, allowing the program to operate normally. A small plug of epoxy resin was placed on the programmer shaft such that it operated the spare switch (No. 806) at position 2.3/4. This switch activated the timer, whose relay contacts were in series with contact S803, which controls the power supply to the programmer motor.

The ladle system supplied by the manufacturer was too small for some of the sediments that we wished to measure because they contained about 0.1% organic matter and thus a large amount was needed. We replaced it with the system shown in Figure 2. The solid sample entrance plug was replaced with a hollow plug having an outer ring which could be quickly turned to alter the pressure on an O-ring around the stainless steel rod as it was being moved (Figure 3). No gas leakage occurred. Its main advantage was that it allowed large sample boats (holding up to 1 g of sediment) to be used without any danger of turning over and spilling sediment in the combustion tube, as often happened with the magnetic system. The output from our instrument was converted to digital form by a panel meter and was stored in a microprocessor-controlled buffer for subsequent transfer to a computer. This allowed the instrument to be left unattended until the program ended.

Determination of operating conditions

Chitin (British Drug Houses) and calcium carbonate (AR grade, 99% min. assay) were used as test substances after oven drying. After trying various temperatures and time periods for combustion in the main furnace we found that 550°C with a hold time of 20 min (total combustion period 23 minutes) gave complete combustion of chitin (Table 1). The expected values for complete combustion are 120 $mgC.g^{-1}$ from carbonate and 455 ± 5 $mgC.g^{-1}$ and 72 ± 5 $mgN.g^{-1}$ from chitin. No further combustion occurred when the time was extended to 33 minutes. At lower temperatures or shorter time periods, chitin was not fully combusted. At temperatures near 600°C carbonate started to decompose, whereas no decomposition occurred at 550°C (Table 1). Incomplete combustion of organic

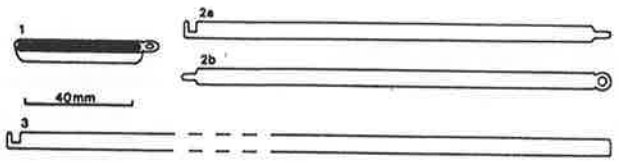

Figure 2. Ladle system. (1) Silica boat about 40 mm × 8 mm with an eye which is hooked by the silica rod. (2) Silica rod (160 mm long) which is interposed between the boat and steel rod and avoids having the steel rod in the high heat zone; a) Side view, b) top view. (3) Stainless steel rod (315 mm long) which passes through sample entrance plug.

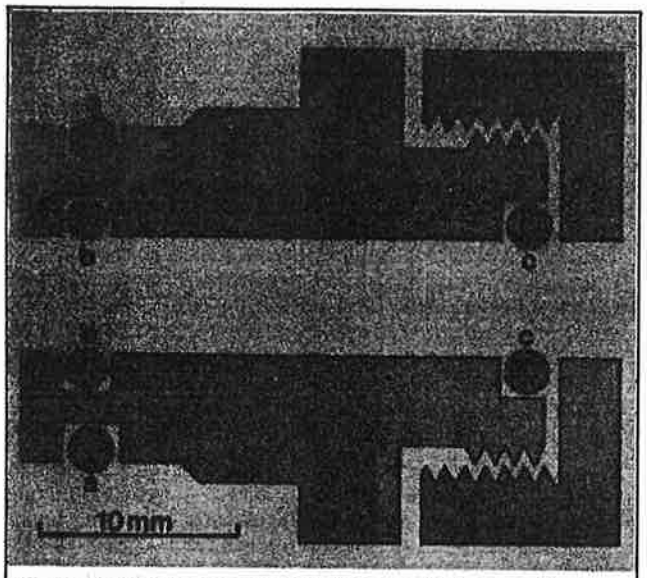

Figure 3. Modified sample entrance plug. The plug has a retaining lug and one O-ring (a) as in the original. It is drilled through the centre and has an O-ring inside at each end (b and c) through which the steel rod passes. The outer knurled ring (d) compresses the O-ring (c) to hold the steel rod in position.

material is manifested by high blanks on a second run, or recovery of nitrogen from a second high temperature burn. As the gut contents of prawns contained chitin, 550°C was selected as the operating temperature.

Carbonate was fully decomposed in samples held in the modified high heat zone (1000°C, 3 minutes) whereas chitin was not (Table 1). No additional carbon was collected from calcium carbonate or coral reef sediments after additional combustion periods. Some of the variability in the carbon values shown in Table 1 was due to instability in the panel meter. To measure the organic C and N in coral reef sediments, it was found that lower temperatures and shorter combustion periods were necessary and results were more reproducible if the samples were first pulverised, but this introduces another problem. Chave and Schmalz (1966) have reported that small calcite particles decompose more readily. Coral sediments contain a variable proportion of magnesium calcite, which decomposes more readily than pure calcite. To determine the optimum operating conditions for measuring organic C in these sediments we tried various temperatures between 490°C and 550°C and time intervals from 5 to 30 minutes. Some examples of results are shown in Table 1. The criterion for completeness of combustion of organic matter was the lack of nitrogen recovery from a second high temperature burn. A temperature of 500 °C for a period of 10 minutes was found to give complete combustion of organic matter in a variety of reef sediments. It is difficult, however, to completely separate organic C from inorganic C. As the combustion period was lengthened, or the temperature raised, more C, but no further N, was released indicating that some inorganic material was decomposing (Table 1). At 500°C for

Table 1. *Recovery of carbon and nitrogen from combustion of chitin, calcium carbonate, coral reef sediments and dolomite. Standard deviations are shown.*

Sample	Combustion Temperature °C	Combustion Time min	Nitrogen $mg.g^{-1}$	Carbon $mg.g^{-1}$	No. of replicates
Chitin	550	23	75.8 ± 4.5	455 ± 7	8
Chitin	550	33	71.6 ± 3.7	454 ± 5	6
Chitin	1000	3	61.4	267	1
$CaCO_3$	550	23	0.5 ± 0.2	0.3 ± 0.2	7
$CaCO_3$	1000	3	0.3 ± 0.2	118 ± 3	9
Coral Sediment	550	20	0.30	7.2	1
	520	20	0.31	5.9	1
	500	20	0.29	4.9	1
	500	10	0.29	4.0	1
	500	5	0.26	4.1	1
Dolomite	500	10	0.13	1.2	1
Dolomite (2nd burn)	500	10	0.01	0.2	1

5 minutes, N values were depressed, indicating that combustion of organic material was incomplete.

Probably the main reason for carbonate decomposition of these reef sediments however, was the presence of magnesium calcite. The problems of determining organic C in reef sediments were mentioned by Hirota and Szyper (1975). To test the analysis with a known source of magnesium calcite, we combusted dolomite (obtained from a bag of garden fertiliser). At 500°C and 10 minutes combustion period, a small amount of C was released, but N was also produced. The C:N ratio of 9:1 indicates that this was probably organic matter. A second burn at 500°C on the same sample generated much less C (Table 1). At higher temperatures however, more C was released indicating that some carbonate was decomposing. Thus the temperature of combustion must be kept at 500°C if this type of carbonate is present in large proportions.

The procedure to measure both organic carbon and carbonate is as follows. After the initial flushing period, the sample boat is pushed into the main furnace (at 500°C or 550°C) and the timer is switched on to hold the combustion for 10 or 20 minutes. The results from this give organic carbon, nitrogen and hydrogen. At the end of the program cycle if carbonate values are required, the sample is placed under the high heat coil, the timer is switched out of circuit and a second program cycle is started. This is not practical in coral reef sediments, because there is so much carbonate that a reasonable sample size for measurement of organic C and N (about 200 mg) is too large for carbonate measurement.

Conclusions

This system has several advantages. It measures organic C in the presence of inorganic C and if the organic C is not too low in proportion to inorganic C, both can be measured on a single weighed sample. Relatively large amounts of material can be combusted, thus reducing errors that are due to small sample sizes or working near the maximum sensitivity of the instrument with sediments in which the organic matter content is low.

Acknowledgements

We are grateful to Mr R. Flynn for his technical assistance in modifying the instrument, and to J. Sokoll and Associates, Kenmore, Queensland, who designed and installed the timer and digital converter/microprocessor system.

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Biogeochemistry of Ancient and Modern Environments

Proceedings of the Fourth International Symposium on Environmental Biogeochemistry (ISEB) and, Conference on Biogeochemistry in Relation to the Mining Industry and Environmental Pollution (Leaching Conference), held in Canberra, Australia, 26 August – 4 September 1979.

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AUSTRALIAN ACADEMY OF SCIENCE,
CANBERRA 1980

MEASUREMENT OF BACTERIAL BIOMASS IN SANDY SEDIMENTS

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INTRODUCTION

Bacteria have important roles in sediment processes such as nutrient cycling, decomposition of organic matter and as a food source for many animals. In shallow marine environments where macrophytes are the main primary producers, bacteria are a dominant component of the food chain because much of the organic matter cannot be digested (Fenchel, 1977). Accurate estimates of bacterial biomass are needed in studies on their trophic role, but these are difficult to obtain in sandy sediments. Direct measurement of bacteria stained with acridine orange has been used after homogenising the sand to release attached organisms, but these are minimum estimates because an undetermined number of bacteria can remain attached (Dale, 1974; Meyer-Reil *et al.*, 1978).

Measuring the volumes of bacteria embedded in sediment is difficult, particularly as there is great variation in size. As it is tedious to count and measure more than a few hundred cells out of a population of perhaps 10^{10} cells g^{-1} of sediment, errors may result from small sample size and patchy distribution. I have attempted to use muramic acid (MA) to estimate biomass, because it should not be subject to errors due to operator fatigue, small sample size or the inability to see or recover all bacteria embedded in or attached to particles (Moriarty, 1975, 1977, 1978). This method, however, has other problems, which relate to the technique and the principle of the method. Measurement of trace amounts of MA in hydrolysed sediment is difficult and detailed procedures are given here for techniques that should overcome problems experienced previously.

Muramic acid was chosen as an estimate of biomass because it is a cell wall component unique to most prokaryotes (Rogers and Perkins, 1968). Being a constituent of the cell wall polymer peptidoglycan, its amount is directly correlated with surface area rather than biomass and thus some estimate of cell size is necessary to convert what is actually a surface area measurement to volume. There are two different types of cell wall structure in bacteria. Most of those that are termed a Gram-negative type (based on a standard staining reaction) have a single thickness of peptidoglycan, whereas the Gram-positive types may have from 2 to 10 layers (Rogers and Perkins, 1968). In earlier work, I had to make an assumption about the proportions of these types of bacteria in sediments because the Gram stain could not be applied directly. A combination of direct microscopy and MA determinations is shown in this paper to provide an estimate of the proportions of Gram-negative and Gram-positive bacteria that are released from the sand grains by homogenisation, and thus to provide more accurate estimates of sediment biomass. One more problem in using MA is that, if the cell wall degrades more slowly than cytoplasm in dead bacteria, an overestimate of biomass will result. In surface marine sediments, the effects of a wet, aerobic environment should lead to rapid autolysis and, combined with predation by animals, few empty wall sacs should accumulate (Moriarty, 1977). This problem is now being investigated by electron microscopy and preliminary results show that few wall sacs accumulate. In deep sediment layers, and in terrestrial soils, cell walls might be expected to accumulate and thus invalidate the use of MA to estimate biomass.

The participation of bacteria in the decomposition of seagrass detritus and in the cycling of nutrients has been discussed by Fenchel (1977). Moreton Bay, Queensland, contains many seagrass shoals and, as part of a study of their productivity, I wished to determine whether bacterial biomass in the sediment was influenced by seagrass cover. Sediments were analysed from two sites with seagrass and one without, although the latter site was surrounded by seagrass.

METHODS AND MATERIALS

Sediments

Sediments were from shoals in Moreton Bay, Queensland that were dominated by *Zostera capricorni* Aschers. The sediments were mainly siliceous sand, median grain size about 0.2 mm, with some heavy minerals. A corer, 50 mm diameter and 250 mm length, was used. For analysis, three layers of each core were placed in weighed containers on ice immediately after collection. The top aerobic layer (0-1 cm), the zone at which the redox potential changed as shown by black colour (usually between 1 and 3 cm), and a deep anaerobic layer (20-21 cm) were sampled. Two cores were taken intact on ice to the laboratory where redox potential was measured 30 min after collection.

At the laboratory, the samples were weighed, a 10 g subsample was removed from each and the remainder was freeze-dried and dry weights were determined.

Correlation of Direct Counts with Muramic Acid

The 10 g subsamples were diluted with 10 ml seawater (previously filtered through 0.2 µm membrane filter), and homogenised at 20,000 r.p.m. for 30 s using an 'Ultra-Turrax' (John Morris Scientific, Sydney). Sand grains were allowed to settle for 1 min and the suspension was decanted into a measuring cylinder. The sand grains were washed twice with filtered seawater and the total suspension of silt and bacteria was made up to 50 ml. A 1 ml sample was removed, diluted to 18 ml with filtered seawater, and 2 ml of concentrated (35%) formalin added. This suspension was stored at 4° until examined microscopically. The remaining 49 ml were centrifuged and the pellet was hydrolysed for MA determination. The technique for direct counts was modified from that of Daley and Hobbie (1975). A portion of the preserved suspension, usually 200 µl, but adjusted if necessary to give a relatively even distribution of bacteria and maximum count rate, was added to a filter funnel containing 5 ml of filtered (0.2 µm) seawater and 20 µl of 0.1% (w/v) acridine orange. After 2 min the mixture was filtered through a black 0.2 µm cellulose acetate (Sartorious) filter, which was then mounted with oil under a coverslip. It was examined with a Leitz Dialux microscope using 12.5X eye pieces and a 50X water immersion objective, the Leitz Ploemopak epifluorescence apparatus and the K filter system. With bacteria embedded in detritus the cellulose acetate filters gave better results than polycarbonate types. Filamentous bacteria and large rods were counted in units of 1 µm length. If a filament was, say, 10 µm long, it was counted as 10 bacteria, not one.

Organic Carbon and Nitrogen

Organic carbon and nitrogen were determined using a Perkin-Elmer model 240 elemental analyser. Carbonates were a negligible proportion of these sediments.

Muramic Acid

Muramic acid values for the total sediment were determined on weighed portions of the freeze-dried sediment. The method has been published elsewhere (Moriarty, 1977; 1978), but as modifications have been made, a complete list of the steps in the procedure is given in Appendix 1.

RESULTS AND DISCUSSION

Relationship of Muramic Acid and Cell Numbers

Muramic acid values in the surface layers of all sediment cores were higher than values at 20 cm depth. Not only were there greater numbers of bacteria in the surface layers than in the deep layers, but there were more bacteria per µg MA at the surface (Table 1). Muramic acid content and numbers of bacteria in the layers at which the sediment changed from aerobic to anaerobic were more variable, although in most cases, the ratio of numbers to MA was less than at the surface. The very low ratio of numbers of cells to MA in deeper layers is probably due to two main factors; viz, an increase in proportion of Gram-positive cells and an increase in dead cells, which do not fluoresce, but which have walls containing MA. Preliminary observations with an electron microscope (unpublished) show that, at a depth of 20 cm, many intact walls are present without contents,

whereas most cells in the surface layers are intact. It was estimated that about 40% of MA in the deep layer might be present in the empty wall sacs. In calculating the ratio of cells to muramic acid and the bacterial biomass, therefore, the MA values of the 20 cm layers were reduced by 40%. Even with this refinement, there were still much lower ratios of cells to MA in the anaerobic layers, which suggests that the proportion of Gram-positive bacteria was higher. It is not possible to use the classical Gram stain on bacteria embedded in clay and organic matter. The approximate proportions of Gram-positive to Gram-negative cells can be estimated from the following formula (Equations 1-2), where n and p are the proportions of Gram-negative and Gram-positive bacteria, respectively (Moriarty, 1977):

$$mgC = \mu gMA/(8n + 44p) \quad (1)$$

where

$$n + p = 1 \quad (2)$$

Thus Equation 1 can be rewritten as follows

$$p = ((mgC/\mu g\ MA) - 8)/36 \quad (3)$$

It has been estimated from microscopic observation that the average biomass of cells in these sediments if 8×10^{-11} mg C. By combining this value with the ratio of the number of cells per µg MA, Equation 2 becomes:

$$p = ((1/(\text{No. cells} \times 10^{8}\ \mu g^{-1}MA \times 8\times10^{-11})) - 8)/36 \quad (4)$$

TABLE 1

MURAMIC ACID (MA) IN RELATION TO BACTERIAL CELL NUMBERS

Depth cm	E_h mV	Total MA µg.g^{-1}	No. Cells Per µg MA cells x 10^8 µg^{-1}	Gram-positive type cells approx. %	Recovery %
Site 1 : Seagrass Sediment					
0 - 1	-	4.4 ± 0.4	8 ± 1	20	33 ± 10
1 - 2	-	3.6 ± 0.6	7 ± 1	30	50 ± 5
20 - 21	-	2.4 ± 0.4	6 ± 1	40	60 ± 5
Site 2 : Seagrass Sediment					
0 - 1	+150 ± 50	3.8 ± 1.4	15 ± 10	0	50 ± 20
1 - 2	+ 40 ± 60	3.0 ± 1.0	8 ± 3	20	60 ± 30
20 - 21	- 80 ± 30	2.3 ± 0.1	6 ± 0	40	70 ± 10
Site 3 : Bare Sand					
0 - 1	-	0.9 ± 0.4	10 ± 2	10	70 ± 30
2 - 7*	-	1.0 ± 0.2	10 ± 3	10	85 ± 15
20 - 21	-	0.3 ± 0.1	6 ± 1	40	20 ± 10

Cells were counted in the supernatant of the homogenised sediment and expressed as a proportion of MA in the same homogenate. The recovery of MA in the homogenised suspension is expressed as a percentage of that in the total sediment. Approximate percentages of cells with walls of a Gram-positive type are estimated as described in the text. Means and ranges of values for 3 cores are shown for sites 1 and 2, and for 4 cores at site 3. The sediment changed from mainly oxidising to reducing at about 1-2 cm depth, as shown by colour change from light to black, at sites 1 and 2. * denotes that 1 cm was sampled in the zone where colour changed from light to black, which varied in these cores from 2 to 7 cm in depth.

The values for the proportion of Gram-positive bacteria in Table 1 have been estimated from this equation. It provides only a very rough guide to proportions, because these values are influenced strongly by variations in the size of cells. Nevertheless, the similarity in results from all cores in three different areas, supports the conclusion that bacteria with cell walls of a Gram-negative type are more common in the surface aerobic layers than in deeper anaerobic sediments. Although it has not been possible to release all bacteria by homogenisation, it is assumed that these results apply to the total bacterial population. Methanogenic bacteria, which do not contain MA and thus could lead to confusing results, are unlikely to be present in the deeper layers studied here, because the sediments are subject to some aeration with tidal movement and thus the redox potential does not fall very low (Table 1). A study is now underway using transmission electron microscopy to investigate the types of cell wall structure in these sediments. Preliminary results indicate that Gram-positive bacteria constitute 12% of bacteria in the top layer and 30% in the 20 cm layer. There is reasonably good agreement between the numbers of bacteria and amount of MA in the homogenised samples, especially in the surface layers. These results verify the methods used to assay MA, and further support is given by the predicted proportions of Gram-positive bacteria and the values thus far obtained by electron microscopy.

King and White (1977) have criticised the method used here because D-lactate dehydrogenase is not entirely specific. As the results presented here show, the MA procedure does work, but ether extraction to remove D-lactate and glycollate formed after acid hydrolysis and the use of internal standards is necessary. The main advantage of the method is that small quantities of sample can be analysed, e.g. 1 mg organic matter. Purification of MA by thin layer chromatography is necessary when the starting material contains large amounts of polysaccharide because cellulose was found to degrade during hydrolysis to yield about 25 µg D-lactate (or the equivalent as glycollate) for each 100 mg cellulose. This error would not be detectable in the sediments analysed here. Fazio *et al.*, (1979) have developed a method using GLC, but it is not simple. A procedure using high pressure liquid chromatography that measures MA without degradation to lactate is being investigated at present, and thus far appears to be very simple and sensitive.

It is difficult to obtain an accurate estimate of bacterial biomass with any technique in sandy sediments. Direct microscopic counting of bacteria on sand grains is almost impossible, so direct microscopy after homogenisation was used by some workers (e.g. Dale, 1974; Meyer-Reil *et al.*, 1978). Counting bacteria embedded in detritus is very difficult, and I have found it even more difficult to measure their sizes. Not all bacteria were removed by the vigorous homogenisation used here; the amounts recovered were variable and unpredictable as shown by MA measurements (Table 1). Some estimates of total biomass based on direct counting only would have been seriously in error. As the results presented here show, MA values only would also not give an accurate estimate without knowledge of average cell sizes and proportions of Gram-positive and -negative cells. In order to estimate the biomass of bacteria from a factor based on surface (such as MA or lipopolysaccharide), the calculation should take into account the size of the cells, as well as the proportion of Gram-negative and Gram-positive bacteria. King and White (1977) proposed a factor, derived from the average MA content of a few organisms of both types, which is arbitrary and could lead to considerable under- or overestimation of biomass, depending on the composition of the population. Some marine Gram-negative bacteria have been shown to contain between 5 and 10 µg MA $mg^{-1}C$. This variation is due to differences in the sizes of the cells, as they all have the same thickness of peptidoglycan. Cells with a diameter of about 1.0 µm have about 5 µg MA $mg^{-1}C$; cells with a diameter of 0.6 µm contain about 10 µg MA $mg^{-1}C$ (Moriarty, 1977). Gram-negative cells 0.2 µm in diameter would contain about 28 µg MA $mg^{-1}C$, but there were few in these sediments.

Thus, as most Gram-negative bacteria in these sediments have diameters within the range 0.5 - 1.0 µm, a value of 8 µg MA $mg^{-1}C$ was selected to convert muramic acid values to biomass. If there were no Gram-positive bacteria present the error in biomass estimation should be less than 25%. Gram-positive bacteria however are present and, from values measured on a few types, an average of 44 µg MA $mg^{-1}C$ has been used in the calculations above. These bacteria also vary greatly in size and thus, for a given thickness of peptidoglycan, in their MA content. They vary much more in MA content, because they may have perhaps 2 to 10 or more layers of peptidoglycan. If they are a low proportion (e.g. 10% as in the surface layers here) the extra variability due to cell size and

composition should not have much effect on the error in estimation biomass. If they are a high proportion of a population, then many more determinations than I made of MA content and variation with size, should be made on cultures from the environment under investigation. Likewise, if other types of prokaryotes, such as sulfur bacteria or blue-green algae, are present in high proportions, the simplified formula (equation 1) may need to be modified. For the seagrass flats studied here, the formula is applicable. The blue-green algal population was low, and would have contributed less than 1% of the MA. Spores were present in very low numbers, compared to bacteria (Moriarty, 1975); this result was confirmed by electron microscopy, where no clearly identifiable spores were seen. The proportions of Gram-positive bacteria probably range from 10% at the surface to 40% at 20 cm, but even if subsequent more detailed work shows that the true values are say 40% at the surface to 80% in deeper layers (and this is unlikely), the biomass calculations would be in error by only a factor of 2.

Bacterial Biomass

To convert total MA values to bacterial biomass, the proportion of Gram-positive bacteria has been taken as 10% at the surface, 20% at 1-2 cm depth and 40% at 20 cm, giving factors of 85, 65 and 45, respectively, by which MA was multiplied to give carbon (equation 1 above). Muramic acid values at 20 cm were reduced by 40% to allow for dead cell walls. Preliminary results with electron microscopy indicate that there is little MA in dead walls in the surface layers. The work discussed here was carried out mainly to test the usefulness of MA as a measure of bacterial biomass. The results below are presented as an indication of the amount and variation in bacterial biomass in these sediments.

Bacterial biomass was greatest at the surface in most cores, although in some cores taken around seagrass roots a higher biomass was found at 1-2 cm, apparently in association with the roots (Table 2). Biomass was lowest in the deeper layers. At the surface, biomass

TABLE 2

BACTERIAL BIOMASS IN RELATION TO ORGANIC CARBON AND NITROGEN

Depth cm	Organic C		Bacterial C			C:N
	mg g^{-1}	g m^{-2}	mg g^{-1}	g m^{-2}	% Org C	
Site 1						
0 - 1	2.1 ± 0.2	30 ± 3	0.37 ± 0.04	5.2 ± 0.5	18 ± 2	10 ± 1
1 - 2	2.5 ± 0.3	35 ± 5	0.23 ± 0.04	3.2 ± 0.5	9 ± 3	10 ± 1
20 - 21	2.4 ± 0.3	34 ± 4	0.11 ± 0.02	0.15 ± 0.2	4 ± 0	11 ± 1
Site 2						
0 - 1	2.0 ± 0.4	27 ± 5	0.32 ± 0.12	4.2 ± 1.0	16 ± 4	9 ± 1
1 - 2	1.7 ± 0	22 ± 0	0.19 ± 0.06	2.6 ± 0.8	12 ± 4	9 ± 1
20 - 21	1.1 ± 0.3	15 ± 4	0.10 ± 0.01	1.2 ± 0.1	9 ± 1	10 ± 1
Site 3						
0 - 1	0.43 ± 0.08	6.9 ± 1.3	0.08 ± 0.02	1.3 ± 0.4	18 ± 5	7 ± 1
2 - 7[a]	0.29 ± 0.01	4.8 ± 0.1	0.06 ± 0.01	0.9 ± 0.1	20 ± 4	6 ± 0
22 22	0.32 ± 0.13	5.1 ± 2.2	0.01 ± 0.005	0.02 ± 0.1	3 ± 2	8 ± 1

Biomass was calculated as described in Results and Discussion. Organic nitrogen values in the sediment are expressed as a ratio of organic carbon. Values per m^2 are calculated over 1 cm depth. Means and ranges of values for 3 cores are shown for sites 1 and 2, and for 4 cores at site 3. [a]denotes that 1 cm was sampled in the zone where the colour changed from light to black.

was up to 10 times and, on average, about 5 times greater in sediments containing seagrass than in sediments from bare sand. The bare sandy areas were a few hundred m^2 in extent and were surrounded by the seagrass flats. The bare sand flat had lower biomass values and in general the oxidising zone penetrated deeper (down to 7 cm). . Total organic carbon and nitrogen values were distributed similarly. Bacterial biomass varied from about 5% up to 25% of organic matter in the upper layers, with an average of about 18% at the surface (Table 2). These high values, together with the rather low redox potential and the presence of sulfide at about 1 cm in depth, especially around the seagrass roots, suggest that the bacteria were actively growing. Thus, there is likely to be a high rate of bacterial productivity in these flats, which is probably supported by exudation from seagrass roots in addition to sources such as leaching from leaves, epiphytes and benthic algae and dead material. As very little organic matter has accumulated in these sediments and the C:N ratios are not high, it seems that most of the organic matter that is not carried away by currents is used by the microbial community and not deposited. No dead leaves or fragments of leaves were observed below the surface of the sediment, so root exudation and decomposition may well be important. Marshall (1970) discussed a similar situation that occurs on shoals with stands of *Zostra marina* in New England. He argued that the low organic carbon content of the sediments was a direct result of high microbial productivity. The low proportion of dead cell walls in the surface layers, which is indicated by the good agreement between direct counts and MA values and is confirmed by preliminary results with an electron microscope, is further evidence that most of the bacteria are active and that there is high predation on them.

ACKNOWLEDGEMENTS

I am grateful to Margaret C. Barclay for carrying out the carbon and nitrogen analyses.

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Microb. Ecol. (1982) 8:1–14

MICROBIAL ECOLOGY

Ultrastructure of Bacteria and the Proportion of Gram-Negative Bacteria in Marine Sediments

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Abstract. Bacteria in sediments from the surface aerobic layer (0–1 cm) and a deeper anaerobic layer (20–21 cm) of a seagrass bed were examined in section by transmission electron microscopy. Bacteria with a Gram-negative ultrastructure made up 90% of bacteria in the surface layer, and Gram-positive bacteria comprised 10%. In the anaerobic zone, Gram-negative bacteria comprised 70% and Gram-positive bacteria 30% of the bacterial population. These differences were highly significant and support predictions of these proportions made from muramic acid measurements and direct counting with fluorescence microscopy. Most cells were enveloped in extracellular slime layers or envelopes, some with considerable structural complexity. The trophic value to animals of these envelopes is discussed. A unique organism with spines was observed.

Bacteria are important as food for many animals in aquatic sediments, and in habitats dominated by macrophytes, such as seagrass beds, bacteria form essential links between the primary producers and animals. Measuring the biomass of bacteria in sediments is necessary in order to quantify the trophic role of bacteria, but it is difficult, particularly in sandy sediments. Direct microscopy with fluorescent staining (usually acridine orange) and an epifluorescent microscope system has proved useful (Zimmerman and Meyer-Reil 1974). Muramic acid measurements have been used to estimate biomass (Moriarty 1975, 1980). Both procedures have advantages and disavantages. Direct microscopy requires that all bacteria be removed from sand grains for a total count, but some bacteria adhere strongly and may not be removed (Moriarty 1980). Bacteria in sediments are usually bound in aggregates of clay and organic matter and cannot be dispersed easily, thus making counting difficult, tedious, and possibly inaccurate. The muramic acid procedure quantifies all bacteria, but unlike direct counting, the method is not simple and rapid. It requires two assumptions: (1) that Gram-negative bacteria predominate or that their proportion is known, and (2) that most of the muramic acid is present in living bacteria, or in other words that bacterial cell walls degrade rapidly after death (Moriarty 1975). In order to check whether these assumptions were valid, muramic acid values were compared with estimates of bacterial biomass using direct light microscopy (Moriarty 1980). It was predicted that about 10% of bacteria in surface sediments (0–1 cm depth) were Gram positive, and that about 40% of bacteria in a deeper anaerobic zone (20–21 cm depth) were Gram positive.

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0095-3628/82/0008-0001 $02.80

The work reported in this paper was carried out to test these predictions using electron microscopy to examine the ultastructure of bacteria in the sediments. Gram-negative and Gram-positive bacteria differ in the ultrastructure of their walls (Cheng and Costerton 1977, Costerton et al., 1974 a, b). Gram-negative bacteria have complex walls, with a lipopolysaccharide membrane outside the cytoplasmic membrane. The outer membrane may appear as a wavy structure under the electron microscope. Gram-positive bacteria lack the double track wall structure and may have a thick wall, either amorphous or with some layers, or in some cases only a thin amorphous wall. Details of other aspects of structure, especially extracellular coats, are also reported. Deposit-feeding animals would ingest the extracellular products of bacteria, and thus such structures may be trophically important.

Methods

Sediments were collected with a corer from intertidal seagrass beds dominated by *Zostera Capricorni* Aschers in Moreton Bay, Queensland, Australia, Samples from 0–1 cm and 20–21 cm layers were removed, and about 2 g was placed in 4% glutaraldehyde in artifical seawater, pH 7.0. Additional samples were placed in the same fixative containing ruthenium red (0.1% w/v). After 12–16 h the fixative was removed by centrifuging, and sediments were washed with a sodium barbiturate-acetate buffer, pH 7.0, 0.1M. The samples were homogenized at 20,000 rpm for 2 min with an Ultra-Turrax blender (John Morris Scientific, Sydney) in 10 ml of buffer in order to release bacteria from sand grains. About 50% of attached bacteria were released by this treatment (Moriarty 1980). The slurry was transferred to a 50 ml measuring cylinder and made up to 50 ml with buffer. After 1 min to allow sand and large silt particles to settle, the top 25 ml was removed and centrifuged. The pellet was warmed to 50°C and suspended in a small volume (about 10 μl) of 2% agar at 50°C, cooled in the refrigerator, and then cut into 1 mm cubes. The cubes of sediment were post-fixed in 2% OsO_4 in artificial seawater (pH 7.0); those that had been initially fixed with ruthenium red also had 0.1% ruthenium red in the post-fixative.

After 16 h the samples were washed 3–4 times with artificial seawater, then soaked for 1 h in uranyl acetate (0.5% w/v) in artificial seawater. They were dehydrated for 1 h each in a series of tert-butanol (from 20% to 100% in steps of 10%). Two more changes of 4 h each of 100% t-butanol (which had been kept over anhydrous $CuSO_4$) were followed by 12 h in a further change of 100% t-butanol to ensure complete dehydration. The cubes were washed for 1 h in propylene oxide, then for 6 h in Spurr's low-viscosity embedding medium, standard hardness, diluted 50% with propylene oxide (Polysciences, Warrington, Pennsylvania, USA). Two changes of Spurr's resin, about 12 h each, were made before final embedding. Samples were left in the final resin for 8 h then heated at 70°C overnight. Diamond knife cut sections were stained with lead citrate and uranyl acetate before viewing on a Philips 300 electron microscope. One section from each of up to 21 different blocks was scanned fully at 25,000X in order to determine the ratio of Gram-negative to Gram-positive bacteria from the ultrastructure of their walls. One example of a coral reef sediment was also examined. The sediment was treated similarly, except that the homogenization step was replaced by treatment with 10% acetic acid overnight at 4°C to remove $CaCO_3$.

The fixation procedure was adapted from Ghiorse (1980), and the embedding procedure was supplied by R. C. Foster (personal communication). Ruthenium red is abbreviated as RR in figure legends. In the figures, all organisms shown stained with ruthenium red were from the surface layers, and all without ruthenium red were from the 20 cm layer.

Results

The distribution of bacteria in the thin sections was patchy, and the density of cells was often low. Most cells were embedded in clay and other inorganic matter, although a few were found in what was probably decomposing organic material. Some bacteria

occurred singly, others were in small colonies in a common slime layer. Fragments of degrading cells could be seen as well as ring-like structures of clay where bacteria had presumably completely degraded. Many of the cells were quite small, with diameters of 0.2–0.5 μm. Most of the cells examined were classified as either Gram negative or Gram positive (Figs. 1 and 2). Some empty cell wall sacs and wall fragments were observed which had clearly been Gram-negative or postive cells, but other wall fragments were difficult to distinguish from capsules (Fig. 3). Doubtful cases were scored as Gram-positive fragments for the purpose of estimating the contribution of wall fragments to muramic acid levels. This gave a conservative estimate, but even so empty cell wall sacs were present in small numbers compared to total cells with intact cytoplasm (Table 1).

Gram-negative bacteria predominated in these sediments. Gram-positive bacteria comprised about 10% of the population in the surface aerobic layers and 30–33% in the deeper anaerobic zone (Table 1). Empty cell sacs, with wall material apparently present, were much fewer in number than complete cells. From the numbers of these sacs, it was estimated that about 7–10% of the total muramic acid may be present in them in the January sample, and up to 12–40% in June (Table 1). The results for the June sample may be an overestimate, because the fixation procedure on that occasion was not sufficiently good to allow wall material to be clearly distinguished from slime layers.

Inclusion bodies were observed in many bacteria. These were mostly transparent (Figs. 1, 4, and 6); others were of medium electron density (Fig. 4c). Electron-transparent inclusions, which were possibly poly-β-hydroxybutyrate, were found in 8% of cells in one analysis (total number of cells examined was 315) and in 6% of cells in a more extensive analysis (total number 658). Internal membranes were also seen (Fig. 6b). In nearly all the bacteria observed, extracellular material was present. Colonies of bacteria were embedded in slime, which in some cases contained electron-dense particles (Fig. 4b). Some cells had single layers of densely staining material in the absence of ruthenium red (e.g., Figs. 1d and 2b). Clay and mineral particles were held away from the cell walls by slime and capsular material (e.g., Figs. 1, 4, and 6). Adhesion of cells to mineral particles is shown in Figs. 1c and 6d. Complex capsules or envelopes with an intricate reticulate or honeycomb structure surrounded some Gram-negative cells (Fig. 5). Other fibrous and more amorphous slime structures were more common (Figs. 4 and 6). Slime layers and capsules with no cells embedded within them were about as numerous as complete cells. These varied from ''halos'' of clay particles apparently held together by slime (Fig. 7e) through obvious slime layers with no cellular contents to slime-containing fragments of cell walls and vesicles of cell membranes (Figs. 3 and 7). A reticulate structure similar to that surrounding complete cells may be a diatom spine (Fig. 7d). Many of the bacteria in the coral reef sediment were also embedded in slime; the example in Fig. 6c shows bacteria in slime around an empty diatom frustule.

Some unusual organisms or structures were observed. These included blebs of membrane or perhaps sheathed flagella protruding from a cell; an organism with a convoluted wall; a Gram-positive organism with electron-dense protrusions (stained with ruthenium red) on the outside of the wall; and a probable immature spore from the 20 cm layer (Fig. 8). One organism from the 20 cm layer showed spines or unusual proturberances from the cell (Fig. 9). These were of different lengths, inserted through the wall apparently onto the inner membrane, and were probably cylindrical as shown by the two cut transversely.

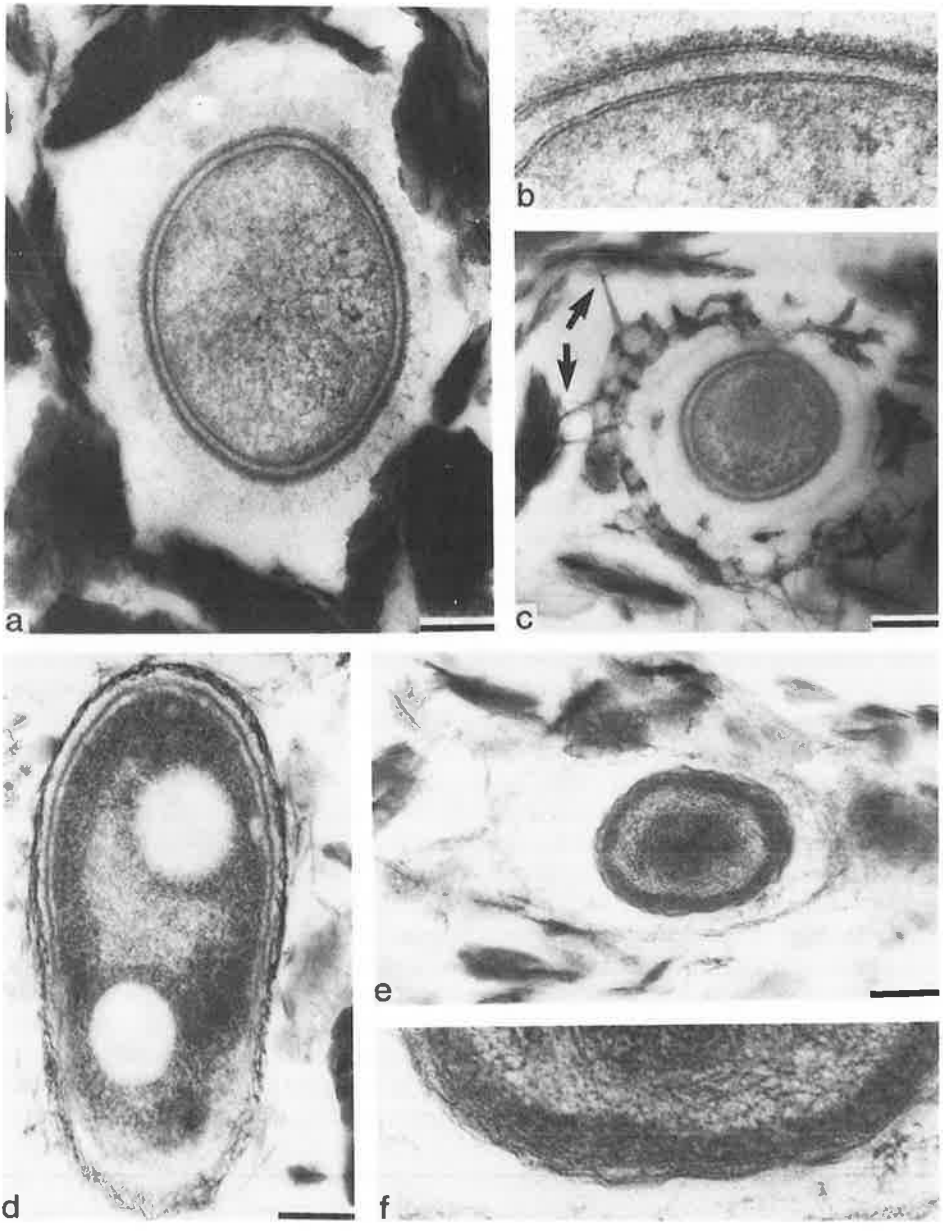

Fig. 1. Examples of Gram-negative bacteria. Bar indicates 0.2 μm. **a** Note slime layers with attached clay or mineral particles; RR stain. **b** 3× enlargement of cell wall in **a** showing inner and outer membrane with large periplasmic space **c** Complex slime layer, densely stained, with attachment to mineral particles (*arrows*); RR stain. **d** Note inclusions and densely stained fibrous capsule; no RR. **e** Convoluted cell wall and lightly stained slime layer; no RR. **f** 3 × enlargement of cell wall in **e**.

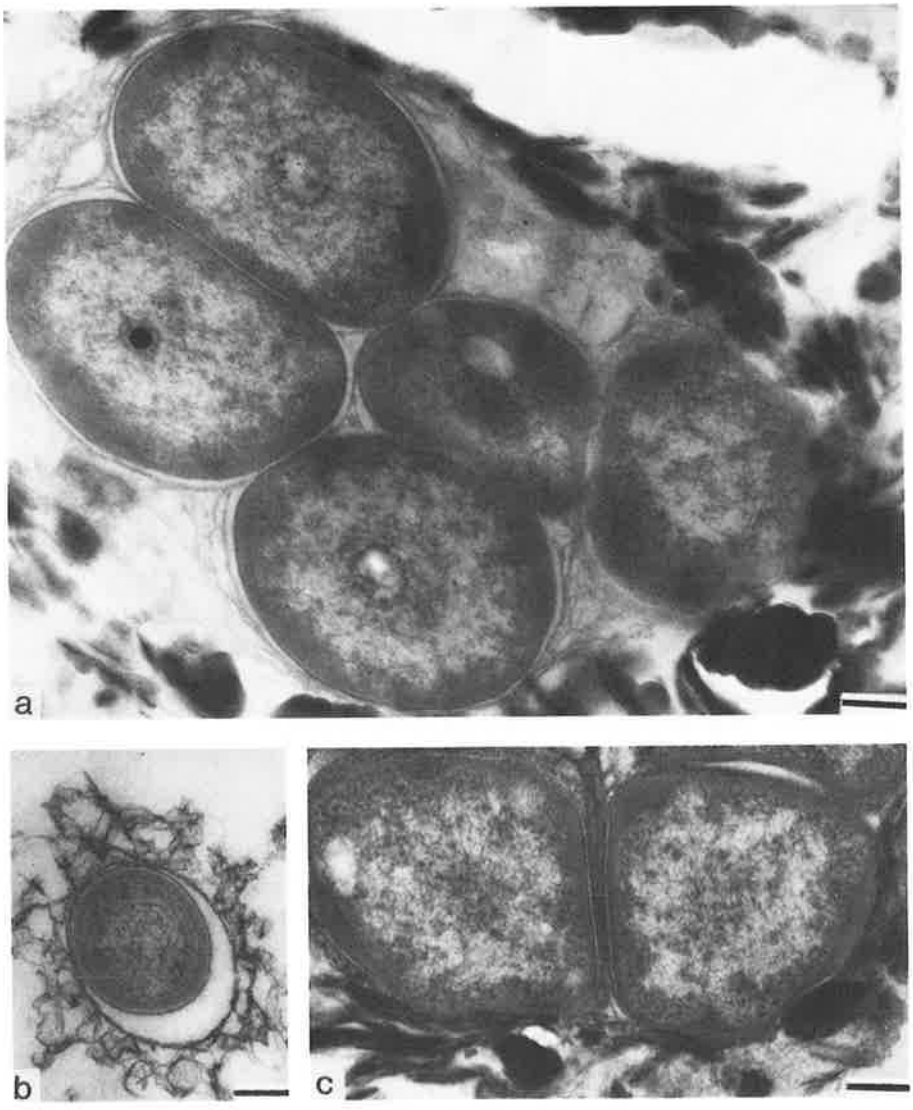

Fig. 2. Examples of Gram-positive bacteria. **a** Microcolony in slime layer with clay around edges; no RR; bar is 0.2 μm. **b** Irregular slime layer; no RR; bar is 0.1 μm. **c** Portion of microcolony showing detail of cell walls; no RR; bar is 0.2 μm.

Discussion

The values of 10% Gram-positive bacteria in the surface layers and 30% in the deeper layers agree well with the predictions made using muramic acid determinations and direct microscopy (Moriarty 1980). From the number and size of bacteria estimated by direct microscopy, the amount of muramic acid present if all bacteria were Gram negative could be estimated. If more muramic acid were present

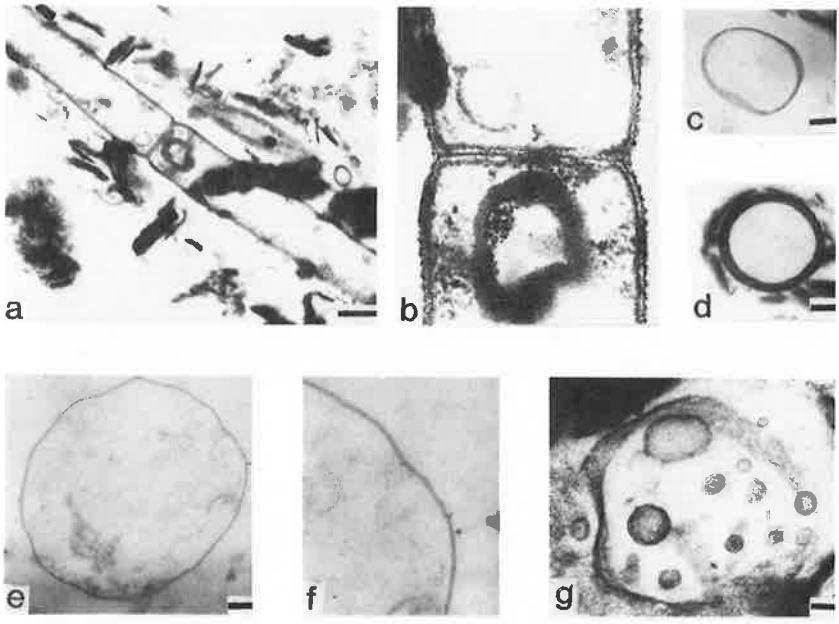

Fig. 3. Examples of empty cell sacs. **a** Cell walls, probably from Gram-positive bacteria; RR stain; bar is 0.2 μm. **b** 3× enlargement of portion of wall in **a**. **c** Probably Gram-positive wall; no RR; bar is 0.1 μm. **d** Probably Gram-positive wall; RR stain, bar is 0.1 μm. **e** Gram-negative wall; no RR; bar is 0.1 μm. **f** 2× enlargement of wall in **e**; **g** degrading Gram-negative cell; no RR; bar is 0.1 μm.

Table 1. Percentages of cells and empty wall sacs with typical Gram-positive wall structure. Two categories of cells were distinguished: (1) those with intact cytoplasm and (2) those with wall sacs or fragments only. The proportion of muramic acid (MA) in empty sacs was estimated by assuming that Gram-positive bacteria contained 5 times more MA than Gram negatives and relating the number of empty sacs to total cell numbers. Thus %MA in Gram-negative sacs = No. Gram-negative sacs/(No. Gram-negative cells + empty sacs + (No. Gram-positive cells and sacs × 5)) × 100.

	Gram-positive cell wall types		MA in empty sacs	Total intact cells	Total empty cell sacs	No. samples
	% intact cells	% empty sacs	%total MA			
June 1979						
0–1 cm	11	33	12	467	39	4
20–21 cm	30	44	40	577	170	10
Jan 1980						
0–1 cm	10[a]	35	7	1,683	69	21
20–21 cm	33[a]	73	10	1,332	100	20

[a] Significantly different $P < 0.001$, unpaired t-test on log transformed ratios of Gram-negative to Gram-positive cells.

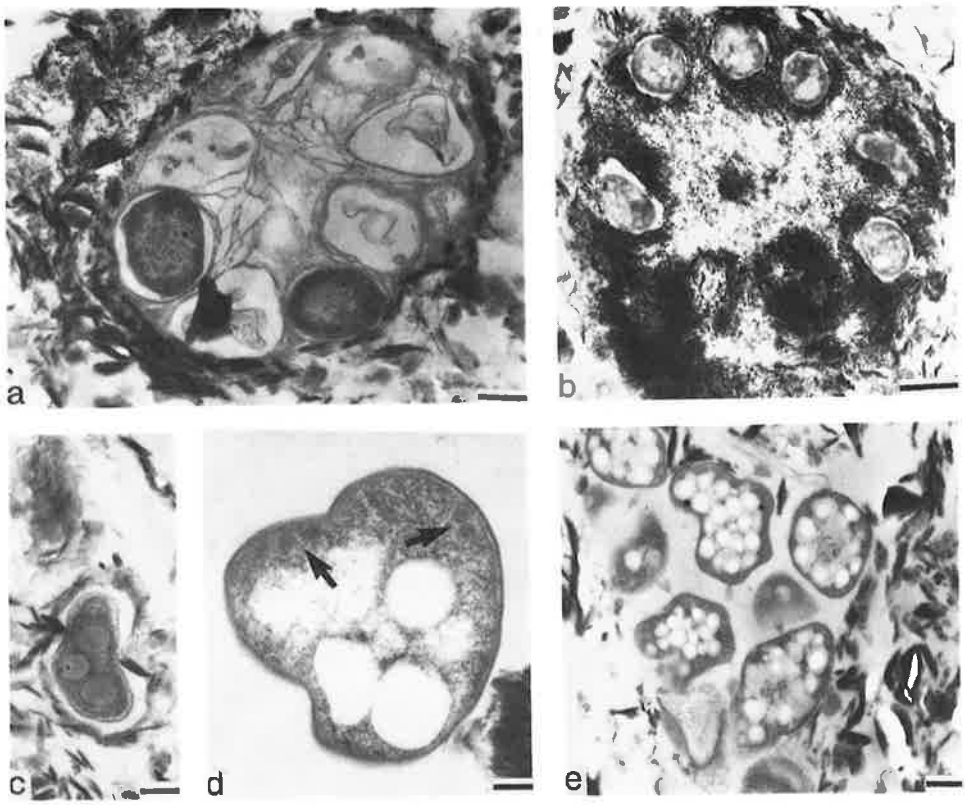

Fig. 4. Colonies of bacteria and inclusions. **a** Gram-negative colony with a number of degrading cells embedded in slime and surrounded by clay and mineral particles; no RR; bar is 0.2 μm. **b** Gram-negative colony in dense slime; no RR; bar is 0.5 μm. **c** Electron-dense inclusions; no RR; bar is 0.1 μm. **d** Large electron-transparent and small electron-dense (*arrows*) inclusions; no RR; bar is 0.1 μm. **e** Colony of Gram-negative bacteria with inclusions; RR stain; bar is 0.2 μm.

than that estimated, the excess was due to that in Gram-positive bacteria and empty wall sacs. Thus from the numbers and size of bacteria, and muramic acid values in the sediment, the proportion of Gram-positive bacteria could be predicted (Moriarty 1980). For comparison with direct microscopy, the sediments were blended and coarse fragments were allowed to settle before taking the suspension for counting and muramic acid determination. Counting the whole sediment after blending was subject to large errors because silt and sand grain fragments obscured many bacteria. Muramic acid measurements showed that generally about 50% (range 10–100%) of bacteria were removed from sand grains and thus direct microscopy on the suspension left after large particles settled out could underestimate total biomass (Moriarty 1980). The correlations between muramic acid and direct microscopy on the blended sediment after settling of silt, leading to the estimates of the proportions of Gram-positive bacteria, are supported by the electron microscopy results. Thus estimation of bacterial biomass by muramic acid measurements is shown to be valid. A further reason for checking the validity of the technique was because high values

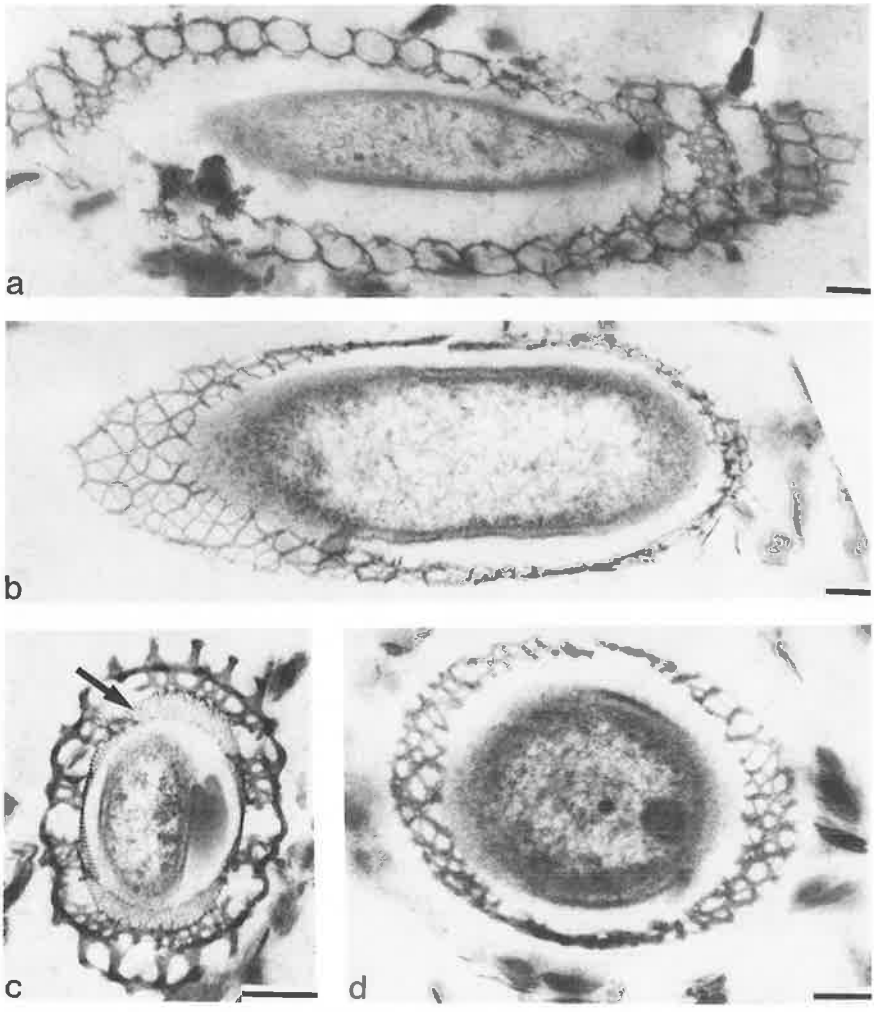

Fig. 5. Reticulate or honeycomb structures around Gram-negative cells. **a, c** Bacteria in possible diatom spine; RR stain; bar is 0.2μm. **b, d** RR stain; bar is 0.1 μm. **c** Tangential section showing fine reticulate structure (*arrow*).

for bacterial biomass were found: 15–25% of organic matter in the surface layers (Moriarty 1980).

To calculate more accurately biomass from muramic acid measurements, it is necessary to know the proportions of Gram-negative and Gram-positive bacteria. From plating and culture studies, there was a widely held belief that most marine bacteria were Gram negative, with perhaps more Gram-positive bacteria occurring in sediments (Hodgkiss and Shewan 1968). Such techniques are selective, and the samples used may not have been representative of the whole bacterial population. Ultrastructural studies on the whole population reported here provide data which agree with the hypothesis that Gram-negative bacteria predominate. Bae et al. (1972) used an exhaustive centrifugal

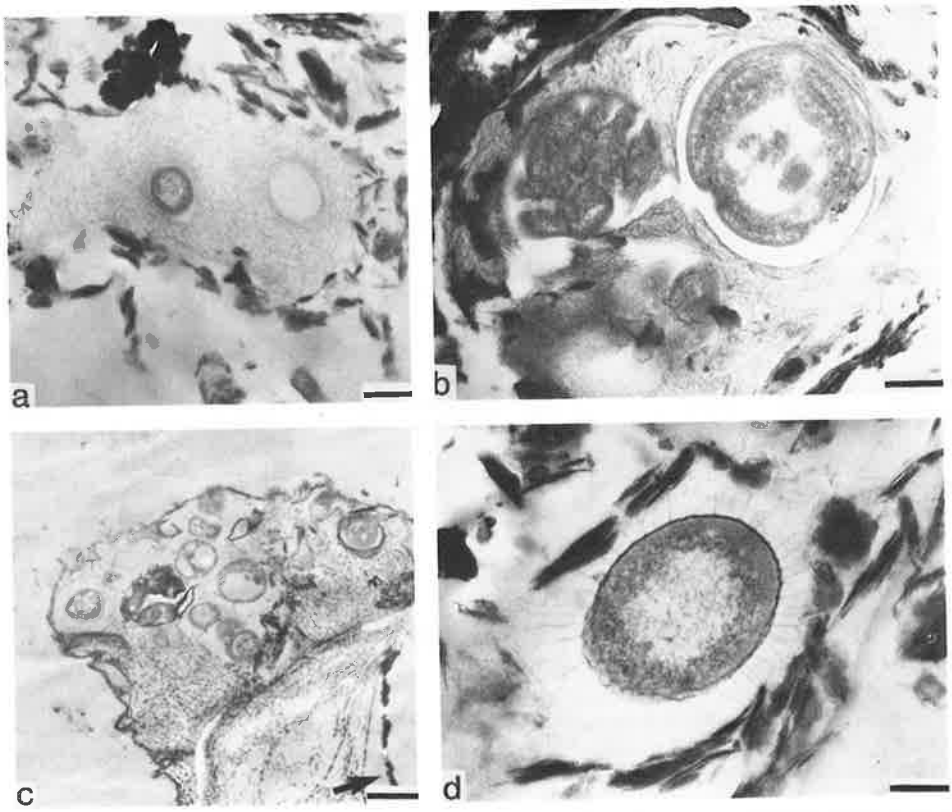

Fig. 6. Amorphous and radial slime layers. **a, b** Extensive amorphous slime layers; note internal membranes in **b**; RR stain; bar is 0.2 μm. **c** Coral reef sediment with empty diatom frustule (*arrow*) surrounded by slime; no RR; bar is 0.5 μm. **d** Radial slime strands attached to clay particles; RR stain; bar is 0.1 μm.

technique to concentrate bacteria from soil for electron microscopy. This technique was tried, but not used here because only a small proportion of the total population was recovered and results might have been biased toward organisms that were not tightly bound in aggregates of clay and organic matter.

An assumption was made that all muramic acid was present in living cells and that cells in surface sediments would be eaten or rapidly hydrolyzed, leaving little muramic acid in wall fragments (Moriarty 1975). An experimental study (Moriarty 1977) and the results reported here (Table 1) substantiate this assumption. In the surface sediment, although slime layers and "cell ghosts" were numerous, few contained fragments of wall material. The blending treatment was unlikely to have caused disruption of cells, so the wall fragments were probably naturally present. If disruption did occur, then even less muramic acid was contained in wall fragments. This suggests that the cells were actively growing and did not persist long enough to die and decompose slowly. Evidence for rapid growth rates has recently been obtained (Moriarty and Pollard 1981). In deeper layers of the sediment bacterial walls did seem to persist for longer as indicated by the larger number of cell ghosts with wall material apparently present (Table 1). Wall material was difficult to distinguish from electron-dense slime layers or capsules in some

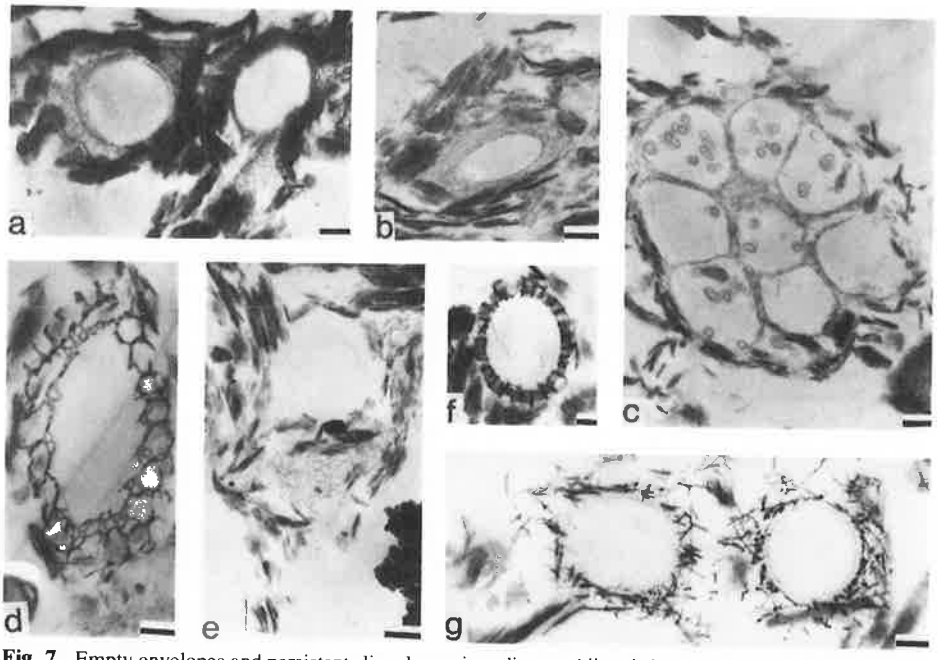

Fig. 7. Empty envelopes and persistent slime layers in sediment. All scale bars are 0.1 μm. **a** Clay particles attached to slime; RR stain. **b** Dense slime layers with attached clay; no RR. **c** Degraded microcolony with membrane fragments; no RR. **d** A possible diatom spine; surface layer. **e** Clay particles apparently held in position by slime from a degraded cell; no RR. **f** Slime layer with densely staining structures; no RR. **g** Slime fibrils with electron-dense inclusions; no RR.

cases. In the earlier study, about 40% of the capsules or slime layers were thought to contain wall material (i.e., muramic acid), but with improved fixation in the second study a much lower value was obtained. Sediment for the earlier study was collected in June (winter) when growth and other processes occur at slow rates. Wall material may well be more persistent in winter. The prediction of 40% Gram-positive bacteria in the deep sediment was made after taking into account the earlier value of 40% of muramic acid being in wall fagments (Moriarty 1980). Thus there may be a small discrepancy between muramic acid measurements in the 20 cm layer and estimates derived from electron microscopy. This may indicate that muramic acid persists in deeper anaerobic layers, perhaps bound to detritus or as small unrecognizable wall fragments. Although the method is valid for the surface sediments for which it was developed, caution should be exercised in using it in anaerobic sediments, where turnover is probably slow and organic matter accumulates. Empty capsules and slime layers were more numerous in the 20 cm layer than on the surface, indicating that bacterial envelopes decomposed slowly.

Most of the bacteria were embedded in slime or had extracellular coats which showed a wide range of variation in structure. Some of these stained with ruthenium red, indicating that they were acidic polysaccharides (e.g., Figs. 1a; 5b, d; and 6a, d). The cell in Fig. 6d shows fibrils extending out to sediment particles similar to those of a soil organism (Balkwill and Casida 1979). As all the bacteria had been attached to sand

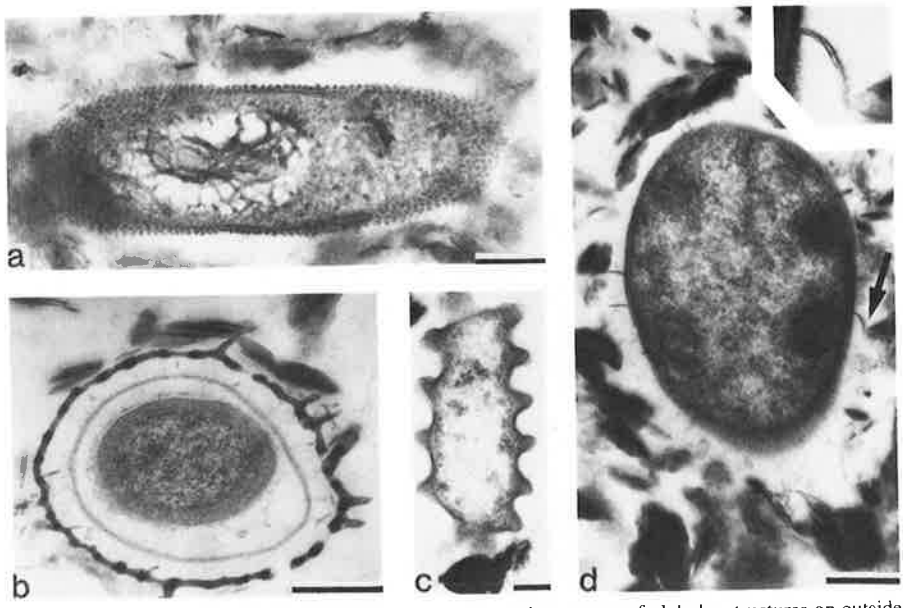

Fig. 8. Some distinctive morphological features. **a** Repeating pattern of globular structures on outside wall layer; RR stain; bar is 0.2 μm. **b** Probably an immature spore with densely stained envelope; no RR; bar is 0.1 κm. **c** A lobed prosthecate bacterium; bar is 0.1 μm. **d** Projections from cell wall, possibly membrane (blebs) or sheathed flagella; inset shows 4× enlargement of arrowed structure; no RR; bar is 0.2 μm.

grains, these polysaccharides were probably involved in adhesion (Fletcher and Floodgate 1973, Marshall et al., (1971). A variety of patterns of slime layers has been described recently, and their role in adhesion discussed in particular (Costerton and Geesey 1978). Cheng and Costerton (1975) have suggested that external coat layers also serve to protect bacteria by providing a relatively constant molecular environment close to the cell wall, although the outside environment may be variable and perhaps deleterious. They also considered that the coating layer held or retained degradative enzymes needed to hydrolyze polymeric substrates. Some of the external envelopes observed here stained densely without ruthenium red, indicating that they may contain other compounds such as protein in addition to polysaccharide (e.g., Figs. 1d and 2b). Both Gram-negative and Gram-positive organisms had extensive slime layers or envelopes. The function of surface polysaccharides has also been discussed by Dudman (1977). In addition to the functions referred to above, he discussed their role in microhomeostasis through their ability to bind water and cations and exert control over the immediate external environment of the bacteria. As polysaccharides also bind heavy metals, preventing their entry into cells, it is possible that some of the electron-dense material was due to heavy metals (e.g., Fig. 4b).

Some of the most intriguing envelopes were those with intricate honeycomb structures, which surrounded some Gram-negative cells (Fig. 5). Some were multilayered (Fig. 5a) and when cut in oblique section were not simple electron-dense strands but an intricate lacework (Fig. 5a, c). They might be diatom spines that have been invaded by bacteria, as they have some similarities to *Chaetoceros* spines (J. McN. Sieburth and P. W. Johnson, personal communication). Thus they may be siliceous

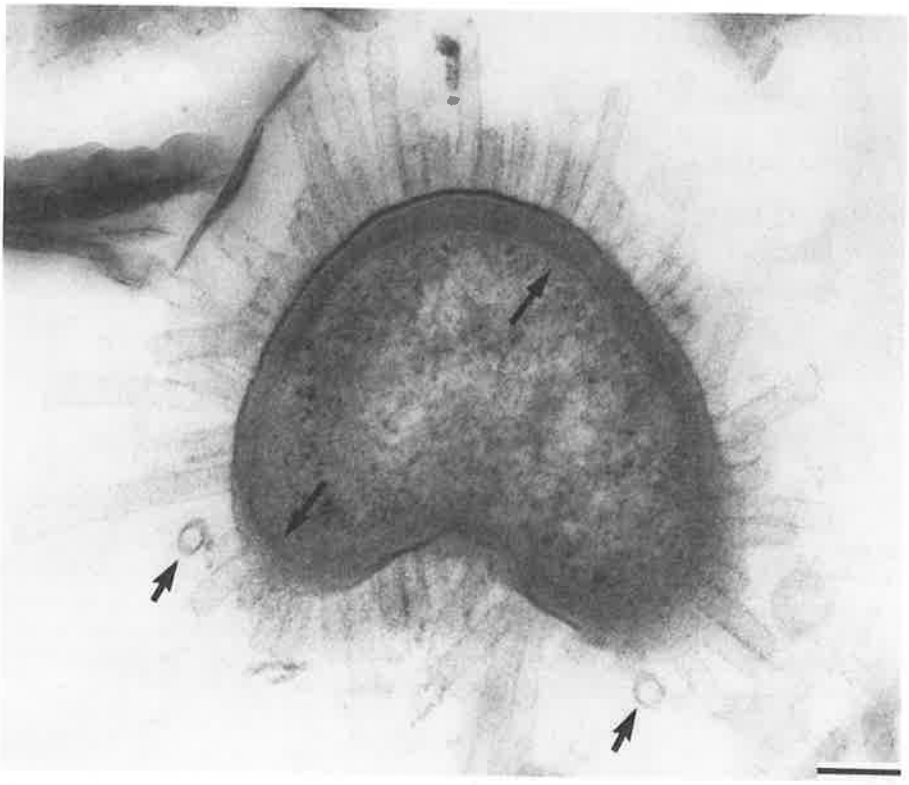

Fig. 9. A spiny bacterium. Large arrows indicate connection with cytoplasmic membrane and small arrows probable transverse section showing hollow spines; no RR; bar is 0.1 μm.

structures and not true bacterial envelopes. Some similar empty structures were observed (Fig. 7d).

The slime layers associated with some cells, especially those in colonies, were extensive (Figs. 4 and 6) and similar to colonies of bacteria embedded in capsular material in soil (Foster and Rovira 1978). The overall impression is that slime and cell envelopes constituted an appreciable amount of organic matter in relation to cell contents. This material is derived from bacteria, and may be as trophically important to deposit-feeding animals as bacteria. At least 50% of the organic matter that was ingested and assimilated by holothurians in coral reef sediments was probably detritus, which would include material from bacterial capsules, and about 10% was bacteria (Moriarty 1982). Electron microscopy has shown that, like the bacteria reported in this paper, most of the coral reef bacteria were embedded in slime (Fig. 6c and unpublished work). Thus although bacterial biomass may be a small percentage of organic matter in many sediments, their biosynthetic products may represent a much larger proportion. Cammen (1980) found that the biomass of microorganisms eaten by the deposit-feeding polychaete *Nereis succinea* satisfied only about 25% of the energy needs of the animals and proposed that detritus may be important as a food source. Bowen (1980) has reported a similar phenomenon in the nutrition of a population of cichlid fish *Sarotherodon*

mossambicus in which total microbial biomass could satisfy only some of the food requirements of the fish. He suggested that polypeptides bound to detrital aggregates were also a source of nutrition. Some of the exopolymers secreted by microorganisms are proteins, glycoproteins, or contain aminosugars and peptides and thus would be nutritionally valuable (e.g., Buckmire and Murray 1973, Costerton et al. 1974a).

Electron-transparent inclusions that were bounded by a nonunit membrane may be either poly-β-hydroxybutyrate or polyglucose. They are similar to inclusions observed in other bacteria (Foster and Rovira 1978, Shively 1971), and with the deformed cell shape are characteristic of poly-β-hydroxybutyrate, which is usually formed when growth is unbalanced, e.g., if nitrogen is limiting. The nature of the medium density and very dense inclusions is not known. The projections from the wall of the organism in Fig. 8d may be membranous blebs, although they are not similar to those on a marine pseudomonad reported by Wiebe and Chapman (1968). They may be sheathed flagella. Cheng and Costerton (1977) studied the ultrastructure of a thin-walled Gram-positive organism (*Butyrivibrio* sp.) from the rumen, which had knobs of ruthenium red-staining polysaccharide on its surface. The organism in Fig. 8a is very similar to this. The irregular organism (Fig. 8c) is similar to *Prosthecomicrobium* (Staley 1968).

No comparable organisms with spines like those shown in Fig. 9 are known to us. The spines, which are hollow, appear to be inserted through the cell wall and have their origin in the cytoplasmic membrane. From the different lengths, it seems that they may have been broken during blending, and thus were longer and are fragile. Structures termed spinae were observed on the surface of a marine pseudomonad (Willison et al. 1977). These spinae had their origin at the outer membrane, had an expanded conical base, and had a definite subunit structure, in contast to those shown in Fig. 9. The spines observed by us are more similar to those on a methane-oxidizing bacterium (Suzina and Fikhte 1977) and those on a *Synechococcus* (Perkins et al. 1981).

Acknowledgments. We thank Dr. R. C. Foster, Division of Soils, CSIRO, Glen Osmond, South Australia, for his assistance in the early stages of this project. We are grateful to Mr. A. McGregor and Ms. Jane Westcott of the Electron Microscope Unit, Department of Microbiology, University of Queensland, who assisted in sectioning and viewing the specimens at the microscope. We also thank Mr. J. D. Kerr, Division of Mathematics and Statistics, CSIRO, Long Pocket, Queensland, for the statistical analysis.

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Arch. Microbiol. 120, 191–193 (1979)

Archives of
Microbiology

Muramic Acid in the Cell Walls of *Prochloron*

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Abstract. Muramic acid has been detected in *Prochloron* with the aid of two different techniques. It was assayed by cleaving D-lactate from muramic acid and then reducing NAD with D-lactate dehydrogenase and measuring the NADH with bacterial luciferase. Gas-liquid chromatography of trimethylsilyl derivatives of cell extracts confirmed that muramic acid was present in about the quantity given by the D-lactate assay. The amount of muramic acid present was 1.7 $\pm$ 0.2 µg/mg dry weight or 1.3 fg/µm^2 of cell surface. This suggests that the thickness of the peptidoglycan layer in *Prochloron* is similar to that in blue-green algae.

Key words: *Prochloron* – Muramic acid – Peptidoglycan.

Muramic acid is known to occur only as a component of peptidoglycan, which is a characteristic constituent unique to the cell walls of prokaryotic organisms (Rogers and Perkins, 1968). I wished to determine whether it occurs in the cell walls of the anomalous alga *Prochloron* which, though clearly prokaryotic, shares several features with the Chlorophyta (eukaryotic green algae)[1].

Prochloron is a genus of unicellular, prokaryotic algae which, because they contain chlorophylls *a* and *b* and lack phycobilin pigments, have been set apart in a new algal division, the *Prochlorophyta* (Lewin, 1976b, 1977) although this has been questioned (Antia, 1977). So far, they have been found only as symbionts of didemnid ascidians. Because they have not yet been grown in culture, studies of their biochemistry have to be based on cells separated from animal hosts collected in nature.

Peptidoglycan consists of chains of alternating units of N-acetylmuramic acid and N-acetylglucosamine, crosslinked with short peptide chains. Acid hydrolysis liberates muramic acid from peptidoglycan, and alkali hydrolysis quantitatively cleaves D-lactate from muramic acid (Tipper, 1968). Two different techniques have been used to show that muramic acid is present in *Prochloron*. One method was based on the assay of D-lactate and the other on gas-liquid chromatography of muramic acid.

Materials and Methods

Prochloron samples. Three collections of *Prochloron* were made from the host species *Lissoclinum patella* in two localities. Sample 1 was collected from near Singapore in July, 1976. Sample 2 was collected on the Great Barrier Reef near Cairns in December, 1976. Sample 3 was collected from near Singapore in April, 1977. Cells were expressed from the animal hosts in the field and then brought to the laboratory on dry ice where they were freeze-dried.

Muramic Acid Assays. The D-lactate was assayed by using D-LDH to generate NADH, and bacterial luciferase to measure NADH. Details of these procedures have been published elsewhere (Moriarty, 1977, 1978). Samples containing about 5 to 10 mg dry weight of whole cells were hydrolyzed with acid and then assayed for D-lactate before and after alkali hydrolysis. Internal standards were used with all assays. Muramic acid values were calculated from the difference in results between assays before and after alkali hydrolysis.

Glycollate and, to a lesser extent, other D-α-hydroxy carboxylic acids also react with D-LDH to reduce NAD. Other compounds may react with other enzymes contaminating the D-LDH assay system to reduce NAD. As these extra reactions occur with the acid hydrolysate, their effect is subtracted from that due to D-lactate derived after alkali hydrolysis. This method for the assay of D-lactate is much more sensitive than other methods that are available.

[1] I agree with the views expressed by Lewin (1976a) in deliberately using "algae" rather than "bacteria" when referring to *Prochloron* and Cyanophyta in this paper. In fact, the similarity of the pigment compositions of *Prochloron* and the Chlorophyta further justifies the informal classification of these prokaryotes with the algae.

Abbreviations. D-LDH = D-lactate dehydrogenase; MA = muramic acid; TMS = trimethylsilyl; TLE = thin layer electrophoresis; GLC = gas-liquid chromatography

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Electrophoresis. A suspension of microcrystalline cellulose was spread to a depth of 1 mm on glass plates (20 × 20 cm). Samples containing about 2 mg dry weight of whole cells were hydrolysed with acid. After removing the acid by freeze-drying, the samples were applied in a band across most of the plate, leaving a strip 3 cm wide for standard muramic acid. The plates were lightly sprayed with a buffer consisting of water:acetic acid:formic acid (975:20:5 (v/v). Electrophoresis was carried out, using the same buffer, at 500 V for 2 h. After development the plates were dried and the standard located with ninhydrin. The band containing muramic acid was scraped off, eluted with water, concentrated by freeze-drying and assayed for D-lactate before and after alkali hydrolysis. The rest of the cellulose on each plate was divided into several bands, eluted and assayed for D-lactate, as a control for the presence of other compounds which might yield D-lactate (or glycollate) after alkali hydrolysis.

Gas-liquid Chromatography. Samples for GLC, containing about 3 mg of hydrolyzed *Prochloron* cells, were freeze-dried then warmed to 60°C before adding 100 µl of trimethylsilylimidazole in pyridine (Tri-Sil-Z from Pierce, Rockford, Ill. USA). After about 10 min 5 µl of the pyridine solution were injected into a Varian series 2700 gas chromatograph. Other details of the chromatography were similar to those described by Casagrande and Park (1977).

Enumeration of bacteria. The method of Daley and Hobbie (1975) was used. Samples were collected on black 0.22 µm Sartorius filters and viewed with Leitz Ploem incident-light fluorescence equipment fitted with the K filter system.

Results

The *Prochloron* cells contained about 1.8 µg MA/mg dry weight when assayed as D-lactate in whole cell hydrolysates (Table 1).

After purification by electrophoresis, a smaller amount of muramic acid was obtained (Table 2). Muramic acid was clearly separated by electrophoresis from glycollic acid, neutral sugars and other compounds which could lead to NAD reduction after alkali hydrolysis. Only the band corresponding to standard muramic acid liberated D-lactate after alkali hydrolysis. A more conclusive demonstration of muramic acid in *Prochloron* is given by the GLC results (Table 2). The double peaks for the two anomeric forms were clearly separated from other peaks. Quantification was difficult with this technique because the TMS derivatives of muramic acid hydrolyse very easily in the crude hydrolysate.

Table 1. Muramic acid content of *Prochloron* measured by the D-lactate procedure

Prochloron sample	Muramic acid[a]		
	µg/mg dry wt.	µg/mg C	fg/µm²
1	1.9	4.2	1.4
2	1.8	4.0	1.3
3	1.6	3.6	1.1

[a] Means for 2 determinations on each of 2 portions of whole cells, about 5 mg to 10 mg dry weight. Accuracy of the assays was about ± 20%. Values for MA per unit surface area were calculated on the basis of the cells being spheres 20 µm in diameter

Table 2. Muramic acid identification with electrophoresis and gas chromatography

Prochloron sample	Muramic acid[a] µg/mg dry wt.		
	TLE	GLC	TLE/GLC
2	0.8	0.7	1.0
3	1.1	1.0	–

[a] The TLE results are for the band corresponding to a standard MA (see Materials and Methods). No other bands contained compounds yielding D-lactate after alkaline hydrolysis. For GLC, TMS-imidazole derivatives were prepared from acid hydrolysates of whole cells, and in one case, from the band corresponding to MA from a TLE plate

Table 3. Bacterial contamination of *Prochloron* preparations[a]

Prochloron sample	Bacteria	*Prochloron*	No. bacteria per *Prochloron* cell	Bacterial biomass as % *Prochloron* biomass[c]
	No. cells/field of view[b]			
1	50 ± 17	12 ± 7	4	0.1
2	35 ± 13	6 ± 3	6	0.1
3	47 ± 14	10 ± 4	5	0.1

[a] Freeze-dried samples, rehydrated in phosphate buffer (0.1 M, pH 7.5), were stained with acridine orange. All green or orange fluorescing particles of bacterial size and shape were counted.
[b] 12 fields of view were counted for each preparation; standard deviation is shown.
[c] Bacterial biomass was estimated as a percentage of *Prochloron* biomass, based on the observation that *Prochloron* cells have a diameter of about 20 µm and bacteria about 0.8 µm

Some bacteria were present in the *Prochloron* samples in numerical ratios of about 5:1 (Table 3). Because the biomass of a typical bacterial cell in these samples is only about 0.02% of that of a *Prochloron* cell, the total biomass of bacteria in these samples amounted to only 0.1% of that of *Prochloron*. In addition to the *Prochloron* and bacteria visible under the microscope, a small amount of unidentified organic matter (perhaps 2% of the total) was seen in sample 3.

Discussion

The experiments reported here clearly show that muramic acid is present in *Prochloron*. The quantities measured in whole cell extracts using the D-lactate assay are likely to be more accurate than those measured after

chromatography or electrophoresis, where losses can occur. These experiments did not rule out the possibility, however, that a small proportion of the D-lactate measured in whole cell extracts came from a source other than muramic acid. In this analysis it was assumed that the only change occurring during alkali hydrolysis was the liberation of D-lactate from muramic acid. Although many sugars can be degraded by acid or alkali to D-lactate and glycollate, this is substantial only when the treatment is carried out under anaerobic conditions for long periods of time (Shaffer and Friedeman, 1930), and would be insignificant in my experiments.

The results of the electrophoresis experiments show that the presumptive muramic acid had a positive charge at pH 2.0 and was the only compound which liberated D-lactate under alkaline conditions. The detection in the gas chromatograph of double peaks with retention times identical to those of standard muramic acid, from the compound that was first separated by electrophoresis, further substantiates the validity of the D-lactate assays. The two peaks are due to the α and β anomeric forms of muramic acid (Casagrande and Park, 1977).

A little of the muramic acid in these samples would have been derived from the contaminating bacteria. If all the muramic acid detected were only from the bacteria they would have needed to contain about 9000 µg MA/mg dry weight, which is clearly impossible. As most marine bacteria are Gram-negative and contain about 4 µg MA/mg dry weight (Moriarty, 1977), the muramic acid in bacteria contaminating these *Prochloron* samples would amount to about 4 ng/mg dry weight; i.e. it is insignificant.

Prochloron contains less muramic acid per unit biomass than most bacteria or blue-green algae (Moriarty, 1977) which is why detection of its presence has proved difficult. The muramic acid content of a cell, however, is a function of its surface area because this compound occurs solely in the peptidoglycan layer of the cell wall. The value of 1.1 to 1.4 fg MA/µm^2 of cell surface, reported here for *Prochloron*, is similar to that obtained for a blue-green alga *Oscillatoria tenuis* (1.6 fg/µm^2) and about 10 times that of a Gram-negative bacterium, *Pseudomonas fluorescens* (0.15 fg/µm^2) (Moriarty, 1977). From these values, we can predict that the peptidoglycan layer in *Prochloron* would be about the same thickness as that in blue-green algae. Electron micrographs show that the structure of the cell walls of *Prochloron* is similar to that of blue-green algae and, indeed, that the electron-dense layer 2 is about the same thickness as that of Chroococcaceae (Schulz-Baldes and Lewin, 1976). Golecki (1977) has shown that this dense layer in the blue-green alga *Anacystis nidulans* is peptidoglycan. Thus, the finding of muramic acid in *Prochloron*, in amounts comparable with those in a blue-green alga, strengthens the hypothesis that *Prochloron* has a typical prokaryotic cell wall, with close affinities to the Cyanophyta.

Acknowledgements. I am most grateful to Dr. Ralph A. Lewin for supplying the freeze-dried samples of *Prochloron* and for his helpful comments on the manuscript.

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Received August 7, 1978

Bacterial Biomass and Productivity in Sediments, Stromatolites, and Water of Hamelin Pool, Shark Bay, Western Australia

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Abstract Heterotrophic bacterial biomass and growth rates were examined in stromatolites formed from four different types of benthic cyanobacterial mats. Bacteria in algal mats were counted using direct microscopy and biomass was estimated from the numbers of bacteria. Heterotrophic bacterial growth rates were estimated from the rate of incorporation of tritiated thymidine into DNA. Pustular mat, which occurs in the upper intertidal zone, contained relatively few bacteria in the surface layers (0–5 mm), having about 0.2×10^6 cells mm^{-3}, or 20 mgC m^{-2} per millimetre depth. Other mats in the lower intertidal and subtidal zones had from 1×10^6 cells mm^{-3} to 8×10^6 cells mm^{-3}. Heterotrophic bacterial productivities were 2.1 to 5.0 mgC m^{-2} h^{-1}. Turnover times were an average of 1 day in the sandy sediment and 5 days in the colloform mat. Although these results are minimum estimates, they indicate that heterotrophic bacteria contribute substantially to the carbon cycle in stromatolites, by utilizing about 20 to 30% of primary production.

Received Aug. 3, 1982; accepted Sept. 14, 1982.

Geomicrobiology Journal, Volume 3, Number 2
0149-0451/83/020121-00$02.00/0

Introduction

Stromatolites have been defined as "organo-sedimentary structures produced by sediment trapping, binding and/or precipitation as a result of growth and metabolic activity of microorganisms, principally cyanophytes" (Walter, 1976). They are abundant in the geological record, particularly the Proterozoic, but now are found in only a limited number of environments of which Hamelin Pool contains the largest array of living stromatolites (Hoffman, 1976; Playford and Cockbain, 1976). Hamelin Pool is a hypersaline lagoon in the southeastern corner of Shark Bay (Lat. 26°S) on the coast of Western Australia. High salinities (55 ‰ to 70 ‰) have inhibited algal grazers and allowed algal mats and stromatolites to proliferate. The stromatolites have been formed from a variety of benthic algal mats. Those examined in the work reported here are pustular mat in the upper intertidal zone and tufted mat in the mid intertidal zone. These mats are subject to considerable desiccation. Smooth mat occurs in the lower intertidal region and colloform mat in the subtidal zone. Each is dominated by different species of cyanobacteria (Golubic, 1976). Because little information is available on the presence of heterotrophic bacteria, their numbers and growth rates were investigated in the work reported below.

This work was carried out as part of a multidisciplinary study of stromatolite biology and geology at Hamelin Pool in November, 1980. A method for determining the growth rates of bacteria in sediment has been applied (Moriarty and Pollard, 1981, 1982). In this method, the rate of incorporation of tritiated thymidine (Tdr) into DNA is measured using an isotope dilution technique. From the rate of DNA synthesis, an estimate of bacterial growth rates can be calculated. Thymidine was chosen for measurement of DNA synthesis in heterotrophic bacteria because cyanobacteria, fungi, and eukaryotic microalgae lack thymidine kinase, the enzyme necessary for its incorporation into DNA (Grivell and Jackson, 1968; Glaser et al., 1973). The advantages and disadvantages of using thymidine incorporation into DNA (not just uptake into cells) to measure growth rates are discussed in detail

elsewhere (Moriarty, 1982). As most, but not all, heterotrophic bacteria possess thymidine kinase, this method gives minimum values for bacterial growth rates. Similar studies undertaken by others show that estimates of bacterial growth rates given by incorporation of thymidine into DNA agree with those made using other techniques (Fuhrman and Azam, 1980, 1982; Riemann and Sondergaard, 1982).

Primary productivity and sulfate reduction in the algal mats and sediments of Hamelin Pool have been studied by Bauld et al. (1979). Measurements of bacterial productivity reported here are compared with their values.

Materials and Methods

Bacterial studies on algal mats were carried out in the locality that was used by Bauld et al. (1979); photographs of this area are shown in Figures 5B and 8 in Playford and Cockbain (1976). The various mat types are described by Golubic (1976) and Hoffman (1976).

Cores (20 mm diameter, 50 mm depth) were taken for enumeration of bacteria. The sediment in the core was divided into portions at depths corresponding to visible laminations (Table 1). The total wet weight of each portion was measured, and then a subsample of about 1 g was weighed. This was treated with 10% acetic acid overnight to remove $CaCO_3$, homogenized with an Ultra-Turrax blender (Janke and Kunkel KG, Breisgau, FRG) at 20,000 rpm, diluted to 18 ml; then 2 ml of 36% formaldehyde solution was added. Samples of 50-100μl were counted with an epifluorescent microscope after staining with acridine orange using the system described earlier (Moriarty, 1980).

Two experimental procedures were used to measure bacterial growth rates. The first was described by Moriarty and Pollard (1981). Sandy sediment from a subtidal area near colloform mat about 50 m (horizontally) from low water level was collected with corers (30 mm diameter). The pale colored surface layer (0–3 mm) was scraped from a series of cores and pooled for analysis using the isotope dilution technique with tritiated thymidine. A sample of sediment was also preserved in formalin for counting bacterial

Table 1
Bacterial numbers and biomass in algal mats.

Sediment core depth (mm)[a]	*Comments*	*No.*[b] $\times 10^6$ *mm*$^{-3}$	*Biomass*[c] (*mgC m*$^{-2}$ *mm*$^{-1}$ *depth*)
Smooth mat			
0– 2	algal mat	3.9 ± 0.2	390
2–10	black, sandy	2.4 ± 0.3	240
10–20	black, sandy	0.5 ± 0.1	50
Tufted mat			
0– 2	algal mat	8.0 ± 2.1	800
2– 4	black, sandy	6.1 ± 0.9	610
4–10	sandy, algal layer	4.0 ± 0.8	400
10–20	sandy, algal layer	1.7 ± 0.2	170
20–30	sandy, old algal mat	2.5 ± 0.5	250
Colloform mat			
0– 3		1.0 ± 0.2	100
Pustular mat			
0– 5	algal mat	0.2 ± 0.1	20[d]
5– 8	black, sandy	2.3 ± 0.7	230
8–12	black, sandy	2.0 ± 0.2	200

[a] Cores were analysed at depths corresponding to laminations.
[b] Numbers are mean values for 10 counts ± standard error.
[c] Biomass was estimated from numbers, assuming 1×10^{-10} mgC cell^{-1}; most bacteria had dimensions of between $0.8 \times 1\mu$m and $1 \times 3\mu$m.
[d] The surface of this mat was very irregular, so the results per unit area are a guide only.

numbers. Colloform mat from the surface of subtidal columnar stromatolites about 100 m from low water level was collected and treated similarly. Samples were collected and analyzed at 0900 h and 1430 h. The weather was calm and thus little or no surface sediment was suspended in the water column.

In the second type of experiment, isotope was injected into individual cores at four levels: 0, 5, 10, and 15 mm depth. Three dilutions (one-, two-, and fourfold) of 9.2×10^5 Bq (25 μCi) of Tdr in 25 μl were injected into duplicate cores through small holes in the sides of the corers and the cores were kept at in situ temperature (23°C) for 15 min. Cores were then extruded and 5-mm sections were placed in 2 ml of 0.6 M NaOH containing 10 mM Tdr and 0.5 ml of a humic acid extract from soil (to assist in precipitation of DNA). They were then treated as described by Moriarty and Pollard (1981). For control experiments, one set of cores was extruded into NaOH, and then 9.2×10^5 Bq (25 μCi) of Tdr was added. Another set of cores was sectioned and preserved in 4% formalin for enumeration of bacteria. Cores from smooth mat and soft sediment about 10 m from the low tide level were analyzed.

The numbers and growth rate of bacteria in seawater were measured in a transect from the shore at the southern end of Hamelin Pool out to about 6 km northwards. Water was collected near the surface (< 10 cm depth) at each station. Within 15 min of collections, 50 ml were incubated with 100 μCi of Tdr (2 nmol) for 30 min at in situ temperature and then filtered through 0.2 μm cellulose nitrate filters and immersed in 1 ml of NaOH (0.6 M) containing 10 mM Tdr and 0.5 ml of a humic acid extract of soil. Samples were then analyzed as described by Moriarty and Pollard (1981). Control samples of 500 ml of water were chilled on ice, then isotope was added and they were filtered immediately. Two samples of 10 ml each from each station were preserved with formalin for enumeration of bacteria by direct microscopy after staining with acridine orange.

Results

Bacteria were very numerous in most zones of the algal mats that were examined (Table 1). Pustular mat was unusual in having

fewer bacteria in the surface layers than other mats. Filamentous bacteria were particularly abundant in all mats, making counting difficult. Most cyanobacteria were readily distinguishable (and were not counted), but some of the filamentous forms that were counted may have been very small cyanobacteria. Filaments were counted as though they were made up of individual bacteria each 2 μm long. Most bacteria were associated with an orange-red fluorescing material (distinct from chlorophyll), which was probably slime stained with acridine orange.

Growth rates of bacteria in sandy subtidal sediment were rapid in the aerobic zone with little difference between morning and afternoon (Table 2). Growth rates were slower in the top 3 mm of colloform mat on subtidal columnar stromatolites, and as bacterial numbers were larger, turnover times were slower (Table 2). The differences between morning and afternoon were not significant. The isotope dilution experiments showed that the specific radioactivity of thymidine incorporated into DNA was diluted to about 50% by other thymine base precursors. Values for bacterial growth rates in core experiments with algal mats (Table 3) were less than those for batch experiments in the subtidal sediments (Table 2). As bacterial numbers in the algal mats were large, the turnover times for the bacterial populations were long (Table 3). Growth rates were most rapid in the top 5–10 mm of sediment. The specific activity of thymidine was diluted by 20–40% in the 0–5 mm zone, and 0–10% in the 5–10 mm zones. No apparent dilution was measurable in the 15–20 mm zone.

The water of Hamelin Pool contained large numbers of bacteria, many of which were large (about 1 by 3 μm) compared with normal marine bacteria. Bacterial density was greatest near the shore in shallow water (Table 4). Growth rates were faster in deeper water, with salinities of around 65 ‰, than in shallow, warm water where the salinity was higher (Table 4). No dilution of isotope incorporated into DNA was found with experiments that were carried out at both an inshore and an offshore site. Growth rates were so slow, however, that small dilution effects would not have been observed.

Table 2
Bacterial Productivity in Surface Sediment.[a]

Sediment	Time of day (h)	No. of bacteria $\times 10^{-8}$ g^{-1} sediment[b]	No. of bacteria dividing: No. $\times 10^{-7}$ $g^{-1}h^{-1}$	No. of bacteria dividing: No. $\times 10^{-10}$ $m^{-2}h^{-1}$	Productivity[c] (mgC m^{-2} h^{-1})	Turnover time (h)
Sediment	0900	3.7 ± 0.2	2.1	5.0	5.0	18
	1400	4.8 ± 0.8	1.4	3.2	3.2	34
Stromatolite	0900	11.8 2.6	1.4	2.1	2.1	84
	1400	22.6 5.6	1.4	2.5	2.5	160

[a] Homogeneous subsamples of sediment were assayed using the isotope dilution technique. The top 3 mm of subtidal sandy sediment and of colloform mat on columnar stromatolites were assayed.
[b] Standard errors of bacterial numbers are shown.
[c] The probable range of variation in productivity is ± 50% of the calculated values, as determined by the deviation of points on the isotope dilution curve from linearity.

Table 3
Bacterial Productivity in Sediment Cores of Intertidal Smooth Mat and Subtidal Soft Sandy Sediment Near Colloform Stromatolites.

Sediment core depth (mm)	*No. of bacteria* $\times 10^{-8} g^{-1}$ *sediment*	*Number of bacteria dividing*		*Productivity*[a] $(mgC\, m^{-2} h^{-1})$	*Turnover time (days)*
		$No. \times 10^{-6}$ $g^{-1} h^{-1}$	$No. \times 10^{-9}$ $m^{-2} h^{-1}$		
Smooth mat					
0– 5	22[b]	5.8	18	1.8	16
5–10	27[b]	2.9	9	0.9	39
10–15	20.5 ± 5.3	4.2	13	1.3	20
15–20	5.2 ± 0.5	1.0	3	0.3	22
Subtidal sediment					
0– 5	15.8 ± 2.3	3.0	9.5	0.9	22
5–10	16.4 ± 3.9	2.8	8.9	0.9	23
10–15	15.8 ± 3.2	1.1	3.5	0.3	60
15–20	9.5 ± 0.9	0.3	0.9	0.1	130

[a] Values are averages of duplicate cores; the range of variation in productivity is about ± 50%.
[b] Samples lost; values estimated from data in Table 1.

Table 4
Numbers and Productivity of Bacteria in Hamelin Pool Water.

Station	*Distance from shore*[a] *(km)*	*Salinity (0/00)*	*Temperature (°C)*	*Depth (m)*	*Number of bacteria* $\times 10^{-8}$ *liter*$^{-1}$[b]	*Bacteria dividing (No.* $\times 10^{-6}$ *liter*$^{-1}$ *h*$^{-1}$*)*	*Productivity*[c] *(μg C liter*$^{-1}$ *h*$^{-1}$*)*	*Turnover time (days)*
Small pool	0.1	107	28	0.05–0.2	15 ± 1.1	1.8	0.18	35
Large pool	0.2	75	27	0.05–0.2	9 ± 0.7	1.4	0.14	27
Main water body	0.3	66	27	0.1	6.5 ± 0.6	2.6	0.26	10
Main water body	0.4	65	27	0.2	5.2 ± 0.4	2.2	0.22	10
Main water body	0.5	66	25	0.2	5.5 ± 0.3	3.2	0.32	7
Over diatom mat	1	65	25	2.5	3.8 ± 0.5	1.3	0.13	12
Over soft sediment	6	62	25	7	4.1 ± 0.5	2.6	0.26	7

[a] Approximate distances from shore at the southern end of Hamelin Pool are shown. Surface samples were taken between 11 A.M. and noon, at low tide, with little or no wind. Nearshore sites were surrounded by pustular mat.

[b] Bacterial numbers were determined on duplicate samples with 10 counts on each; standard errors are shown.

[c] Productivity was calculated from the growth rates of bacteria by assuming an average biomass of 10^{-10} mgC cell^{-1}.

Discussion

The abundance of bacteria in the water column was similar to that in other marine environments (Ferguson and Rublee, 1976; Fuhrman and Azam, 1980; Moriarty, 1979). The cells were large compared with other marine bacteria, and their average growth rate was a little slower. Fuhrman and Azam (1982) have reported turnover times of 1–2 days for marine bacteria and we have found turnover times of 2–6 days for areas on the east coast of Australia (Moriarty and Pollard, unpublished results). Probably not all the bacteria were growing, but what proportion this might be of the whole population is not known. Therefore the total number of cells present was used to calculate turnover time, and so the turnover times are maximum values, and may well be much less for some bacteria. The amount of incorporation of isotope into DNA was low and, therefore, no dilution of isotope in DNA was measurable although it probably occurred (Moriarty and Pollard, 1981). Karl (1979) has advocated the use of tritiated adenine as a better alternative to thymidine for this purpose, but unlike thymidine, adenine is readily incorporated into DNA by diatoms, blue-green algae and other microalgae. Measuring the productivity of all bacteria in natural environments is difficult. It is unlikely that one procedure will be applicable to many different environments or to all types of bacteria within one environment. Thymidine is the best available substrate for measurement of growth rates of heterotrophic bacteria (Kornberg, 1980; Moriarty, 1982).

Bacterial growth rates in the subtidal surface sediment and stromatolite layers were high, and similar to values obtained for sediments in seagrass beds (Moriarty and Pollard, 1981). Growth rates in cores of smooth mat and sediment were considerably lower, due to heterogeneity of microbial populations within the various layers of sediment, particularly in the smooth mat where bacteria were subject to desiccation and salt stress. There was a noticeable trend to slower growth rates in the deeper layers of the sediment and mat. Sulfate reduction rates were also greater in the surface layers than deeper in the sediment (G. W. Skyring, personal communi-

cation). Thus most heterotrophic bacterial activity occurs in the top 10 mm or so of sediment or algal mat.

The productivity values show only the amount of carbon incorporated into bacterial biomass. Most of the bacteria were embedded in slime layers and extra carbon is required for synthesis of the extracellular slime. Extracellular polysaccharides can amount to many times cellular biomass (Wilkinson, 1958). Heterotrophic bacteria also require reduced carbon compounds for respiration and it is assumed that, for the comparisons presented below, respiration needed to support slime production is equivalent to that required for biomass production. Accurate values for growth yields and the proportion of assimilated carbon used for respiration are not available for bacteria in natural systems. For the calculations here, a 50% conversion efficiency is within the ranges found by a number of workers (Payne and Wiebe, 1978). The total amount of carbon needed to support the productivity of bacteria in sediment is probably at least three times the amount occurring in new cell biomass. In the sediment and stromatolite surface layers (Table 2) the total carbon required could be as high as 3–7 mgC $m^{-2}h^{-1}$. These values apply to the top 3 mm only. As the core experiments show, bacterial productivity is similar in the top 10–15 mm, so that probably another 2–7 mgC $m^{-2}h^{-1}$ would be required to support the whole bacterial population.

These experiments indicate that about 5–14 mgC $m^{-2}h^{-1}$ was needed to support the bacterial production in the subtidal colloform mats, and about 3–5 mgC $m^{-2}h^{-1}$ was required by bacteria in the smooth, intertidal mat. Bauld et al. (1979) reported primary productivity in smooth mat to be 17 mgC $m^{-2}h^{-1}$ and in the colloform mat to be 113 mgC $m^{-2}h^{-1}$. In other words, about 20–30% of the primary production is utilized by the heterotrophic bacteria. Very little dilution of labelled thymidine by other precursors of thymidine bases in DNA was found in the cores, which was consistent with the low rates of incorporation of thymidine into DNA. The results for bacterial productivity are minimum values, because any errors in the measurement of the rates of thymidine incorporation into DNA are likely to result in underestimation of the true rate. Some bacteria, particularly anaerobes with strict and

limited nutrient requirements may not be able to utilize thymidine for DNA synthesis. The results indicate, however, that heterotrophic bacteria are very important in the carbon cycle of stromatolites. Longer term diel studies on primary and bacterial production are needed in order to quantify the carbon cycle links between the algae and bacteria under submerged and exposed conditions.

Acknowledgments

I am grateful to Peter C. Pollard for assisting in preparations for the isotope dilution experiments. The work was carried out during a field trip organized by M. R. Walter, Baas Becking Geobiological Laboratory, Canberra, and W. J. Wiebe, Division of Fisheries Research, CSIRO Marine Laboratories, Perth. I am grateful to W. J. Wiebe and staff at the Perth laboratory for their support.

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C. DNA Synthesis and Bacterial Growth Rates

CSIRO Marine Laboratories reprint No. 1172

Vol. 5: 151–156, 1981 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published May 31

DNA Synthesis as a Measure of Bacterial Productivity in Seagrass Sediments

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ABSTRACT: Bacterial growth rates in sediments have been calculated from measurements of the rate of incorporation of tritiated thymidine into deoxyribonucleic acid (DNA). The dilution of isotope in DNA was used to estimate the sum of the pools of thymidine in the sediment and of other cellular precursors of thymidine in DNA. Growth rates of bacteria in the surface zone of seagrass bed sediments varied from 3.7×10^8 cell divisions h^{-1} g^{-1} dry weight of sediment on a hot autumn day to 3.3×10^6 cell divisions h^{-1} g^{-1} in winter. By combining growth rate measurements using the isotope dilution procedure with biomass measurements, it is now possible to obtain reasonably reliable estimates of bacterial productivity in sediments.

INTRODUCTION

Seagrass beds are highly productive plant communities, in which much of the primary production is not utilized directly by animals, but enters higher trophic levels through microorganisms, especially bacteria (Phillips and McRoy, 1980). To quantify this process, we need to know not only the biomass of the bacterial population, but also its growth rate. Methods are available for measuring biomass in these sediments, which show that bacteria in seagrass beds of Moreton Bay, Queensland constitute about 20 % of the sediment organic matter, excluding that which seagrass roots contribute (Moriarty, 1980). The measurement of growth rates of the whole population has not been possible by classical microbiological techniques. By measuring the rate of synthesis of deoxyribonucleic acid (DNA), we hoped to be able to estimate the growth rate of bacteria in the sediment. Bacteria in sediments take up [methyl-^{3}H] thymidine (thymine-2-deoxyribose: Tdr) and use it for DNA synthesis (Tobin and Anthony, 1978). Fuhrman and Azam (1980) have used the rate of incorporation of Tdr into DNA in seawater to estimate growth rate of planktonic bacteria. They assumed that by adding a large excess of isotope, the contribution of Tdr from other pools or pathways would be negligible. In fact this may not be the case as Rosenbaum-Oliver and Zamenhof (1972) found that exogenous Tdr contributed only a portion of the Tdr in DNA, varying from 35 % to 63 % in a normal strain of *Escherichia coli*, depending on the culture conditions. The highest value of 63 % of exogenous Tdr was obtained with 1 mg Tdr ml^{-1} of culture medium. If natural populations of bacteria behave similarly, then to estimate the rate of DNA synthesis, and thus obtain the rate of bacterial division, it is necessary to measure the dilution of added Tdr by pools in the cells and in the sediment. Thymidine-5′-triphosphate (dTTP), the final precursor in DNA synthesis, is synthesised only partly from exogenous Tdr and partly via other pathways within the cell (Rosenbaum-Oliver and Zamenhof, 1972). An isotope dilution experiment can be used to determine the effect of added 'cold' precursor on the amount of labelled precursor incorporated into a macromolecule (Forsdyke, 1968). In this paper we show how, using this technique, the total sum of pools contributing thymine bases to DNA synthesis may be estimated, and thus calculate the growth rate of the bacterial population.

MATERIALS AND METHODS

We took a series of cores (25 mm diameter) of sediment from a seagrass bed in Moreton Bay, Queensland (Australia), which was dominated by *Zostera capricorni*, and combined the top 3 mm. For routine isotope dilution experiments, a series of 25 mm diameter centrifuge tubes (usually 7, but more for some experiments) were set up containing 7.4×10^5 Bq (20

0171-8630/81/0005/0151/$ 02.00

μCi) of tritiated Tdr. Unlabelled Tdr was added to give a series with the isotope diluted by progressively more Tdr as shown in the figures. For some experiments, more tritiated Tdr was used. Portions of the mixed slurry were dispensed with a small plastic spoon into the tubes and incubated at the *in situ* temperature in a water bath on the boat. The incubation was terminated by addition of 2 ml of 0.6 M NaOH, which gave a final concentration of about 0.4 M NaOH (we now add 0.6 M NaOH containing 10 mM Tdr). Sediment was dispensed into control tubes and treated with NaOH prior to isotope addition. Normally milder conditions for the extraction of DNA have been used (Thomas et al., 1974; Tobin and Anthony, 1978). We found, however, that the more rigorous conditions described here extracted more DNA from these sediments. Samples were heated at 100 °C for 4 h, centrifuged at 5000 × *g* for 10 min and the supernatants were dialysed overnight against running water, then 100 μl of Tdr and DNA, each 5 mg ml^{-1}, was added. The pH was adjusted to 0.7 with concentrated HCl. The acidified solutions were cooled for 40 min on ice and the precipitate immediately collected on a Whatman GF/C filter. After washing with 2 ml cold 5 % (w/v) trichloracetic acid (TCA) the DNA was hydrolysed in 2 ml 5 % TCA at 100 °C for 30 min. After centrifuging, 0.5 ml of supernatant was counted in 4 ml scintillant (PCS II, Amersham Australia Pty Ltd).

Forsdyke (1968), in studying RNA synthesis, showed that the observed counts (x) appearing in a macromolecule were given by the relation $x = n/(p+y+1)$, where n was the maximum count that would be incorporated with no dilution, and p was the dilution factor due to the endogenous pool of precursors, and $(y+1)$ the dilution by the added pool. In the case of DNA synthesis, $(y+1)$ is the dilution by added Tdr and p is the dilution by Tdr in the sediment and in the cell. Another factor (μ) is added to these pools, the contribution from *de novo* synthesis via deoxyuridine-5′-monophosphate (dUMP). Thus the equation becomes $x = n/(p+y+u+1)$ and is rearranged to give $y+1 = (1/x)n - (p+u)$. Forsdyke (1968) plotted the actual amounts of pyrimidine nucleoside added, rather than the dilution, against $(1/x)$, but as $1/x$ is the dependent variable we have plotted it against the amount of Tdr present. The negative intercept on the abscissa gives the size of the pools that effectively dilute the isotope in dTTP, the final precursor of DNA.

The growth rates (G) of the bacterial populations were calculated from the relation $G = R \times 1.3 \times 10^{18}/SA$, where R = rate of incorporation of Tdr into DNA in disintegrations min^{-1} (dpm) · min^{-1} g^{-1} sediment, and SA = the specific activity of the Tdr, corrected for dilution in dpm $mole^{-1}$. The factor 1.3×10^{18} was derived from the assumption that thymine constitutes an average of 25 % of the bases in bacterial DNA (range 12 % to 36 %) and that the genome size is 2.5×10^9 daltons (range 1×10^9 to 3.6×10^9, Gillis et al., 1970; Wallace and Morowitz, 1973). Bacterial numbers were counted by direct microscopy (Moriarty, 1980).

RESULTS

The initial rate of incorporation of Tdr into DNA was linear, indicating that there is very rapid mixing of label with the various pools inside and outside the bacteria (Fig. 1). The linear period was 8 min for undiluted labelled thymidine in a sample collected in the autumn when water temperatures were high (Fig. 1A) and over 20 min in at lower temperatures in winter (Fig. 1C). Departure from linearity probably occurred when the adsorption of thymidine onto sediment particles lowered the concentration so that an excess was no longer available to the bacteria. This conclusion is supported by the longer period of linearity that occur-

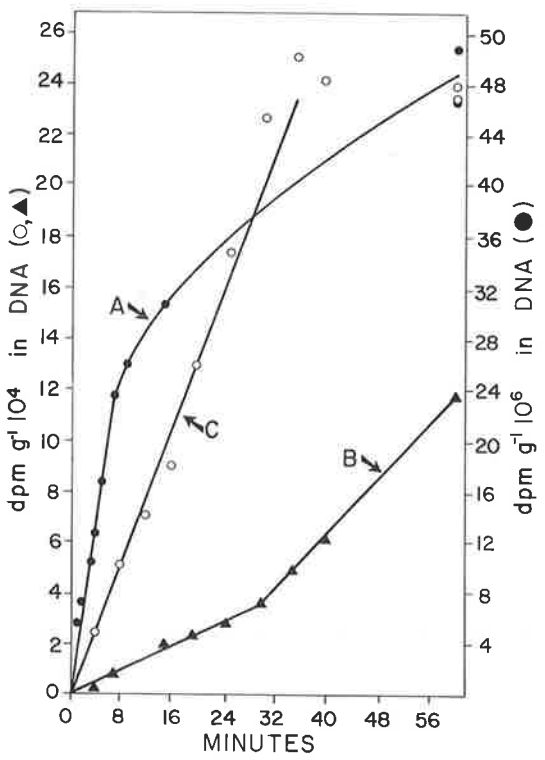

Fig. 1. Time course of incorporation of [methyl-^{3}H]-Thymidine (Tdr) into DNA. A: Autumn samples (water temperature 31 °C) 1.8×10^6 Bq Tdr (50 μCi, SA = 1.6×10^{12} Bq/mmol). B: Same samples but 1.5×10^6 Bq Tdr (40 μCi, SA = 5.9×10^{10} Bq/mmol). C: Winter samples (18 °C), 7.4×10^5 Bq Tdr (20 μCi, SA = 1.6×10^{12} Bq/mmol). Autumn samples, 4–5 g wet weight of surface sediment slurry, were combined with isotope and held at 31 °C. Incubation was terminated by addition of 2 ml of 0.6 M NaOH. Winter samples, 2.05 ± 0.05 g wet weight sediment, were assayed at 18 °C

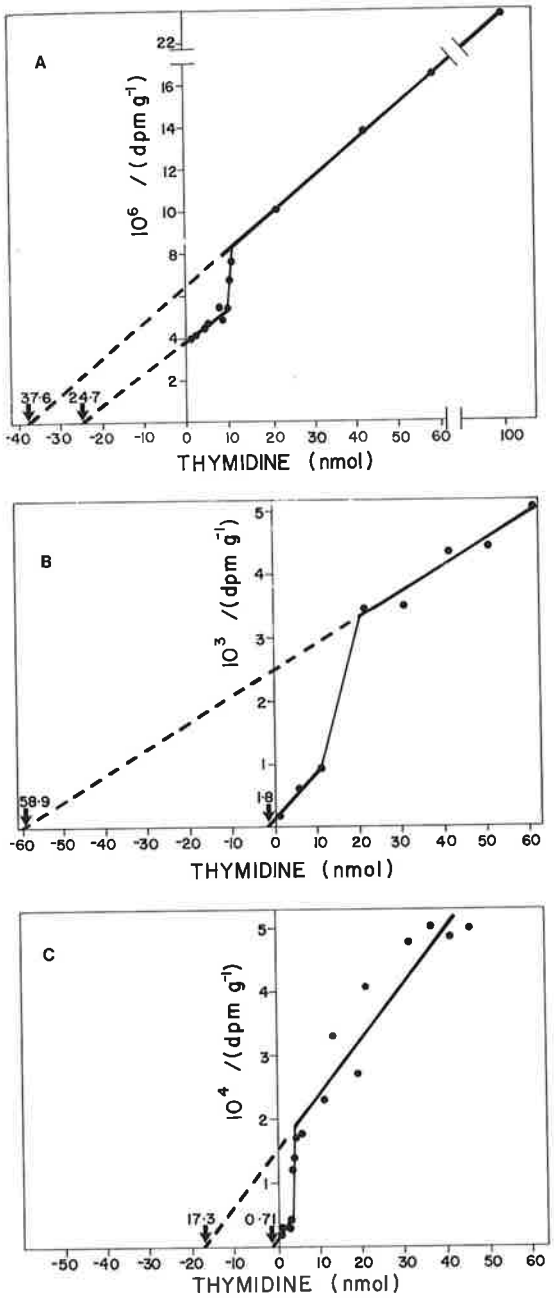

Fig. 2. Isotope dilution plot of incorporation of [methyl-^{3}H] Tdr into DNA. Each sample of 4–6 g wet weight of sediment incubated with isotope plus added Tdr for 8 min at 31 °C. Results from 3 separate sampling occasions. A: 1.8 × 10^6 Bq (50 µCi) isotope added. B and C: 1.5 × 10^6 Bq (40 µCi) isotope added

red when the label was diluted with thymidine (Fig. 1B).

Plots of the dilution of isotope incorporated into DNA by added thymidine indicate that substantial pools of thymidine or other precursors of thymidine-5′-monophosphate (dTMP) are present. Biphasic plots were obtained for three samples, in which two pools of dTMP precursors are apparent (Fig. 2). Two growth rates were calculated for each of these samples, using the two pool sizes and respective rates of incorporation of isotope. Within each sample, the two growth rates were similar (Table 1). For most samples simple linear plots were obtained, some examples of which are shown in Fig. 3.

It is not possible to estimate the pool size of dTMP precursors using undisturbed cores, because the varia-

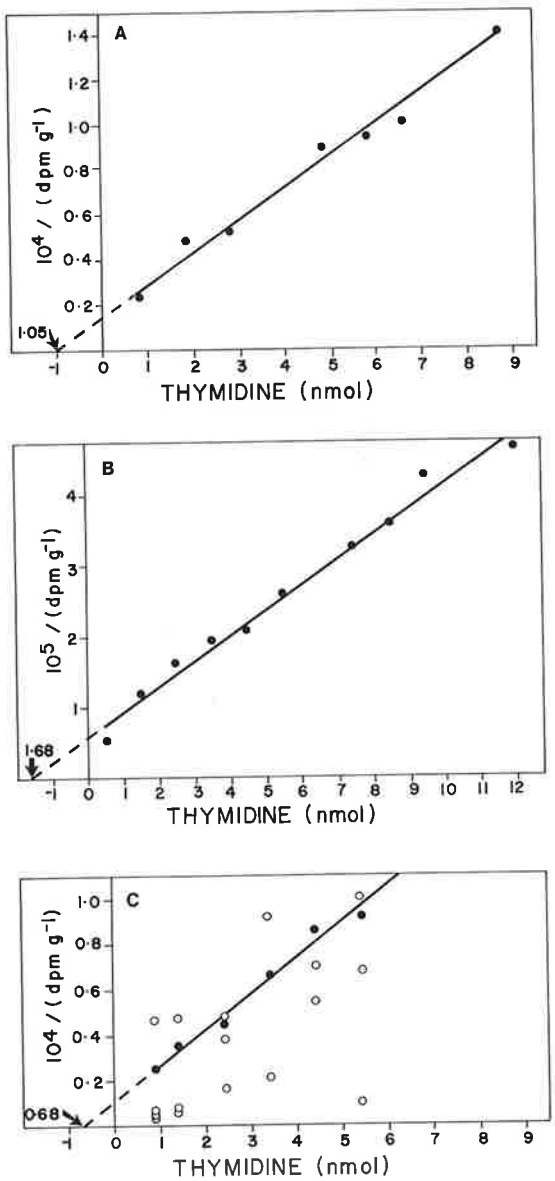

Fig. 3. Isotope dilution plots of incorporation of [methyl-^{3}H]-Tdr into DNA. Each sample of 2 g wet weight of sediment slurry incubated with isotope plus added Tdr at ambient temperature. A: 1.3 × 10^6 Bq (40 µCi) isotope incubated for 15 min at 17 °C. B: 7.4 × 10^5 Bq (20 µCi) isotope incubated for 30 min at 18.5 °C. C: closed circles, 7.4 × 10^5 Bq isotope incubated for 15 min at 24 °C, regression line calculated for closed circles only; open circles, 7.4 × 10^5 Bq isotope incubated with top 3 mm of individual cores for 15 min at 24 °C

Table 1. Growth rates of bacteria. For each sediment sample shown in Fig. 2, two pools of thymidine were apparent. Growth rates calculated for each are listed as number of cell divisions h^{-1} g^{-1} dry weight of sediment. Tdr = tymine–2–deoxyribose

Sample	Tdr pool size (nmol)	Growth rate (cells h^{-1} g^{-1})
A	37.6	5.2×10^8
A	24.7	2.5×10^8
B	58.9	2.6×10^6
B	1.8	1.5×10^6
C	17.3	1.2×10^7
C	0.7	1.1×10^7

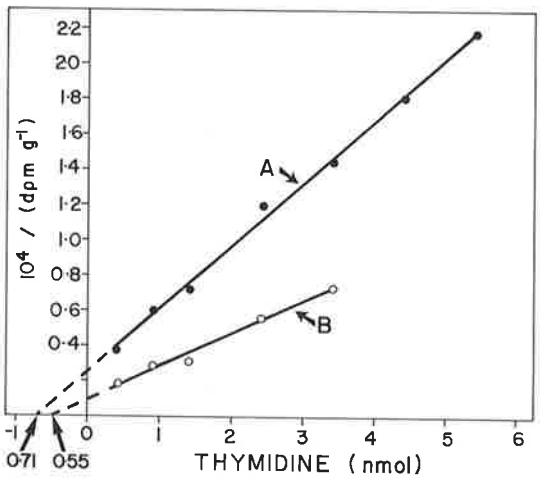

Fig. 4. Effect of delay in analysis on isotope dilution plot. Each sample of 2 g wet weight of sediment slurry incubated with 7.4×10^5 Bq isotope for 15 min at 20 °C. A: Incubations were carried out immediately after collection and mixing on the boat. B: Incubations carried out after 1 h on return to the laboratory

bility is too great for a meaningful regression line to be drawn (Fig. 3C, open circles). A precise estimate of pool sizes is possible only with a mixed slurry (Fig. 3C, closed circles). The results from this particular experiment demonstrate the patchiness in bacterial activity. It is necessary to carry out the incubation with thymidine as soon as possible after sample collection. The pool size of dTMP precursors was larger, and the rate of incorporation of Tdr lower in samples that were analysed on the boat immediately after collection, compared to samples that were brought to the laboratory and analysed under more controlled conditions about 1 h later (Fig. 4). The growth rates of bacteria in the seagrass flat sediments collected in a hot autumn period were two orders of magnitude higher than those collected in winter (Table 2). The turnover time for the whole population varied from about 5 h to over 1 week.

DISCUSSION

The estimation of bacterial activity or growth rates in natural environments by measurement of the incorporation of tritiated thymidine into DNA has been used and discussed by a number of workers (e.g. Brock, 1971; Thomas et al., 1974; Fuhrman and Azam, 1980). We have assumed that only bacteria were able to utilize nanomolar quantities of Tdr for DNA synthesis in the short time periods of these experiments. In fact, many blue-green algae and small eukaryotic algae and fungi lack thymidine kinase, and thus cannot incorporate Tdr directly into DNA (Grivell and Jackson, 1968; Glaser et al., 1973). Most bacteria probably do contain thymidine kinase, which is a necessary enzyme of the 'salvage' pathway for DNA synthesis (Cleaver, 1967). The *de novo* pathway proceeds via dUMP to dTMP and thus bypasses thymidine. The interactions between these pathways are complex and may not be the same in all bacteria, so it cannot be assumed that the addition of a large amount of thymidine will lead to a cessation or even a considerable lowering of the contribution of the *de novo* pathway. The assumption that all bacteria have thymidine kinase is a conservative one. If, as Fuhrman and Azam (1980) point out, some bacteria lack this enzyme, then the productivity estimates will be lower than the actual production. Fuhrman and Azam (1980), in their study of the production of bacteria in seawater, commented that because it was not possible to measure internal

Table 2. Growth rates and turnover times of bacterial populations in seagrass sediments. Growth rates and population sizes expressed per g dry weight of sediment. All experiments were carried out on samples collected in the early afternoon

Month	Temperature (°C)	Population growth rate (cells h^{-1} g^{-1})	Population size (cells g^{-1})	Turnover time (h)
April	31	3.7×10^8	2×10^9	5.5
June	18.5	4.6×10^6	6.4×10^8	58
July	17	4.6×10^6	6.4×10^8	140
August	20	3.3×10^6	6×10^8	180
October	24	7.3×10^6	1×10^9	130
October	24	2.1×10^7	1×10^9	48

pool sizes, they were making the conservative assumption that the internal pools and the biosynthetic pathways leading to DNA were saturated by the added Tdr. In fact, as Rosenbaum-Oliver and Zamenhof (1972) showed with *Escherichia coli*, exogenous Tdr contributed only 63 % of thymine bases to DNA when supplied at a concentration of 1 mg ml^{-1} in the presence of amino acids, and 42 % in the absence of amino acids. Fuhrman and Azam (1980) reported that the dilution of labelled thymine incorporated into DNA appeared to be small. The isotope dilution technique described here, in which the dilution of isotope incorporated into DNA is measured, provides a way to estimate the degree of participation of exogenous thymidine in DNA synthesis. It is assumed that the rate-controlling step occurs after the synthesis of dTMP; if this were not so, then the addition of increasing quantities of Tdr would not give a linear plot. Rosenbaum-Oliver and Zamenhof (1972) found that the growth rates of 4 strains of *E. coli* were not affected by the concentration of exogenous Tdr. The linearity of the plot shown in the initial minutes of Fig. 1, in each phase of Fig. 2 and in Figs 3 and 4, indicates that the complex population of bacteria in the sediment can be treated as a single entity. Only the activity of fast growing bacteria is measured with this technique. The turnover times for the whole population are an average of fast growing cells and cells that are not dividing (Table 2).

An interpretation of the biphasic graphs shown in Fig. 2 is that two pools of thymidine or other precursors of dTMP exist in the sediment. This interpretation is supported by the similarity in growth rates calculated for each sample using both pool sizes and the respective rates of isotope incorporation (Table 1). The second pool was exposed at higher concentrations of Tdr, and as this occurred on only three occasions, it is likely to have been a pool in the sediment rather than the bacteria. The demonstration of such large pools means that any comparative study of bacterial activity or growth rates in such environments must take these pools into account, otherwise only the dilution of isotrope rather than a difference in activity of bacteria may be measured.

The procedure that we have described for the isolation of DNA was developed to give maximum recovery of labelled DNA from these sediments, which are mainly sand with some clay and heavy metals. For other sediments and seawater, a lower concentration of NaOH (0.3 M) should be used. Purification of DNA from other cellular components is necessary, because some isotope was incorporated into other macromolecules. The technique is based on the Schmidt-Thannhauser procedure (reviewed by Munro and Fleck, 1966). With this method RNA is hydrolyzed and DNA solubilized in a hot alkali solution. Low background levels of radioactivity were obtained when unlabelled Tdr was added and the solution was dialysed. Acidification of the dialysis residue and then hydrolysis of DNA in TCA separates DNA from protein. As Tobin and Anthony (1978) showed, DNA is the only source of label after purification by the above procedure.

The accuracy of the growth rate calculations is affected by two assumptions, *viz.* that thymine constitutes 25 % of the bases in DNA and that the genome size is 2.5×10^9 daltons. If only one or two species of bacteria were actively growing, then the results given in Tables 1 and 2 could be in error by a factor of 2 or 3. If, however, many species are using the Tdr that was supplied, it is likely that the average quantities used above provide a close estimate of the true values. Work is in progress to check G+C ratios of the actively growing cells.

The growth rates obtained for benthic bacteria in the seagrass sediments show considerable variability, some of which is probably a seasonal trend (Table 2). The very rapid growth rates and large pool sizes on the hot April day may indicate a site of rapid decomposition in the sediment. As these values represent only one measurement during the warmest period of day, they cannot be extrapolated to a full 24 h. The turnover times for the bacterial population in the winter months are similar to the value of 91 h obtained by Meyer-Reil et al. (1980) with an indirect technique, for a microbial population on a sandy beach with a similar temperature regime. Further work is now in progress to assess diurnal and seasonal changes in bacterial productivity. Preliminary results show that there is a large fluctuation in diurnal growth rates in the seagrass beds, which is why it is not valid to extrapolate the results reported here. It is clear, however, that in quantifying the importance of rapidly growing bacterial populations in food chains, measurement of productivity as well as biomass is essential. The biomass of bacteria in sediments generally remains relatively constant, whereas productivity can vary by more than an order of magnitude (Table 2). The difference between the productivity and change in biomass over a given time interval is a measure of the amount of bacteria removed from the system by grazing, or perhaps loss to the water column. The productivity measurements provide a measure of total bacterial activity, unlike assays for heterotrophic activity which measure the activity of that portion of the population that are able to use the substrate provided. Thus it will be possible to investigate processes such as the effect of deposit-feeders on bacterial growth and the proportion of primary productivity that is cycled directly through bacteria to animals.

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From: HETEROTROPHIC ACTIVITY IN THE SEA
Edited by John E. Hobbie and
Peter J. LeB. Williams
(Plenum Publishing Corporation, 1984)

MEASUREMENTS OF BACTERIAL GROWTH RATES IN SOME MARINE SYSTEMS USING THE INCORPORATION OF TRITIATED THYMIDINE INTO DNA

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INTRODUCTION

Heterotrophic microorganisms, especially bacteria, play an important part in decomposition processes, nutrient cycling and food chains in aquatic systems. In quantifying their role, measurement of the growth rate of the whole bacterial population is necessary, but has proved difficult. A number of different methods have been proposed for measuring microbial growth rates, but many are not specific for bacteria or do not include the whole population.

Some techniques have involved culture and enumeration of bacteria. The best approach for this type of technique is to alter natural conditions as little as possible and to use direct microscopy to count bacteria (Meyer-Reil 1977). Another technique based on direct microscopy uses the frequency of dividing cells to calculate growth rate of bacteria in seawater (Hagström et al. 1979). Besides the problem of determining whether a pair of cells is dividing, there are difficulties with this technique, particularly in the relationship between the frequency of dividing cells and growth rate (Newell and Christian 1981). This technique is not applicable to sediments, where many cells remain attached to each other and are bound in large aggregations of slime and particulate matter.

Azam, in this volume, has discussed in some detail the requirement for measuring the growth rate of natural microbial populations. It is evident that the ideal method should involve minimal handling of the bacterial population and be applied quickly enough so as not to alter natural or in situ growth rates or to be influence by bacterial grazers. The use of radioactive nucleic acid precursors, especially thymidine, to measure the rate of DNA synthesis, has many

of the prerequisites of the ideal method. As with all other techniques, there are disadvantages as well as advantages in using measurements of nucleic acid synthesis. This paper considers the measurement of growth rates calculated from the rate of tritiated thymidine incorporation into DNA. A more extensive review of methods based on the synthesis of RNA and DNA is published elsewhere (Moriarty, in press).

RELATIONSHIPS BETWEEN DNA AND RNA SYNTHESIS AND GROWTH

Growth in microorganisms is a complex process involving synthesis of protein, RNA, and DNA, usually culminating in cell division. DNA synthesis is directly proportional to division rate in bacteria. The regulation of DNA synthesis occurs primarily at initiation (Lark 1969). Once initiated, DNA synthesis proceeds to completion, and the termination of replication triggers a cycle of division. Initiation of replication is affected by growth conditions. Under conditions of rapid growth, more than one replication fork may proceed along the chromosome at one time, but the rate of travel is not affected by growth rate (Lark 1969). This close relationship between growth and DNA synthesis means that measurement of the rate of DNA synthesis is a good measure of bacterial growth rates. Unlike bacteria, eukaryotes do not synthesize DNA continuously throughout a growth cell cycle, but only at one stage. DNA synthesis in eukaryotes is dependent on continued protein synthesis, and replication stops if protein synthesis stops (Lark 1969). Thus growth in eukaryotes is more complex. The method chosen to measure DNA synthesis in a natural environment should, therefore, be reasonably specific to bacteria for ease of interpretation.

The relationship of RNA synthesis to growth is much more complex than that of DNA synthesis. Cells that are growing rapidly (generation time of 1-2 h) do show a direct correlation between RNA synthesis and growth rate. In cells that are growing more slowly, however, there is some relationship between growth rate and RNA synthesis, but it is not a simple linear function (Nierlich 1974). This is further complicated by the different rates at which the main types of RNA are synthesized. About 97% of total RNA is ribosomal and transfer RNA, but these account for only about 50% of RNA synthesis in rapidly growing cells. Messenger RNA, which is unstable, comprises the rest (Nierlich 1974). In slowly growing cells, net synthesis of stable RNA may cease completely when an excess of these RNA forms is present; however, as they do turn over, a slow rate of synthesis still occurs (Nierlich 1978).

In order to calculate division rates of cells in a natural population, accurate information is needed on the rate of synthesis of each form of RNA, the amount of RNA present and the growth state of the cells. The amount of RNA per cell is variable and depends on

the growth state (Maaloe and Kjeldgaard 1966). Cells that are growing slowly generally have an excess of stable RNA. As most cells in natural populations are not likely to be in a state of rapid growth, the lack of a clear relationship between growth state and RNA content as well as synthesis rates means the at measurement of the total amount of RNA synthesis is not possible, as discussed by Fuhrman and Azam (1980). Several reviews on growth and RNA synthesis in bacteria discuss the complexities of the processes and it is only selected strains of bacteria growing under defined conditions and at particular growth rates, that show precise relationships (Edin and Broda 1968; Nierlich 1974, 1978; Maaloe and Kjeldgaard 1966). Thus there are severe problems associated with the use of RNA synthesis for estimating microbial growth rates (Karl 1979, 1981).

The utilization or potential for utilization of exogenous adenine for nucleic acid synthesis by algae as well as heterotrophic bacteria, is a real disadvantage in trying to estimate bacterial growth rates. As explained below, the great advantage of thymidine is that may be used to measure heterotrophic bacterial growth rates in the presence of microalgae. In order to study the growth and activity of particular members of the complex microbial community, we need techniques that are specific rather than general in their application.

Enzymology of Thymidine Incorporation into DNA

Thymidine (thymine-2-deoxyribose; Tdr) is unique among nucleosides because the only function of its nucleotides in cells is participation in the synthesis of DNA (O'Donovan and Neuhard 1970). Thymidine is readily incorporated into DNA via a salvage pathway, but in some bacteria the incorporation stops after a short time due to breakdown of thymidine (O'Donovan and Neuhard 1970). De novo synthesis proceeds via dUMP directly to dTMP (Fig. 1). Catabolism of thymidine starts with conversion to thymine and ribose-1-phosphate by the action of an inducible phosphorylase. The best radioactive label is [methyl-^{3}H] because subsequent conversion to uracil removes the label. The tritiated methyl group can be transferred to a wide variety of compounds, but DNA is not labelled, as demonstrated in microorganisms that lack thymidine kinase (Fink and Fink 1962). [2-^{14}C] Thymidine, on the other hand, does label DNA after catabolism, because the label is retained in the resulting uracil (Grivell and Jackson 1968). The absence of tritium incorporation into DNA in some eukaryotic microorganisms led Grivell and Jackson (1968) to show that these organisms lacked thymidine kinase. As Kornberg (1980) pointed out, thymidine meets reasonable well the criteria for pulse labelling. These are that the precursor should be rapidly and efficiently taken up by bacteria, be stable during uptake, be converted rapidly into the nucleotides and specifically label DNA with little dilution by intracellular pools. He also outlined pitfalls in its use, of which some are particularly relevant to

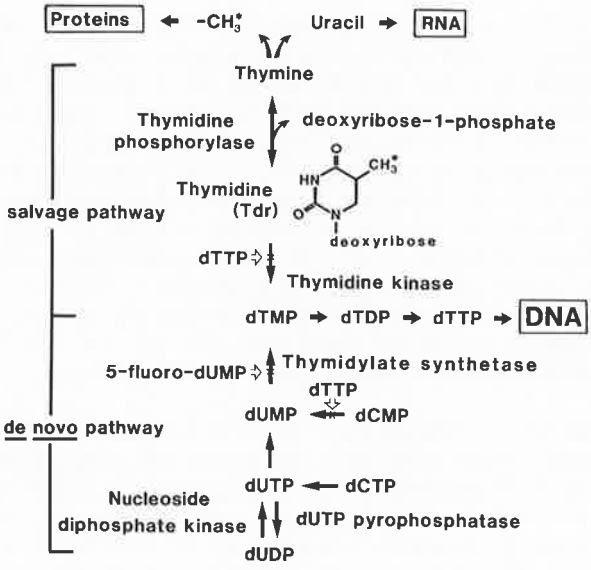

Figure 1. Some pathways of thymidine metabolism. The asterisk shows the position of tritium labelling in thymidine. Sites of feedback inhibition of dTTP and inhibition by 5-fluoro-dUMP are indicated (adapted from Kornberg 1980).

environmental studies and are discussed in detail below.

Thymidine is converted to dTMP by thmidine kinase (Fig. 1). This enzyme must be present for labelling of DNA to occur to a significant extent. Thymidine kinase was thought to occur in most organisms (Kornberg 1980), but some groups of microorganisms are now known not to contain it. These include fungi (Neurospora crassa, Aspergillus nidulans and Saccharomyces cerevisiae) and Euglena gracilis (Grivell and Jackson 1968), and a number of cyanobacteria (blue green algae) (Glaser et al. 1973). It is also absent from the nuclei of various eukaryotic algae, but may be present in chloroplasts although the amount of label incorporated from tritiated thymidine into chloroplast DNA was slight and required hours or days of incubation to be shown by autoradiography (Stocking and Gifford 1959; Sagan 1965; Steffensen and Sheridan 1965; Swinton and Hanawatt 1972).

We have been unable to obtain significant incorporation of [methyl-^{3}H] Tdr into DNA of four species of marine microalgae (Thalassiosira, Isochrysis, Platymonas and Synechococcus), which suggests that they lack thymidine kinase (Pollard and Moriarty, in preparation). As there are no reports of the presence of thymidine kinase in the nuclei of small eukaryotic algae, fungi or

cyanobacteria, it seems reasonable to generalize, and conclude the this salvage pathway is lacking in all members of these groups of microorganisms. The lack of thymidine kinase in blue green algae and many eukaryotic microorganisms is a considerable advantage for studies on heterotrophic bacterial production in the marine environment. Protozoa probably do contain the enzyme (Plant and Sagan 1958; Stone and Prescott 1964), but as explained below their contribution to label DNA in short term experiments is probably small. Thus the use of thymidine provides specific information about the growth of heterotrophic bacteria which has not been available previously.

Most bacteria that lack thymidine kinase are mutants specially selected for biochemical studies. Two wild type strains of Pseudomonas have been reported not to incorporate thymidine into DNA (Ramsay 1974). The technique used to demonstrate this was autoradiography, which is insensitive compared to liquid scintillation counting of purified DNA. Ramsay's results could mean that these bacteria lacked thymidine kinase, or that they had a deficient membrane transport mechanism. A few species of Pseudomonas have been found not to utilize thymidine, due to a deficient cell membrane transport system (Pollard and Moriarty, in preparation). Fuhrman and Azam (1980) have found good agreement between bacterial growth rates in seawater measured by the incorporation of thymidine and by counting the increase in cell number. The results of an autoradiographic study on bacteria in sea water support the view that most aerobic marine heterotrophic bacteria can utilize thymidine (Fuhrman and Azam 1982). Anaerobic bacteria with strict and limited nutrient requirements may not be able to utilize thymidine, particularly if they can transport only a limited range of metabolites. Desulfovibrio, for example, does not appear to be able to utilize exogenous thymidine (G. W. Skyring, personal communication).

The possibility that some bacteria in seawater may be unable to incorporate thymidine into DNA means that estimates of bacterial productivity may be too low, but this disadvantage is considerably outweighed by the advantages of using thymidine to measure DNA synthesis over other techniques for estimating growth rates of bacteria in natural populations.

Kinetics

Bacteria, with their active transport systems, take up organic molecules much more rapidly than do algae or protozoa, and can utilize nanomolar concentrations of organic molecules in their environment more effectively than algae or protozoa (Wright and Hobbie 1966; Fuhrman and Azam 1980). Thus in a short time period (e.g., 10 min at 25°C, 20 min at 15°C) tritiated thymidine should be taken up preferentially by bacteria in a mixed community. We have conducted autoradiography on surface sediment and epiphytic populations of microorganisms and have found that bacteria were

heavily labelled, whereas very few silver grains were formed over diatoms and cyanobacteria after 15 min (Moriarty and Pollard 1982). Autoradiography of the same populations with tritiated adenine, however, showed more label in microalgae than with thymidine; bacteria also were more heavily labelled.

Uptake of labelled thymidine by organisms should not be confused with incorporation into DNA, although in bacteria the latter may be the main fate of labelled thymidine assimilated intact (Hollibaugh et al. 1980; Fuhrman and Azam 1980). As mentioned above, thymidine is readily incorporated into DNA in bacteria, but thymidine phosphorylase soon converts thymidine to thymine and deoxyribose-1-phosphate (Fig. 1). Labelled thymidine concentration within cells may be rapidly depleted, so it is important to measure the rate of label incorporation into DNA and not simply label uptake. In our work with sediments we have found that label incorporation into DNA proceeds linearly for 5-8 min at high temperatures (27°-31°C) and 20-30 min at lower temperatures (15°-18°) in sediments, and over 1 h in seawater (Fig. 2). We had interpreted the change from the linear rate to be due to adsorption of thymidine by clay in sediments, but although this undoubtedly is a factor, degradation by thymidine phosphorylase may also have occurred. Experimental studies to determine growth rates must be carried out in the initial linear period of incorporation of label into DNA. Uptake of thymidine by cells and incorporation into TCA-insoluble fractions are different processes, probably with different kinetics which may be uninterpretable in a mixed population.

The kinetic studies (Fig. 2) show that thymidine is very rapidly taken up and is incorporated into DNA in less than 1 minute. We presumed that this was indicative of bacterial activity (Moriarty and Pollard 1981), because protozoa, the other main group of microorganisms with thymidine kinase, are generally particulate feeders and probably would not have membrane transport mechanisms that are as efficient as those of bacteria. We are not aware of any literature on this topic and so are conducting studies with cycloheximide, an inhibitor of DNA synthesis in eukaryotes (Cooney and Bradley 1962; Venkatesan 1977). Preliminary results indicate that cycloheximide has no effect on ^{3}H-thymidine incorporation into DNA in sediments (Moriarty and Pollard 1982). In other words, although bacteria are not the only organisms that utilize thymidine, they are the only ones that do so significantly in a short experimental period.

Purification of DNA

In those organisms that do not contain thymidine kinase, degradation is the only fate of thymidine. The amount of tritium appearing in DNA is negligible, but other compounds are labelled and thus DNA must be purified when working with mixed populations, particularly if bacteria are minor components. In seawater, Fuhrman

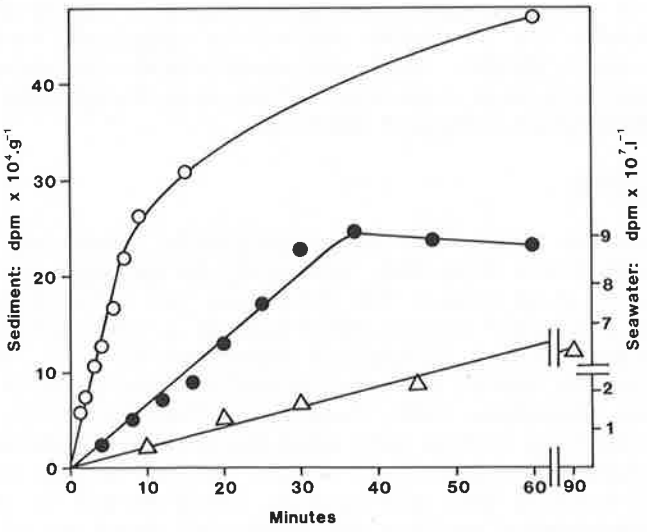

Figure 2. Time course of incorporation of tritiated thymidine into DNA. Δ = seawater; ● = sediment (17°C); ○ = sediment (31°C) (from Moriarty and Pollard 1981, 1982).

and Azam (1980) have found that 90% of the total uptake of tritiated thymidine was by microorganisms that passed through a 1 μm filter and about 80% of that was incorporated into DNA. The proportion incorporated into DNA will vary with different samples, so DNA should be purified, or the proportion of tritium in DNA should be checked for each environment.

We based our methods on the Schmidt-Thannhauser procedure (reviewed by Munro and Fleck 1966) but modified them to obtain maximum recovery of DNA from sediment with low background radioactive contamination (Moriarty and Pollard 1981). Recovery of standard amounts of DNA varied from about 40% to 70%; it was higher when humic acid was present. One problem with the technique is that DNA has to be precipitated from solution by ice-cold strong acid, and we have found that losses occurred, probably due to hydrolysis. Recoveries of DNA were generally in the range of 55% $\pm$ 5%. In order to calculate growth rates of bacteria, it is essential, therefore, to measure the actual recovery of DNA from environmental samples.

The problem of measuring background values due to adsorption of radioactivity is an important one. It is important to ensure that control experiments, in which bacterial DNA synthesis is prevented, contain the same amount of labelled thymidine during extraction of DNA. We achieved this in sediments by mixing control sediments in NaOH first, then adding the same amount of thymidine as in the experimental samples. Background values could also be due to

adsorption of or exchange of tritiated water or other products of thymidine metabolism in active cells onto compounds such as DNA, protein and humic acids. Such processes would not occur in zero-time or formalin-killed controls. Thus true background values may be higher than those actually measured.

Isotope Dilution

The specific radioactivity of exogenous thymidine is diluted during incorporation into DNA, primarily by de novo synthesis of dTMP (Fig. 1). A technique for measuring the dilution of label from an exogenous precursor during synthesis of a macromolecule is to add different quantities of unlabelled precursor as well, and to measure the effect on the amount of label actually incorporated into the macromolecule (Forsdyke 1968, Hunter and Francke 1974). We showed that this technique worked well with bacterial populations in sediments (Moriarty and Pollard 1981). A plot of the reciprocal of isotope incorporated into DNA against total amount of thymidine added is extrapolated to give the amount of dilution of isotope in DNA itself (Fig. 3). We referred to the negative intercept on the abscissa rather loosely as pools of thymidine and other endogenous precursors that dilute the added labelled thymidine (Moriarty and Pollard 1981). In fact, true pool sizes can be calculated only if the actual amount of label taken up by the cells is known. We used the negative intercept on the abscisssa to determine the specific activity of tritiated thymidine actually incorporated into DNA.

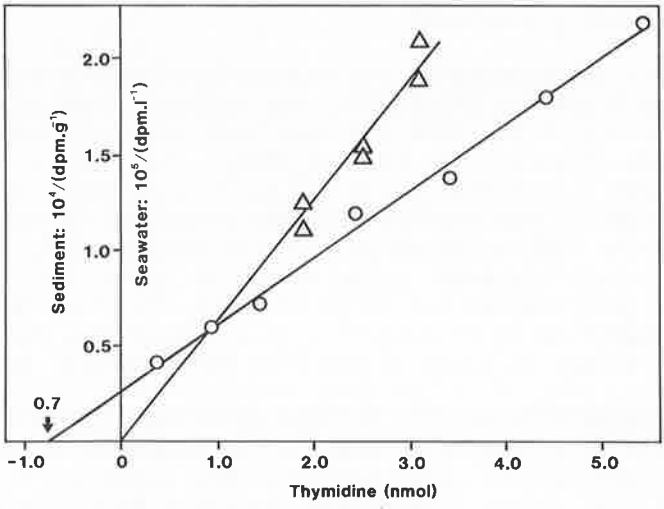

Figure 3. Isotope dilution plots for incorporation of tritiated thymidine into DNA. Δ = seawater; O = sediment (from Moriarty and Pollard 1981).

This technique measures the dilution of labelled thymidine in dTTP (the final precursor for DNA synthesis) by all other precursors of dTTP, including de novo synthesis. Provided DNA is purified before counting radioactivity that is incorporated, it doesn't matter if only a small proportion of the thymidine that is taken up by cells is used for DNA synthesis, because only the dilution of isotope in thymidine pools that are actually being used for DNA synthesis in growing cells is measured. Thus this method is not subject to errors inherent in trying to extract and quantify nucleotides from cells.

The amount of dilution of labelled thymidine incorporated into DNA in various marine systems varies considerably. Fuhrman and Azam (1980) initially assumed that there was little dilution in seawater samples that they analyzed. We have used our technique on seawater from a number of environments, and have found that there was no significant isotope dilution in planktonic bacteria when a high concentration of thymidine (16 nM) was used (Fig. 3; Moriarty 1983; Moriarty and Pollard 1982).

Subsequently, Fuhrman and Azam (1982) reported that isotope dilution may be as high as 4 to 7-fold in seawater bacteria. Their reasons for suggesting this were based on the relative difference in incorporation of labelled thymidine and ^{32}P into DNA in samples in the presence and absence of the inhibitor mitomycin C. The difference between the rates of incorporation of the labelled substrates, however, may have been due to the synthesis of RNA, and not to dilution of thymine in DNA. This is because tritiated thymidine is incorporated only into DNA and not RNA (any results to the contrary would be due to imperfect extraction and separation of the macromolecules), whereas ^{32}P will label both nucleic acids, and furthermore, mitomycin C may block RNA synthesis as well as DNA synthesis. A better procedure to test for isotope dilution is the one that we have used (Moriarty and Pollard 1982). These problems with measuring the amount of isotope dilution by de novo synthesis of dTMP can be avoided by using a high enough concentration of thymidine to supply all the thymine required for DNA synthesis, in which case de novo synthesis is switched off by feedback inhibition (for more detail see Moriarty, in press). About 10–20 nM thymidine is generally sufficient to do this (unpublished observations). Once this concentration is determined for a particular environment, only one incubation is needed with thymidine to estimate a growth rate, which considerably simplifies ecological work.

In sediments there appears to be a correlation between growth rate of bacteria and dilution of isotope in DNA. Dilution is greater in systems with fast growth rates than in systems with slowly growing bacteria (Table 1). These results suggest that in slowly growing bacteria the exogenous thymidine is sufficient for DNA synthesis, and de novo synthesis of dTTP is inhibited. In rapidly growing bacteria, however, the supply of exogenous thymidine presumably is insufficient

Table 1. Degree of participation (DP) of exogenous thymidine in DNA synthesis by bacteria in a seagrass sediment (Moriarty and Pollard 1982).

DP %	Production rate No. cells produced·h^{-1}·g^{-1}
16	2×10^7
18	1×10^7
25	7×10^6
33	6×10^6
57	2×10^6
80	8×10^5

to maintain the level of dTTP required, and thus de novo synthesis proceeds as well. The degree of participation of exogenous thymidine in DNA synthesis was generally in the range of 20% to 70% in sediment populations. Similar values were found by Rosenbaum-Oliver and Zamenhof (1972) for E. coli DNA synthesis.

Small pools of thymdine inside and outside cells may also exist, but are probably unimportant. Since publishing our original studies, in which we reported evidence suggesting that substantial pools of thymidine occurred in three sediment samples (Moriarty and Pollard 1981), we have analyzed many different environmental samples and have found no further evidence for substantial dilution of tritiated thymidine (see e.g., Moriarty and Pollard 1982). Those experiments which showed substantial dilution were carried out with comparatively large amounts of sediment. Probably most of the thymidine was adsorbed to sediment, as the actual concentration available to bacteria was very small. The kinetics of transport into the cell may be different at very low substrate concentrations. With less sediment dilution is low, and no biphasic curves are obtained (Moriarty and Pollard 1982).

Growth Rate Calculation

To calculate growth rate from the rate of synthesis of a macromolecule measured by incorporation of a precursor, the total amount of the macromolecule per cell and the amount of precursor in the macromolecule must be known. As pointed out by Fuhrman and Azam (1980), the RNA content of bacterial cells is variable, and depends on growth rate, whereas DNA content of all bacteria varies little over a wide range of growth rates. Thus to estimate growth rate of bacteria DNA synthesis is more suitable than RNA synthesis. We assumed that thymine constituted an average of 25% of the bases in DNA, and the average genome size was 2.5×10^9 daltons

(range 1 x 10^9 to 3.6 x 10^9) (Moriarty and Pollard 1981); these values are similar to those used by Fuhrman and Azam (1980).

Another way to determine a conversion factor is to estimate it directly, by measuring the growth rate of bacteria in seawater culture by direct microscopy and measuring the rate of incorporation of thymidine into DNA. This has been done by Kirchman et al. (1982) and has one particular advantage. The conversion factor calculated with their procedure takes into account any bacteria that cannot utilize thymidine for DNA synthesis. There are two conditions that need to be met in both the seawater culture and the natural marine environment, viz., (1) the time period for assay with tritiated thymidine must be short enough to ensure that only DNA is labelled, and (2) isotope dilution should be measured, or better still, prevented by using a high thymidine concentration. If these conditions are not met the conversion factors in culture would be different from those in the sea.

BACTERIAL PRODUCTIVITY IN WATER AND SEDIMENTS

Some examples of the range of bacterial growth rates and productivities that we have measured in seawater are shown in Table 2. Diurnal variation of growth rates in the water column occurs in some bodies of water. In the surface sediment of seagrass beds there is a marked diurnal cycle in bacterial growth rates (Fig. 4).

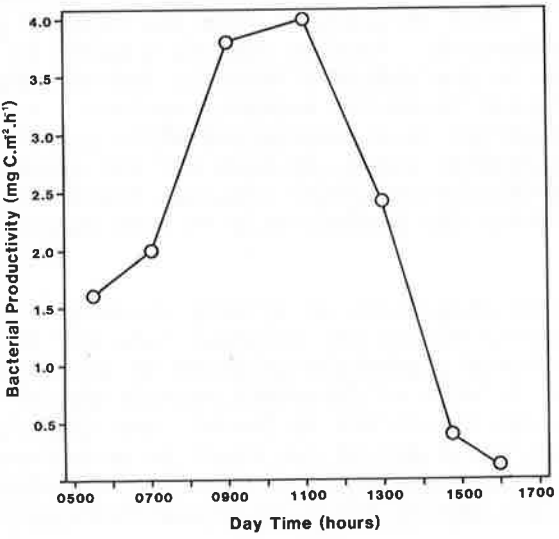

Figure 4. Diurnal variation in bacterial productivity in surface sediment of seagrass beds (adapted from Moriarty and Pollard 1982).

Table 2. Bacterial productivity in seawater.

Environment	Productivity $\mu g\ C \cdot l^{-1} \cdot h^{-1}$	Reference
Moreton Bay, Queensland	1 - 3	Moriarty and Pollard 1982
Great Barrier Reef, lagoon off Lizard Is.	0.2 - 0.6	Moriarty and Pollard, in preparation
Hamelin Pool, Shark Bay, W.A. (hypersaline)	0.1 - 0.3	Moriarty 1983

Although not shown here, we have carried out studies in a number of environments and over a full diel period. The large increase in growth rates (often 5-10 fold) occurred only in sediments closely associated with seagrass during daylight hours (Moriarty and Pollard 1982). Bacterial productivities in the seagrass beds in autumn were about 80 mg $C \cdot m^{-2} \cdot d$ in the surface sediment (0-3 mm depth) and about 50 mg $C \cdot m^{-3} \cdot d$ in the water column (average depth 1 m). If we assume the bacterial conversion efficiency is 50% then a total of 260 mg $C \cdot m^{-2} \cdot d^{-1}$ would be required from the primary producers to support this production. Further organic C would be required for slime production by the sediment bacteria and to support the bacterial population below the sediment surface. As there are technical difficulties in measuring bacterial growth rates accurately in anaerobic cores, we have not yet obtained many results for bacterial productivity around seagrass roots. Preliminary results suggest that the productivity is less than in the surface layers.

A substantial proportion of primary production probably is utilized by bacteria within the seagrass beds and through them by animals. The primary production utilized by bacteria includes dead roots and leaves as well as dissolved organic matter excreted by roots, rhizomes and leaves and by benthic and epiphytic algae. The primary producers would depend, at least to some extent, on release of nutrients from decomposing organic matter by bacteria. We are trying to quantify some of these interrelationships.

Measurement of bacterial growth by DNA synthesis rates indicates how many new cells are produced. It does not tell us how much total organic matter, especially extracellular products such as slime, is

produced by bacteria. Bacteria that are not dividing and synthesizing DNA may also produce extracellular products. Such products are probably trophically important to deposit-feeders, and accurate measurement of their production in natural environments is a challenge awaiting investigation.

ACKNOWLEDGMENTS

I am grateful to Peter C. Pollard for preparing the figures.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1984, p. 1076–1083
0099-2240/84/121076-08$02.00/0

Vol. 48, No. 6

Validity of the Tritiated Thymidine Method for Estimating Bacterial Growth Rates: Measurement of Isotope Dilution During DNA Synthesis

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Received 22 March 1984/Accepted 6 August 1984

The rate of tritiated thymidine incorporation into DNA was used to estimate bacterial growth rates in aquatic environments. To be accurate, the calculation of growth rates has to include a factor for the dilution of isotope before incorporation. The validity of an isotope dilution analysis to determine this factor was verified in experiments reported here with cultures of a marine bacterium growing in a chemostat. Growth rates calculated from data on chemostat dilution rates and cell density agreed well with rates calculated by tritiated thymidine incorporation into DNA and isotope dilution analysis. With sufficiently high concentrations of exogenous thymidine, de novo synthesis of deoxythymidine monophosphate was inhibited, thereby preventing the endogenous dilution of isotope. The thymidine technique was also shown to be useful for measuring growth rates of mixed suspensions of bacteria growing anaerobically. Thymidine was incorporated into the DNA of a range of marine pseudomonads that were investigated. Three species did not take up thymidine. The common marine cyanobacterium *Synechococcus* species did not incorporate thymidine into DNA.

In the past, techniques were not available for measuring growth rates of most heterogeneous bacterial communities in nature. Considerable advances have been made recently in the measurement of bacterial growth rates by determining the rate at which [*methyl*-^{3}H]thymidine is incorporated into bacterial DNA (2, 3, 5, 6, 11, 12, 26, 27).

There are two principal pathways for the synthesis of thymidine nucleotides. The first is the de novo pathway by which nucleotides are synthesized from precursor molecules such as carbamyl phosphate and aspartic acid. Ultimately, dUMP is methylated through the action of thymidylate synthetase (EC 2.1.1.45), thereby producing dTMP (22). The second is the salvage pathway, in which thymidine is phosphorylated to form dTMP by the enzyme thymidine kinase (EC 2.7.1.21) (22).

With further phosphorylation, dTMP is converted to dTDP and then to dTTP. The enzyme DNA polymerase (EC 2.7.7.7) utilizes dTTP to incorporate thymine into DNA with the other three bases. Extracellular and intracellular deoxyribonucleotide pools and biosynthetic pathways can contribute thymine to DNA as well as dilute the radioactively labeled thymine that is incorporated into DNA from the supplied precursor ([^{3}H]thymidine).

The DNA polymerase is probably bound into a multienzyme complex together with the kinases needed to synthesize the deoxyribonucleotides (24, 25). Small pools of dTTP and other precursors in the complex are the immediate source of nucleotides for DNA synthesis. They turn over very rapidly and are functionally separate from the general cellular pools of the nucleoside triphosphates. Thus, it is impossible to measure the size of such pools and any change in specific radioactivity of an exogenous precursor by extracting the nucleotides. A technique known as isotope dilution analysis is available for measuring the change in the specific radioactivity of a precursor at the site of incorporation. This method was developed for use with mammalian cell cultures by Forsdyke, Scott, and Sjostrom (9, 10, 35–38). We have used isotope dilution analysis in marine sediment and seawater to estimate bacterial growth rates (26, 27).

Because the technique has been criticized (13, 20), we report here results of studies with a chemostat, which show that the rate of tritiated thymidine incorporation into DNA gives a good estimate of growth rate and thus that isotope dilution analysis measures the dilution of radioactivity in dTTP. Furthermore, we show that de novo synthesis of dTMP from dUMP can be inhibited by high concentrations of thymidine, thus eliminating the need to carry out isotope dilution analysis with every sample.

An explanation of isotope dilution theory and methodology is given here (for a review, see references 9, 10, 26, 27, 36, 37; D. J. W. Moriarty, Adv. Aquat. Microbiol., in press). A fixed concentration of radioactively labeled thymidine and various concentrations of nonradioactive thymidine were incubated with growing bacteria. The DNA was extracted, and the total concentration of added thymidine was plotted against the reciprocal of the radioactivity in the DNA fraction (see Fig. 1). If there was no dilution of the isotope incorporated into DNA by any sources other than the unlabeled thymidine that was added, the plot should pass through zero. A negative intercept on the ordinate is an estimate of the amount of isotope dilution by other sources of thymine in DNA. It is not strictly a pool of thymidine but instead represents the influence of pools that diluted the tritiated thymidine before incorporation into DNA.

The isotope dilution method measures the dilution of labeled thymine in dTTP, the final precursor to DNA, in all sources of thymine, because the effect of added thymidine on the incorporation of radioactivity into DNA itself is measured. A necessary condition is that the rate-limiting step for the incorporation of thymidine be the final one, i.e., the DNA polymerase step. The following explanation of the concepts of the isotope dilution methodology has been adapted from the work of Forsdyke and his colleagues (9, 10, 35–38). In the simplest model, the incorporation of a labeled precursor into a macromolecule may be represented as a

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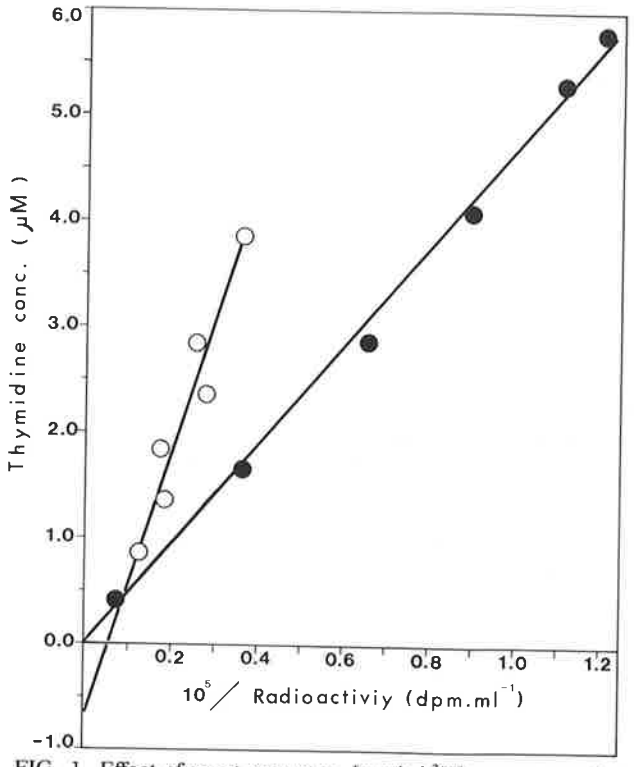

FIG. 1. Effect of yeast extract on [*methyl*-^{3}H]thymidine isotope dilution in a culture from a chemostat containing *A. undina*. Symbols: ●, yeast extract present; ○, yeast extract absent. The ordinate is total thymidine concentration (labeled plus unlabeled).

one-step transfer from a pool into a stable product; two assumptions were made.

First, the rate of labeling of the macromolecule should not be influenced by the concentration of the precursor. As indicated above, DNA synthesis is a complex process which depends on many factors and is linked ultimately to the supply of energy and nutrients to the cell. In a growing cell, with DNA synthesis initiated, the tight regulation of nucleotide biosynthetic pathways ensures that an adequate supply of precursors are available and that this supply of precursors does not limit or stimulate DNA synthesis.

Second, the rate of isotope transfer into the precursor pool should be constant; however, there need not be equilibrium between the exogenous and endogenous pools, which would occur only if there were a free two-way exchange between the two pools. In theory, the simple model for isotope dilution analysis requires that the final precursor pool be in chemical equilibrium with the pool of added radioactive precursor. This would be the case in an ideal reaction vessel in which there was, in effect, one pool and all chemical reactions were reversible. The actual situation is very complex, with transport across the cell wall occurring in only one direction and with many enzymatic reactions, particularly in multienzyme complexes, also occurring in one direction. The model is still valid in this situation, provided that the rate of isotope transfer through an enzymatic pathway and the rate of isotope mixing with unlabeled pools along the pathway remain constant. In addition, the rate-limiting step for isotope incorporation into a macromolecule must be the final one. Isotope dilution analysis measures dilution up to a rate-limiting step. If this step is at the replication fork, i.e., the DNA polymerase step, then the dilution of tritiated dTTP by other sources of dTTP in the pool at the replication fork is measurable.

Given these assumptions, a number of counts (n) should be incorporated into DNA in a given time from a precursor of given specific activity and should be a measure of the rate of synthesis if there is no dilution by a pool of similar compounds in the system. If there is a pool of similar compounds and its size happens to equal that of the added precursor (dilution pool, p = 1), the observed counts (x) in DNA will equal $n/2$. Thus, $x = n/(p + 1)$. If, in addition to the labeled precursor, a known quantity of unlabeled precursor is added to give a further dilution, y, then $x = n/(p + y + 1)$. The added pool is $y + 1$. Upon rearrangement, the expression becomes $y + 1 = (1/x)n - p$. Thus, if the reciprocal of the observed counts ($\times$) is plotted against the dilution ($y + 1$), a linear relationship is obtained with an intercept of $-p$, the dilution given by the pool in the system. In practice, it is easier to plot actual quantities of the precursor (Fig. 1), but it should be remembered that the "pool sizes" found are relative quantities and are used only to calculate the dilution of specific radioactivity of the precursor. For example, in the experiment without yeast extract (Fig. 1), 9.1×10^5 dpm [$(1/(0.11 \times 10^{-5})$] of radioactivity was incorporated into DNA from 0.8 nmol of labeled thymidine. The specific activity of the added thymidine was 50 μCi/nmol. The intercept was −0.5 nmol. The specific activity of the final precursor was therefore 50 μCi/(0.5 + 0.8 nmol) in that sample. Another way to express this is as the extent of participation of labeled thymidine in DNA synthesis, which in the above example was 0.8/1.3 = 61%.

Because there has been some controversy about the use and specificity of thymidine to measure growth rates of heterotrophic bacteria in natural environments (20), we carried out experiments on the ability of some organisms to take up thymidine and incorporate it into DNA. Further justification for the use of thymidine is desirable, although it has been accepted as a means to measure heterotrophic bacterial production (2, 12, 26). Because some *Pseudomonas* species cannot incorporate thymidine into DNA, we have investigated this in a range of marine pseudomonads, in a marine cyanobacterium, and in some eucaryotic algae.

MATERIALS AND METHODS

DNA from herring sperm and crystalline thymidine were purchased from Sigma Chemical Co., St. Louis, Mo. Amersham Australia Pty. Ltd. Sydney; Australia, supplied aqueous solutions (containing 2% ethanol) of [*methyl*-^{3}H]thymidine at a concentration of 1 mCi/ml and specific activity of 40 to 60 Ci/mmol. Purified and sterilized thymine-2-^{14}C-DNA, extracted from *Escherichia coli* at 15 to 50 μCi/mg, was also purchased from Amersham, whose phase-combining system, PCS II, was used for liquid scintillation counting of radioactive samples.

Microorganisms. The marine pseudomonads and *Synechococcus* species were kindly donated from the Australian Collection of Marine Microorganisms (Sir George Fisher Centre, James Cook University of North Queensland, Townsville) by J. L. Reichelt. The freshwater *Pseudomonas* species were supplied by K. C. Marshall, School of Microbiology, University of New South Wales, Sydney; three unicellular algal species were obtained from S. W. Jeffrey, Commonwealth Scientific and Industrial Research Organization Marine Laboratories, Cronulla, Australia. Pseudomonads and *Synechococcus* species were maintained in modified luminous medium and MN medium, respectively, made

with 80% sterilized sea water (1, 42). Algae were grown on nutrient "f" medium (14).

The marine anaerobic bacterial culture was a mixture of organisms isolated from surface sediment in a sea grass bed (*Halodule wrightii*) in the northeastern Gulf of Mexico. Sediment was incubated under an atmosphere of 95% nitrogen (oxygen free)–5% carbon dioxide in a sterile nutrient medium (1% [wt/vol] glucose, 0.1% [wt/vol] yeast extract) for 2 days. Then fresh medium was inoculated with this suspension. Before and after assays with tritiated thymidine, microbial growth was monitored with a spectrophotometer (540 nm) to ensure that cells were in the log phase of growth during all experiments.

Ability of microorganisms to incorporate [^{3}H]thymidine into DNA. *Pseudomonas* species were subcultured in nutrient medium containing (per liter) glucose (0.3 g), yeast extract (0.3 g), and peptone (0.6 g) in 80% sterile seawater. A portion (2 ml) of the suspension was sampled and incubated with 10 μCi (0.2 nmol) of tritiated thymidine. After 10 min, 2 ml of 0.6 M NaOH was added, and then DNA was extracted (26). Algal and cyanobacterial samples (2 ml) were incubated with 5 μCi of tritiated thymidine. After 8 min, 2 ml of 0.6 M NaOH was added, and DNA was extracted (26).

Time course assays and isotope dilution analysis were carried out with the mixed anaerobic cultures as follows. Tritiated thymidine (18 μCi; 0.4 nmol) was added to three culture tubes (20 ml), each fitted with a rubber septum in the cap through which syringe needles could be inserted. An additional 0.8, 1.0, and 1.2 nmol of unlabeled thymidine were added to the three tubes. The tubes were flushed with N_2-CO_2 gas mixture, and then 18 ml of the culture was added. Subsamples were removed at time intervals ranging from 1 to 40 min during purging with N_2-CO_2 gas mixture to maintain anaerobic conditions. The subsamples were injected into 10 ml of ice-cold trichloroacetic acid (TCA; 2% [wt/vol]) and filtered within 1 h.

Thymidine uptake kinetics. Batch cultures (13 ml) of the species *Pseudomonas bathycetes*, *P. marina*, and *Alteromonas undina* (subcultured as above) were incubated with 30 μCi of tritiated thymidine. The population density of each species was similar at the time of the experiments; in optical density terms, *A. undina* was 0.87, *P. marina* was 0.84, and *P. bathycetes* was 0.85. All were in the log phase. Every 30 s, 1 ml was filtered through a 25-mm cellulose acetate filter (pore size, 0.2 μm). The radioactivity in the filtrate was measured.

Chemostat. A chemostat (volume, 475 ml) was set up, containing a continuously mixed suspension of the marine bacterium *A. undina*. It was supplied at a constant rate (100 ml/h) with an aseptic nutrient medium containing (per liter) glucose (0.3 g), yeast extract (0.3 g), and peptone (0.6 g) in 80% seawater. The culture volume was kept constant; in some experiments, the yeast extract was omitted. The growth rate was calculated from data on nutrient flow rate, culture volume, and the number of cells per milliliter. Bacteria were counted by direct microscopy with a modification of the method of Zimmerman and Meyer-Reil (43) by using acridine orange staining (15) and with equipment described elsewhere (25).

Under steady-state growth conditions, the specific growth rate is equivalent to the dilution rate (30): specific growth rate = flow rate (milliliters per hour)/volume (milliliters); and bacterial growth rate = (flow rate [milliliters per hour]/volume [milliliters]) × number (cells per milliliter) = growth rate (cells per milliliter per hour).

A 15-ml suspension of *A. undina* (15 ml) from the chemostat was used as the sample. The time course was started by adding 300 μCi of tritiated thymidine, and two samples (2 ml each) were removed at each assay time. The incubation in one sample was stopped by the addition of 2 ml of 0.6 M NaOH, and the DNA was then extracted. In the second sample, the incubation was stopped by the addition of 2 ml of ice-cold 5% TCA, and then the samples were filtered and washed as described below.

Isotope dilution analysis. *A. undina* was grown rapidly in two chemostats, one with yeast extract (in the nutrient medium) and the other without. Samples were taken from each chemostat (0.4 and 1.0 ml), incubated with tritiated thymidine (10 μCi and 40 μCi, respectively), and diluted with increasing amounts of unlabeled thymidine. After 2 min, samples were immediately filtered and washed with cold 5% TCA.

The rate of DNA synthesis, and hence cell division, was calculated from the rate of thymidine incorporation as described by Moriarty and Pollard (26, 27), except that a conversion factor of 2×10^{18} cells dividing per mol of thymidine incorporated was used (Moriarty, in press).

The number of bacterial cells dividing per hour (dX/dt) equals the rate of DNA synthesis (molecules per hour): $dX/dt = (5.6 \times 10^4)$(dpm incorporated into DNA)/(incubation time [min]/specific activity [Ci/mmol]). Specific growth rate (μ) = $(dx/dt)(1/x)$, where x is the population density.

Sea water (containing particulate material from decaying sea grass leaves) was collected from an inshore sea grass bed (*H. wrightii*) in the northeastern Gulf of Mexico. Three isotope dilution experiments were set up in polypropylene bottles with 5, 20, and 50 nM of tritiated thymidine and increasing concentrations of unlabeled thymidine. The assays were started by adding 25-ml portions of the seawater at defined time intervals (generally 30 s apart). After a 20-min incubation with a radioisotope, each sample was immediately filtered. Filters were then washed five times with ice-cold 3% TCA and treated further as described below.

Extraction of radioactively labeled DNA. For some experiments (see Fig. 1 and 3), bacterial DNA was extracted in hot NaOH, cooled, and then precipitated in ice-cold TCA as described by Moriarty and Pollard (27). ^{14}C-DNA was added to a few blank samples, with NaOH, in the initial stage of DNA extraction to check recovery.

In the majority of experiments, the radioactively labeled macromolecules that were insoluble in cold TCA, but hydrolyzed in hot TCA were collected. Incubations were stopped by adding ice-cold TCA to small volumes (<2 ml). Large samples (e.g., seawater) were filtered first, and filters were washed five times with ice-cold 3% TCA. The suspensions were immediately filtered through 25-mm polycarbonate filters (pore size, 0.2 μm). This filter type was used in preference to cellulose acetate and cellulose nitrate because it minimized nonspecific isotope adsorption. Filters, unless otherwise stated, were washed five times with 5 ml of ice-cold 3% TCA and hydrolyzed in 2 ml of 5% TCA at 100°C for 30 min. Radioactivity was counted in a sample of the hydrolysate. Before using this method, we checked to ensure that there was no difference between the radioactivity of macromolecules insoluble in cold TCA (and soluble in hot TCA) and DNA extracted as above (see below).

RESULTS

Tritiated thymidine uptake and incorporation into DNA. About 50% of the radioactively labeled thymidine (added to an *Alteromonas* batch culture) was removed from the extra-

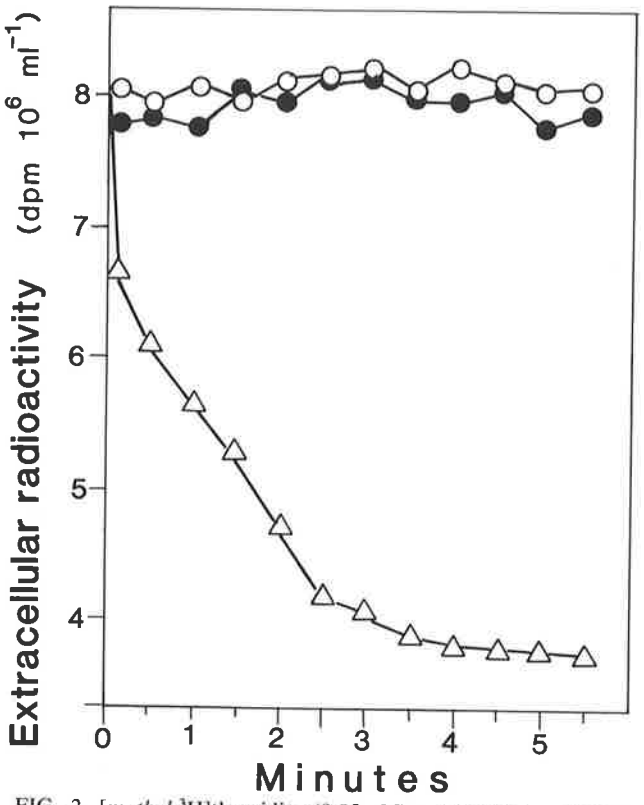

FIG. 2. [*methyl*-^{3}H]thymidine (0.05 μM) uptake from extracellular medium of rapidly growing batch cultures of *A. undina* (△), *P. marina* (○), and *P. bathycetes* (●).

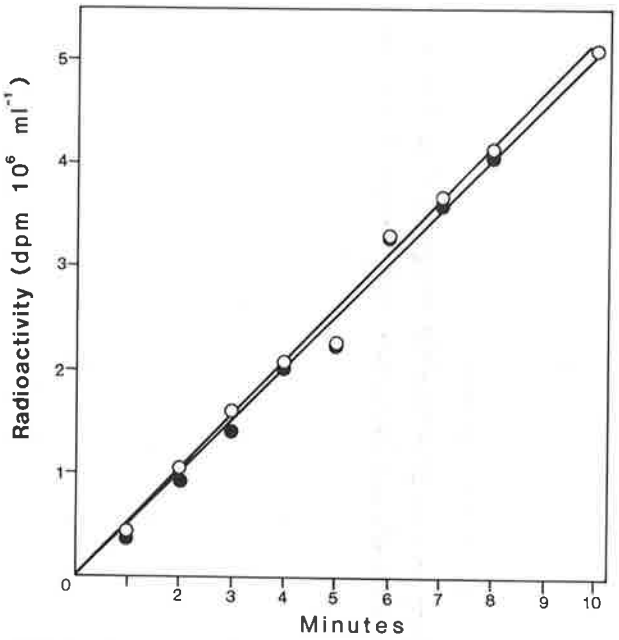

FIG. 3. Time course for incorporation of [*methyl*-^{3}H]thymidine (0.43 μM) into cold TCA-insoluble macromolecules (○) and extracted DNA (●).

cellular medium within 2.5 min (Fig. 2). Concomitant with rapid uptake was the initiation of the radioactive labeling of DNA (Fig. 3). Radioactivity was incorporated into DNA at a linear rate which was independent of the rate of uptake of tritiated thymidine (cf. Fig. 2 and 3). The rate of incorporation of the isotope into macromolecules that were insoluble in cold TCA was the same as the rate of incorporation in purified DNA (Fig. 3). Thus, DNA was the only macromolecule that was labeled by tritiated thymidine for at least 10 min in the chemostat culture.

Growth rate measurement. The growth rates of *A. undina* determined by isotope dilution analysis agreed very well with growth rates calculated by direct microscopy (Table 1). Agreement was good both in experiments in which there was dilution of the isotope as well as in those in which there was no dilution (Fig. 1). Therefore, the rate of labeled thymidine incorporation into DNA can be used to calculate accurately the rate of DNA synthesis.

Patterns of isotope dilution in seawater. The degree of isotope dilution in bacteria growing in seawater could be altered by starting with three different concentrations of radioactive thymidine (Fig. 4). At 50 nM, the plot was linear, with no indication of isotope dilution (zero intercept at the ordinate). At 5 nM, the plot could be described as linear below 35 nM total thymidine, with a negative intercept on the ordinate. Above 35 nM, the slope increased, which means that dilution had decreased. With 20 nM tritiated thymidine, data points for total thymidine concentrations of greater than 35 nM fell on a line of best fit having an ordinate intercept of zero (implying no isotope dilution). Values for both of the duplicates of 20 nM tritiated thymidine were displaced to the right of the line, indicating that some dilution had occurred at this concentration. Because the seawater was not homogeneous but contained particulate detritus, variation was not unexpected; some points did not fit any pattern.

TABLE 1. Comparison of growth rates measured by isotope -dilution analysis and direct microscopy in aerobic chemostats (containing *A. undina*) and an anaerobic batch culture (heterogeneous bacterial population)

Environment	Isotope dilution (%)	Specific growth rate/h by [a]:	
		Direct microscopy	DNA synthesis
Aerobic chemostats	0[b]	0.30 ± 0.04 (21)	0.28 ± 0.03 (6)
	0[b]	0.34 ± 0.04 (21)	0.32 ± 0.04 (6)
	44[c]	0.29 ± 0.08 (21)	0.21 ± 0.02 (6)
	44[c]	0.22 ± 0.04 (21)	0.29 ± 0.07 (12)
Anaerobic batch culture	0	0.53 ± 0.1 (10)	0.72 ± 0.1 (6)

[a] Specific growth rate is expressed as the mean plus or minus standard error (*n*). See the text for details of this experiment.
[b] Yeast extract present.
[c] Yeast extract absent.

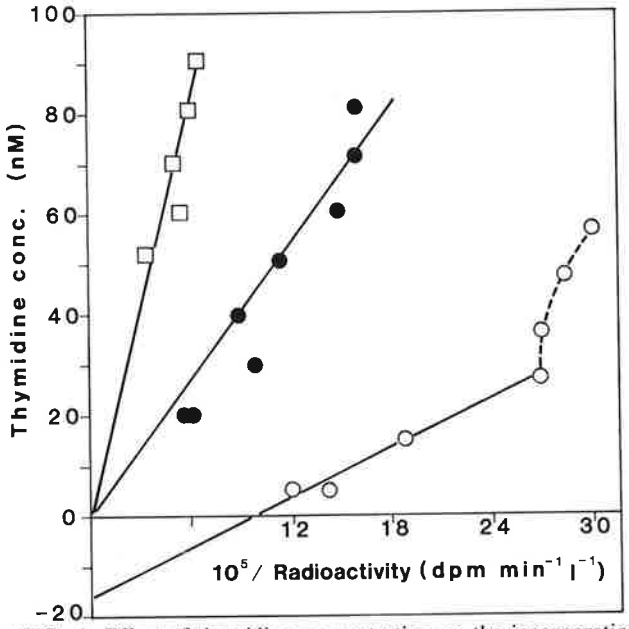

FIG. 4. Effect of thymidine concentration on the incorporation of tritiated thymidine into DNA in seawater over a sea grass bed (*H. wrightii*) in the Gulf of Mexico, Fla.; [^{3}H]thymidine concentrations of 50 nM (□), 20 nM (●) and 5 nM (○) were used. The ordinate is total thymidine concentration (i.e., labeled plus unlabeled).

The bacterial growth rates calculated for these plots did not differ significantly (Table 2). The abrupt changes in the linearity (Fig. 4) at 35 nM were probably due to changes in the contribution of dTMP by the de novo pathways. In this system of rapidly growing bacteria on detritus in seawater, thymidine concentrations of greater than 35 nM were sufficient to maintain the level of dTTP needed for DNA synthesis, and thus de novo pathways were inhibited.

Ability of microorganisms to incorporate thymidine into DNA. Most of the bacteria studied here incorporated tritiated thymidine into DNA (Table 3). In only two species, *P. marina* and *P. bathycetes*, was DNA not labeled; because these species were unable to remove tritiated thymidine from the extracellular medium, it is likely that they lack transport enzymes for thymidine (Fig. 2). These organisms were growing during the experiment as indicated by the change in optical density.

There was no significant incorporation of tritiated thymidine into three species of microalgae, namely *Thalassiosira pseudonana*, *Isocrysis galbana*, *Platymonas suecica*. Algal cultures were not axenic. The low levels of tritiated thymidine incorporated into DNA were probably due to bacterial growth. No thymidine was incorporated into the DNA of a marine *Synechococcus* species (Table 4)

DISCUSSION

Regulation of de novo thymidine nucleotide synthesis. Enzymes in the biosynthetic pathway for thymidine nucleotides and other nucleotides are probably organized in a multienzyme complex associated with DNA polymerase (24). Thymidylate synthetase, which methylates dUMP, is a key enzyme in the de novo synthesis of thymidine nucleotides (22). The supply of dUMP is regulated through the feedback inhibition by dTTP of enzymes in the pathways leading to dUMP (reviewed by Moriarty [in press]). The pool of dTTP used for control is functionally separate from the small, rapidly turning over pool used for DNA synthesis in the DNA polymerase complex (24), but the larger pool is possibly derived from that in the complex. An increase in the size of the cellular dTTP pool can quickly be followed by inhibition of de novo synthesis, leading to rapid depletion of the biosynthetic pool of dTTP. The cellular dTTP cannot penetrate the multienzyme complex and serve as a precursor for DNA synthesis (24). It is by this mechanism that excess thymidine can cause inhibition of de novo pathways and provide the sole source of dTTP for DNA synthesis. If the thymidine supply is insufficient to maintain dTTP at the level required for DNA synthesis, de novo synthesis is not fully inhibited; thus, dilution of the isotope would result. Kuebbing and Werner (23) have shown that when thymidine was added to growing mammalian cells, it was almost immediately incorporated into DNA at full specific activity, thereby blocking any further action of the de novo pathway. A similar mechanism probably operates in bacteria, as our results indicate with the chemostat when yeast extract was used. High concentrations of thymidine (ca. 1 μM) were used in the chemostat compared with natural seawater, because the cell density was very high and growing rapidly. The low concentrations used in seawater (ca. 10 nM) would have been sufficient for only a few seconds of DNA synthesis in the chemostat.

The discussion above illustrates a point that should be

TABLE 2. Bacterial production rates in seawater at various concentrations of tritiated thymidine[a]

[*methyl*-^{3}H]thymidine concn (nM)	Isotope dilution (%)	Bacterial production rate (10^7 cells/h per liter)
5	76	2.9 ± 0.4
20	0	2.4 ± 0.4
50	0	3.6 ± 0.4

[a] Bacterial density was 9.8 (±25) × 10^8 cells per liter; n = 10. Bacterial production rate is expressed as the mean plus or minus standard error. n = 7.

TABLE 3. Survey of the ability of some marine pseudomonads to incorporate tritiated thymidine into DNA[a]

Bacterial strain (Australian Collection of Marine Microorganisms no.)	Growth rate (ΔA_{550}/Δtime)	Radioactivity incorporated (10^3 dpm/min per ml)
Alcaligenes aquamarinus (135)	0.08	0.06 (3)
A. cholinophagum (822)	0.09	7.71 (3)
A. venustus (168)	0.01	0.55 (2)
A. pacificus (127)	0.07	17.07 (3)
Alteromonas communis (157)	ND	12.92 (3)
A. haloplanktis (129)	ND	85.52 (3)
A. macleodii (827)	0.05	66.37 (3)
A. undina (816)	0.12	146.40 (5)
A. putrefaciens (530)	0.13	30.18 (3)
Pseudomonas bathycetes (136)	0.24	0.003 (2)
P. doudoroffii (167)	0.11	2.27 (3)
P. marina (132)	0.08	0.01 (2)
P. nautica (171)	0.09	0.90 (3)
P. nigrafaciens (821)	0.20	3.17 (3)
Pseudomonas species (freshwater organism)	0.05	0.20 (2)

[a] During the log phase of growth, each culture was incubated with 0.63 μM [*methyl*-^{3}H]thymidine for 10 min (*Pseudomonas* sp. for 100 min). Background radioactivity has been substracted. All cultures were in the log phase of growth during the experiments; a relative measure of growth during each experiment is given by the change in absorbance at 550 nm (ΔA_{550}/Δtime). Radioactivity incorporated is expressed as the mean (n). ND, Not determined.

TABLE 4. Ability of three genera of algae and a marine cyanobacterium to incorporate [*methyl*-^{3}H]thymidine into DNA[a]

Algae (CSIRO Culture Collection no.)	Cell density (10^6)/ml	Growth rate ($\Delta E_{665}/\Delta$time)	Radioactivity incorporated (10^3 dpm/min per ml)
Thalassiosira pseudonana (CS-20c)[b]	1.20	0.56	0.06
Isocrysis galbana (CS-22)[c]	1.15	0.96	0.13
Platymonas suecica (CS-56)[d]	0.2	0.58	0.07
Synechococcus species[e]	0.01	0.69	0.00

[a] Eucaryotic algae were incubated with 50 and *Synechococcus* sp. with 20 nM [*methyl*-^{3}H]thymidine for 10 and 120 min, respectively. Relative growth rates during each experiment are given by the change in fluorescence at 665 nM ($\Delta E_{665}/\Delta$time). Mean values for duplicate determinations for the eucaryotic algae and the mean of nine determinations for *Synechococcus* species are shown. CSIRO, Commonwealth Scientific and Industrial Research Organization.

[b] *Bacillariophyceae* (diatom).

[c] *Prymnesiophyceae* (golden-brown flagellate).

[d] *Prasinophyceae* (green flagellate).

[e] Cyanobacterium (Australian Collection of Marine Organisms [N100]).

noted when applying the thymidine method in natural environments. The minimum concentration of thymidine that should be used will depend on the bacterial density and growth rate. Higher concentrations will be needed for higher densities and faster growth rates. Furthermore, the concentration chosen should be high enough to inhibit de novo synthesis and should eliminate the need to carry out isotope dilution analysis on every sample. Preliminary experiments with isotope dilution analysis would be needed to determine the minimum concentration of thymidine needed. Some workers have attempted to do this by increasing the amounts of radioactive thymidine added at the same specific activity and using the concentration at which an asymptote for incorporation into DNA is reached (2, 13). This method works, provided that there are no endogenous or exogenous pools of thymidine itself. A double-reciprocal plot is needed to show dilution with this technique (16). It is better and easier to use the isotope dilution analysis described previously (9, 10).

An example of the effect of tritiated thymidine concentrations on isotope dilution in a natural situation is given here (Fig. 4). At a high concentration (50 nM), de novo synthesis was inhibited; at the lowest concentration of tritiated thymidine (5 nM), however, insufficient dTTP was synthesized from thymidine, and so de novo synthesis of dTMP also occurred, thus diluting the isotope. At 20 nM tritiated thymidine, the thymidine concentration was not quite enough to supply all of the dTTP required, but above 40 nM total thymidine concentration was sufficient; thus, the middle plot could be extrapolated through point zero for the higher points only (Fig. 4). These experiments show that nonlinear isotope dilution plots will be obtained if the thymidine concentration is only a little less than that needed to supply all of the requirements for DNA synthesis. If it is much less, plots will be linear, at least over a small range of concentrations. This suggests that discrete concentrations of dTTP are needed to effect control of the allosteric enzymes in the biosynthetic pathways.

Isotope dilution analysis has been criticized on the grounds that the rate of uptake into DNA was dependent on the concentration of substrate supplied (20). As the discussion above shows, the rate of DNA synthesis is independent of the concentration of thymidine supplied, because the de novo synthesis pathways can be turned on to make up any shortfall in supply. The processes of thymidine uptake (i.e., transport into the cell) and its incorporation into DNA are quite distinct processes and should not be confused.

Isotope dilution and the rate-limiting step. The multienzyme complex associated with DNA polymerase contains a small but rapidly turning over dTTP pool that is dedicated to DNA replication and is functionally separate from the main cellular dTTP pool (23). It is the in vivo specific activity of this pool that the isotope dilution analysis must measure. This analysis will only measure isotope dilution before the rate-limiting step in this pathway (38). If other steps, such as those involving thymidine transport enzymes or the thymidine kinase, become rate-limiting, any measure of bacterial growth will be seriously underestimated. Since this did not happen with the chemostat experiments, the rate-limiting step must have been at the level of DNA polymerase. The results reported here show that thymidine transport appears to be much faster than the rate of thymidine incorporation into DNA.

Under some circumstances, it is possible that thymidine kinase may become rate limiting for the incorporation of labeled thymidine into DNA. The end product of this biosynthetic pathway, dTTP, has been shown to be an inhibitor of thymidine kinase (17–19, 28, 29). Thymidine competes with dTTP and can reverse the inhibition if its concentration is high enough. In the presence of dTTP and low concentrations of thymidine, the V_{max} of thymidine kinase is low, its K_m is high, and it is rate limiting for thymidine incorporation (38). A probable example of this effect in studies on bacterial growth rates in sediments was observed by us in earlier work, although we did not correctly interpret it then (26). Biphasic plots were obtained from isotope dilution experiments (Fig. 5) in which it seems likely that thymidine kinase was inhibited by dTTP originating from de novo synthesis from dUMP. This inhibition was not influenced by the labeled thymidine supplied because the concentration in the cell was likely to have been low. The actual concentration around the cells in these experiments was very low because the large amounts of sediment that were used adsorbed most of the thymidine. At higher concentrations of added thymidine, this inhibition was reversed, and the rate-limiting step moved from thymidine kinase to DNA polymerase; thus, the effect of isotope dilution by thymidine nucleotide synthesis became apparent. Even after the reversal of thymidine kinase inhibition, the degree of participation by added thymidine in DNA synthesis was low; i.e., the concentration supplied was not sufficient to meet the needs of DNA synthesis. In later work, biphasic plots were avoided because less sediment was used, thus, effective concentrations of thymidine were higher (27).

The inhibition of thymidine kinase and its reversal by extra thymidine may be seen in an isotope dilution analysis when biphasic plots are obtained that bend to the right (see Fig. 5). If a plot bends to the left (see Fig. 4), it indicates that the contribution of de novo synthesis of dTMP is decreasing. No isotope dilution occurred when *Alteromonas* species was grown in yeast extract nutrient medium (Fig. 3). Sufficient exogenous thymidine was probably available for DNA synthesis via the salvage pathway. Without yeast extract, however, the radioactive label was substantially diluted in DNA (44%), but this dilution was not due to extracellular or intracellular thymidine pools; otherwise, dilution would have been apparent in the experiment with yeast extract. We

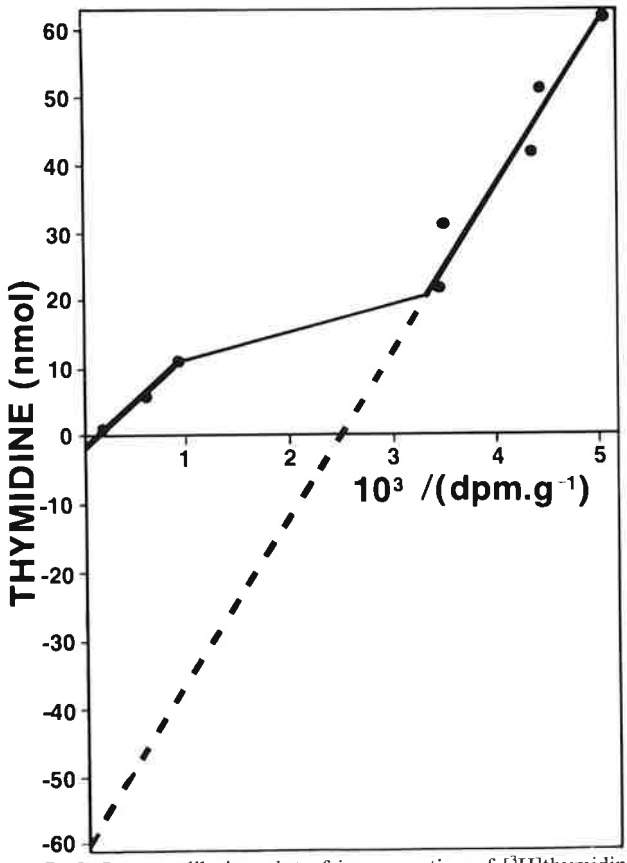

FIG. 5. Isotope dilution plot of incorporation of [^{3}H]thymidine into DNA of marine sediment dominated by sea grass (*Zostera capricorni*). (Redrawn from reference 26).

conclude, therefore, that the de novo biosynthetic pathway contributed to the pools of compounds that competed as thymine precursors for DNA synthesis when yeast extract was omitted.

We have established that correct estimates of growth rates were obtained by isotope dilution analysis in the presence and absence of yeast extract; therefore, thymidine kinase in both cases could not have been rate limiting. The dilution in the absence of yeast extract may be due to the activity of the catabolic enzyme thymidine phosphorylase. *Escherichia coli* lacking the enzyme thymidine phosphorylase readily incorporates 98% of exogenous thymidine into DNA (7). The yeast extract may contain either a phosphorylase inhibitor or sufficient deoxyribose to obviate the need for the degradation of thymidine; thus, enough exogenous thymidine may have been available to supply the requirement of dTTP for incorporation into DNA via the salvage pathway. Conversely, in the absence of yeast extract, phosphorylase may have competed with thymidine kinase for thymidine, thus decreasing the amount of thymidine available for DNA synthesis. De novo synthesis then contributed some of the dTTP needed as a DNA precursor, causing the dilution of tritium in DNA (Fig. 1).

Thymidine uptake by bacteria. Because there are reports of *Pseudomonas* species which cannot utilize thymidine (32), we investigated a range of marine pseudomonads and found that most incorporated thymidine into DNA (Table 3). It seems likely that most bacteria in the sea do utilize thymidine since good agreement has been found between other methods for estimating growth rates and the thymidine method (2, 13, 21). In the mixed culture of anaerobic bacteria from a sea grass bed, there was good agreement between growth rates determined by direct microscopy and the thymidine method (Table 1). Thus, thymidine is probably used by many common anaerobic bacteria. Those with specialized nutrient requirements, e.g., sulfate reducers, are probably not likely to be able to take up thymidine. The DNA in acetate-utilizing sulfate reducers could not be labeled with tritiated thymidine (unpublished observations). Because these bacteria do not grow efficiently, bacterial production in sediments would not be seriously underestimated with the thymidine method.

Specificity of the thymidine technique. One of the important advantages of using thymidine in studies on microbial ecology is that the growth rates of heterotrophic bacteria can be measured specifically (12, 26). This is because all cyanobacteria and eucaryotic algae and fungi that have been investigated lack thymidine kinase or at least have been shown not to incorporate thymidine directly into DNA. The work reported here with *Synechococcus* species and algae further supports this argument. Although protozoa contain thymidine kinase, their DNA is unlikely to be labeled in short-term experiments with low concentrations of thymidine (Moriarty, in press). Labeling of nuclear and cytoplasmic DNA in protozoa has been reported, but long time periods were needed (31, 34). A comment has been made that microbial ecology literature is "replete with reports of [H^3] thymidine incorporation into the DNA of eucaryotic algae, protozoa, yeasts, fungi and slime molds" (20). The first of the reports listed (4) makes no mention of thymidine. In other reports concerning algae and fungi, it is the inability of these organisms to incorporate thymidine into nuclear DNA that is discussed. Autoradiography was used to show that tritiated thymidine did label the chloroplast or cytoplasm of several different genera of these eucaryotes over a long time period (8, 33, 39–42). In every case, no significant labeling of the nuclei was reported. Indeed, Steffensen and Sheridan (39), using three genera of marine algae, *Dictyota*, *Padina*, and *Bryopsis*, found almost all of the radioactivity to be in the cytoplasm, reporting that "nucleii did not incorporate tritiated thymidine even though cells were dividing rapidly in the three genera examined." Sagan (33), in his study of *Euglena* species and [^{3}H]thymidine incorporation, reviewed some of the previous work in this field. In those reports in which the cytoplasm of microalgae was labeled with tritiated thymidine, he concluded that the investigators were observing chloroplast-related metabolism of exogenous thymidine over the long-term incubation periods.

ACKNOWLEDGMENTS

We are grateful to D. C. White, Florida State University, for providing facilities and assistance with work carried out with material from sea grass beds in the Gulf of Mexico. The mixed culture of anaerobic bacteria was grown by Jim Guckert and Carol Mancuso in the laboratory of D. C. White, and we gratefully acknowledge their help. We also thank Robert Pendrey in our laboratories, who cultured the algae.

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Journal of Microbiological Methods 3 (1985) 321–330
Elsevier

JMM 00108

A convenient method for measuring rates of phospholipid synthesis in seawater and sediments: its relevance to the determination of bacterial productivity and the disturbance artifacts introduced by measurements

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(Received 28 November 1984) (Revised version received 21 February 1985) (Accepted 22 February 1985)

Summary

A rapid and convenient method for estimating microbial activity based on radioactive isotope incorporation into lipids is presented. The methods, utilizing disposable equipment, quantitatively measure bacterial and microeukaryotic activity separately. Bacterial activity was measured from the rate of incorporation of [^{32}P]phosphate into phospholipids during the first 10 to 20 min of an incubation when rates were linear. This rate of phospholipid synthesis was unaffected by cycloheximide. When isotope dilution methods were used to define specific activity, the productivity of bacterial suspensions, measured with the phospholipid method, was equivalent to productivity of suspensions measured by rates of [^{3}H]thymidine incorporation into DNA. The rate of [^{35}S]sulfate incorporation into sulfolipids was inhibited by cycloheximide. This provided an estimate of microeukaryotic activity. As DNA synthesis is not affected by disturbance as rapidly as phospholipid synthesis, the differences in bacterial production rates given by these two methods can be utilized to estimate disturbance artifacts induced by adding labelled precursors to sediments.

Key words: *Bacterial activity – Bacterial productivity – Disturbance artifact – Phospholipid synthesis – Sediment – Sulfolipid synthesis*

Introduction

Bacteria in aquatic sediments are essential components of food chains, particularly in areas such as seagrass beds where few animals feed directly on the primary producers. Although many methods are available for estimating bacterial biomass, activity or productivity [1] difficulties remain in applying or interpreting them. Further developments

or techniques are needed, especially in the measurement of production. A very useful method at present is based on the incorporation of tritiated thymidine into DNA [2].

The measurement with [^{32}P]phosphate of relative rates of bacterial lipid synthesis on particulate detritus in water has been proposed as a way to study bacterial activities [3]. Phospholipids are the major lipids in bacteria and their concentration is well correlated with biomass [4]. There is also a correlation between the rate of phospholipid synthesis and growth rate of bacteria [5]. In order to quantify the absolute rates of phospholipid synthesis, and thus estimate growth rates, we show in this paper how the specific activity of [^{32}P]phosphate at the site of synthesis may be measured using an isotope-dilution procedure [6–8]. Although all organisms synthesize phospholipids, short incubation times favour bacterial activity as shown here in experiments with cycloheximide, an inhibitor of growth in eukaryotes but not in prokaryotes [9, 10]. As a control for the effect of cycloheximide, a primarily eukaryotic activity [11], viz., ^{35}S incorporation into lipids, was also measured. We give a comparison of the bacterial productivities estimated from the rates of phospholipid synthesis and DNA synthesis. The combination of phospholipid biosynthesis and DNA synthesis can give a quantitative estimate of the disturbance artifact that occurs when labelled precursors are added to highly stratified sediments.

Materials and Methods

Materials

$H_3{}^{32}PO_4$ and $H_2{}^{35}SO_4$ (carrier free) were supplied by Amersham, U.K. and New England Nuclear, Boston, MA, U.S.A. [5-*methyl*-^{3}H]thymidine (40 Ci/mmol) was supplied by Amersham Inc., U.K. Cycloheximide was supplied by Sigma Chemical Company, St. Louis, MO, U.S.A. Radioactive incorporations with $^{32}PO_4$ and $^{35}SO_4$ were made in 50 ml disposable polypropylene syringes with eccentric luer tips, to each of which a 10 cm length of polyvinyl tubing was attached.

Lipid extraction

Glass fibre filters or sediments were transferred to disposable syringes from which plungers had been removed and with tubing fastened to the eccentric luer tip. The plungers were inserted and 4 ml chloroform and 8 ml methanol were drawn up through the plastic tube into the syringe. The syringe was shaken and then left standing with the vinyl tubing clamped to the syringe with a rubber band. After at least 2 h in ice, a mixture of 4 ml chloroform and 4 ml water, containing a suspension of $Ca(OH)_2$ (40 mg) to precipitate unincorporated phosphate or sulfate, was drawn into the syringe through the tubing. The syringe was shaken well and left to stand overnight at an angle of 50° with the luer tip uppermost so that precipitates settled away from the opening. The lower (chloroform) phase was expelled through the tubing into pre-weighed scintillation vials and the volume was calculated from the weight. Pigments were destroyed by bleaching in sunlight or strong photographic flood lights. The chloroform was then removed by evaporation. Fifteen ml of scintillation fluid (Aquasol; New England Nuclear) were added and the radioactivity was determined in a scintillation spectrometer. Quenching was corrected in ^{35}S samples with an external standard procedure.

Addition of isotopes to sediments

(a) Slurry method

Sediments were collected in 25 mm diameter corers and the upper 10 mm of each were transferred to a beaker, mixed gently with an equal volume of water and one ml spooned into syringes (from which plungers were removed) containing 20 μCi $^{32}PO_4$ or $^{35}SO_4$ and, in some experiments, cycloheximide (100 μg in 10 μl) in 0.5 ml filtered seawater. The plungers were replaced. The syringes had been fitted previously with a tube attached to the luer tip, which formed a valve when fastened to the side of the syringe with a rubber band. After incubation at ambient seawater temperature for various time intervals, the reaction was stopped by drawing in 4 ml of chloroform and 8 ml methanol through the tube as described above. In one experiment, the slurry was mixed for 15 min before the incubation with [^{32}P]phosphate.

(b) Mild slurry method

Sediments were collected in 8 mm diameter corers and the upper 10 mm were immediately transferred to syringes containing 20 μCi of [^{32}P]phosphate in 0.1 ml of filtered seawater. The plungers were then inserted and tubing, fixed previously to the luer tip, was clamped to the side with a rubber band. After swirling the sediment briefly to mix it, the syringes were incubated at ambient seawater temperature for 15 min. For isotope dilution experiments, syringes were prepared with 0, 20 or 50 nmol of phosphate in addition to the radioactive phosphate.

(c) Injection method

Cores of sediment were collected in 8 mm diameter corers (3 ml disposable syringes with base cut off). A total of 150 μl of isotope (20 μCi $^{32}PO_4$) and, in some cores, an extra 50 nmol phosphate, were injected through the core and the cut end was then closed with a rubber stopper. Cores were incubated at ambient seawater temperature for 15 min. Each core was then expelled into the 50 ml syringe and lipids were immediately extracted.

Measurement of DNA synthesis

Procedures for determining rates of DNA synthesis in sediment were similar to those described by Moriarty and Pollard [12, 13] with the following modifications. Cores were collected in 8 mm diameter corers, and, for some experiments, the top 10 mm were transferred to polypropylene centrifuge tubes containing 0.5 ml filtered seawater and 50 μCi [^{3}H]thymidine. The sediments were mixed briefly by swirling, and then incubated at ambient seawater temperature for 15 min. Reactions were stopped by adding 10 ml of 80% (v/v) ethanol and the tubes were stored on ice. For other experiments, the tritiated thymidine was injected into the cores, which were incubated for 15 min and then expelled into 10 ml of 80% (v/v) ethanol. In the laboratory, the ethanol was removed after centrifuging, and the sediments were extracted in 0.3 M NaOH as described elsewhere [12, 13]. The extracted DNA was dialyzed and radioactivity was measured in portions of the extracts. Experiments were carried out to show that recoveries of DNA with this method were usually complete, and that results were the same as corrected results ob-

tained with the earlier procedure [12, 13]. Thus, in the short time interval of the incubations, DNA was the only macromolecule that was detectably labelled.

Suspensions of microbes

Seawater (1 l) and about 50 leaves of *Halodule wrightii* were placed in a bottle and shaken for a minute. Leaves and large particles were removed by filtering the water through a screen of 150 μm mesh.

Bacterial growth rates were measured with the thymidine method on 20 ml subsamples as described elsewhere [12] with the following modifications. Reactions were stopped by adding 1 ml of 36% (v/v) formaldehyde. Samples were chilled on crushed ice, then sufficient 100% (v/v) trichloroacetic acid (TCA) was added to give a final concentration of 5%. After standing on ice for 15 min, the samples were filtered through 25 mm Whatman GF/F filters and washed 5 times with 3% (v/v) TCA.

Rates of phospholipid synthesis were measured as follows: Disposable syringes (30 ml) with eccentric luer tips were prepared containing a triplicate series of 0, 10, 20 and 100 nmol of phosphate and 20 μCi of [^{32}P]phosphate in a final volume of 100 μl. Plungers were inserted into the syringes. Incubations were started by drawing up 20 ml of water into the syringes and capping the luer tips. Blanks, which contained 100 μmol phosphate to dilute out the uptake of radioactive phosphate, were immediately filtered through GF/F filters in a holder attached to the syringe. Other incubations were stopped after 15 min by filtering. Filters were immediately transferred to a second set of disposable syringes that were fitted with plastic tubing on the luer tips. Plungers were inserted and the lipid was extracted by drawing a mixture of chloroform (4 ml) and methanol (8 ml) into each syringe. The syringes were sealed by clamping the tubing to the side of the syringe with a rubber band. Glass-fibre filters were necessary, because cellulose acetate filters degraded in the chloroform, causing high and variable blank values.

Calculations

Specific activity of [^{32}P]phosphate at the site of lipid synthesis was calculated from the results of an isotope-dilution analysis [6–8, 12, 13]. The concentration or amount of phosphate added was plotted against the reciprocal of radioactivity in phospholipid. The negative intercept is a measure of the relative pool size in the cell at the site of synthesis.

Bacterial production was calculated from rates of thymidine incorporation into DNA as described by Moriarty and Pollard [13], using conversion factors of 2.5×10^{-11} mg C per cell and 2×10^{18} cells produced per mol thymidine incorporated. Production was calculated from rates of phospholipid synthesis using the conversion factor of 50 μmol P per g dry weight of bacteria [5], and a carbon content of 50%. Thus, μmol P incorporated $\times 10$ = mg C bacterial biomass produced.

Results

Phospholipid synthesis

Phosphate was incorporated into lipid at a linear rate for about 20 min in sediments

from a seagrass bed and a coral reef. Cycloheximide had no effect during this period, but did inhibit phospholipid synthesis after 20 min (Fig. 1a, c).

Sulfolipid synthesis

Sulfate was incorporated into sulfur lipids at a linear rate, and this was markedly inhibited after 5 min by cycloheximide (Fig. 1b, d). There was no incorporation of ^{35}S into lipid in anaerobic sediment in the seagrass beds.

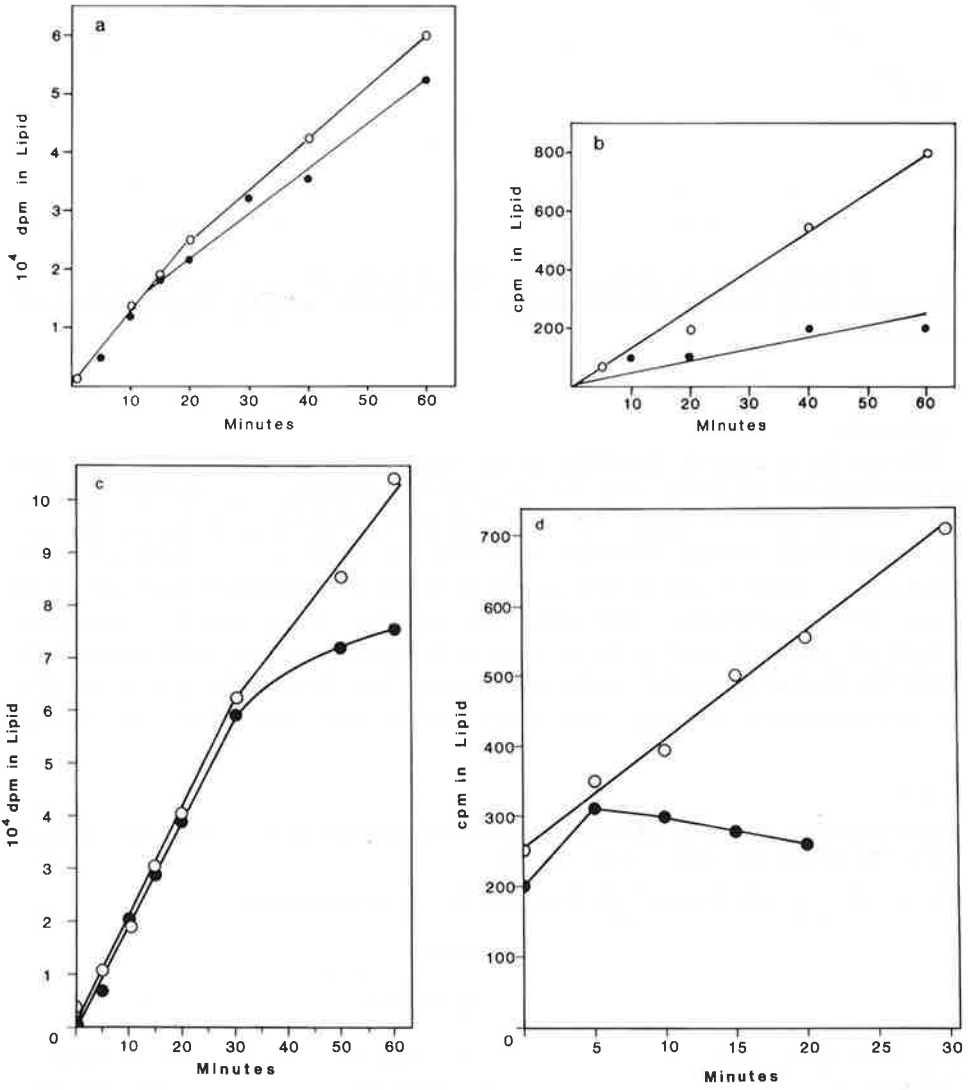

Fig. 1. Effect of cycloheximide on lipid synthesis. Rate of ^{32}P incorporation into phospholipids (a), and ^{35}S in sulfolipids (b) are shown in seagrass bed sediments from Moreton Bay, Queensland (153°20′W, 27°30′S) and rates of ^{32}P incorporation into lipids (c) and ^{35}S into lipids (d) in a coral reef sediment from Lizard Island, Queensland (145°20′W, 14°30′S) are shown. Control, ○; cycloheximide, ●.

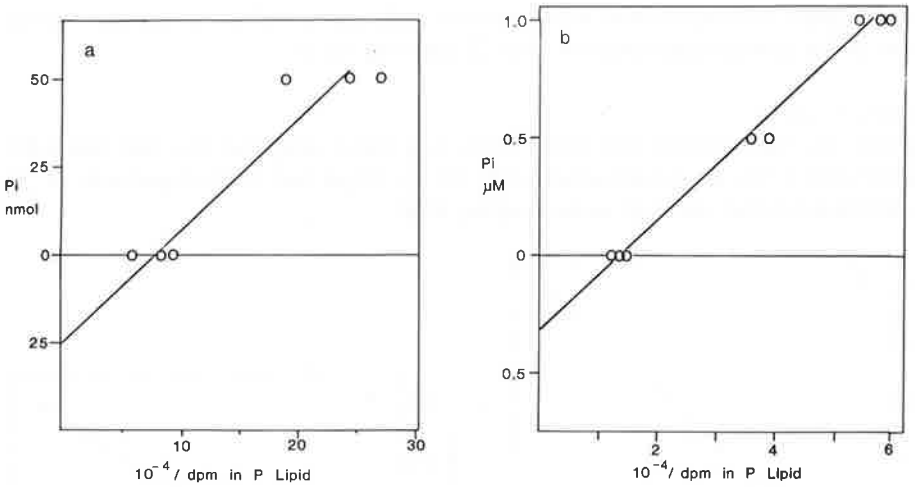

Fig. 2. Isotope-dilution plots for incorporation of [^{32}P]phosphate into lipid. a, each point was the result from an individual core of sediment injected with isotope; b, an experiment with water containing detritus from seagrass leaves.

Productivity

The specific activity of phosphate at the rate-limiting step for incorporation into phospholipid was determined with isotope dilution experiments (Fig. 2). More variation was observed when individual cores were injected or used for mild slurries (Fig. 2a), than when mixed slurries of sediment were used. The specific activity is the ratio of ^{32}P (dpm or μCi) added to each sample divided by the value of the negative intercept on the y-axis, in terms of amounts of P added. In the water column, which contained suspended detritus from seagrass leaves, rates of bacterial production estimated with the thymidine method agreed well with rates estimated from the phospholipid method (Table 1). Compared to sediment, these water samples were homogeneous and aerated.

TABLE I

COMPARISON OF RATES OF BACTERIAL PRODUCTION MEASURED USING RATES OF PHOSPHOLIPID SYNTHESIS AND DNA SYNTHESIS

Measurements were made in seawater, which contained detritus from seagrass leaves.

	Method	
	Phospholipid	DNA
Incorporation (nmol l^{-1} h^{-1})	0.47 ± 0.03 (5)[a]	0.14 ± 0.02 (6)
Production (μg C l^{-1} h^{-1})	4.7 ± 0.3 (5)[b]	5.8 ± 0.8 (6)[b]

[a] Data given as $\bar{x} \pm$ standard deviation (n).

[b] Analysis of variance: not significantly different.

TABLE 2

COMPARISON OF BACTERIAL PRODUCTION RATES ESTIMATED FROM RATES OF PHOSPHOLIPID AND DNA SYNTHESIS IN SEDIMENT SLURRIES AND CORES

Sediments were from a seagrass bed in August; water temperature 28 °C.

	Bacterial production (mg C m^{-2} h^{-1})	
	Phospholipid method	Thymidine method
Mixed slurry (n=8)	12.0 ± 0.3(8)[a]	9.4 ± 0.8(8)[c]
Injected cores (n=5)	9.1 ± 1.5(5)[b]	8.9 ± 1.6(5)[b]
Well-mixed slurry (n=6)	50.0 ± 2.0(6)	not determined

Data are presented as $\bar{x}$ ± standard deviation (n).
Analysis of variance: a > b ($P < 0.05$); a > c ($P = 0.05$).
Values labelled b, c not significantly different.

Bacterial productivities in surface sediment, when measured with the phospholipid and thymidine methods by injection into cores agreed well (Table 2).

Disturbance artifact

There was no significant effect on bacterial growth rates measured with the thymidine method when a slurry was made, whereas when the phospholipid method was used there was a significant difference ($P<0.05$). In this experiment, the surface sediment was collected from cores, gently mixed and then dispensed within a period of about 10 min. In another experiment, when the surface sediment was well mixed and aerated for 15 min, the rates of phospholipid synthesis increased by about 5 times (Table 2).

Discussion

Bacterial activity

As all organisms contain phospholipids, the measurement of the rate of phospholipid synthesis in a mixed community such as sediment is a useful measure of microbial activity [3]. During incubation periods of up to 2 h with [^{32}P]phosphate, it was predominantly bacterial activity that was measured [11]. Because the eukaryote inhibitor, cycloheximide, had no effect on the rate of ^{32}P incorporation into lipid for 20 min, we conclude that phospholipids were being synthesized mainly in bacteria (Fig. 1a, c). A control experiment to show that the cycloheximide was active, and not adsorbed to sediment, was the effect of cycloheximide on ^{35}S incorporation into lipid. The incorporation of [^{35}S]sulfate slowed or stopped within 5 min in the presence of cycloheximide. Results were similar in both coral reef sediments and siliceous sand (with some silt and clay) (Fig. 1b, d).

Further evidence that most of the phospholipid synthesis was due to bacteria is given by the good agreement with growth rates measured by the thymidine technique in the water samples (Table 1). The thymidine method specifically measures the growth of heterotrophic bacteria [2].

In order to use the rates of phospholipid synthesis as measures of growth or production, it is necessary to show that turnover does not account for a significant portion of the [^{32}P]phosphate incorporation. In monocultures of *Haemophilus*, 1.7–3.9 (1–3 h) generations were required for the ^{32}P of the most labile phospholipid, phosphatidyl glycerol (PG), to turnover in pulse-chase experiments [4]. In a microbial biofilm formed on estuarine detritus, the phosphate of PG, which is the most metabolically active of all the phospholipids, lost 50% of its ^{32}P in 2 h [14]. In this system, muramic acid showed a biphasic turnover with half times of 3.2 and 78.5 h. In sediments phospholipids lost half their ^{32}P in 2 days when incubated aerobically and in 12 days when incubated anaerobically [15]. Consequently in the short incubations used here, the ^{32}P incorporation represented phospholipid biosynthesis.

Sulfolipid synthesis

Rates of sulfolipid synthesis are correlated with growth and activity of microeukaryotes. This has been shown in biofilms of estuarine microbiota that were manipulated with antibiotics and nutrients to stimulate either prokaryotic or microeukaryotic growth [11]. Sulfolipid synthesis from $^{35}SO_4^{2-}$ was significantly slowed within 5 min in the presence of cycloheximide in both coral reef and siliceous sand sediments (Fig. 1b, d). Thus, sulfolipid synthesis in these sediments is a convenient marker for microeukaryotic activity. This has been shown for fungi in biofilms incubated in darkness [11] or in diatoms when incubated in the light [16].

Productivity

To utilize either thymidine or phospholipid synthesis as a measure of bacterial productivity, the specific activity of the precursor at the rate-limiting step in synthesis must be determined. The isotope-dilution procedure of Forsdyke provides a simple method to determine the specific activity at the site of synthesis [6, 7]. The problems of $^{32}PO_4$ binding in the sediment results in decreases in the isotope available for phospholipid synthesis, but it does not change the specific activity. Using the specific activities of $^{32}PO_4$ from Fig. 2, and a value of 50 μmol phospholipid/g dry weight of bacteria [5], the rate of phospholipid synthesis gives values for microbial productivity close to those determined from the rate of [^{3}H]thymidine incorporation into DNA. As tritiated thymidine incorporation into DNA in short-term experiments is an exclusively bacterial process [2], this is additional evidence that short-term phospholipid synthesis is a measure of bacterial activity. Because all microorganisms form phospholipids, the comparison of rates of DNA synthesis and phospholipid synthesis could provide insight into activity of those bacteria that do not incorporate [^{3}H]thymidine into DNA. Sulfate-reducing bacterial monocultures do not incorporate [^{3}H]thymidine into DNA (Moriarty, unpublished experiments). Consequently a measure of these anaerobic processes may be possible with a combination of phospholipid and DNA synthesis.

Disturbance artifact

Findlay et al. [17] have shown that the method of adding labelled precursors to highly stratified microbial environments such as sediments can have marked effects on rates of synthesis of macromolecules.

The data in Table 1 show that it is possible to detect a disturbance artifact by a combination of [^{3}H]thymidine incorporation into DNA and $^{32}PO_4$ incorporation into phospholipid.

The injection method of adding isotope showed significantly less phospholipid synthesis than the slurry method. When the slurry procedure was preceded by vigorous mixing for 10–15 min, the rate of phospholipid synthesis increased 5-fold in these sediments. The initial rate of [^{3}H]thymidine incorporation into DNA was not immediately affected by the method of addition of labelled precursor, because there is a lag in changes to rates of DNA synthesis in a 'shift up' experiment [18].

The technique described in this paper represents a rapid and convenient method of measuring short-term phospholipid or sulfolipid synthesis. It can give insight into bacterial and microeukaryotic activity and, when combined with [^{3}H]thymidine assays of DNA synthesis, can give a measure of disturbance artifacts. It also has potential for measuring anaerobic bacterial productivity in sediments.

Acknowledgements

We thank the Director and staff of the Lizard Island Research Station for their assistance. The following grants to D.C. White are acknowledged: OCE-80-19757 from Biological Oceanography, National Science Foundation; NAG-2-149 from the Advanced Life Support System Office, National Aeronautics and Space Administration; and Contracts N0014-82-C-0404 and N0014-83-K0056 from the Department of the Navy, Office of Naval Research; and CR-80-9994 of the Robert S. Kerr Environmental Research Laboratory of the Environmental Protection Agency.

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Arch. Hydrobiol. Beih. Ergebn. Limnol.	31	211–217	Stuttgart, Mai 1988

Accurate conversion factors for calculating bacterial growth rates from thymidine incorporation into DNA: Elusive or illusive?

By DAVID J. W. MORIARTY

With 3 tables in the text

Abstract

Growth rates of heterotrophic bacteria in water and sediment can be estimated from measurements of the rate of tritiated thymidine incorporation into DNA. The accuracy of this estimation is dependent on the accuracy of the factor used to convert rates of thymidine incorporation into rates of DNA synthesis and thus rates of cell division. Many different conversion factors have been published, and values range over more than 1 order of magnitude although most values are between 1×10^{18} and 4×10^{18} cells produced per mol thymidine incorporated. Variables affecting the conversion factor values are discussed.

Introduction

Quantitative studies on carbon cycling in aquatic environments cannot be complete without information on the amounts of organic matter transferred between different trophic levels by heterotrophic bacteria. Measurements of bacterial production, which can be made from measurements of bacterial growth rates, are now providing new information on the role of bacteria in the carbon cycle of aquatic systems.

Of the methods available for estimating bacterial growth rates, the thymidine method is the simplest to apply in aquatic environments (Moriarty 1986). In principle, it depends on the measurement of rates of DNA synthesis, which are coupled to rates of cell division. Thymidine has been used by biochemists to measure rates of DNA synthesis because it is used in the cell almost entirely for DNA synthesis; it is not incorporated into other macromolecules (Kornberg 1980). There are, however, problems in its use (Kornberg 1980, Moriarty 1986). As this is discussion meeting, I wish to put forward some topics to stimulate discussion of the thymidine method and current problems in interpretation of results.

0071-1128/88/0031-0211 $ 1.75

Theoretical conversion factor

In order to calculate the rate of DNA synthesis, we need to know the proportion of the four bases that thymidine constitutes; the genome size or the amount of DNA in each cell; and the amount of thymine triphosphate (TTP) that is incorporated into DNA.

This may be expressed as follows:

$$N = \frac{\text{mol T} \times 318 \times 1/p}{W}$$

where N = number of DNA molecules synthesised, i.e. number of cells produced.

mol T = mol thymidine incorporated
318 = average molecular weight of the four nucleotides in DNA
p = proportion of TTP in DNA
W = weight of DNA per genome
(W = molecular weight of DNA x 1.67×10^{-24})

A factor (F) for converting mol T incorporated to number of cells dividing is therefore:

$$F = \frac{318 \times 1/p}{W}$$

There are three variables (T, p, W) that need to be measured. The proportion of TTP in DNA is the one that varies the least: p can range from 0.12 to 0.35, but for a mixed population of bacteria the mean is about 0.25, i.e. the average G + C ratios are about 50 %. If the growing population had a G + C content that was 10 % higher or lower, this would make only a slight difference to the final result.

Genome sizes of bacteria occur mostly in the range of 1×10^9 to 3×10^9 daltons (Riley & Anilious 1978). Thus the weight of DNA per cell ranges from 1.7 to 5 fg. An average rounded-off value of 2 fg $cell^{-1}$ can be used for approximate calculations for marine bacteria (Fuhrman & Azam 1982). Thus values for the theoretical conversion factor (F) would range from 2.5×10^{17} to 8×10^{17} when p = 0.25. An average value for marine systems would be 6×10^{17}.

If p = 0.2, which would be the case if *Pseudomonas* species only were dividing, then the average value would be about 8×10^{17}, and the range 3×10^{17} to 1×10^{18}. Thus for a mixed population of growing bacteria in the sea, an average factor of 7×10^{17} is likely to give a value that is within a factor of 2 of the true value for growth rates, provided the true rate of TTP incorporation is known.

Fuhrman & Azam (1980) give a range of 2×10^{17} to 1.3×10^{18} for the conversion factor. The value of 1.3×10^{18} is applicable to mycoplasmas and other bacteria with a small genome size, about 5×10^8 daltons (see Masover & Hayflick 1981). If the very small marine bacteria also have small genome sizes, then a higher conversion factor than the average would be more accurate.

The third variable that affects the value of the conversion factor is the rate of TTP incorporation into DNA. It is the measurement of this rate that is likely to be the cause of most error in the conversion factor. When tritiated thymidine is supplied to the growing bacteria TTP and consequently DNA become labelled and it is critically important to determine the specific activity of the labelled thymidine in TTP (Moriarty 1986).

Empirical conversion factors

In order to circumvent the problems in determining whether theoretical conversion factors are valid for given experimental conditions, many workers have measured conversion factors empirically. These range from 5×10^{17} to 6.8×10^{19}, mostly with means between 1.1×10^{18} and 2.6×10^{18} (Table 1). Why is there such a large discrepancy between these values and the theoretical values? As pointed out above, it is more likely to be due to the problem of measuring accurately the rate of TTP incorporation into DNA than to variations in genome size or composition. In other words, general statements about conversion factors in particular environments cannot be made without paying close attention to experimental details.

Table 1. Some empirical factors for converting mol thymidine incorporated into DNA to number of cells dividing.

Conversion factor ($\times 10^{18}$)		Source material	Reference
range	mean		
	2.0	Lab cultures	Pollard & Moriarty (1984)
1.7–2.4		Coastal marine	Fuhrman & Azam (1982)
1.9–8.9	4.4	Marine estuary	Kirchman et al. (1982)
17 –68		Coastal marine	Kirchman et al. (1982)
0.5–5.9		Freshwater pond	Kirchman et al. (1982)
1.9–2.2	2.0	Freshwater lake	Bell et al. (1983)
2.8–6.2	4.0	Open ocean	Ducklow & Hill (1985)
0.3–5.8	1.1	Coastal marine	Riemann et al. (in press)
2.0–7.2	4.5	Freshwater lake	Riemann (1985)
0.9–7.0	2.6	Freshwater lakes	Riemann (1984)
1.6–7.3	2.2	Freshwater lakes	Lovell & Konopka (1985)
5.8–8.7	6.9	Freshwater marsh	Murray & Hodson (1985)

Factors affecting measured rates of thymidine incorporation

There are many factors that may affect the rates of tritiated thymidine incorporation into DNA and comparisons with changes in numbers of bacteria. These are:

1. Dilution by intracellular pools of TTP or de novo synthesis.
2. Slow rates of uptake into some bacteria.
3. Lack of synchronisation between times for measuring changes in cell numbers and rates of thymidine incorporation.
4. Grazing of bacteria by protozoa.
5. Lack of distinction between tritium incorporation into DNA and into protein, carbohydrate and other macromolecules.

The first two points listed above are likely to be the main sources for error, and I will discuss them briefly here, as they have been reviewed in detail elsewhere (Moriarty 1986). In my experience, thymidine pools outside the bacteria in aquatic environments are unlikely to be concentrated enough to affect the specific radioactivity of tritiated thymidine, even in sediments. Within the bacteria, however, significant dilution can occur if de novo synthesis of thymidine monophosphate

(TMP) is not inhibited or if there are large pools of TTP or TMP. The TTP that is incorporated into DNA is synthesised in a multienzyme complex and is probably separate from any (possibly larger) cellular pool of TTP. In growing bacteria, the TTP in the multienzyme complex is continually replenished, so only a short time should be needed for it to be synthesised entirely from the exogenous tritiated thymidine, if de novo synthesis is inhibited completely. Complete feedback inhibition of de novo synthesis would occur only if tritiated thymidine were transported into the cell faster than TTP was incorporated into DNA. That is, uptake should be at a faster rate than incorporation. Even if this condition were met, dilution of tritiated TTP would occur if the general cellular pool of TTP was large. In the latter pool TTP may be degraded and supply unlabelled thymidine or TMP for resynthesis of TTP in the multienzyme complex. Short term time course experiments would be needed to show whether this effect was important. Thus there are at least two functionally separate pools of TTP in the cell, and these turnover rapidly. We have to determine whether the pools are turning over at a much faster rate than the time period used for incubations and, therefore, that the TTP in them is replenished from the added thymidine. The term "saturate with tritiated thymidine" has been misused by some workers in referring to these pools.

If uptake of thymidine is the rate-limiting step, dilution will always occur, and will not be measurable. Some species of aquatic bacteria cannot take up thymidine (e.g. Pollard & Moriarty 1984). These may be a low proportion of the total: most active bacteria in two marine samples were found to take up thymidine also (Fuhrman & Azam 1982). Further work is clearly needed to determine not so much whether all aquatic bacteria take up thymidine, but whether the rate of uptake is faster than the rate of DNA synthesis.

Another problem is the breakdown of thymidine by thymidine phosphorylase. If thymidine phosphorylase activity is so great that thymidine is degraded as fast as it is taken up, this will have the same effect as slow or absent transport mechanisms. That is, little tritiated thymidine will enter the biosynthetic pathway for DNA and isotope dilution experiments may not reveal dilution of tritiated TTP by de novo synthesis. The tritium from the thymidine would label other macromolecules. Thus autoradiography would show that the bacteria were taking up thymidine, but isotope dilution could be occurring and not be measurable, with protein being labelled.

The effect these processes have on the empirical determination of conversion factors would be evident as higher values than determined theoretically. Experimental procedures should be designed to minimise or eliminate istotope dilution. Where transport enzymes or thymidine phosphorylase are not limiting, this can be done by using concentrations of thymidine that are high enough to cause feedback inhibition of TMP synthesis. Thymidine phosphorylase is an inducible enzyme, so it is possible that short term incubations (5 to 10 minutes) will give more accurate results than longer term (e.g. 30 to 60 minutes). Experimental studies with natural populations are needed on this problem.

Are the problems with interpretation of results from rates of tritiated thymidine incorporation too great for the method to be useful ecologically? I do not think so. In a recent extensive study of two different marine sites, Riemann et al. (1987) found that conversion factors for 60 samples were mostly within the range or close to the maximum of the range for theoretical values. Their average value was 1.1×10^{18}. They used thymidine concentrations of 10 nM or 20 nM,

which were high enough to prevent measurable dilution by de novo synthesis of TMP, and they integrated rates of thymidine incorporation over the whole time during which numbers were counted. It is apparent from their data that maximum rates of tritiated thymidine incorporation into DNA occurred earlier than (i.e. out of phase with) rates of cell division, as would be expected.

Much of my work has been with sediments, where it is far more difficult than in the water column to record changes in numbers of bacteria under natural conditions, and not have confounding effects such as protozoan grazing. Thus empirical conversion factors have not been determined. Instead, we have compared the production of bacteria measured with the thymidine method with that measured by an independent technique, phospholipid synthesis. The first results from these two techniques correlated very well, when a conversion factor of 1.3×10^{18} was used (Moriarty et al. 1983). In the second comparison, the results for the thymidine method were a little lower than with the phospholipid method (Moriarty et al. 1985). A conversion factor of 2.0×10^{18} was used then. With a lower factor, there was a significantly lower rate of production for one set of samples, but not for a second (Table 2). Although there are also assumptions made with the phospholipid method, it can be seen that that both methods give similar results, and thus that results from the thymidine method may be better with the lower conversion factor.

Such comparisons are complicated by another conversion factor that is needed, namely, the amount of carbon per cell. Our results, like most others, have been calculated on the assumption that C constitutes 10 % of the cell wet weight, but this has been challenged recently by Bratbak & Dundas (1984), who measured C contents of around 20 %. If they are right, it means that estimates of bacterial production need to be doubled. It also means that the bacterial cells are a lot drier than supposed; they would have a water content of only about 50 or 60 %. It is important, therefore, for any discussion of bacterial contribution to aquatic carbon cycling to include a discussion of the carbon content per cell.

The production of bacteria in sediments can be compared with the total amount of organic matter made available by primary producers. We have established carbon budgets for bacterial production over diel and seasonal cycles in tropical seagrass beds (Table 3). With a conversion factor of 1×10^{18}, about 25 % of the seagrass production is converted into bacterial biomass. This percentage can be justi-

Table 2. Comparison of bacterial production in sediment and water measured using rates of phospholipid synthesis and DNA synthesis. A cellular carbon content of 10 % wet weight was assumed. Conversion factor for thymidine was 1.3×10^{18} cells per mol Tdr incorporated. Values are $\mu g\ C\ l^{-1} h^{-1}$ for water and $mg\ C\ m^{-1} h^{1}$ for sediment ± standard error.

Source	Phospholipid	Thymidine	Reference
Water, suspended detritus	4.7 ± 0.3	3.8 ± 0.5	Moriarty et al. (1985)
Sediment (slurry)	12.0 ± 0.3	6.1 ± 0.5	Moriarty et al. (1985)
Sediment (injected cores)	9.1 ± 1.5	5.8 ± 1.0	Moriarty et al. (1985)
Sediment	1.6	2.1	Moriarty et al. (1983)
Sediment (injected cores)	1.6 ± 0.2	1.1 ± 0.2	Moriarty et al. (1983)

Table 3. Relationships between production of bacterial biomass and primary production in some tropical seagrass beds (Moriarty, Pollard & Roberts, unpublished data). Bacterial production was calculated using a conversion factor of 1×10^{18} cells produced per mol T incorporated and cellular carbon content of 10 % of wet weight. Total production in water column and sediment to a depth of 5 cm was measured.

Season/Site	Primary Production (PP)	Bacterial Production	
	$g\,C\,m^{-2}d^{-1}$ (± S.D., n = 6)	$g\,C\,m^{-2}d^{-1}$ (± S.E., n = 25)	% PP
Sheltered Bay			
Summer	11.0 ± 4.0	2.6 ± 0.4	24
Winter	2.8 ± 1.0	0.7 ± 0.1	25
Estuary			
Summer	3.5 ± 0.5	1.1 ± 0.2	31
Winter	2.0 ± 0.4	0.27 ± 0.04	13
Reef flat, *Caulerpa*, *Cymodocea*, *Thalassia*			
Summer	6.0 (± 1)	1.13 ± 0.18	19
Winter	3.1 (± 0.5)	0.27 ± 0.04	9

fied by assuming that on average the bacterial growth efficiency is 25 % and that all seagrass production is eventually decomposed by bacteria. If we used a conversion factor of 2×10^{18}, the average bacterial growth efficiency would need to be 50 %. These calculations are based on a cell C content of 10 % of wet weight. If 20 % is correct, then a conversion factor of 5×10^{17} would be more appropriate. This latter value certainly agrees best with the theoretical values.

A better independent check of the values obtained for bacterial production in the sediment, and better information on carbon content of the bacteria are needed to check the results obtained with the thymidine method.

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Advances in MICROBIAL ECOLOGY

Volume 9

Edited by

K. C. Marshall

University of New South Wales
Kensington, New South Wales, Australia

PLENUM PRESS · NEW YORK AND LONDON

Chapter 6

Measurement of Bacterial Growth Rates in Aquatic Systems from Rates of Nucleic Acid Synthesis

D. J. W. Moriarty

6

Measurement of Bacterial Growth Rates in Aquatic Systems from Rates of Nucleic Acid Synthesis

D. J. W. MORIARTY

1. Introduction

Marine microbiology has expanded rapidly as a scientific discipline in the last 10–20 years. A change in experimental approach, from isolation of individual organisms and pure culture studies to whole-community studies, has helped foster this expansion. New techniques, such as epifluorescence microscopy and the use of radioisotopes, have shown that bacteria are more numerous and active than had been generally accepted. Early work with radioisotopes showed that bacteria were actively metabolizing organic matter in the sea, but accurate measurements of growth rates and production were needed in order to quantify fully the role of bacteria in food chains and cycles of organic matter. Perhaps the ultimate expression of bacterial activity is cell division. If we can quantify this, then we can confidently make statements about other activities of bacteria.

It is the growth of heterotrophic bacteria that will be considered here. The growth of other microorganisms that fix carbon dioxide for their primary source of carbon, such as chemoautotrophic bacteria and cyanobacteria, can in principle be measured using $^{14}CO_2$. There has been no satisfactory technique until recently for measuring the growth of heterotrophic bacteria in aquatic environments. Laboratory methods for mea-

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suring growth rates do not work in natural environments, due to the small size of heterotrophic bacteria, their aggregation on or in particles and in mixed species groups, and the effects of predation. Brock (1971) stressed that microbial ecologists need to study what bacteria are doing in nature. He discussed a number of techniques that could be used, but no generally useful methods were available then. With the development over the last few years of methods for calculating growth rates from the rates of incorporation of radioactive precursors into nucleic acids, considerable advances have been made. In his review on microbial growth rates, Brock (1971) discussed aspects of the labeling of DNA with radioactive thymidine, which showed that it could be applied as a technique to study growth rates. This review examines in detail methods for measuring bacterial growth rates in the natural environment using the rates of synthesis of nucleic acids, especially DNA. A general review of biomass and activity measures has recently been published (van Es and Meyer-Reil, 1982), so other techniques will not be discussed here.

Brock (1967) used tritiated thymidine and autoradiography to estimate the growth rate of *Leucothrix mucor* in various aquatic habitats. His method is applicable only to bacteria that can be grown in pure culture and can be recognized in their natural environment. When applied to the microbial populations in general, autoradiography can be used as an indication of metabolic activity or to estimate the number of growing cells, but not growth rates. For example, autoradiography has been used by Ramsay (1974) to quantify active bacteria on *Elodia canadensis* leaves and by Hoppe (1976) to count active bacteria in seawater. More recent work has centered on the measurement of radioactivity in extracted DNA as a measure of the growth rate of the whole community of aquatic bacteria.

Kunicka-Goldfinger (1976) used semicontinuous culture on membrane filters to show that there was a linear relationship between incorporation of tritiated thymidine into macromolecules insoluble in trichloroacetic acid (TCA) and bacterial growth. She stated that the measurement of DNA synthesis with tritiated thymidine would be a useful measure of bacterial growth in water. Tobin and Anthony (1978) demonstrated that DNA was labeled with tritiated thymidine by bacteria in lake sediments. The incorporation of tritiated uridine into RNA was used by La Rock *et al.* (1979) to show that there was a zone of active bacterial growth in a deep anoxic basin in the Gulf of Mexico.

At about the same time, Fuhrman and Azam (1980) and Moriarty and Pollard (1981) developed the use of tritiated thymidine for estimating bacterial growth rates in water and sediment, respectively. Fuhrman and Azam (1980, 1982) showed that growth rates of bacteria in coastal waters could be estimated from the rate of incorporation of tritiated thymidine into DNA in seawater samples. Moriarty and Pollard (1981,

1982) measured the growth rate of bacteria in marine sediments using tritiated thymidine incorporation into DNA. These methods hold considerable promise for advancing our knowledge of the productivity of bacteria and the flux of organic carbon in aquatic ecosystems and have been eagerly applied by other research workers. As it is only a short time since these methods were introduced, methodologies and interpretations of results need improvement.

As an alternative to using radioactive thymidine for labeling DNA, Karl (1981, 1982) has proposed the use of adenine. Adenine also labels RNA in addition to DNA, and rates of RNA synthesis have been used to estimate overall microbial growth rates in freshwater ponds and the sea (Karl, 1979; Karl *et al.*, 1981). As will be discussed below, the microbial populations studied with adenine include microalgae and other groups in addition to heterotrophic bacteria.

Azam and Fuhrman (1984) have discussed the criteria necessary for the ideal method for measuring growth rates of bacteria in nature. These may be listed as follows: (1) The method should be specific for heterotrophic bacteria; (2) the method should not rely on balanced growth, or if it does, balanced growth should be shown to occur in the environment in question; (3) the growth rate should not be altered by any incubation or other procedures involved in the measurement of the growth rate; and (4) if the method requires a conversion factor, there must be a means of determining that factor with confidence for the sample.

The measurement of rates of DNA synthesis with tritiated thymidine complies with these criteria to a better extent than measurements using other precursors, provided certain conditions are met. There are, however, problems in obtaining accurate and ecologically meaningful results with any nucleic acid precursor that is used to measure growth rates. This is because methods that depend on measurement of nucleic acid synthesis do not in fact measure growth or cell division directly, but the rate of incorporation of a radioactive precursor into the macromolecule. The processes of uptake, or transport of the precursor into the cell, as well as metabolism within the cell, need to be taken into consideration in order to convert rates of precursor incorporation into a macromolecule to rates of cell division. Some aspects of the biochemistry of DNA and RNA synthesis that are relevant to the interpretation of results will be discussed below.

2. Bacterial Growth Processes

A good general introduction to the principles of bacterial growth and its measurement is available (Pirt, 1975). This text is aimed primarily at measurement of growth in pure cultures, but the basic principles dis-

cussed there are relevant to natural populations. The main phases in a classical bacterial growth cycle in batch culture are well known: lag, exponential, stationary, and, finally, death. These ideal stages can be identified with cultures in the laboratory, but in a natural environment the situation is more complex, with different bacteria in varying stages of growth at the same time. In marine environments many bacteria do not die, but develop what is known as a "starvation-survival" phase (Morita, 1982). When energy substrates are depleted, the bacteria divide rapidly and form many small cells. The final expression of growth of a bacterium is cell division and an increase in numbers and biomass of viable cells. In natural systems it is very difficult (or even impossible) to measure growth accurately by counting new cells formed. Before cells divide, there must be synthesis of new cellular components: walls, membranes, proteins, RNA, and, of course, DNA. Thus, growth may be defined as the increase in a particular component of the biomass of cells, which culminates in cell division.

The measurement of the rate of DNA synthesis is particularly useful, because it is related to cell division. Once DNA synthesis is initiated, it proceeds to completion, and triggers a cycle of cell division (Lark, 1969). Although metabolic turnover of DNA does not occur in a manner analogous to that of enzymes or messenger RNA, some turnover does occur due to processes such as excision and repair of incorrect bases. The rate of thymidine incorporation due to such processes would be insignificant compared to normal replication. Variations in the rate of synthesis of other cellular components, such as RNA or protein, may not reflect rates of cell division. A direct correlation between rates of cell division and increase in mass of most cellular components, such as RNA and protein, occurs only when growth is balanced (Campbell, 1957). According to Campbell, growth is balanced over a time interval if, during that interval, every extensive property (e.g., protein, RNA, DNA, cell number) increases by the same factor. This state of growth applies during the exponential phase of an ideal growth curve. In other words, during balanced growth the relationship between growth rate and the rate of increase of biomass or any cellular substance (x) is an exponential one. The rate of formation of any component x is proportional to the amount of x at any given time, and may be expressed as follows (Pirt, 1975):

$$dx/dt = \mu x$$

where μ is the specific growth rate; it has dimensions of time^{-1}. The value of μ will be the same for every extensive property during balanced growth. The DNA method for measuring microbial growth rates gives an estimate of the number of bacteria dividing per unit time. Knowing the actual

number present, one can estimate the doubling time for the population. Some workers use specific growth rate; this is related to doubling time or generation time g as follows:

$$g = (\ln 2)/\mu$$

During balanced growth, the rate of synthesis of any component; e.g., RNA, may be chosen to measure growth rate. Conditions for balanced growth have been defined for particular enteric bacteria. Detailed studies of the physiological states and chemical composition of *Salmonella typhimurium* have been made by Schaechter *et al.* (1958) and Kjeldgaard *et al.* (1958). They pointed out that if the growth rate was increased, by supplying better quality nutrients for example, the rates of RNA and protein synthesis immediately increased, whereas DNA synthesis and cell division rates continued at the old rate for some time and then abruptly increased. Conversely, when growth rate was lowered by withdrawing nutrients, RNA and protein synthesis slowed or stopped, but DNA synthesis and cell division continued at the fast rate for some time, and then rapidly slowed to the new rate. Rapidly growing cells were larger and contained more RNA, protein, and DNA than slowly growing cells.

Brunschede *et al.* (1977) found a similar situation in *Escherichia coli.* After an increase in growth rate, RNA and protein synthesis increased immediately, but DNA synthesis was stable for 30 min and then increased. The rates of synthesis of all three macromolecules took a period of 2 hr or longer to stabilize.

DNA synthesis is regulated primarily at the site of initiation. Faster growth rates are achieved by having more than one replication fork proceeding along the chromosome. The rate of travel of a replication fork is constant (Lark, 1969). Thus, provided the rate of thymidine incorporation is measured over a shorter time interval than is required for one cycle of DNA synthesis, small changes in the external environment due to sampling should not affect the measurement. There are difficulties, of course, in extrapolating from the laboratory to the field, and obviously major changes, such as aerating an anaerobic sediment, will affect energy metabolism and thus directly affect the rate of thymidine incorporation. For this and other reasons, studies on bacterial growth processes in sediments are particularly difficult, but not impossible. The thymidine technique is a promising one, although present results need to be accepted with caution.

The ratios of rates of stable RNA (transfer and ribosomal RNA) to DNA synthesis in *E. coli* were directly related to growth rate during balanced growth and at fast growth rates (Dennis and Bremer, 1974). At slow growth rates (less than 0.67 hr^{-1}), the relationship between rates of stable

RNA and DNA syntheses did not hold in their experiments. This growth rate, although slow for a culture, is much faster than is observed in many natural systems. There may be problems, therefore, in using techniques for measuring growth that depend on the ratio of RNA synthesis to DNA synthesis unless it is shown that balanced growth occurs (e.g., Karl, 1981). This illustrates one of the problems confronting microbial ecologists. Much of the laboratory work from which conclusions are drawn and extrapolated to the natural environment has been undertaken on organisms such as *E. coli*. The need to move between the laboratory and nature and carry out complementary experiments has been emphasized by Brock (1971).

Caution must be observed when applying the principles of the growth processes described above to all bacteria, especially in the natural environment, where nutrient supply is usually small and variable. Even in a chemostat, an organism growing at a constant rate may not be in a state of balanced growth. For example, the fermentative bacterium *Zymomonas* exhibits unbalanced or uncoupled growth (Swings and de Ley, 1977). The rate of substrate dissimilation per unit weight of organism is independent of growth rate. The amounts of glucose metabolized and ATP produced do not vary with growth rate in minimal media where a growth factor is limiting. Swings and de Ley (1977) suggest that the uncoupling occurs because *Zymomonas* lacks adequate mechanisms to control energy charge levels and the link between ATP production and its consumption during biosynthesis. In the well-studied *E. coli*, however, such mechanisms do exist. This illustrates the danger in generalizing to all bacteria from studies with *E. coli*. If some marine or freshwater bacteria are more akin to *Zymomonas* in their lack of fine cellular control mechanisms, the measurement of processes such as protein or RNA synthesis or oxygen uptake may not be related to growth rate, even if there is a constant supply of nutrient. Thus, relating growth rate to most extensive properties (such as RNA content) can only be done under well-defined conditions and such studies cannot be extrapolated to all marine bacteria. It is unlikely, therefore, that methods for measurement of growth rate based on RNA synthesis meet the second criterion listed in Section 1. DNA synthesis, on the other hand, is more closely correlated to cell division and growth rates. Because there are more than one or two replication forks present in rapidly growing cells, multiple copies of the genome will be present; thus, the DNA content per cell is prone to vary with growth rate. The DNA content per unit biomass, however, does not vary to the same degree, because rapidly growing cells are larger than slowly growing ones (Maaloe and Kjeldgaard, 1966).

Where bacteria live in conditions of very low nutrient supply, such as in the ocean, the assimilated nutrients may be needed more for cell maintenance (e.g., protein turnover and osmotic balance) than for

growth. Thus, at very low growth rates, the extra energy required for maintenance will alter the relationship between growth rate and energy production, which could lead to uncoupled growth (Pirt, 1975). In dealing with growth processes in natural systems, the principles gained from the study of pure cultures may not be applicable. Christian *et al.* (1982) have demonstrated this need for caution in their studies with mixed batch cultures from natural populations. They found that growth rate constants calculated from a variety of different measures of growth were variable. Referring to the criteria listed above, we see from the discussion in this section that the measurement of DNA synthesis appears to meet the second and third criteria better than the measurement of rates of synthesis of other macromolecules.

3. Biochemistry of Nucleic Acid Synthesis

3.1. Introduction

In principle, the measurement of growth rates using nucleic acid synthesis is simple, involving the measurement of the rate of incorporation of radioactive precursor into a macromolecule. Several conditions need to be observed: (1) The specific radioactivity of the precursor immediately before incorporation into the macromolecule must be known; (2) the radioactivity measured at the end of the experiment must only be in the macromolecule under consideration; and (3) the added radioactive molecule should be incorporated into the macromolecule by only one biosynthetic pathway, to avoid complications due to differing kinetics or degrees of participation. The processes of uptake of thymidine, assimilation into cellular constituents, and incorporation into DNA are all distinct, and the rates of occurrence of each vary between types of microorganism.

It is the final process, the immediate incorporation of thymidine into DNA through the action of thymidine kinase, that enables us to distinguish bacterial activity from that of other microbes, and thus makes thymidine so useful as a measure of heterotrophic growth rates. Thymine nucleotides, unlike those of other nucleic acid bases, have only one function in cells, participation in DNA synthesis (O'Donovan and Neuhard, 1970). In this respect, thymidine meets the first criterion listed in Section 1, in contrast with adenine or other precursors.

These points will be elaborated in the section below, where aspects of the biochemistry of DNA and RNA synthesis relevant to use of nucleic acid bases and nucleosides in natural aquatic systems are discussed. Most knowledge of these processes has been gained from studies on enteric bacteria, particularly *Escherichia coli.* As discussed above (Section 2), conclusions drawn from such work may suggest what happens in a natural

community, but are not necessarily directly applicable. Furthermore, the application of rates of nucleic acid synthesis to growth rates in the sea involves studying the net activity of a whole community of different bacteria. By reporting a single growth rate we are making the simplifying assumption that the community behaves as a single species or population of a species.

A detailed description of DNA synthesis and pathways of nucleotide biosynthesis is given by Kornberg (1980). Some of the following is based on Kornberg's book, which may not always be cited specifically in the text.

3.2. Measurement of DNA Synthesis with Thymidine

3.2.1. Biochemistry of Thymidine Incorporation into DNA

Thymidine meets the criteria for pulse labeling DNA in bacteria reasonably well. It is rapidly and efficiently taken up by bacteria, is stable during uptake, is converted rapidly into nucleotides, and labels DNA with little or no dilution by intracellular pools (Kornberg, 1980). There are, however, pitfalls in its use, which must be considered. For environmental studies, the main problems are (1) dilution of the labeled thymine moiety during incorporation into DNA by other sources of thymine nucleotides; (2) degradation of thymidine within cells and subsequent random distribution of label; (3) the possible uptake of thymidine by microorganisms other than bacteria and, conversely, the lack of uptake by some bacteria; and (4) effects on the rate of DNA synthesis caused by disturbing the interactions between different microorganisms and their environment during experimental manipulation. These points are considered below.

There are two principal pathways for nucleotide biosynthesis in cells: (1) the *de novo* route, in which the nucleotides are synthesized from basic cellular components; and (2) the salvage pathway, in which free bases and nucleosides arising from breakdown of excess nucleotides or nucleic acids are converted back to nucleotide triphosphates.

Thymidine itself does not occur in the *de novo* pathway. Thymidine monophosphate (TMP)* is synthesized directly from deoxyuridine

*A note on nomenclature is necessary here. Deoxyribonucleotides are generally abbreviated as, e.g., dAMP for deoxyadenine monophosphate, and ribonucleotides as, e.g., AMP. Thymidine was originally known only as the deoxy form, as it is not (or rarely) found in RNA, and so the terms thymidine, deoxythymidine, or thymidine deoxyribose are used interchangeably, as are TMP, dTMP, etc. The following abbreviations are used in this chapter: A, adenine; Ad, adenosine; dAd, deoxyadenosine; dR-1-P, deoxyribose-1-phosphate; R-1-P, ribose-1-phosphate; I, inosine; IMP, inosine monophosphate; PRPP, phosphoribosylpyrophosphate; Tdr, thymidine; dTMP, dTDP, and dTTP, thymidine mono-, di-, and triphosphate, respectively; dUMP, dUDP, and dUTP, deoxyuridine mono-, di-, and triphosphate, respectively; dC, deoxycytidine; dCMP, dCDP, and dCTP, deoxycytidine mono-, di-, and triphosphate, respectively.

monophosphate by the enzyme thymidylate synthetase (Fig. 1). In the salvage pathway, thymidine is phosphorylated to form TMP by the enzyme thymidine kinase (Figs. 1 and 2). The base (thymine) is converted into the nucleoside (thymidine) by the action of the enzyme thymidine phosphorylase (Fig. 2). Deoxyribose-1-phosphate is needed, which is often supplied by another nucleoside; e.g., deoxyadenosine. Unless there is an adequate supply of deoxyribose-1-phosphate, this reaction does not occur, and in fact the reverse reaction may predominate. This is why thymidine and not thymine is used as a precursor to measure rates of DNA synthesis.

The relevance of these pathways to the use of labeled thymidine in aquatic environments is twofold. Firstly, much of the label may be lost by catabolism to thymine and then by loss of the labeled methyl group during further degradation of thymine (Fig. 2) (Fink and Fink, 1962; Vogels and van der Drift, 1976). There are no direct routes for tritium label to be incorporated into RNA or DNA after degradation of thymine. The tritiated methyl group enters the general pool of metabolites in the cell and eventually tritium may be distributed into all compounds, including RNA and DNA, but it will be considerably diluted. The amount of isotope incorporated into nucleic acids in such a way would be small compared to direct incorporation of thymidine into DNA, and would take some time (see Section 3.2.3). In short-term experiments (generally 10–30 min), such nonspecific labeling is insignificant (Pollard and Moriarty, 1984; Moriarty *et al.*, 1985a; Riemann, 1984). If the bacteria are not growing, not only DNA, but other macromolecules also are not labeled significantly above background levels (D. J. W. Moriarty, unpublished results). The effects of catabolism on the kinetics of labeling will be discussed below (Section 3.2.5).

Secondly, the main mechanism for the dilution of isotope in DNA is the action of thymidylate synthetase, in which TMP is derived from dUMP and is mixed with TMP formed by thymidine kinase from thymidine. In order to calculate the rate of DNA synthesis using radioactive thymidine, the specific radioactivity of the thymidine triphosphate pool must be known. Other sources of the thymidine inside and outside the cell may dilute the isotope, but on present experience this is not usual in bacteria from aquatic habitats. During conversion to thymidine triphosphate, the isotope may be diluted by synthesis of nucleotides from sources other than the added precursor. As the salvage and *de novo* pathways converge at the synthesis of dTMP (Fig. 1), it is here that the major dilution of isotope is likely to occur in growing cells. As dTDP and dTTP are synthesized only from dTMP, isotope in dTDP and dTTP would not be diluted further. There are pathways by which cytidine nucleotides can be converted to thymidine nucleotides via dUTP or dUMP (Fig. 1). Thus, dUTP and dUMP are key intermediates in the biosynthesis of thymidine nucleotides.

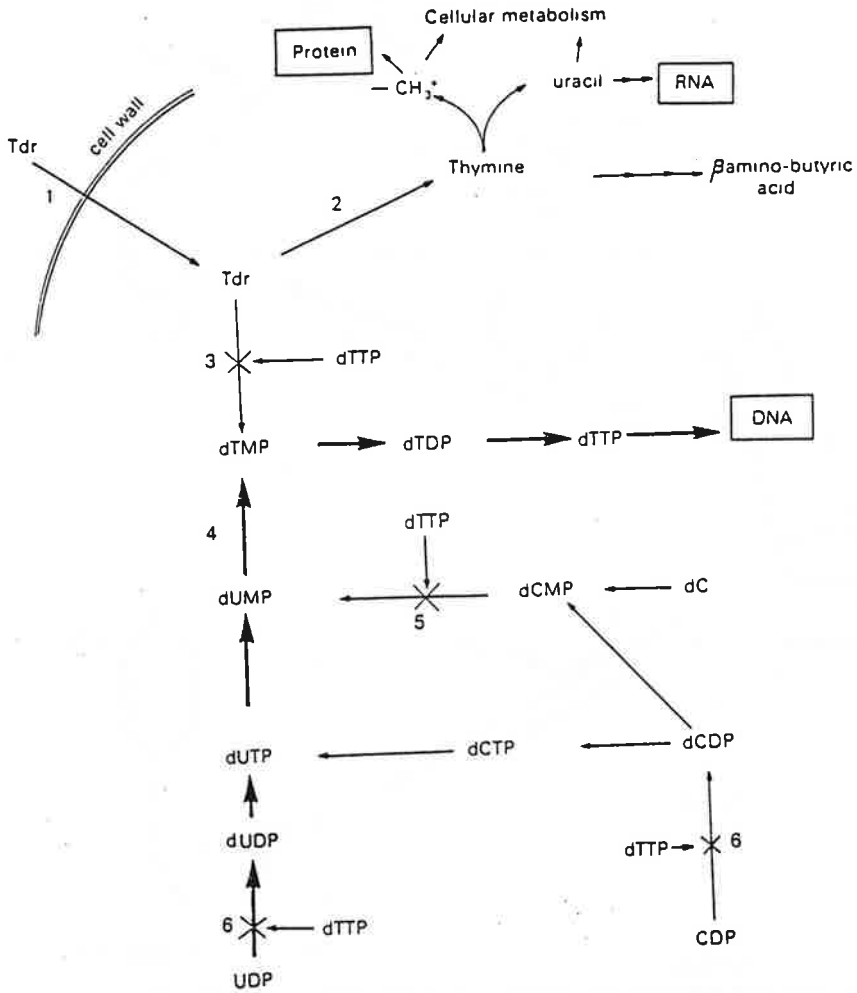

Figure 1. Pathways of thymine nucleotide metabolism. Tdr, Thymidine; dTMP, dTDP, and dTTP, thymidine mono-, di-, and triphosphate, respectively; dUMP, dUDP, and dUTP, deoxyuridine mono-, di-, and triphosphate, respectively; dC, deoxycytidine; dCMP, dCDP, and dCTP, deoxycytidine mono-, di-, and triphosphate, respectively. Enzymes: (1) active transport mechanism; (2) thymidine phosphorylase; (3) thymidine kinase; (4) thymidylate synthetase; (5) deoxycytidylate deaminase; (6) ribonucleoside reductase. The sites of feedback inhibition by dTTP are shown. Salvage and degradative pathways are shown with thin arrows; *de novo* pathways are shown with bold arrows.

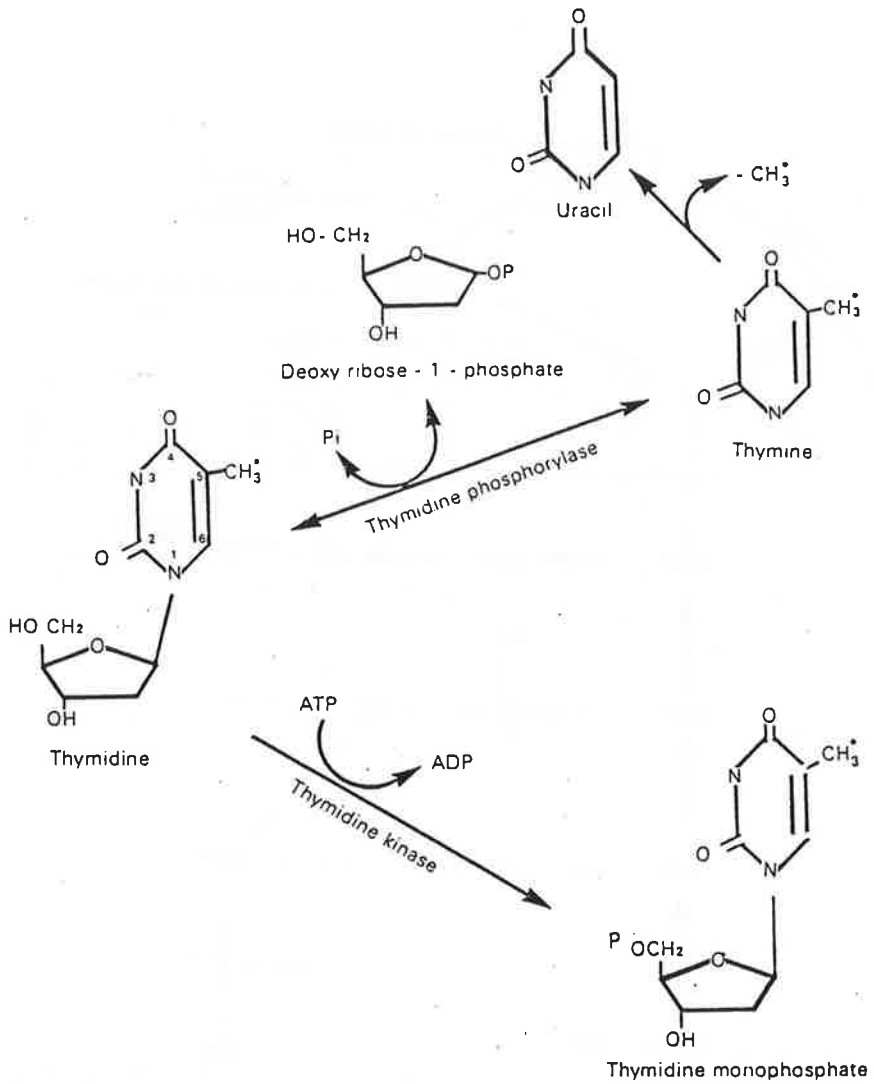

Figure 2. Reactions and structures of some compounds involving thymidine. The location of the tritium label is shown as an asterisk.

There is evidence that DNA polymerase is closely associated with a number of nucleotide kinases and that its activity is very much greater when the enzymes are organized together (Mathews *et al.*, 1979). This means that there are functional compartments of nucleotide precursors inside the multienzyme complexes in which high concentrations of precursors are available at the site where they are used; i.e., the replication

fork on the DNA. These pools would be small and turning over rapidly, in contrast to larger pools of the same nucleotides that would be dispersed more generally in the cytoplasm and be available for repair of DNA and perhaps regulation of nucleotide biosynthesis, but not normal DNA synthesis. There must, therefore, be more than one pool of dTTP (and other trinucleotides) in cells. Evidence for such pools of dTTP in bacteria was provided by Werner (1971), who showed that the rate of labeling of DNA by thymine or thymidine was much faster than the rate of labeling of the extractable dTTP pool. Thus, the specific activity of the dTTP pool that is used for DNA synthesis cannot be measured by extracting dTTP; it can, however, be measured using an isotope-dilution analysis (see Section 3.2.6).

Probably all organisms regulate nucleotide biosynthesis to prevent unnecessary buildup of nucleotide triphosphates. There are complex interactions between many nucleotides involving activation or inhibition of certain key biosynthetic enzymes. Thymidine triphosphate is an important regulator in pyrimidine nucleotide biosynthesis and this means that the pool size of dTTP must be closely regulated in order for it to act in this way (Maley and Maley, 1972). There are two routes for the *de novo* synthesis of thymidine nucleotides, one via cytidine nucleotides and one via uridine nucleotides (Fig. 2). Both are regulated by dTTP, which acts as an inhibitor of ribonucleoside reductase and deoxycytidylate deaminase. An increase in the dTTP pool size will slow down or turn off the supply of dTMP by *de novo* synthesis, depending on the size of the pool (Kuebbing and Werner, 1975). When measurements are made of bacterial growth rates in natural systems, sufficient thymidine can be added to inhibit *de novo* synthesis completely (Pollard and Moriarty, 1984). Thus, isotope dilution can be prevented. This makes ecological work simpler, because the specific activity of dTTP used for DNA synthesis is not altered from that of the tritiated thymidine supplied, and only one measurement is needed. As noted below (Section 3.2.6), this conclusion is based on laboratory cultures with copiotrophs. It may not apply to oligotrophs and needs to be checked in oligotrophic environments. [Oligotrophs are bacteria that are adapted to growth at very low concentrations of nutrients, whereas copiotrophs are bacteria that grow only when copious quantities of nutrients are supplied, for example, in the usual laboratory media; see Poindexter (1981).]

3.2.2. Transport of Thymidine

Before DNA can be labeled with radioactive thymidine, the thymidine needs to be rapidly and efficiently taken up by the cells. Furthermore, the enzyme thymidine kinase must be present, as shown in Section

3.2.1 and discussed in detail in Section 3.2.3. Very little information is available on pyrimidine nucleoside transport into most bacteria. Thymidine, uridine, cytidine, and deoxycytidine are all transported chemically intact into *E. coli* by an energy-dependent process. The purines adenosine and guanosine are transported similarly. At least two different transport mechanisms have been identified (Munch-Petersen *et al.*, 1979). In normal wild-type cells, the processes of transport and catabolism are closely linked but separate. The catabolic enzymes, although located close to the cell surface, were inside the membrane. A similar separation of transport and catabolism could be expected to occur in other bacteria, but no information is available.

There are interactions between nucleosides during uptake, indicating that at least one mechanism is shared by different nucleosides. Thymidine competed with uridine during transport (Roy-Burman and Visser, 1981). Mygind and Munch-Petersen (1975) also showed that nucleosides competed for the same transport mechanism, or at least part of the transport mechanism. Thus, the extent of labeling of DNA with tritiated thymidine will be influenced by the presence of other nucleotides in the environment outside the cells. The rate of tritiated thymidine incorporation into DNA in sediment bacteria was depressed by uridine (a pyrimidine), but not by hypoxanthine (a purine) (D. J. W. Moriarty and P. C. Pollard, unpublished results). The most likely explanation for this is that uridine competed for uptake sites with thymidine in the population of sediment bacteria.

Probably not all bacteria have mechanisms for efficient transport of nucleosides or bases. Some species of marine *Pseudomonas* do not incorporate labeled thymidine into DNA because they are unable to take up thymidine into the cell (Pollard and Moriarty, 1984). Bacteria with very limited nutrient requirements, such as chemolithotrophic bacteria, may also lack such transport systems. Sulfate-reducing bacteria have limited nutrient requirements, and it has been found that tritiated thymidine was very poorly incorporated into their DNA (D. J. W. Moriarty and G. W. Skyring, unpublished results). Bacteria that cannot transport nucleosides may not be able to transport free bases either.

3.2.3. Thymidine Kinase

After transport into the cell, the next stage in incorporation of thymidine into DNA is its conversion to TMP by the salvage pathway enzyme thymidine kinase (Fig. 2). Although most organisms were once thought to have this salvage pathway (Kornberg, 1980), some groups of microorganisms are now known to lack it. Thymidine kinase is not present in the fungi *Neurospora crassa, Aspergillus nidulans,* and *Saccharo-*

myces cerevisiae, the alga *Euglena gracilis* (Grivell and Jackson, 1968), and the cyanobacteria *Anacystis* and *Synechocystis* (Glaser *et al.,* 1973). The nuclei of the eucaryotic algae *Bryopsis, Chlamydomonas, Dictyota, Euglena, Padina,* and *Spirogyra* lack thymidine kinase; although a small amount of label was incorporated, it required hours or days of incubation to be shown by autoradiography (Stocking and Gifford, 1959; Sagan, 1965; Steffensen and Sheridan, 1965; Swinton and Hanawalt, 1972). According to Sagan (1965), this low rate of labeling was probably due to degradation of thymidine and incorporation into RNA and protein. We have examined species from three genera of microalgae (*Thalassiosira, Isochrysis,* and *Platymonas*) and a marine *Synechococcus* and have found no significant incorporation of tritiated thymidine into their DNA (Pollard and Moriarty, 1984). Similarly, Bern (1985) has shown that bacteria took up tritiated thymidine from lake water, whereas a variety of blue-green and eucaryotic algae did not.

It was the lack of tritium incorporation into DNA in some eucaryotic fungi and algae and the nonspecific labeling of DNA and RNA by [^{14}C]thymidine that led Grivell and Jackson (1968) to show that these organisms did not have thymidine kinase. Since all cyanobacteria, eucaryotic algal nuclei, and fungi that have been investigated do not contain thymidine kinase, it seems reasonable to conclude that that is a general phenomenon in these groups and thus that they do not incorporate thymidine into DNA. In support of this generalization, Fuhrman and Azam (1980) reported that over 90% of total incorporation of thymidine into TCA-insoluble matter was into particles less than 1 μm in size. There are reports of thymidine incorporation into DNA in algae and protozoa. Some of these (e.g., Grivell and Jackson, 1968) used [2-^{14}C]thymidine, not [^{3}H-methyl]thymidine. Much longer time periods and higher concentrations of thymidine than are used to measure bacterial growth rates were needed to demonstrate incorporation of radioactivity into DNA in these eucaryotes.

Even if some algae do take up thymidine, they cannot incorporate the tritium label on the thymidine into DNA immediately. The thymidine is catabolized, and after some time the labeled methyl group is distributed among various cellular components, including protein and RNA. Protozoa, however, probably do contain thymidine kinase (Plaut and Sagan, 1958; Stone and Prescott, 1964). They do not feed primarily on dissolved organic compounds, and thus are unlikely to have efficient transport mechanisms that can take up the nanomolar concentrations of thymidine supplied during experimental manipulation. Cycloheximide, an inhibitor of DNA synthesis in eucaryotes, has no effect on the incorporation of thymidine into DNA in marine sediments and seawater (Moriarty and Pollard, 1982; and unpublished observations). Thus,

although bacteria may not be the only organisms that can utilize thymidine for DNA synthesis, they are the only ones that do so over a short period.

The experimental evidence discussed in this review shows clearly that thymidine is useful as a measure of bacterial growth rates, but there are pitfalls. In particular, experiments should be for short time intervals at nanomolar concentrations. Under these circumstances, the rate of labeling of DNA in eucaryotes will be slow compared to that in bacteria.

3.2.4. Uptake of Thymidine by Different Organisms

Many microorganisms other than bacteria may be able to take up thymidine, but unless they also have the enzyme thymidine kinase, DNA will not be rapidly labeled. As discussed in Section 3.2.3 on thymidine kinase, this means that labeling of DNA by thymidine is specific for bacteria in short-term experiments. The use of short-term experiments needs to be stressed here. Over a long period (2–24 hr) label may be incorporated into DNA of eucaryotes, including protozoa. In at least one seawater sample examined, it seems that only a small proportion of the active bacteria cannot utilize thymidine. This conclusion was reached by Fuhrman and Azam (1982) after a careful autoradiographic study. Ramsay (1974) found that fewer bacteria were labeled with tritiated thymidine than with glucose in a freshwater habitat. It is possible that some of the difference may have been due to a different amount of isotope taken up, as discussed by Fuhrman and Azam (1982), but this cannot be the full explanation. *Pseudomonas fluorescens* and a pseudomonad strain isolated from a freshwater lake could not be labeled with [^{3}H]thymidine (Ramsay, 1974). A few species of aquatic pseudomonads have been found not to incorporate tritiated thymidine into DNA (Pollard and Moriarty, 1984). Thus, not all marine or freshwater heterotrophic bacteria can utilize thymidine, probably due to a deficiency in transport of thymidine. Those bacteria that cannot utilize thymidine probably constitute only a small proportion of the total number of bacteria in aquatic systems. Measurements of growth rates with tritiated thymidine agree well with measurements made using direct counts of bacterial numbers in containers (Bell *et al.*, 1983; Fuhrman and Azam, 1982; Kirchman, *et al.*, 1982). The work of Kirchman *et al.* (1982) indicates that most bacteria in the system they studied utilized thymidine.

3.2.5. Degradation of Thymidine

Thymidine is rapidly degraded within cells, initially by the inducible enzyme thymidine phosphorylase. The tritium on the methyl group is

transferred to other compounds, including water, and eventually will be incorporated by *de novo* pathways into RNA, DNA, and protein. It will be considerably diluted during these processes, but these processes would become apparent during any long-term experiments. Thus, other compounds insoluble in cold trichloroacetic acid (TCA), in addition to DNA, would be labeled in mixed populations of microorganisms, but in the short term (usually less than 1 hr) specific labeling of bacterial DNA predominates. Karl (1982) disputes this statement, but unfortunately, he used long-term incubations and used acid in the initial extraction procedure. Deoxyribonucleic acid is very labile to acid, and readily fragments on subsequent treatment with alkali, thus appearing in RNA fractions (Munro and Fleck, 1966). Riemann (1984) has studied the distribution of tritium from thymidine into various macromolecules and showed that, in general, most was incorporated into DNA.

In marine sediments, thymidine was incorporated into DNA at a linear rate for an initial period of 8 min at warm (31°C) temperatures and up to 20 min at colder (18°C) temperatures (Fig. 3). Slower rates of incor-

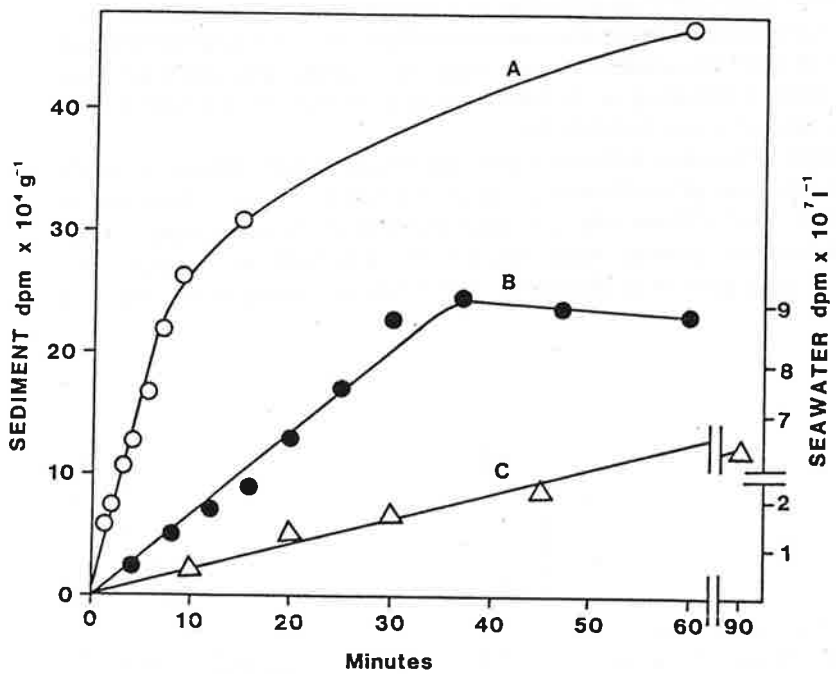

Figure 3. Time course of incorporation of [methyl-^{3}H]thymidine into DNA. (A) Sediment, temperature 31°C; (B) sediment, temperature 18°C; (C) seawater, temperature 18°C. [Redrawn from Moriarty and Pollard (1981, 1982).]

poration often followed the initial period. These kinetic studies indicate that the supply of labeled thymidine became limiting after a short period, due to adsorption of thymidine in the sediment (D. J. W. Moriarty and P. C. Pollard, unpublished results). Thus, in working with sediments, higher concentrations of thymidine and short-term analyses are needed (over a period of 5–20 min). Only results based on the initial linear period of incorporation can be used to calculate growth rates.

Linear rates of incorporation occurred for a longer time in seawater, probably because the concentration of thymidine was higher than in sediment interstitial water (Fig. 3). Effects due to labeling of other macromolecules may become apparent after a short time. In one experiment (Fig. 4) the rate of incorporation of label into extracted DNA differed from the rate of incorporation of label into TCA-insoluble compounds after about 30–40 min. In the first 30 min, the two rates agreed well, indicating that all label was being incorporated into DNA (i.e., bacterial DNA) initially. Hollibaugh *et al.* (1980) found that 82% of the tritium in macromolecules was in DNA in the first hour of incubation of a seawater sample. As Riemann (1984) has shown, the chemical methods for separating DNA from other macromolecules do not give clear-cut results. The relative differences between the amounts of label in DNA and protein and the rates of label incorporation into these macromolecules differ between water bodies, depending on factors such as temperature, microbial composition, and nutrient availability.

Correct ecological interpretations cannot be made without accurate values for the rate of incorporation of thymidine into DNA. If the rate of incorporation of tritium into any other macromolecules or organic matter is measured, growth rates cannot be calculated with confidence because, unless growth is balanced, only DNA synthesis is directly cor-

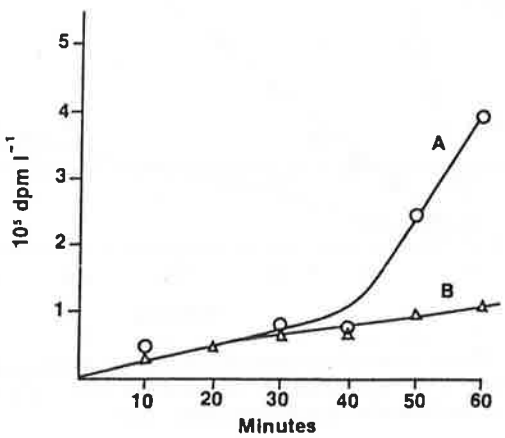

Figure 4. Time course for incorporation of tritiated thymidine into all macromolecules insoluble in (A) trichloroacetic acid and (B) purified DNA in coral reef waters. [From Moriarty *et al.* (1985b).]

related with cell growth (see Section 2). In the water column it is sufficient to use incorporation into TCA-insoluble matter over a short time interval, because mostly DNA is labeled initially (Moriarty *et al.*, 1985a; Pollard and Moriarty, 1984). A correction factor for tritium in other macromolecules may give incorrect results; this factor can change with time. The validity of the technique for each new environment is best established using a time course experiment.

3.2.6. Isotope Dilution

It is not possible to measure the specific activity of any nucleotide at the site of DNA replication simply by extracting the nucleotides and measuring it directly, because there are functionally separate pools of nucleotides in the multienzyme complexes (see Section 3.2.1).

The specific radioactivity of precursors at the site of macromolecule synthesis can be determined using an isotope dilution analysis (Forsdyke, 1968, 1971; Sjostrom and Forsdyke, 1974; Scott and Forsdyke, 1976, 1980). This procedure has been used to measure rates of DNA synthesis in marine environments (Moriarty and Pollard, 1981; 1982).

The principle of the technique is as follows: a series of samples are incubated with a constant amount of radioactive thymidine to which increasing amounts of unlabeled thymidine are added. The DNA is extracted and the reciprocals of the amounts of radioactivity in DNA are plotted against the amounts of thymidine present (Fig. 5). If there is no dilution of the isotope incorporated into DNA by any sources other than the unlabeled thymidine that was added, the plot will pass through zero (e.g., Fig. 5A). A negative intercept on the ordinate is an estimate of the amount of dilution of isotope by other sources of thymine in DNA (e.g., Fig. 5B). It is not strictly a pool of thymidine, but represents the sum of all pools that dilute the tritiated thymidine prior to incorporation into DNA.

The isotope dilution method measures the dilution of labeled thymine in dTTP, the final precursor to DNA, by all sources of thymine, because the effect of added thymidine on incorporation of radioactivity into DNA itself is measured. A necessary condition is that the rate-limiting step for incorporation of thymidine be the final one, i.e., DNA polymerase. Provided the concentration of thymidine is sufficiently high, this condition is met in bacteria with normal regulatory mechanisms (Pollard and Moriarty, 1984). Oligotrophic bacteria may not, however, regulate DNA synthesis in the normal way, and dilution could still occur (see this section below).

Isotope dilution experiments need not be carried out on every sample if a sufficiently high concentration of labeled thymidine is used (Pol-

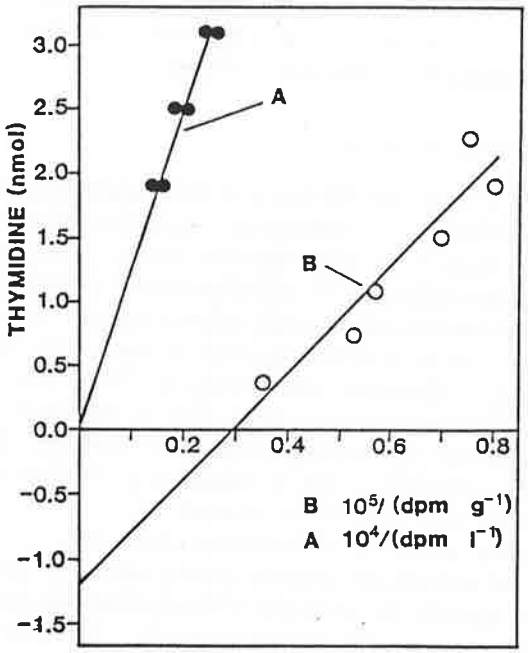

Figure 5. Isotope dilution plots of incorporation of tritiated thymidine into DNA in (A) seawater and (B) epiphytic flocculent sediment on seagrass. [From Moriarty and Pollard (1982).]

lard and Moriarty, 1984). Isotope dilution was more apparent in sediments, especially when growth rates were high, because thymidine was adsorbed to the sediment and thus the effective concentration was much less than that expected from the level of addition (Moriarty and Pollard, 1981, 1982). Large amounts of thymidine, compared to the amounts used in water column work, need to be added to sediment to block *de novo* synthesis and thus prevent isotope dilution. Alternatively, small amounts of sediment can be used.

An alternative procedure for checking whether isotope is being diluted as a result of *de novo* synthesis is to add increasing amounts of tritiated thymidine at the same specific activity. The concentration of thymidine at which no further radioactivity is incorporated should be used. *De novo* synthesis should be fully inhibited at this concentration; typical values for this are 5 nM in lake water (Bell *et al.*, 1983), and 5–10 nM in seawater (Fuhrman and Azam, 1982; Kirchman *et al.*, 1982). From personal observations and discussions with H. W. Ducklow and J. A. Fuhrman, I would recommend that a minimum of 20 nM thymidine be used. Where there are no large pools of thymidine (i.e., >1 nM), this experiment measures the effect of *de novo* synthesis. If, however, pools of thymidine are present, a double reciprocal plot would be needed to mea-

sure the pool size (Hunter and Francke, 1974), but this is not accurate, because a very large pool will have a value close to zero when plotted as a reciprocal. In my experience, pools of thymidine are rarely, if at all, large enough to have any effect and thus the alternative procedure does work.

Hagstrom (1984) has suggested that a better approach is to extract pure DNA from natural samples and measure the dilution of tritiated thymidine directly. It may be possible to purify DNA from water, but it is very difficult to do so from sediment, because humic compounds adsorb strongly to DNA (Torsvik, 1980). There is, however, a problem in deciding what proportion of purified DNA is from growing bacteria and what is from other organisms or inactive bacteria. Unless this proportion is known, a growth rate cannot be estimated.

The best method for measuring dilution of radioactive thymidine is being debated in the literature. This debate is unresolved because no method that has been proposed thus far is free of methodological problems when applied to field samples. It is better to attempt to minimize or measure dilution than to assume an arbitrary value that has been published for other environments or populations.

The process of tritiated thymidine incorporation into DNA is distinct from the process of uptake of an isotopically labeled substrate into cells. The velocity of uptake is dependent on substrate concentration, but, as stressed above, the synthesis of DNA is independent of uptake and biochemical conversions of precursors, i.e., the rate of DNA synthesis is independent of added precursor concentration. Bacteria take up thymidine very much faster than they incorporate it into DNA and thus the rate of incorporation of label into DNA is not influenced by radioactive thymidine concentration while zeroth-order kinetics applies. Laws (1983) has incorrectly criticized the work of Forsdyke and his colleagues (cited above) by concluding that the mathematics was faulty and thus that the isotope dilution analysis could not be used as proposed here. The mathematics is correct, but it is only a tool and cannot prove or disprove the hypothesis concerning measurement of isotope dilution. It is the biological premise on which the mathematics is based that must be disproved, and, as Laws stated, that rests on the assumption that the velocity of uptake is completely independent of the concentration of the radioactive precursor. In the case of DNA synthesis the premise is true for bacteria with normal regulatory mechanisms.

The isotope dilution methodology has been validated by experiments with the marine bacterium *Alteromonas undina* grown in a chemostat (Pollard and Moriarty, 1984). Growth rates measured directly compared well with rates calculated using the isotope dilution technique with tritiated thymidine (Table I). Two different levels of isotope dilution were

Table I. Comparison of Two Methods for Measuring Bacterial Growth Rates[a]

Percentage dilution	Specific growth rate (hr^{-1})	
	Direct microscopy	DNA synthesis
0	0.3 ± 0.04	0.28 ± 0.03
44	0.22 ± 0.04	0.29 ± 0.07

[a]From Pollard and Moriarty (1984). *Alteromonas undina* was grown in a chemostat and growth rates were measured by direct microscopy of acridine orange-stained preparations and by the rate of incorporation of tritiated thymidine into DNA, using the isotope dilution methodology.

used. Therefore, in those experiments the isotope dilution method did give an adequate estimate of the specific activity of dTTP, the final precursor of thymine in DNA. Such experiments, where growth rates measured using direct microscopy agree with those estimated from the rate of thymidine incorporation into DNA, show that uptake and thymidine kinase were not rate-limiting steps. The rate-limiting step for tritiated thymidine incorporation must have been at the level of DNA polymerase, otherwise the thymidine method would have underestimated growth rates.

Isotope dilution is not invariant in particular populations of bacteria, but is dependent on a number of factors, particularly the concentration of tritiated thymidine around the bacteria. To summarize the discussion above on the biochemistry of DNA synthesis, we see that bacteria regulate the concentration of dTTP. If thymidine is supplied at a sufficiently high concentration, it is used preferentially for DNA synthesis (provided it can be taken up), and *de novo* synthesis of thymidine nucleotides is inhibited. A note of caution is necessary here, however. Further work is needed to check the validity of the thymidine method in the field. For example, if a growing population is dominated by bacteria that cannot easily take up thymidine, transport may be the rate-limiting step, and thus isotope dilution would still occur, but not be measurable. Oligotrophic bacteria may not regulate DNA synthesis in the same way as copiotrophic bacteria, such as *Alteromonas undina,* and thus would not respond in the same way to added thymidine. Ideally, metabolism in oligotrophic bacteria is regulated by the concentration of substrates (Poindexter, 1981). Thus, studies of growth with thymidine (or adenine and other precursors of nucleic acids) in environments where oligotrophs might predominate, such as the open ocean, need to be interpreted with caution. The good correlations between values for bacterial production in the North Sea estimated with the thymidine method and two other methods indicate that isotope dilution did not occur (Lancelot and Billen,

1984). These and other studies (see Section 3.2.7.), which show a conversion factor of about 2×10^{18} cells dividing/mole thymidine incorporated, support the argument that isotope dilution is prevented by a sufficiently high concentration of thymidine.

Fuhrman and Azam (1982) measured DNA synthesis in bacteria from coastal waters and open ocean using the thymidine technique and a [^{32}P]phosphate technique. They concluded that the thymidine was being diluted, particularly in the open ocean bacteria. Although it is difficult to measure rates of DNA synthesis accurately with ^{32}P (Fuhrman and Azam, 1982; Moriarty, 1984) and thus decide how great the thymidine dilution is, the difference between coastal and open ocean bacteria is difficult to explain. Ducklow and Hill (1985) have reported that rates of thymidine incorporation could not be reconciled with changes in the numbers of bacteria growing in seawater cultures in the open ocean. It may be that oligotrophic bacteria are common in open ocean water and differ significantly in their biochemistry from "ordinary" bacteria.

Some workers have reported nonlinear isotope dilution plots and hence uncertainty in interpreting results (Riemann and Sondergaard, 1984; Riemann *et al.*, 1984). Such problems may be methodological, because there is continual improvement in the methods used for measuring thymidine incorporation into DNA (Pollard and Moriarty, 1984). It is also possible that there are populations of bacteria that have different biochemical regulatory mechanisms, as suggested above, and thus do not respond as predicted to changes in thymidine concentration. More studies are necessary on these problems in the use of tritiated thymidine.

3.2.7. Calculation of Growth Rates

In order to calculate growth rates of organisms from the rate of synthesis of a macromolecule, we need to know the amount of that macromolecule per cell; the amount should also remain constant per unit biomass, or nearly so. For this reason, DNA synthesis is much better than RNA synthesis as a measure of growth. As pointed out in Section 2, RNA content may be very variable and its rate of synthesis cannot be used as a measure of growth in natural systems. The genome size of most bacteria is within the range $(1–3.6) \times 10^9$ daltons (Gillis *et al.*, 1970; Wallace and Morowitz, 1973). The average genome size is 2.5×10^9 daltons, or 4×10^{-15} g DNA cell^{-1}. Fuhrman and Azam (1982) measured the DNA content of a mixed population of marine bacteria and obtained a value of 2.6×10^{-15} g cell^{-1}.

Thymine bases in DNA are assumed to be 25% of the total number of bases. For the average of a mixed population this is a reasonable value. Most GC ratios (guanine + cytosine) of marine bacteria lie in the range

35–70%. A factor of 1.3×10^{18} to convert moles of thymidine incorporated into number of DNA molecules synthesized (i.e., number of bacteria dividing) was derived by Moriarty and Pollard (1981, 1982). Fuhrman and Azam (1980) used a range of 2.0×10^{17} to 1.3×10^{18} and later increased this to an average of 1.7×10^{18} for nearshore waters and 2.4×10^{18} for offshore waters, which led to better agreement with other measurements (Fuhrman and Azam, 1982). Using the average amount of DNA cell^{-1} determined by Fuhrman and Azam (1982), I conclude that a conversion factor of 2×10^{18} is a good round figure in agreement with information currently available, at least for nonoligotrophic environments.

Some recent studies by Bell *et al.* (1983) show that this conversion factor is applicable to freshwater bacterial growth as well. They measured bacterial growth in lakewater cultures using direct microscopy and tritiated thymidine incorporation and calculated conversion factors that ranged from 1.9×10^{18} to 2.2×10^{18} cells dividing mole^{-1} thymidine incorporated. Further work is needed to improve the accuracy of conversion factors and to determine whether they differ significantly between environments. Ducklow and Hill (1985) have found that a conversion factor of 4.0×10^{18} best fitted data obtained from seawater cultures of natural populations in warm core Gulf Stream rings, which are oligotrophic.

The value of 2.0×10^{18} is based solely on the size of the DNA molecule and its thymine content. It is assumed either that the radioactivity measured in the experiments is in DNA only, and that there is no dilution of the radioactivity, or that adequate corrections have been made for these sources of error. Where very different conversion factors are found, it may indicate that one of the above assumptions is not valid. For example, isotope dilution could occur, but not be measurable as discussed in the previous section (3.2.6).

As Fuhrman and Azam (1982) point out, errors arise in the calculation of conversion factors because assumptions and extrapolations are needed. They attempted to calculate a factor directly by measuring the incorporation of thymidine into DNA and the increase in number of bacteria in two experiments with samples of water treated to remove predators on bacteria. In one experiment, water was passed through a 3-μm filter and allowed to incubate; in the other, water sterilized by filtration was inoculated with a natural population and incubated. Their results gave a conversion factor of 1.3×10^{18} cells produced/mole thymidine incorporated.

A somewhat different approach was used by Kirchman *et al.* (1982). They diluted seawater tenfold with filtered seawater and compared the rate of increase in cell numbers with incorporation of tritium from thy-

midine into TCA-insoluble matter. They found conversion factors ranging from 1.9×10^{18} to 6.8×10^{18} cells produced $mole^{-1}$ thymidine incorporated into TCA-insoluble matter. They developed a mathematical model to relate growth and cell division to the rate of incorporation of a radioactive precursor into a macromolecule. An assumption was made that the bacterial population growing in nature was the same as that growing in the seawater culture. Two conditions need to be met for the application of their method: (1) the time period for assay in culture and the environment should be short enough to ensure that DNA is the only macromolecule labeled; and (2) isotope dilution should be measured or, better still, the concentration of thymidine should be high enough to eliminate it. These conditions are necessary because growth rates and the rates of synthesis of different macromolecules may be different in the seawater culture than in the natural environment. An advantage of their procedure for estimating a conversion factor for a particular environment is that it takes into account bacteria that cannot utilize thymidine, as well as avoiding the need to make assumptions about the genome size and proportion of thymine in DNA.

In calculating conversion factors from data obtained with culture studies, further problems arise in determining the type of growth curve to be analyzed. Growth may be linear or exponential and, in diluted water from natural aquatic systems, it may be difficult to decide which form the growth curve takes and therefore the time period over which the conversion factor should be estimated. In addition, a lag phase may occur (Christian *et al.*, 1982).

3.3. Biochemistry of Adenine Incorporation into DNA and RNA

In contrast to pathways for thymidine involvement in DNA synthesis, those of adenine and its nucleotides are very complex. Adenine nucleotides have many functions in cellular metabolism, particularly in regulation of biosynthesis and in energy transfer and storage. For the sake of clarity only some of the pathways of nucleic acid synthesis are shown in Fig. 6. These may differ in detail for different microorganisms. Salvage pathways are very important in supplying nucleotides for nucleic acid synthesis. There is constant degradation of nucleic acids, mainly various forms of RNA and especially messenger RNA, which has a short half-life. Some of the salvage pathways involving adenine, and interconversions between nucleosides and nucleotides are shown in Fig. 6. The *de novo* route of synthesis is via inosine monophosphate to AMP for ribonucleotides. Deoxyribonucleotides are synthesized by reduction of the corresponding ribonucleotide diphosphate in most organisms. Some bacteria

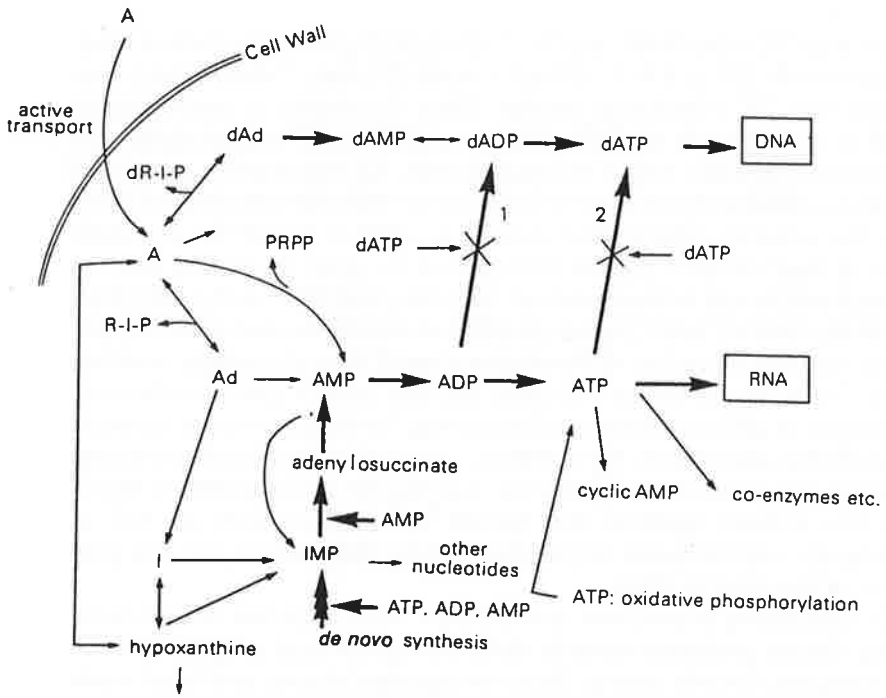

Figure 6. Pathways of adenine nucleotide metabolism. The principal pathways for *de novo* synthesis are shown by heavy arrows. Unlabeled arrows indicate other reactions not shown here. A, Adenine; Ad, adenosine; dAd, deoxyadenosine; I, inosine; IMP, inosine monophosphate; R-1-P, ribose-1-phosphate; dR-1-P, deoxyribose-1-phosphate; PRPP, phosphoribosylpyrophosphate. Enzymes: (1) ribonucleoside reductase, usual type; (2) ribonucleoside reductase, less common. Sites of feedback inhibition by dATP and other nucleotides are shown.

have an enzyme that reduces the ribonucleotide triphosphate (Thelander and Reichard, 1979).

Adenine is transported into the cell intact by active transport (i.e., it is energy dependent), which is facilitated by rapid conversion to adenosine or adenosine monophosphate (Roy-Burman and Visser, 1975; Burton, 1977). The label from the adenine would be distributed among many adenine compounds (Fig. 6). Tritiated adenine (labeled in the [2-H] position) would not give rise to labeled guanosine nucleotides, because the 2 position is substituted differently.

The complexity of the pathways for distribution of labeled adenine and compartmentalization of different processes, as well as individual differences between different microorganisms, cannot be adequately

depicted here (Fig. 6). For example, the synthesis of ATP is shown here mediated by the enzyme adenylate diphosphate kinase as a precursor to RNA or DNA synthesis. Elsewhere in the cell, however, there are other mechanisms for ATP synthesis, particularly substrate-level phosphorylation and oxidative phosphorylation in heterotrophs and photophosphorylation in algae. Labeled adenine would undoubtedly enter the pools of adenine nucleotides used in energy metabolism.

Because there are separate sites for DNA synthesis, RNA synthesis, and various energy supply processes and the reaction kinetics vary, particularly where multienzyme complexes are involved, rapid equilibration of labeled adenine in various pools would not be expected to occur. Indeed, Karl *et al.* (1981) show that the specific activity of ATP pools in a complex freshwater community and in a pure culture of a marine bacterium do not equilibrate for up to 4 hr. They found that pools of AMP and ATP were separated in different compartments. Winn and Karl (1984a) have discussed the problems in determining rates of nucleic acid synthesis with tritiated adenine when the specific activity of ATP changes during the incubation. They showed that the specific activity did stabilize if adenine was always present to excess in the medium and a sufficiently long incubation time (10% of the generation time) was used. If, therefore, tritiated adenine is used as a short-term pulse label, results will be difficult to interpret. There is, however, a possibility that the confinement of microorganisms in a bottle for a long time may alter growth rates, and this effect may be difficult to recognize.

The specific activity of the ATP pool that is used for RNA synthesis cannot be measured directly by extracting ATP from cells, because the different pools of ATP cannot be extracted separately (Fuhrman and Azam, 1980). Karl (1981) has attempted to circumvent the problems of actually measuring the specific activity of ATP in natural environments by relating the ratio, (rate of RNA synthesis/rate of DNA synthesis), to specific growth rate. The disadvantage of this technique is that the incorporation of adenine into nucleic acids is not specific to bacteria and if growth is not balanced, the rate of RNA synthesis may not be proportional to DNA synthesis or growth, particularly in mixed communities of microorganisms.

In separate, but not mixed, cultures of rapidly growing bacteria and algae, the adenine procedure did give good estimates of the true growth rates (Winn and Karl, 1984a). In order to explain their results, the authors had to demonstrate that bacterial activity was minimal in the algal culture. More work is needed to show not only whether the rates of nucleic acid synthesis measured in the field can be reliably related to growth, but also the microorganisms to which the rates apply. At this stage, it is my conclusion that the difficulties with interpretation are too

great for adenine to be a useful precursor for measuring microbial or bacterial growth rates in natural environments.

There are a number of reasons why adenine cannot be used to measure accurately growth rates of microbes in the natural environment; some of these have been mentioned above and are summarized here. Problems in the measurement of specific activity of ATP, the final precursor to RNA synthesis, and dATP, the precursor to DNA synthesis, by extraction of ATP are further compounded in the natural environment. Even if there were no compartments within a cell, in a community of organisms there would be many that were not growing or that had very different pool sizes of ATP and differing growth rates.

Bacteria, with their efficient transport mechanisms, are likely to take up adenine more rapidly than are algae or protozoa, and as their ATP pools are smaller, the specific radioactivity of ATP would be higher. The rates of turnover and synthesis of new nucleic acids would differ between eucaryotes and procaryotes, which would complicate interpretation of results. Techniques are needed that allow measurement of processes within smaller ecosystem compartments and thus let us refine models for ecosystem function. The microbial world includes primary producers using light or chemical energy, herbivores, decomposers, and carnivores; in other words, many different trophic levels are included. The adenine method lumps all these together.

There may be particular environments where one group of microorganisms (e.g., bacteria) predominates, and thus results of the adenine method could be meaningfully interpreted. If, however, bacteria predominate, the thymidine method is preferable. A direct comparison between the two methods in such environments would be interesting.

Adenine, or precursors other than thymidine, may be useful in restricted environments where information is needed on RNA synthesis, or perhaps DNA synthesis in eucaryotes (e.g., fungi). The isotope dilution procedure of Forsdyke (see Section 3.2.6) would be preferable for determining specific radioactivity. Selective inhibitors could also be useful.

4. Ecological Significance of Bacterial Growth Rate Measurements

4.1. Introduction

Many research workers are now using the tritiated thymidine method to determine bacterial growth rates in aquatic systems. Most workers agree that the thymidine method gives a good measure of bacterial growth, although there is some discussion about how close the mea-

sured values are to the real values. Continual improvements in methodology and interpretation in ecological terms are occurring. It should be noted, however, that conversion factors are not necessarily universal, and may vary between different aquatic systems or microbial communities. Independent, corroborative data on bacterial growth in nature are necessary, but are difficult to obtain (Ducklow and Hill, 1985). In their original paper, Fuhrman and Azam (1980) commented on the need for caution in calculating growth rates from the thymidine data; this comment still applies. Nevertheless, the thymidine method is very promising and deserves further development. It has not only provided information that supports estimates of bacterial growth rates from other methods, but has also enabled microbial ecologists to study bacterial growth in environments (e.g., sediments) where other techniques do not work. Some of the applications and results obtained from the use of the tritiated thymidine and adenine methods are discussed below.

4.2. Comparison of Thymidine and Other Methods

Values for bacterial growth rates in natural systems determined with the thymidine method have been compared directly with those made using other techniques. Fuhrman and Azam (1980, 1982) have compared growth rates measured with tritiated thymidine and direct counts of bacteria in enclosed water samples and found good agreement. Newell and Fallon (1982) also tried to compare the two techniques, but did not obtain consistent growth with enclosed samples. In order to measure growth rates with direct counts in culture, predators of bacteria have to be removed (usually by filtration with 3-μm filters). Algae will also be removed, and because there is a coupling between bacterial growth and supply of nutrients from algae, filtration is likely to disrupt bacterial growth rates. Furthermore, many apparently free bacteria may be utilizing nutrients on surfaces, and may be separated from surfaces by shear forces during filtration (Hermansson and Marshall, 1985). This problem illustrates one of the advantages of using tritiated thymidine. Experiments may be carried out in a time short enough to avoid the effects of predators or change in supply of substrates. As pointed out in Section 2, DNA synthesis is unlikely to be immediately affected by a change in nutrient status, but should continue without change until replication is completed. Toxic effects (e.g., contaminants in sample bottles or O_2 in a very anaerobic environment) are likely to have an immediate effect, however. Oligotrophic bacteria may respond immediately to changes in substrate concentration. Thus, changes in growth rates may be induced by the enclosure of water in bottles and such effects need to be considered.

Ducklow and Hill (1985) showed that short-term (15–45 min) assays of thymidine incorporation agreed well with measured increases in cell numbers. They concluded that the thymidine method was a valid and useful technique for determining rates of bacterial growth in the sea. They also pointed out that there were some discrepancies between changes in direct counts and thymidine incorporation rates in oligotrophic waters. Reasonable agreement between the thymidine method and direct microscopy has also been reported by Bell *et al.* (1983) and Bell and Kuparinen (1984) and for some experiments by Riemann *et al.* (1984). In other experiments, the thymidine technique underestimated the growth rates (Riemann *et al.*, 1984).

A number of comparisons have been made between the thymidine and frequency-of-dividing-cells methods (Hagstrom, 1984; Newell and Fallon, 1982; Riemann *et al.*, 1984; Riemann and Sondergaard, 1984). Good correlations between values for growth rates in the water column were found in most instances, indicating that both methods were measuring growth. Discrepancies were found in many cases, however, in the absolute values of growth rate. Newell and Fallon (1982) found that values were two to seven times lower with the thymidine method. Values calculated from the frequency-of-dividing-cells method were unrealistically high (5–50 g C m^{-2} d^{-1}) compared to measured values for oxygen utilization. It is very difficult in sediments to distinguish dividing cells from cells that have divided but remain attached to each other in a filament. Riemann and Sondergaard (1984) also reported that the thymidine method gave lower values for growth rate than the frequency-of-dividing-cells method. Reasonably close correspondence has been reported by Hagstrom (1984) and Riemann *et al.* (1984).

Very good agreement has been found between the thymidine method and two other methods for estimating heterotrophic bacterial production or utilization of carbon (Lancelot and Billen, 1984). The other two methods involved determination of the uptake of dissolved sugars, amino acids, and carboxylic acids by bacteria and an estimate from exoproteolytic enzyme activities. The thymidine method measures cell division rates with reasonable accuracy, but not production, because it is difficult to measure bacterial cell sizes accurately to determine their carbon content and thus estimate production. Furthermore, not all bacteria may be growing, so an average cell size may over- or underestimate production. The work of Lancelot and Billen (1984) is valuable, therefore, in helping decide whether production estimates from thymidine incorporation rates are accurate. Information is also needed on conversion efficiencies before a complete assessment can be made. More studies on conversion efficiencies in natural systems are necessary.

4.3. Water Column

The growth rates of bacteria that have been measured in various seas are generally all within two orders of magnitude (Table II). These growth rates agree quite well with rates measured by other techniques, some examples of which are given in Table II. Doubling times of 7–37 days have been reported for a freshwater lake (Riemann *et al.* (1982). In a eutrophic lake in Sweden, bacterial doubling times ranged from 0.2 to 2.9 days in summer (Bell *et al.*, 1983).

The growth rates that have been measured are composite ones for the whole community. The thymidine method does not distinguish between different populations with different growth rates. They ought to be a true average because the relationship between thymidine incorporation and growth is linear. Although the proportion of active bacteria in a community may be assessed by autoradiography, it is difficult to determine the proportion that are actually growing. Kirchman *et al.* (1982) have suggested a way to determine the proportion of growing bacteria. By analyzing mathematically the relationship between the change in bacterial abundance in culture with time, they estimated that at least 50% of bacteria present were dividing.

Several factors contribute to the variability in growth rates shown in Table II. The most important ones are temperature and nutrient supply to bacteria. Growth rates are faster in warm water than in cold water, and so seasonal temperature differences will be directly correlated with growth rates. Heterotrophic bacteria require a supply of organic matter, which in the open ocean comes originally from phytoplankton. Thus, high bacterial growth rates are found in seasons and regions where phytoplankton are present. Fuhrman *et al.* (1980) found that bacterial growth rates off the coast of California were correlated more with abundance of phytoplankton than with primary production. They suggested that in this case, bacterial growth probably depended on organic matter released from algal cells as a result of zooplankton feeding. Evidence to show that bacterial growth rates were faster in the presence of zooplankton has been reported (Eppley *et al.*, 1981). They suggested that this was due to the release of organic matter from the phytoplankton by the zooplankton. Bacterial growth in the sea is also dependent to some extent on excretion of organic matter from phytoplankton during photosynthesis (Smith *et al.*, 1977; Williams and Yentsch, 1976). Using the tritiated thymidine method, Bell *et al.* (1983) showed that algal exudates supported between 10 and 80% of bacterial growth in a freshwater lake. During a spring bloom in a Swedish freshwater lake, bacterial growth (including respiration) accounted for about 20% of gross primary production. The bacterial growth was supported by algal excretory products (Bell and Kuparinen,

1984). Ducklow and Kirchman (1983) found that there was a coupling between bacteria and phytoplankton density in shelf waters in the New York Bight.

A very tight coupling occurred between bacterial growth and the spring phytoplankton bloom in the North Sea (Lancelot and Billen, 1984). Bacteria utilized 44–68% of total primary production, up to 100% of dissolved organic matter production, and some particulate organic production. Rapid recycling of nitrogen via bacterial activity was necessary to support the primary production.

Not all the variation in bacterial growth rates is due to seasonal changes in temperature or phytoplankton density. Diel variation may be quite marked in some environments. Doubling times varied from 1.4 days at noon to 12 days at night in water over a seagrass bed (Table III) (Moriarty and Pollard, 1982). A similar variation of one order of magnitude was found in the water column near coral reefs (Table III). Diel variation of bacterial growth in planktonic communities may be influenced by excretion of organic matter from algae during photosynthesis as well as by the release of organic matter during feeding of zooplankton. Thus, various factors may influence bacterial growth rates in planktonic communities, and if these do not act in concert, simple diel cycles linked to photosynthetic production may not occur. Small diel cycles in bacterial growth rates in a freshwater lake were found with the use of tritiated thymidine (Riemann *et al.*, 1982). The thymidine method has been used to show diel variations in growth rates in coastal seawater and freshwaters by Hagstrom (1984), Riemann and Sondergaard (1984), and Riemann *et al.* (1984).

More detailed studies on small-scale temporal and spatial variation in bacterial growth rates are needed because bacteria respond quickly to changes in nutrient concentration. The tritiated thymidine technique is ideally suited for conducting such studies because incubation times need only be short and small samples are sufficient.

Values for bacterial productivity ranging from 5 to 45% of primary productivity have been obtained (Table II). If the efficiency of utilization of organic matter is taken to be 50% (Payne, 1970), these values show that from 10 to 90% of primary production is needed to support the bacterial production. Ducklow and Kirchman (1983) suggested that their high value of 35% for bacterial production as a proportion of primary production was due to utilization of allochthonous organic matter in a river plume. The proportion of primary production that is utilized by bacteria in the water column probably depends on the growth state of the phytoplankton. In the early stages of a bloom, excreted dissolved organic matter is likely to be the main source of carbon for bacteria, and this is not a high proportion of primary production. In a senescing bloom, where a

Table II. Growth Rates of Bacteria in Marine Water Columns[a]

Location	Season	Production (ng C/liter per hr)	Specific growth rate (day^{-1})	Bacterial production as percent primary production	Reference
Thymidine technique					
North Sea	Spring	80–240	—	5–20	Lancelot and Billen (1984)
Antarctic	Summer	0.2–121	0.007–0.46	10–12	Fuhrman and Azam (1980)
New York Bight	Spring (3°C)	294–556	0.09	23–35	Ducklow *et al.* (1982)
California coast	Spring	196–2210	0.5–2.3	—	Fuhrman and Azam (1980)
California coast	Spring	80–800	0.2–1.0	5–25	Fuhrman and Azam (1982)
Chesapeake Bay	Spring	292–3130	1.1–6.9	—	Ducklow (1982)
Georgia coast	Summer	208–2080	0.07–0.3	—	Newell and Fallon (1982)
Moreton Bay, East Australia	Autumn, day	300	1.3	5–10	Moriarty and Pollard (1982)
Moreton Bay, East Australia	Autumn, night	90	0.3	—	Moriarty and Pollard (1982)
Great Barrier Reef	Summer	375–1830	0.5–6.9	—	Moriarty *et al.* (1985b)
Great Barrier Reef	Winter	10–151	0.01–0.3	—	Moriarty *et al.* (1985b)
Hamelin Pool, West Australia	Spring	130–320	0.02–0.1	—	Moriarty (1983)
Frequency of dividing cells					
Antarctic	Summer	0.02–1.4	0.8–2.3	15–45	Hanson *et al.* (1983)
Baltic Sea	Spring, Summer, Autumn	175	0.2–1.7	25	Hagstrom *et al.* (1979)
Georgia coast	Summer	1700–5600	0.5–0.8	—	Newell and Fallon (1982)
Growth in enclosed chambers					
California coast	Spring	416–1416	0.4–1.4	—	Fuhrman and Azam (1980)
California coast	Spring	—	0.7–3.5	—	Carlucci and Shimp (1974)
Growth in diffusion cultures					
North Atlantic	—	—	2.3–3.5	—	Sieburth *et al.* (1977)
Baltic	Summer	416–2375	0.2–1.4	29	Meyer-Reil (1977)
North Atlantic	—	25–1817	—	—	Delattre *et al.* (1979)
Growth in continuous culture (single species)					
North Atlantic	—	—	0.1–0.3	—	Jannasch (1969)

[a]Where conversions were needed the following factors were used: biomass, 20 fg C/cell nearshore and 10 fg C/cell offshore; growth rate, 2×10^{-18} cells/mole thymidine incorporated.

Table III. Daily Fluctuations in Bacterial Growth Rates in Seawater Over a Seagrass Bed in March and Over Three Zones of a Coral Reef in July[a]

Time of day	Seagrass bed		Coral reef[b]					
	Specific growth rate (day^{-1})	Number of bacteria (10^9 $liter^{-1}$)	Specific growth rate (day^{-1})			Number of bacteria (10^9 $liter^{-1}$)		
			Outside	Crest	Flat	Outside	Crest	Flat
0600	0.07	6.8	—	—	—	—	—	—
0900	0.14	4.2	0.6	0.2	0.5	6.0	5.0	3.8
1100–1200	0.35	2.8	0.5	0.2	0.4	7.1	7.7	3.7
1400	0.06	3.6	—	—	—	—	—	—
1600	—	—	0.1	0.4	2.8	5.6	6.4	2.9
1900	0.03	4.5	—	—	—	—	—	—

[a]Data from Moriarty and Pollard (1982) and Moriarty *et al.* (1985b).
[b]Zones studied are 500 m outside the reef, the reef crest, and the reef flat.

large amount of organic matter is available to bacteria as a result of cell death, lysis, and feeding by zooplankton, bacterial production may be high in proportion to primary production. Lancelot and Billen (1984) found that bacteria utilized 44–68% of primary production during a spring bloom in the North Sea. They estimated from comparisons of thymidine incorporation rates and uptake of dissolved organic carbon that the bacterial growth efficiency was 10–30%.

There is enough comparable information from different sources and different methodologies to show that bacterial growth in seawater generally utilizes at least 25% of primary production. Growth rates are faster inshore than offshore (Fuhrman *et al.*, 1980; Newell and Fallon, 1982). The reasons for these differences are complex and require further study. Runoff from land supplies nutrients directly for bacterial growth and for phytoplankton growth. More particulate matter from rivers and disturbance of sediments in shallow coastal regions would provide greater surface area for concentrating nutrients and thus providing a site for bacterial growth (Kjelleberg *et al.*, 1982).

Particulate matter in the water column may be an important site for bacterial growth, not only as a surface for concentrating nutrients, but also as a source of organic nutrients. The thymidine method was used by Ducklow *et al.* (1982) to show that the sedimentation of particles with attached bacteria in the Hudson River plume removed from 3 to 67% of daily bacterial production. Bacterial growth on particles that are probably derived from mucus accounted for at least 50% of bacterial production over coral reefs. About 50% of tritiated thymidine incorporation occurred

on particles in water from a coral reef lagoon and over a seagrass bed on a reef flat. About 30% of tritiated thymidine incorporation occurred on particles in water outside the reef area (Moriarty *et al.*, 1985b). In a freshwater pond, only a small proportion (2.8%) of thymidine incorporation into DNA occurred on particles. Thus, the estimated production of particle-bound bacteria and the mineralization of organic matter by attached bacteria was low compared to the activities of free bacteria (Kirchman, 1983). The thymidine method has proved to be useful for studying the effects of particles on bacterial growth rates because it can be used over a short enough time interval to minimize changes due to sampling.

If the sampling procedure includes filtration, it is likely that bacterial activity at particle surfaces will be underestimated because shear forces will easily remove reversibly attached bacteria (Hermansson and Marshall, 1985). By labeling bacteria with tritiated thymidine and substrate (stearic acid) with ^{14}C, Hermansson and Marshall (1985) showed that many apparently free bacteria were able to utilize substrates bound to particle surfaces.

The adenine method has been used to measure microbial growth rates in the tropical north Pacific Ocean by Winn and Karl (1984b). They estimated production in the photic zone (0–150 m) to be 400 mg C m^{-2} day^{-1}, which was four times higher than previous estimates of primary production in that area (Bienfang and Gundersen, 1977). Because phytoplankton and bacterial production cannot be discriminated with the adenine technique, such values cannot be compared with those from the thymidine technique. In deeper waters (150–900 m), however, bacteria were probably the main microbial producers and in this depth zone, Winn and Karl (1984b) estimated production to be 790 mg C m^{-2} day^{-1}. If bacteria are 30% efficient in utilizing carbon in the sea (Lancelot and Billen, 1984), the bacteria in the deep zone would require an input of 2.6 g C m^{-2} day^{-1}, which is far greater than any measured or estimated values [e.g., 3–24 mg C m^{-2} hr^{-1} (Beinfang and Gundersen, 1977)], particularly when much of the primary production is probably utilized in the upper 150 m. These results support the conclusion (Section 3.3) that tritiated adenine is not a useful precursor to use for determining microbial or bacterial growth rates in natural environments.

4.4. Sediments

The measurement of bacterial biomass and growth rates in sediments is more difficult than in water. Perhaps for this reason few quantitative studies have been carried out, and yet in shallow environments benthic productivity is important in food chains and in nutrient cycles. Macrophytes are significant primary producers in many coastal waters,

and much of their production has to be cycled through bacteria before it becomes available to animals.

Thymidine is strongly adsorbed to particulate matter, so concentrations of added thymidine in sediments need to be 100 to 1000 times greater than those used for experimental work in the water column. Likewise, if there is a large amount of particulate matter in the water column, higher concentrations of thymidine will be needed. If thymidine concentrations are not high enough, isotope dilution will be substantial, making results difficult to interpret correctly (Pollard and Moriarty, 1984).

Bacterial production measured with the thymidine method compares well with estimates from an independent technique using [^{32}P]phosphate incorporation into phospholipid (Table IV) (Moriarty *et al.*, 1985d). Close agreement was not expected because phosphate is used by all microbes, although phospholipids were mainly labeled in bacteria (Moriarty *et al.*, 1985d). Furthermore, phosphate does not disperse as readily in sediment as does thymidine. Support for the use of phospholipid synthesis as an alternative measure of growth is shown by the close agreement with the thymidine method where aerobic bacterial growth on seagrass leaf detritus suspended in the water column was measured (Table V). Because the results of the two methods agreed well for sediment also, it may be concluded that the thymidine method measures the growth of most bacteria in the sediment, including anaerobic bacteria. These experiments were carried out with care to ensure as little disturbance as possible. Rates of DNA synthesis did not respond immediately to disturbance of sediment, which makes the technique a useful one for sediments. Rates of phospholipid synthesis, however, increased rapidly

Table IV. Comparison of Measurements of Bacterial Production in Sediments and Water Made Using the Thymidine Technique and Rates of [^{32}P]Phosphate Incorporation into Phospholipid[a]

Type of sample	Depth (cm)	Thymidine[b] (mg C m^{-2} hr^{-1})	[^{32}P]lipid[b] (mg C m^{-2} hr^{-1})
Sand bank	0–1	9.0± 1.4	12.3± 1.1
Halodule sediment	0–2	7.6± 0.7	8.4± 2.3
Halodule sediment	2–4		1.2± 0.1
Halodule sediment	8–10	0.5± 0.14	0.3± 0.03
Water column, seagrass leaf detritus	—	6.0± 1.2[c]	4.7 ± 0.3[c]

[a]Moriarty, *et al.* (1985d, and unpublished work).
[b]Standard errors are shown for 6–12 measurements each.
[c]Value is g C/liter per hr.

in a well-mixed slurry (Moriarty *et al.*, 1985d). Further work is needed to determine the responses of DNA synthesis in sediments to handling.

Most bacterial production occurs at or near the surface of sediments, as seen in the example in Table IV. Bacterial production was most rapid in the upper 2 cm of sediment in a seagrass bed and was more than an order of magnitude lower at 8–10 cm.

The productivity of bacteria in sediment from a number of environments is shown in Table V. Results have been normalized to 10 mm depth for comparison, which may have over- or underestimated some values. Seagrasses are very productive, and in turn they support an active bacterial community.

High rates of bacterial productivity have been found in sediments of seagrass *(Zostera capricorni)* beds, with a marked diel variation (Fig. 7). Growth rates at noon were about an order of magnitude faster than at night (Moriarty and Pollard, 1982). Growth rates were low, and showed no diel variation in sediments without seagrass. It was estimated that bacterial growth in the water and sediments utilized between about 10 and 20% of net primary production of the seagrass system.

Thymidine can be used in anoxic sediments to estimate the growth rates of many anaerobic bacteria. This has been demonstrated in an experiment using an enrichment culture of a mixed community of bacteria from a seagrass bed sediment growing on glucose and yeast extract.

Table V. Bacterial Productivity in the Top 10 mm of Sediment from Different Environments

Site	Production (mg C m^{-2} day^{-1})	Actual depth[a] (cm)	Reference
Halodule bed, Gulf of Mexico, U.S.	85	2	D. J. W. Moriarty (unpublished results)
Spartina creek bank, Georgia	10.5	20	Fallon *et al.* (1983)
Nearshore, Georgia	40.5	25	Fallon *et al.* (1983)
Six km offshore, Georgia	16.5	25	Fallon *et al.* (1983)
Zostera bed, Moreton Bay, Australia	60	0.5	Moriarty *et al.* (1985a)
Aquaculture ponds, Malaysia	150–500	1	Moriarty (1986)
Mangrove creek bank, Malaysia	230	1	D. J. W. Moriarty (unpublished results)

[a]Because different workers studied different depths of sediments, results were normalized to the top 10 mm for comparison; actual values can be obtained using these depth values.

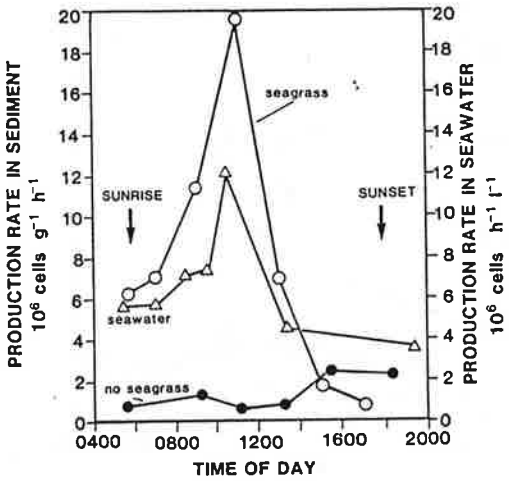

Figure 7. Diurnal variation in bacterial cell production rates in sediments and water column associated with seagrass beds. [From Moriarty and Pollard (1982).]

The growth rate of the culture measured by direct microscopy agreed well with that estimated using the thymidine method (Pollard and Moriarty, 1984). Fermentative bacteria, an important group in sediments, were probably predominant in the culture, and thus it is likely that the thymidine method measures their growth. Experiments with pure cultures are needed to check this. Not all anaerobic bacteria can take up thymidine. Tritiated thymidine does not label DNA in *Desulfovibrio* (G. Skyring, personal communication) or acetate-utilizing, sulfate-reducing bacteria (D. J. W. Moriarty, unpublished results). Because these bacteria can use only a very limited range of substrates, they may lack pyrimidine transport enzymes. The thymidine method thus gives minimum values for growth rates in anoxic sediments.

Bacterial productivity in coral reef sediments is high and shows seasonal and diel variations (Moriarty *et al.*, 1985c). Holothurians feed on bacteria in reef sediments (Moriarty, 1982), and it was estimated that they ate 10–40% of daily bacterial production in summer (Moriarty *et al.*, 1985c). Heavy grazing by them depresses bacterial production (Fig. 8). Benthic microalgae probably contribute some nutrients to bacteria, but mucus and slime settling from the water column are also likely to be significant sources of nutrients for bacteria (Ducklow and Mitchell, 1979). Bacterial productivity is too high in proportion to primary production in reef sediments to be linked closely to the growth of microalgae. Other sources of organic matter, such as mucus from animals (especially corals) are needed to support some of the high rates of bacterial production (Table VI).

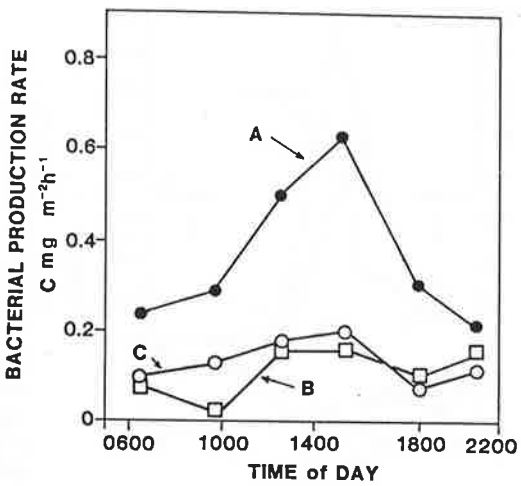

Figure 8. Bacterial cell production rates in coral reef sand, measured using the rate of tritiated thymidine incorporation into DNA. (A) Sediment from which holothurians were excluded. (B) Sediment on which holothurians were confined by a cage. (C) control area, no cage. [From Moriarty *et al.* (1985c).]

Table VI. Comparison of Bacterial and Primary Productivities in the Top 10 mm of Coral Reef Sediments[a]

	Primary productivity (mg C /m² per day)	Bacterial productivity (mg C /m² per day)	Respiration rate (mg C/ m² per day)
Normal area	350	190	630
Cage without holothurians	670	370	870
Cage with holothurians	230	190	600

[a]From Moriarty et al. (1985c). Bacterial productivity was measured using tritiated thymidine and microalgal productivity with $^{14}CO_2$ incorporation or O_2 changes in chambers.

Microbial production in coral reef sediments in Kaneohe Bay, Hawaii has been estimated with the adenine method to be 1.1–5.8 g C m^{-2} hr^{-1} (Burns *et al.*, 1984). This production would require an input of about 30–250 g C m^{-2} day^{-1}, which seems very unlikely. Sediment to a depth of 5 cm was collected and homogenized, so it is likely that only bacterial DNA synthesis was measured with the adenine. By comparison with bacterial DNA synthesis measured with thymidine at Lizard Island (see this section above), these rates are about two orders of magnitude too high. These rates are also one to two orders of magnitude higher than those measured on the Kaneohe Bay reef by Sorokin (1978), who used a dark ^{14}C-fixation method. Thus, these results of Burns *et al.* (1984) also indicate that adenine is not useful as a measure of microbial or bacterial growth rates.

4.5. Growth State

The growth state of bacteria is relevant to the methodology of growth rate measurement. For balanced growth (see Section 2), a constant supply of nutrients is needed. Bacteria in seawater and sediments are generally not in an environment where there is a copious and continual supply of nutrients. Many bacteria exist as small, starved cells and are adapted to respond quickly to change in the environment (Kjelleberg *et al.*, 1982). If they are starved, nutrients may be used for maintenance rather than growth and division. This conclusion is supported by studies on bacterial growth rates in the Antarctic. More tritium from thymidine was incorporated into protein than into DNA in bacteria in deep Antarctic water compared to bacteria in surface waters, where a lower proportion of tritium was used for protein synthesis (Hanson and Lowery, 1983).

The diel variation that occurs in growth rates shows that bacteria do respond rapidly to changes in nutrient concentration. It is unlikely, therefore, that they will be in a state of balanced growth as would occur for bacteria in culture with a constant supply of nutrients. Hanson and Lowery (1983) attempted to determine whether bacteria in Antarctic waters were in a state of balanced growth by comparing rates of [^{3}H]adenine incorporation into RNA and DNA and [^{3}H]thymidine incorporation into DNA. Rate ratios of RNA/DNA synthesis are difficult to interpret when two different precursors are used, because adenine may be utilized by most microbes [i.e., algae, protozoa, and bacteria (Karl *et al.* 1981)], whereas thymidine is incorporated into DNA of bacteria only. It does seem likely, however, that oceanic microplankton are in various growth states. This conclusion is supported by the wide variation in doubling times for bacteria in both water and sediment from one environment (see Tables III and VII) (Moriarty and Pollard, 1982). Short doubling times (0.1–0.3 day) indicate that probably most bacteria in a community are growing, whereas long times suggest that many may be dormant.

4.6. Food Chain Dynamics

Tritiated thymidine has been used to label bacteria and study their utilization by animals (Hollibaugh *et al.*, 1980). The advantage of this technique is that a short-term pulse label can be employed during which bacterial DNA is labeled specifically (see Section 3.2.3). Furthermore, natural assemblages of bacteria may be labeled, thus avoiding the need to work with cultures of possibly unnatural species. Hollibaugh *et al.* (1980) used this technique to study grazing rates of microzooplankton on bacteria. Bacteria have been shown to constitute only a low proportion

of the carbon requirements of an isopod that fed on leaf detritus (Findlay *et al.*, 1984).

The generally large proportion of primary production that is utilized by bacteria implies that bacteria should have an important role in aquatic food chains. The biomass or number of bacteria in sediments without seagrass is not much lower than those with seagrass, yet the growth rates of bacteria around seagrass may be considerably higher (Moriarty, 1980; Moriarty and Pollard, 1982). This suggests that grazers on bacteria are more active in the seagrass zone than in bare sediments, and that the bacteria are being grazed at a rate approximately equal to the growth rate. Bacterial numbers or biomass in sediments from a wide diversity of environments are similar (e.g., Dale, 1974; Moriarty, 1980; 1982; Newell and Fallon, 1982). A possible explanation for this is that these values for bacterial biomass are near the minimum for effective grazing by bacteriovores. The expenditure of energy in searching for food may be greater than that gained at this level of bacterial biomass. A correlation was observed between bacterial biomass and doubling time and meiofauna numbers in an aquaculture pond (Moriarty, 1986). Three pens within a pond were fertilized daily with chicken manure for periods of 1, 2, and 3 weeks, respectively. After 1 week, bacterial biomass was high, but after 2 weeks, bacterial biomass fell twofold and after 3 weeks, it had fallen fivefold (Table VII). Conversely, bacterial doubling times increased fourfold over the 3-week period. These changes were correlated with increased numbers of meiofauna. Although meiofauna clearly have a role both in controlling bacterial numbers and, in this case, stimulating growth rates, protozoa are probably also important in sediments.

A similar situation occurs in the water column where microflagellates limit bacterial populations (Fenchel, 1982). Detailed studies of the interactions between bacterial production and grazers can now be studied

Table VII. Effect of Meiofauna on Bacterial Biomass and Growth Rates in the Upper 1 cm of Sediment in a Tropical Aquaculture Pond[a]

Pond treatment	Bacteria		Meiofauna number (per 10 cm^2)
	Biomass (g C m^{-2})	Specific growth rate (day^{-1})	
Untreated	3.4	6	140
Manure, 1 week	4.3	8	300
Manure, 2 weeks	1.9	6	1500
Manure, 3 weeks	0.8	2	1500

[a]From Moriarty (1986). Bacterial biomass was measured with muramic acid and growth rates with the tritiated thymidine technique.

because the thymidine technique permits short-term measurements of growth rates under almost natural conditions with algae and bacteriovores present.

The results of Ducklow and Kirchman (1983) for bacterial growth rates in the Hudson River plume suggest that bacterial grazers play a significant role in limiting bacterial populations. During a spring bloom in a Swedish lake, the actual numbers of bacteria that accumulated were about 50% less than the numbers predicted by the thymidine method. A likely explanation for this effect was considered to be grazing by protozoans (Bell and Kuparinen, 1984). Hagstrom (1984) reached a similar conclusion when he compared rates of bacterial growth, measured by both the frequency-of-dividing-cells and thymidine methods, with the actual increase in bacterial numbers in coastal seawater samples. Now that we can measure bacterial growth rates in natural systems, another major problem that can be investigated is the quantitative contribution of protozoa and other larger animals that feed on bacteria to the food chain and nutrient cycles. Ducklow (1983) and Williams (1984) have both reviewed this problem, and suggest that bacteria contribute significantly to the nutrition of protozoa and microzooplankton. Whether bacteria are an important food source for higher organisms in the water column is still open to question, but seems doubtful. In the sediments, however, bacteria are an important food source for large animals (e.g., Moriarty, 1982; Moriarty *et al.*, 1985c).

ACKNOWLEDGMENTS. I am grateful to many colleagues for helpful discussions. I wish to thank Hugh W. Ducklow and Peter C. Pollard for comments on an early draft of the manuscript, and especially Peter J. leB. Williams for much help and detailed criticism.

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D. Bacterial Productivity and Carbon Cycling in Seagrass Beds

Marine Biology 72, 165–173 (1982)

CSIRO Marine Laboratories Reprint No. 1316

Marine Biology

Diel Variation of Bacterial Productivity in Seagrass (*Zostera capricorni*) Beds Measured by Rate of Thymidine Incorporation into DNA

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Abstract

Diurnal variations occurred in bacterial growth rates in the sediment and water column associated with seagrass (mainly *Zostera capricorni* Aschers) beds in Moreton Bay, Queensland, Australia. Studies were carried out in March and June 1981. Cell production rates increased by 5- to 10-fold during the morning and decreased during the afternoon. No nocturnal variation was observed. Daily bacterial cell production rate in the aerobic zones of the seagrass bed was estimated to be 43 mg C m^{-2}. A minimum of 100 mg C m^{-2} d^{-1} would be required to support the bacterial production. This represents about 10% of net primary production. The incorporation of tritiated thymidine into DNA was used to measure bacterial growth. The validity of the method is discussed.

Introduction

Our understanding of the ecology of marine bacteria depends very much on the methods that are available. Improvements in microscopy and the application of biochemical techniques have transformed views on the role of bacteria in marine systems. Bacteria are far more abundant and active and occupy a more important position in the food chain than previously thought (see Sieburth, 1979). Reliable measurements of growth rates and cell production of bacteria without greatly disturbing the *in situ* environment are now possible. Probably the most useful technique is the estimation of DNA synthesis rates from measurements of the rate of tritiated thymidine incorporation, which has been used in the water column by Fuhrman and Azam (1980, 1982) and in sediments by Moriarty and Pollard (1981). Karl (1979, 1981) and Karl *et al.* (1981) have proposed the use of tritiated adenine incorporation into RNA or DNA as measures of microbial growth. Their technique, however, does not distinguish heterotrophic bacterial growth from that of other microorganisms, including microalgae, because all microbes utilize adenine. Other disadvantages in the use of adenine have been discussed elsewhere (Moriarty, in press, b). Thymidine is a more specific measure of the growth of heterotrophic bacteria (see "Discussion").

To quantify fully the role of bacteria in the carbon cycle, their productivity must be measured. In this paper, the productivity of bacteria in seagrass beds (mainly *Zostera capricorni* Aschers) in Moreton Bay, Queensland, Australia, was measured over diel periods with the tritiated thymidine technique (Moriarty and Pollard, 1981). Bacterial biomass in seagrass bed sediments is high and the bacteria are important in the food chain (Moriarty, 1976, 1980).

Materials and Methods

Eight cores of sediment were collected at each time period and assayed as described earlier (Moriarty and Pollard, 1981). Leaves of *Zostera capricorni* Aschers with a coating of flocculent sediment, which was loosely attached mainly to older leaves, were collected and shaken vigorously with seawater in a container. The suspended material from about 15 leaves was diluted to 100 ml, and 10 ml portions were added to tubes each containing 740 kBq (20 μCi) of tritiated thymidine (45 to 55 Ci $mmol^{-1}$). The reaction was stopped by adding 0.5 ml of 10 *M* NaOH containing 165 m*M* thymidine after 10 min. Seawater was collected with a long tube to give an integrated column sample. Portions of 250 ml were dispensed into flasks and tritiated thymidine (100 μCi) was added. All assays were carried out in a waterbath at ambient temperature for 10 min. At some time periods, isotope dilution experiments were carried out by adding tritiated thymidine of different specific activities to a series of flasks. The assay was terminated by filtering the water through cellulose nitrate filters (Sartorious, 0.2 μm pore size) and immersing the

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filters in 0.3 *M* NaOH containing 5 m*M* thymidine. Cellulose nitrate was found to be the best filter material because it degraded with alkali, and greater proportions of DNA were recovered from these filters, presumably because DNA was bound to the degraded cellulose nitrate.

All assays were carried out in the field immediately after sample collection. Further samples were preserved in formalin (4% v/v formalin) for direct microscopy with acridine orange (details given in Moriarty, 1980).

In the laboratory, DNA was purified as described by Moriarty and Pollard (1981), except that the samples were autoclaved at 120 °C for 30 min instead of heating for 4 h at 100 °C. After dialysis, 0.5 ml of an extract of humic compounds was added to aid in recovery of DNA. The humus extract was prepared by autoclaving top soil and litter in 0.6 *M* NaOH for 30 min, and then neutralizing it.

Recovery of DNA was checked using two procedures. In one, 1 mg of DNA (herring sperm, Sigma Chemical Co. Missouri, USA) was added to sediment in alkali before autoclaving. From the final solution used for counting radioactivity in DNA, 0.5 ml was removed and assayed for deoxyribose using the Dische method (Burton, 1956). The difference in absorbance between samples with and without added DNA gave the recovery of added DNA. In another method to check DNA recovery, ^{14}C-DNA (Amersham) was added to sediment with alkali. This was to check the accuracy of the Dische assay.

The relationship between the rate of bacterial cell division and the rate of thymidine (Tdr) incorporation into DNA was described earlier (Moriarty and Pollard, 1981). This relationship may also be described by the following equation:

$$N = 0.96 \times \text{dis/min} \times \text{nmol}/(\text{Bq} \times t),$$

where N is the number of bacteria dividing per hour; dis/min is the disintegrations per minute of Tdr incorporated into DNA; nmol is the total amount of thymidine present (including corrections from isotope dilution plots); Bq is the amount of radioisotope added; and t is time of incubation in minutes. The relationship was derived from the earlier one as follows:

$$\text{No. DNA molecules synthesized } (x) = 1.3 \times 10^{18} \times \text{mol Tdr incorporated},$$

$$x = 1.3 \times 10^{18} \times \text{dis/min} \div \text{specific activity (corrected for isotope dilution)},$$

$$x = 1.3 \times 10^{18} \times \text{dis/min} \times \text{nmol} \times 1 \times 10^{-9}/(\text{Bq} \times 8.14 \times 10^{10}).$$

$$\text{Rate of DNA synthesis } (x\ \text{h}^{-1}) = x \times 60/t = 0.96 \times \text{dis/min} \times \text{nmol}/(\text{Bq} \times t).$$

The specific activity in the above equation should be that of the final precursor of thymine in DNA, viz. thymidine triphosphate. As it is not possible to measure this directly, we have used an isotope dilution technique in which increasing quantities of unlabelled thymidine were added to a constant amount of radioactive thymidine (Moriarty and Pollard, 1981). If there are no other sources of unlabelled thymidine, or other precursors of thymidine triphosphate to dilute the labelled thymidine, then a plot of the reciprocal of radioactivity in DNA against thymidine should pass through zero. A negative intercept on the ordinate is an estimate of the amount of dilution of isotope by other sources than that added. As regression analysis is used to fit lines in the plots of isotope dilution experiments, thymidine concentration should be plotted on the ordinate.

The effect of cycloheximide on DNA synthesis was measured by carrying out kinetic experiments. Surface sediment was added to tubes containing tritiated thymidine (1480 kBq) and cycloheximide (100 μg). A series without cycloheximide was also assayed. The reaction was stopped at different time intervals and DNA was purified as described above.

Autoradiography was carried out as described by Meyer-Reil (1978), except that 4′,6-diamidino-2-phenylindole dihydrochloride was used as the fluorescent stain (Porter and Feig, 1980). Samples of flocculent sediment loosely attached to leaves (see above) were incubated for 10 min with either tritiated thymidine or adenine and then chilled on ice and brought to the laboratory for further processing. [Methyl-^{3}H]-thymidine was obtained from Amersham, UK.

Results

Recovery of DNA from Sediment and Water

When an extract of humic compounds was added as a co-precipitant after dialysis, the recovery of radioactive label in DNA was increased three-fold (Table 1). About 40% of DNA was lost during purification by the Schmidt-Tannhauser procedure. With standardized procedures, this loss was constant as shown by the agreement of two different measures (Table 1). The Dische colorimetric assay was less precise, because humic compounds contributed a background colour which varied slightly.

Diurnal Variation in Isotope Dilution

The dilution of tritiated thymidine incorporated into DNA was greatest around midday and often low or not measur-

Table 1. Recovery of DNA from sediment around *Zostera capricorni* beds. Effect of adding extract of humic compounds on amount of labelled DNA extracted is shown. Actual recoveries were determined by colorimetric assay of added DNA and recovery of ^{14}C-DNA (see "Materials and Methods"). Means and ranges of variation and numbers of determinations (n) are shown.

^{3}H-DNA (net dis/min g^{-1} sediment)		% recovered using:	
Without humic extract	With humic extract	^{14}C-DNA method	Dische assay
3 185±500 (n=3)	9 053±1 700 (n=3)	63±2 (n=3)	60±10 (n=3)

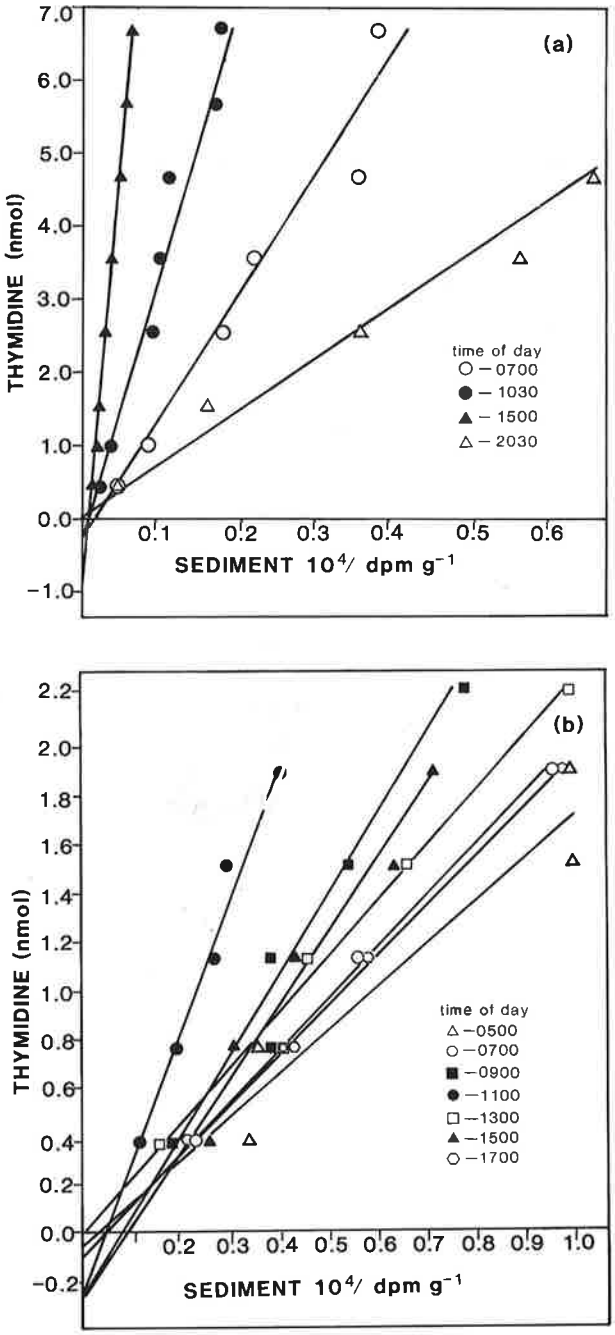

Fig. 1. Isotope dilution plots of incorporation of [methyl-^{3}H]-thymidine into DNA in surface sediments associated with *Zostera capricorni*. Each sample of 1.5 g wet weight of sediment was incubated with 1.48×10^6 Bq (40 μCi) of isotope at ambient temperature. (a) Winter, 17 °C; samples incubated for 15 min. (b) Autumn, 26 °C; samples incubated for 10 min

able at night in surface sediment (Fig. 1). Only some examples, from two diurnal studies, are shown in Fig. 1, but similar patterns have been observed in every study. Variability in the assays was generally greater at low rates of incorporation of thymidine into DNA, and this is demonstrated by the greater scatter of values during the early morning or evening (Fig. 1). More variability occurred usually when rates of incorporation of radioisotope into DNA approached the background values. Precision was better at higher rates of incorporation and low or zero dilution of isotope by added non-labelled thymidine, and therefore the fit of the lines is more precise near the origin. Lines were fitted using a least-squares regression analysis; in a few cases, where variability was high, a positive intercept on the ordinate was obtained. In such cases, these lines were drawn through zero by eye.

The extent of participation of added thymidine in DNA synthesis may be calculated by dividing the amount of thymidine supplied by the total (apparent) amount used for DNA synthesis, e.g. 0.4/1.4 at 15.00 hrs in Fig. 1a. Expressed as a percentage of total thymidine incorporated into DNA, the extent of participation of added thymidine was 28% at 15.00 hrs, 40% at 10.30 and 07.00 hrs, and 100% at 20.30 hrs (Fig. 1a).

Diel Variation in Production Rates

In the surface sediment around seagrass plants (*Zostera capricorni*), the rate of production of bacterial cells increased markedly during the morning to a peak at midday and then decreased to lower values which remained constant overnight (Fig. 2). The diurnal variation in bacterial productivity always occurred in sediments associated with seagrass, and other examples are shown in Fig. 3. Where the seagrass cover was less dense, there was less variation (Fig. 3a). There was no diurnal variation in sediments where seagrass was more than 2 m away (Fig. 3b). No correlation was apparent between diurnal production rates and water temperature or tide height.

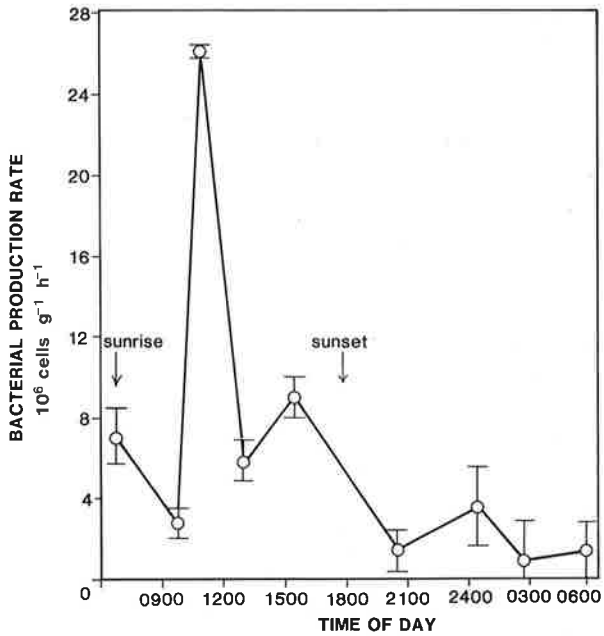

Fig. 2. Diel variation in bacterial cell production rate in surface sediment around *Zostera capricorni* plants. Error bars indicate maximum range of variation in calculation of growth rate from isotope dilution plots

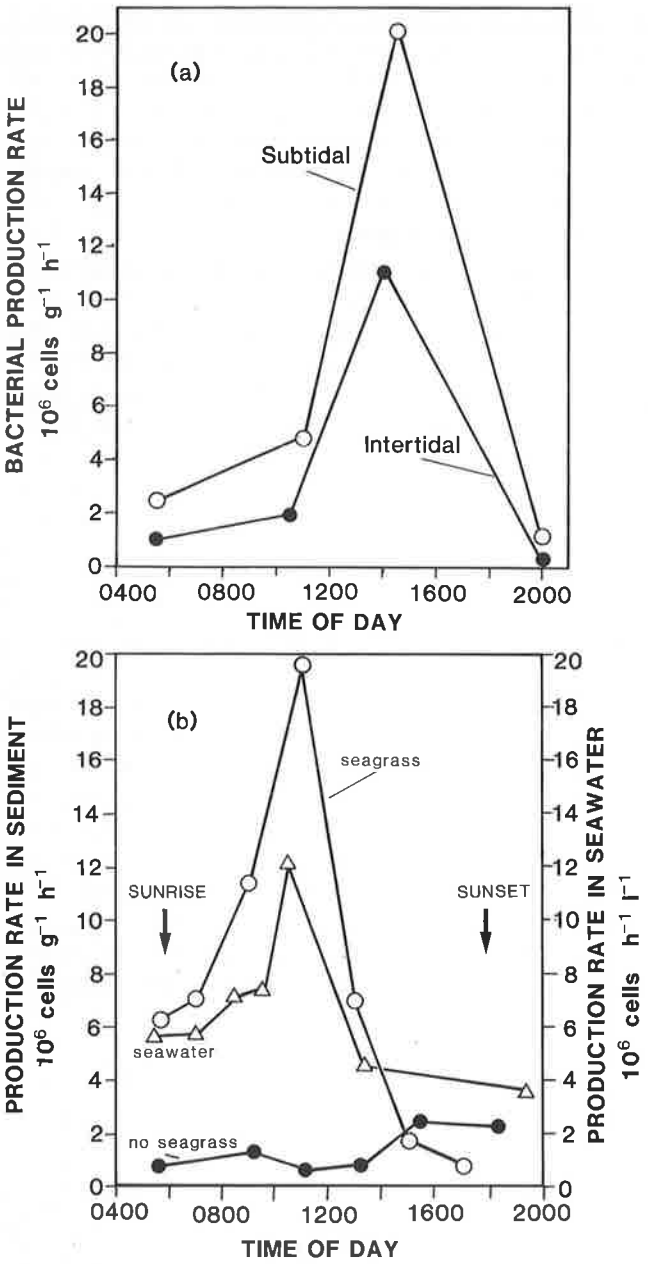

Fig. 3. Diurnal variation in bacterial cell production rate in surface sediments and water column associated with *Zostera capricorni*. (a) Comparison of subtidal sediment with dense seagrass cover and intertidal sediment with sparse seagrass cover; winter, temperature 17 °C. (b) Surface sediment and water column in area of dense seagrass cover compared with sediment in an area (4 m diam) with no seagrass; autumn, temperature 26 °C

It is difficult to provide estimates of error in the values for production rates of bacterial cells because the isotope dilution plots are more precise near the origin. Some indication of error was obtained by drawing lines of fit that encompassed the full range of variation of individual points in each isotope dilution plot and from which a range of variation in production rates was calculated. The error bars in Fig. 2 are examples of such variation.

On the surfaces of the seagrass leaves, particularly older leaves with their dense covering of epiphytes, bacteria in loosely attached flocculent material bound together with fine sediment particles and microalgae also exhibited diurnal variation in production rates. Dilution of tritiated thymine incorporated into DNA was large. The added exogenous thymidine contributed 21% of thymine in the DNA (Fig. 4). The bacteria on the leaf surfaces were growing rapidly, with a doubling time for the whole population of about 6 h.

Bacterial production rates in the water column over the seagrass beds varied in a similar way over a diurnal period (Fig. 3 b). As a different water mass was sampled at each time, due to tidal movement, the similarity in diurnal variation between sediment and water column indicates that a peak in bacterial production at noon occurred over the whole seagrass bed.

No significant dilution of tritiated thymidine in DNA was measurable during growth of bacteria in the water column (Fig. 4). That is, the added thymidine contributed about 100% of the thymine needed for DNA synthesis, although the precision was such that the contribution could have been 90% or even a little lower. The rate of incorporation of tritiated thymidine into DNA in the water column remained linear for over 1 h (Fig. 5), whereas in the sediment it was linear for only 10 min.

Doubling Times of Bacteria

In the sediment and water column, the biomass doubling time for the whole bacterial population depends on the time of day the measurements are made. Some examples during a June (winter) day in the sediment and an autumn day in the water column are shown in Table 2. The

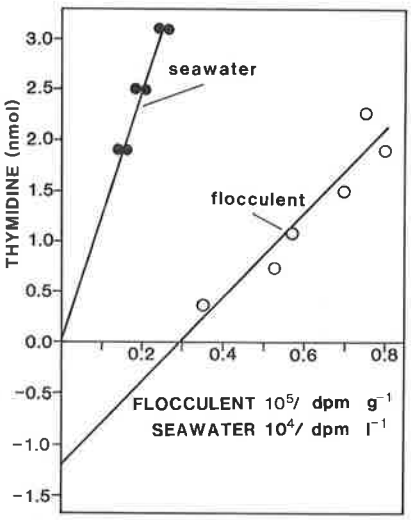

Fig. 4. Isotope dilution plots of incorporation of [methyl-^{2}H]-thymidine into DNA in epiphytic flocculent sediment and in seawater associated with *Zostera capricorni*. Flocculent sediment was assayed in autumn, temperature 26 °C; seawater was assayed in winter, temperature 18 °C

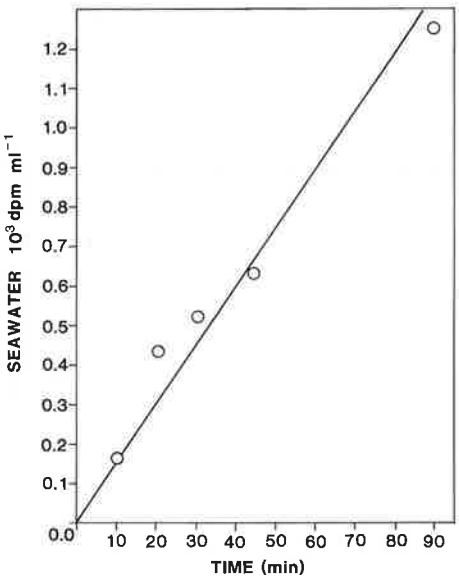

Fig. 5. Time course of incorporation of [methyl-^{3}H]-thymidine into DNA in seawater over *Zostera capricorni* beds

Table 2. Doubling times of bacteria in sediment and water associated with *Zostera capricorni* beds. Sediment values were determined on a winter (June) sample and water on an autumn (March) sample. An integrated water column was analysed; depth at low tide was 0.3 m (05.00 hrs, 17.00 hrs), at high tide 2.5 m (11.00 hrs). Sediment was not analysed at 06.00 hrs

Time of day (hrs)	Surface sediment		Water column	
	Doubling time (d)	No. bacteria (10^9 g^{-1})	Doubling time (d)	No. bacteria (10^9 l^{-1})
06.00	–	–	10	6.8
09.00	12	1.7	5	4.2
11.00	6	1.4	2	2.8
14.00	17	1.2	13	3.6
19.00	42	1.2	21	4.5

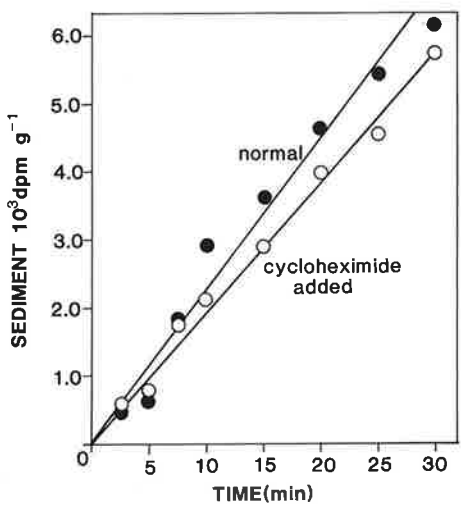

Fig. 6. Effect of cycloheximide on incorporation of [methyl-^{3}H]-thymidine into DNA in surface sediment around *Zostera capricorni* plants

doubling time for the population cannot be obtained from one measurement, but as the average of a diel series. For the surface sediment sample, the bacterial population would be replaced about every 20 d in this winter sample. The doubling time in the water column is about 10 d for the autumn sample. Some sediment was present in the water column in the early morning and evening samples, due to turbulence at low tide. Larger numbers of bacteria were present at these times, but growth rates were not raised. Hence, the average doubling time of the usual water-column bacteria is probably faster than indicated here.

Effect of Cycloheximide

Cycloheximide had no effect on the rate of DNA synthesis as measured by the incorporation of tritiated thymidine (Fig. 6).

Autoradiography

Autoradiograms, exposed for 4 d and 7 d, of the flocculent material loosely attached to seagrass leaves showed that ordinary bacteria had taken up thymidine. Cyanobacteria (blue-green algae) and diatoms had not taken up thymidine in measurable quantities. It was not possible to determine the proportion of active bacteria, because they were in clumps and thus silver grains could not be related to particular bacteria. With tritiated adenine, on the other hand, diatoms and cyanobacteria as well as heterotrophic bacteria were heavily labelled.

Discussion

Methodology

Measurement of the production of all bacterial cells in natural environments is difficult. It is unlikely that one procedure will be applicable to many different types of environment or to all types of bacteria within one environment. In sediments, where bacteria are bound together in aggregates and cannot be separated from each other or from eukaryotes and bacterial predators, the incorporation of tritiated thymidine into DNA may be used as a measure of production rates (Moriarty and Pollard, 1981). It is important to purify the DNA, because isotope binds to other compounds. Our extraction procedure maximized the recovery of labelled DNA. The addition of an extract of humic compounds improves recoveries. In addition, we measured the amount of DNA recovered (Table 1).

In our earlier paper, we used the term "pool sizes" rather loosely, as well as the more cumbersome but correct term "degree (or extent) of participation" of labelled thymidine in DNA synthesis (Moriarty and Pollard, 1981). We reported that, on three occasions, the extent of

participation of added exogenous thymidine in DNA synthesis was low, which we interpreted as indicating the presence of large pools of thymidine in the sediment. We now think that only one of those experiments may have been due to a pool of thymidine (or related exogenous precursors) in the sediment (see Fig. 2A in Moriarty and Pollard, 1981). The biphasic curves and lower extent of participation of thymidine (2%) in DNA synthesis at high concentrations of thymidine may have been due to an inhibition of the pyrimidine transport mechanism or to a stimulation of thymidine catabolism by the excess thymidine. In the work reported here, the extent of participation of thymidine in DNA synthesis varied from 100% in seawater and sediment at night to as low as 30% at 11.00 or 12.00 hrs. In other words, when the bacterial populations were growing slowly, exogenous thymidine supplied all that was required for DNA synthesis and we presume that *de novo* synthesis of deoxythymidine monophosphate (dTMP) was inhibited (Moriarty, in press, b). When the bacteria were growing rapidly, however, the salvage pathway and/or the uptake of thymidine did not function rapidly enough to supply thymidine for dTMP, and thus *de novo* synthesis proceeded to dilute the labelled thymidine that was incorporated into DNA.

Fuhrman and Azam (1980), in their study of bacterial production in seawater using tritiated thymidine, assumed that by adding a large excess of thymidine, the pools of thymine precursors of DNA would be saturated and thus the isotope would not be diluted. Although only one example is shown here (Fig. 4) we have carried out isotope dilution experiments on many water bodies and have only occasionally found measurable dilution, which supports the conclusions of Fuhrman and Azam (1980). An hypothesis that would explain this is that in the water column, bacteria were growing slowly enough for exogenous thymidine to have supplied all the requirement for DNA synthesis and that feedback inhibition of *de novo* synthesis of dTMP occurred.

Fuhrman and Azam (1982) have criticized our isotope dilution procedure and subsequently modified their own view by saying that isotope dilution did occur in seawater bacteria, although it could not be measured directly. They misquoted our paper (Moriarty and Pollard, 1981) and misinterpreted the isotope dilution technique. We did not assume that precursor pools were in isotopic equilibrium nor that this assumption (which we did not make) was proved by the observation of linear plots. The isotope dilution procedure that we adopted has been used to study the synthesis of macromolecules (Forsdyke, 1968; Hunter and Francke, 1974). Where a compound in a biosynthetic pathway has more than one route to its synthesis (e.g. dTMP in this case), and if the steps are not reversible, isotopic equilibrium between all components of the pathway does not occur. The ratio of isotope to unlabelled substrate will decrease at each branch point in the pathway. It is necessary, however, for the ratio of isotope to unlabelled substrate at each point to remain constant during the experiment and thus kinetic experiments are necessary to show that the period of the assay does not exceed the period of linear rate of incorporation of isotope into DNA (Moriarty and Pollard, 1981). It is also a necessary condition that the labelled exogenous precursor does not stimulate DNA synthesis, which would occur if the precursor were a limiting substrate. That is, the rate-limiting step should occur after the synthesis of dTMP, which is a reasonable assumption because there are other substrates needed for DNA synthesis, all of which have to be incorporated in a particular order. The rate-limiting step for DNA synthesis is far more likely to be at the level of DNA polymerase rather than prior to dTMP synthesis, as Fuhrman and Azam (1982) imply in their criticism of our work.

From their independent verification of the procedure for measuring the rate of synthesis, they concluded that isotope dilution must occur, because they found higher rates of DNA synthesis with ^{32}P than with tritiated thymidine (Fuhrman and Azam, 1982). There are alternative explanations for their results, the most likely being that ^{32}P measured DNA synthesis in small cyanobacteria as well as heterotrophic bacteria. Polycarbonate filters of 1 μm pore size retain only about 50% of particles 1 μm in diameter and 70% of 2 μm particles (Sheldon, 1972). Small chroococcoid cyanobacteria are now known to be abundant in ocean waters (Johnson and Sieburth, 1979; Sieburth, 1979; Waterbury *et al.*, 1979; Perkins *et al.*, 1981), and as many of these are less than 2 μm in diameter they would pass through the 1 μm filter used by Fuhrman and Azam (1982). As cyanobacteria lack thymidine kinase, the difference between DNA synthesis measured by ^{32}P and ^{3}H-thymidine would be due to the presence of growing chroococcoid cyanobacteria. Fuhrman and Azam (1982) reported that discrepancies between the two methods were greater in open ocean water rather than near the shore. This could be due simply to higher populations of cyanobacteria offshore rather than to some unexplained difference between the biochemistry of marine bacteria inshore and offshore.

Thymidine was selected as a measure of DNA synthesis for many reasons which have been discussed in detail by Moriarty (in press, b). One important attribute is the ability to distinguish heterotrophic bacterial production from that of photosynthetic microorganisms. Cyanobacteria, eukaryotic microalgae and fungi lack thymidine kinase, the enzyme necessary for its incorporation into DNA, and so if they did take up thymidine, it would be catabolized and the label would not enter DNA (see Moriarty, in press, b, for references and full discussion). Although some eukaryotes do have thymidine kinase (e.g. protozoans and higher animals), we expected that our procedure would still be a measure of bacterial production only, because in the short time periods of each experiment (5 to 15 min) and with the low concentrations of thymidine added, larger organisms than bacteria could not effectively take up and use thymidine for DNA synthesis.

The experiments with cycloheximide and autoradiography support this hypothesis. Cycloheximide inhibits

DNA synthesis in eukaryotes, but has little or no effect on prokaryotes (Cooney and Bradley, 1962; Venkatesan, 1977). The DNA synthesis that was measured with tritiated thymidine was not affected by cycloheximide (Fig. 6). Karl (1981) and Karl *et al.* (1981) have measured DNA synthesis with adenine and have shown that adenine is incorporated into macromolecules in a wide variety of microorganisms, including microalgae. Our autoradiograms showed that cyanobacteria and microalgae were labelled with tritiated adenine, but not with thymidine.

Fuhrman and Azam (1982) showed that in the water column all or most metabolically active heterotrophic bacteria took up thymidine. From this and other work they concluded that the rate of thymidine incorporation into DNA was a good measure of bacterial production in the water column. They derived experimentally a factor of 1.4×10^{18} cells produced per mole thymidine incorporated, which was in good agreement with the factor that both they and we estimated previously (Fuhrman and Azam, 1980; Moriarty and Pollard, 1981). Unfortunately, they then modified their factor to account for the discrepancy of ^{32}P and ^{3}H-thymidine results as discussed above.

Sediments have a greater variety of bacteria, including strict anaerobes that have limited nutrient requirements. It is possible that some of these lack the ability to take up thymidine. Indeed, some sulphate-reducing bacteria incorporated very little thymidine into DNA when it was added to their culture medium (G. W. Skyring, personal communication). The work reported here was confined to aerobic environments and, until more studies are undertaken with anaerobic sediments, the values obtained should be considered as minimum estimates of bacterial production.

Kinetic experiments with seawater showed that the rate of incorporation of thymidine into DNA was linear for 30 to 60 min, which is much longer than in sediment (10 to 20 min). Binding of thymidine to clay or other substances in sediment is probably one reason for the short period (Moriarty and Pollard, 1981). Another factor which may affect the kinetics of incorporation is the presence of thymidine phosphorylase. This enzyme cleaves thymidine to thymine and may be more active in bacteria that are growing rapidly, such as those in close association with seagrass.

Diurnal Variation in Bacterial Production

The occurrence of a diurnal change in bacterial growth and the lack of any nocturnal variation suggests that the bacterial activity is linked to photosynthesis. As the effect occurred in bacteria closely associated with seagrass, we presume that the seagrass (*Zostera capricorni*) itself is the main contributory factor. Epiphytic and epibenthic microalgae may be involved also. Oxygen concentration in the water was near saturation and varied only a little. It did not reach a peak until mid-afternoon (unpublished observations), so the stimulation of aerobic bacteria by increased oxygen tension is not a likely mechanism to explain the diurnal variation in growth. As bacteria in the water column, in flocculent sediment loosely attached to seagrass leaves, and in the surface layer of the sediment, all showed similar patterns in diurnal growth rates, it is probable that excretion of nutrients from leaves and epiphytic microalgae during photosynthesis stimulated the bacterial growth. Excretion from the rhizomes or roots may also contribute to the bacterial production. The sediment that was used here did not contain large fragments of dead leaves or roots, and thus the only bacterial growth measured was that dependent on dissolved organic compounds in the water column or interstitial water. Diel cycles in bacterial production in the water column have been discussed by Sieburth *et al.* (1977) who used diffusion culture chambers to show that bacterial growth was linked to algal production. Karl (1981), using adenine, showed that the rate of DNA synthesis in a planktonic microbial population (i.e., bacteria and microalgae) increased four-fold during the day. Diel cycles in planktonic bacterial production have been found in a freshwater lake by Riemann and Sondergaard (in press), who used the tritiated thymidine method.

Carbon Flow

By integrating the area under a full diel curve for bacterial cell production, the total number of cells produced per day can be estimated. A value of 2.5×10^{-11} mg C cell^{-1} is about average for the size of bacteria in the seagrass beds, so the daily bacterial biomass production as carbon can be calculated. At present, we are carrying out a seasonal study to relate bacterial productivity to primary productivity, but the results from one series of experiments, carried out in autumn (March), are given here to illustrate the importance of bacteria in the cycle of organic matter. Diurnal curves for the sediment and water column on that day are shown in Fig. 3 b. Daily production was estimated as 12 mg C m^{-2} for surface sediment, 15 mg for flocculent sediment on old leaves and 16 mg in the water column. Due to tidal flow, the same water mass could not be sampled each time, so the water column value is approximate and based on an average depth of 2 m. These values give a total of 43 mg C m^{-2} d^{-1} in bacterial cell production in the aerobic zones. If a value of 43% is assumed for conversion efficiency in bacteria, which is within the range found by other workers (Payne and Wiebe, 1978), 100 mg C m^{-2} d^{-1} would be required to support that bacterial population. The actual conversion efficiency may be lower, because many bacteria may not be actively growing but still utilize organic compounds for cell maintenance. Bacteria in the sediments produce much slime (Moriarty and Hayward, 1982), and this also requires carbon compounds; perhaps even an amount equal to the cell biomass (Wilkinson, 1958).

Other populations of bacteria that have not been studied are the aerobic ones in the phylloplane and

rhizosphere, and the anaerobic bacteria in the sediment. We attempted to study phylloplane bacteria by blending leaves, but found that a factor was released from the leaves which completely inhibited DNA synthesis. Using intact cores, preliminary results show that DNA synthesis measured with thymidine is about one order of magnitude lower per gram of sediment in the anaerobic zone than in the aerobic zone. These results are similar to those found by Moriarty (in press, a) for a different environment. Over a 5 cm depth of sediment, bacterial production in the anaerobic sediment would be about equal to that in the aerobic zone if the preliminary values are correct.

Seagrass productivity in the area is being measured; preliminary results indicate that net production is about 1 g C m^{-2} d^{-1}. Values obtained thus far show that at least 10% of net daily production is cycled through bacteria, and possibly another 10% would be needed to support the production of anaerobic, rhizosphere and phylloplane bacteria. In cyanobacterial mats of Hamelin Pool, Shark Bay, Western Australia, heterotrophic bacterial production was found to be about 20 to 30% of net primary production (Moriarty, in press, a). Bacterial production in the water column has been shown by others to account for a relatively large proportion of net primary production (Sieburth, 1979). Fuhrman and Azam (1982) estimated that from 10 to 50% of net primary production was utilized by bacteria, although as discussed above, their results may be a little overestimated.

Doubling Times of Bacteria

The doubling times for the bacterial populations in the sediment and water are mostly rather long (Table 2), but not very dissimilar from values reported by Jannasch (1969) who used a chemostat. They are slower than values reported by Fuhrman and Azam (1982) of 8 h to 2 d for bacteria in temperate ocean water. As discussed above, these authors may have overestimated the production rate and hence doubling times of bacteria. They do not mention at what time of the day their samples were taken, but if it was within a few hours of noon, the rates they measured would be higher than the average daily rate if a diurnal cycle in growth rates is a common feature of marine bacterial populations. We have found short doubling times on two occasions (see "Results", and Moriarty and Pollard, 1981) but these do not occur over a diel period. If these very fast rates were a normal occurrence, or even if we had underestimated doubling times by a factor of 2 or 3, the proportion of net production of the seagrass and its epiphytes that was utilized by bacteria would be unrealistically high. The increased bacterial production during the morning is probably due to dissolved organic matter excreted during photosynthesis, whereas the nocturnal production may be controlled by dissolved organic matter from a variety of sources, including decomposition of particulate matter. The proportion of bacterial production that may be dependent on excretion during photosynthesis is about half of the total diel production, so about 50 mg C m^{-2} d^{-1} would be needed to support it; this amount is roughly 5% of net primary production of the seagrass community. It agrees well with the minimum values of 1.5 and 2.1% average excretion rates for *Zostera marina* in North Carolina, USA (Penhale and Smith, 1977). Although more work is needed on the community in Moreton Bay, it seems that our results for bacterial doubling times are not very much underestimated. If they were underestimated, a greater amount of carbon would be needed, but this does not appear to be available.

These rather slow times do not mean that each bacterial cell is doubling every week or 2 wk, but rather that most of the bacteria observed microscopically are probably dormant. Fuhrman and Azam (1982) found that 50 to 80% of bacteria in the water column were actively growing. Bacteria in sediments may be less active, as they are embedded in aggregates of slime and mineral particles and attached to surfaces. Unless they are close to a source of nutrition, e.g. in the rhizosphere or in the turbulent zone at the sediment surface, they may have periods of starvation and when nutrient does become available, it may be used for maintenance rather than growth and division. Bacterial cell numbers in the sediment did not vary significantly during a diel period, but this would not be expected because of the effect of bacterial grazers and the fact that an overall doubling time of 1 wk or so was too slow to observe a diel change.

Conclusions

The use of tritiated thymidine to estimate bacterial production rates is a valuable tool in microbial ecology, because it can be used for short-term experiments with minimum manipulation of the natural environment and hence *in situ* activities. Significant variation in diurnal rates of bacterial production occurs in seagrass (*Zostera capricorni*) beds, apparently linked to excretion of dissolved organic matter during photosynthesis. The values for bacterial production reported here are only part of the total for the seagrass bed, but indicate that a substantial proportion of primary production must be cycled through bacteria.

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Date of final manuscript acceptance: October 13, 1982.
Communicated by G. F. Humphrey, Sydney

Microbial Biomass and Productivity in Seagrass Beds

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Abstract Different methods for measuring the rates of processes mediated by bacteria in sediments and the rates of bacterial cell production have been compared. In addition, net production of the seagrass *Zostera capricorni* and bacterial

Received December 15, 1983; accepted April 28, 1984.

Geomicrobiology Journal, Volume 4, Number 1
0149–0451/85/010021–00$02.00/0

production have been compared and some interrelationships with the nitrogen cycle discussed. Seagrass productivity was estimated by measuring the plastochrone interval using a leaf stapling technique. The average productivity over four seasons was 1.28 ± 0.28 g C m^{-2} day^{-1} (mean ± standard deviation, n = 4). Bacterial productivity was measured five times throughout a year using the rate of tritiated thymidine incorporated into DNA. Average values were 33 ± 12 mg C m^{-2} day^{-1} for sediment and 23 ± 4 for water column (n = 5). Spatial variability between samples was greater than seasonal variation for both seagrass productivity and bacterial productivity. On one occasion, bacterial productivity was measured using the rate of ^{32}P incorporated into phospholipid. The values were comparable to those obtained with tritiated thymidine. The rate of sulfate reduction was 10 mmol $SO_4^=$ m^{-2} day^{-1}. The rate of methanogenesis was low, being 5.6 mg CH_4 produced m^{-2} day^{-1}. A comparison of C flux measured using rates of sulfate reduction and DNA synthesis indicated that anaerobic processes were predominant in these sediments.

An analysis of microbial biomass and community structure, using techniques of phospholipid analysis, showed that bacteria were predominant members of the microbial biomass and that of these, strictly anaerobic bacteria were the main components.

Ammonia concentration in interstitial water varied from 23 to 71 μM. Estimates of the amount of ammonia required by seagrass showed that the ammonia would turn over about once per day. Rapid recycling of nitrogen by bacteria and bacterial grazers is probably important.

Introduction

In many shallow tropical and temperate regions, seagrasses are major primary producers. Large and active microbial populations are present in the sediments and thus must influence sedimentary chemical processes. Seagrasses are effective in stabilizing sediments and in promoting sedimentation (McRoy and Helfferich, 1980). They contribute large amounts of organic matter to their environments, yet few animals feed on them directly. A detrital food chain mediated by microorganisms is important in seagrass ecosystems upon which many fish are

dependent (e.g., see Klug, 1980; Ogden, 1980). Bacteria are important food components for species such as mullet *(Mugil cephalus)* and oysters, and other commercially important species, such as penaeid prawns, are dependent on bacterial-based food chains.

In order to determine the flow of carbon and nitrogen in seagrass sediments through degradative pathways, it is necessary to know the major functional groups of microbes and their rates of growth and activity. The seagrass sediments in Moreton Bay are mainly anoxic, although there is a narrow oxidized layer on the surface and around the roots of the seagrass. Thus anaerobic as well as aerobic microbial processes will be important. The biomass and community structure of the sedimentary biota can be determined by biochemical analyses. The fatty acids esterified to phospholipids are particularly valuable as markers of different types of organisms. Phospholipids occur primarily in membranes and thus are a good measure of microbial biomass (White, 1983). An analysis of phospholipids and relationships to microbial biomass is reported here.

Aerobic bacteria generally oxidize their organic substrates completely to carbon dioxide. In anaerobic food chains fermentative bacteria and denitrifying bacteria can utilize complex organic substrates. Denitrifiers completely oxidize the organic substrates to carbon dioxide and convert nitrate to ammonia and N_2 in the process. Fermenters release simple organic end products and CO_2. These end products provide substrates that are subsequently degraded by sulfate-reducing and methanogenic bacteria to CO_2 and CH_4. The rates of sulfate reduction and methanogenesis can be determined by measuring the end products of these processes, but the quantitative aspects of fermentation and respiration cannot be easily measured. It is possible, however, to measure the growth rates of bacterial communities in nature, and then to estimate the overall amount of organic carbon needed to sustain the amount of biomass that is produced.

Growth rates of a substantial proportion of the bacterial community may be determined from measurements of the rate of tritiated thymidine incorporated into DNA. Such measurements in seagrass beds have shown that bacterial productivity is high

and that large diurnal variations occur, apparently coupled to photosynthesis in seagrasses (Moriarty and Pollard, 1982). In this paper, seasonal variation in bacterial productivity was measured and compared to seagrass productivity. A ten-day workshop with fieldwork was organized to carry out studies on other measures of biomass and microbial activity, particularly sulfate reduction, methanogenesis, nitrogen cycling, and phospholipid analysis. As the project was only a short-term one, the study of these processes could not be detailed, but rather was aimed at determining whether the results obtained from the thymidine method, a new technique for measuring bacterial growth, were compatible with other measures of C flow in a seagrass bed. The origin of organic matter for bacterial growth and metabolism is, of course, the seagrass, so measurements of seagrass production were made in order to determine what proportion of that production was being used by bacteria associated with the seagrass. Processes of sulphate reduction and methanogenesis were studied. Since the nitrogen and carbon cycles are interlinked, some aspects of the nitrogen cycle are reported here.

Locality

Seagrass communities in Moreton Bay, Queensland, Australia, have been described by Young and Kirkman (1975). The particular community studied here is the one they described as consisting of *Zostera capricorni* Aschers and *Halophila ovalis* (R.Br.) Hook.f. and which occurs in the Pelican Banks area (Fig. 1 in Young and Kirkman, 1975). *Halodule uninervis* (Forsk.) Aschers also occurs in our study area on the eastern side of Pelican Banks. The sediment was primarily sand, with a small amount (2–6%) of silt and clay.

Materials and Methods

Primary Production of Zostera capricorni

The growth of *Z. capricorni* was estimated by measuring the plastochrone interval (the time interval between the initiation of

two successive leaves on one shoot) at various times (Table 1) over a ten-month period. The plastochrone interval was measured using a leaf stapling technique (Zieman, 1974). Measurements followed the procedures outlined by Jacobs (1979) and Poiner (1983) and were made four times between September 1981 and June 1982, one meter below mean low water datum.

The production of the different plant parts (leaf blades, leaf sheaths, and rhizomes) was calculated from the plastochrone interval according to the methods developed by Jacobs (1979) and Tomlinson (1972). Root production was not included due to difficulties in separating live and dead root material from the samples.

Thymidine Method for Measuring Bacterial Growth Rates

Measurements of bacterial growth rates in the aerated surface sediment and in the water column were carried out as described by Moriarty and Pollard (1981, 1982). Bacterial growth on leaves was measured by cutting off all the leaves of individual shoots at their base and placing them in a small tube containing 10–20 ml of filtered seawater. Tritiated thymidine (40 μl or 1.48 M Bq; specific activity 1.85 M Bq $nmol^{-1}$) was added and the leaves were incubated for 15–20 min at *in situ* temperature. The reaction was terminated by adding sufficient 10 M NaOH to give a final concentration of 0.3 M NaOH. The DNA was extracted and radioactivity was measured (Moriarty and Pollard, 1981; 1982). Six replicate shoots were assayed; six replicate blanks were prepared by adding NaOH at zero time to another set of shoots. Estimates of the areal density of shoots were also made.

Cores of sediment were assayed with minimal disturbance by using 2-ml plastic syringes with the ends removed. Cores of 40 mm depth were taken, and the top aerated layer was removed. Tritiated thymidine (100 μl, 3.7 M Bq) was distributed throughout the length of the core with a syringe and then the cores were capped. After incubating at *in situ* water temperature for 15–20 min, the cores were expelled into centrifuge tubes containing 2 ml of 0.6 M NaOH. The DNA was extracted as described by Moriarty and Pollard (1981, 1982). Six replicate cores were as-

sayed at each time interval. Six replicate blanks were prepared by injecting tritiated thymidine into each core and then immediately expelling the core into NaOH.

Recovery of DNA was measured in all experiments by the use of ^{14}C-DNA (Moriarty and Pollard, 1982).

For each seasonal study, bacterial growth was measured at about 2–3 hr intervals throughout the day. Daily productivity was calculated by integrating the results thus obtained over a 24-hr period. It was necessary to measure growth rates at several intervals because there was a marked diurnal variation in bacterial growth in seagrass beds (Moriarty and Pollard, 1982).

Phospholipid Method for Measuring Growth Rates

The method was adapted from that of White et al. (1979*c*). Sediment was collected as described for the thymidine method. Spoonfuls of the mixed slurries were added to 30-ml polypropylene disposable syringes containing 0.5 μl filtered seawater and 740 k Bq of ^{32}P-orthophosphate (carrier free). Plastic tubing had earlier been placed on the luer outlet of the syringes; it was equal in length to the syringe, was bent up alongside the syringe body, and was held in place with a rubber band to seal the syringe–luer port. After the tubing was loosened the syringe was tilted so that no sediment or fluid was over the exit port, the plunger was inserted and pushed down until it was near the sediment. The tubing was then clipped into place under the rubber band, the sediment was well mixed with isotope, and the sample incubated in a water bath at *in situ* temperature. The incubation was terminated by drawing up a mixture of 4 ml of chloroform and 8 ml of methanol through the tubing. The tubing was bent over again to seal the luer port and the mixture was left to extract for at least 2 hr. Then an additional 4 ml of chloroform and 4 ml of water was drawn up into each syringe, mixed, and left to stand at an angle with the port on the upper side so that it was covered by the chloroform layer and not by the sediment. After standing overnight, the chloroform layer was gently expelled through the tubing into phase-separating filters (Whatman) over collecting tubes. The phase-separating filters were used to ensure that none of the sediment or aqueous phase was

included in the chloroform phase. Measured portions of the chloroform phase (usually about 3 ml) were transferred to glass scintillation vials. If plant pigments were noticeable, these were bleached by exposure to strong sunlight for an hour or so. The chloroform was evaporated off. The sample and stock ^{32}P solutions were counted at the same time to avoid decay corrections.

Cores of sediment in 2-ml syringes were injected with 100 μl of ^{32}P (3700 k Bq) and incubated in a water bath. The incubation was stopped by expelling the cores into 30-ml syringes and drawing up a chloroform and methanol mixture as described above. Interstitial water was collected by centrifuging cores of sediment at 3000 × *g* in 5-ml syringes plugged with Whatman GF/F filters. Inorganic phosphate was measured in replicate samples of interstitial water as described by Strickland and Parsons (1968). The specific activity could then be calculated. Rates of phospholipid synthesis were then calculated, and these were converted to bacterial growth rates using the factor of 50 μmol phospholipid per gram dry weight of bacteria, which is equivalent to 1 μmol P per 10 mg C of bacteria (White et al., 1979*a*). The assumption was made that only growing bacteria were synthesizing phospholipid and that there was equilibration of isotope with phosphate inside and outside the cells.

Sulfate Reduction

Sulfate reduction rates were determined by the method of Skyring and Chambers (1980) and Skyring et al., 1983. The samples were not assayed for pyrite.

In vitro Methane Production. Sediment cores 20 cm deep were taken within the seagrass beds. Cores were extruded from the corer and transferred to the laboratory for subcoring. *In vitro* methane production from four sediment depths (0–5 cm, 5–10 cm, 10–15 cm, and 15–20 cm) was assayed by the method of King and Wiebe (1978). Subcores (5 ml), obtained with a cut-off 10 cc plastic syringe, were placed in 60-ml serum bottles and capped with Venoject tops (Terumo). The serum bottles had been flushed previously with nitrogen gas scrubbed of oxygen with a zinc-methylviologen trap. The bottles also contained 5 ml

of deoxygenated artificial seawater (Lyman and Fleming, 1940) with 0.03% cysteine. Sediment samples were added to the serum bottles, which were quickly recapped and flushed again with nitrogen. The bottles were incubated at 27°C in the dark. Samples of the gas headspace were withdrawn from the bottles and injected into Venoject evacuated tubes (Terumo) for subsequent methane analysis.

The effects of added substrate on methane production were also assessed. Samples were prepared as described above. In one set of samples 5 mM (final concentration) of sodium acetate was added to the soil slurry. In a second set of samples a H_2/CO_2 (20:80) gas phase was used instead of nitrogen.

Methane samples were analyzed on a Pye Unicam Series 204 flame ionization gas chromatograph with a 6′ × ⅛″ stainless steel Poropak R column. The column temperature was 60°C and carrier gas flow was 32 ml min^{-1}.

Sediment Gas

Sediment gas bubbles were collected by the method of Oremland and Taylor (1977). An inverted funnel with an attached 10-ml glass tube was held close to the sediment surface by a diver. The sediment beneath the funnel mouth was gently agitated by hand, and released gas bubbles displaced the water in the tube. When the tube was filled with gas it was detached from the funnel and capped. On the boat, inverted vials were uncapped in a beaker of water and a gas sample was withdrawn with a syringe and injected into an evacuated tube for analysis later. Methane was determined as described above. Nitrogen, carbon dioxide, and argon-oxygen were analyzed on a Carle III thermal conductivity gas chromatograph. Nitrogen and argon-oxygen were measured on a 1 m × 3 mm stainless steel Molecular Sieve 5A column. CO_2 was measured on a 6′ × ⅛″ stainless steel Chromosorb 102 column. Column temperature was 60°C and carrier gas flow was 60 ml min^{-1} in both columns.

Interstitial Water Chemistry. Tightly fitting discs of acid-washed GF filter paper were placed in the bottom of 5-ml polypropylene

syringes and the syringes were then filled with freshly collected sediment. Each syringe was suspended over a 10-ml centrifuge tube and spun at 1000 × *g* for 5 min. Interstitial water in the centrifuge tube either was diluted with deionized water for ammonia analysis and then frozen with dry ice or was frozen undiluted for primary amine analysis. Ammonia analysis was carried out with the automated phenol-nitroprusside–dichloroisocyanurate method of Ryle et al. (1981). Total primary amines were determined with fluorescamine and the results are given in glycine equivalents. To 2.5 ml of 0.2 M H_3BO_3 buffer (made to pH 9.0 with NaOH) 200 μl of interstitial water sample or glycine standard was added. While the sample was vortexed, 250 μl of fluorescamine reagent (30 mg fluorescamine in 100 ml acetone) was added. After this solution was mixed for 1 min, approximately 2 ml was removed and manually injected into a Waters 420-C fluorescence detector.

Nitrogen and Carbon Content of Seagrasses. Seagrasses were collected by hand and, after sorting, dried at 50°C for 24 hr. The total nitrogen content was determined using a salicylic acid-sulfuric acid Kjeldahl digestion, followed by steam distillation into boric acid and titration with dilute sulfuric acid. The total carbon content was determined with a Perkin-Elmer Model 240 elemental analyzer modified according to Moriarty and Barclay (1981).

Extraction and Analysis of the Phospholipids. Sediments were collected with polyvinylchloride corers 5 cm in diameter and 5 cm deep. The top 2 cm were removed and extracted from the fresh sediment with a one-phase, chloroform-methanol extraction. Then additional chloroform and water were added, and after thorough mixing the phases were separated (White et al., 1979*c*). The chloroform was filtered through Whatman 2V fluted filter paper (Whatman Inc., Clifton, NJ) to dehydrate the lipid for further analysis.

After extraction and digestion with perchloric acid, the total phospholipid was determined by colorimetric analysis of the lipid phosphate (White et al., 1979*c*). The lipid was then quan-

titatively partitioned into neutral, glyco-, and phospholipid fractions by chromatography on silicic acid (Unisil^R 100–200 mesh) (King et al., 1977; Gehron and White, 1982). The phospholipid was subjected to mild alkaline hydrolysis, and the aqueous phase was analyzed for phosphate to determine the diacylated phospholipid content. One portion of the organic phase was recovered, methylated, and purified by thin layer chromatography. The alkyl fatty acid methyl esters and monohydroxy fatty acid methyl esters were recovered quantitatively and analyzed by capillary gas–liquid chromatography on a 50-m fused silica column with chemically bonded SE-30 (Bobbie and White, 1980). The structural identification of the fatty acid methyl esters was made by determining the quantitative response to catalytic hydrogenation on polar and non-polar columns, by their behavior on thin layer chromatography plates impregnated with silver nitrate, and by mass spectrometry of fragments generated by electron impact (Bobbie and White, 1980; Bobbie et al., 1981).

Another portion of the organic phase of the mild alkaline hydrolysis was subjected to mild acid hydrolysis and partitioned against water. The phosphate recovered in the water phase was determined; it is a measure of plasmalogens (White et al., 1979*b*).

Results

Seagrass Production

The values for *Zostera* production indicate a trend towards lower values in winter (June) (Table 1). There was considerable variability in the results, and it is not possible to give an estimate of error because sample sizes were too small. Root production could not be estimated. The mean value for *Z. capricorni* production is 1.28 g C m^{-2} day^{-1}.

Bacterial Production

Bacterial productivity in the aerated surface layers of the sediment (0–3 mm) was about equal to that in the anaerobic zone

Table 1
Production of *Zostera capricorni*

	Plant Components (mg C m^{-2} day^{-1})			
Month	*Leaf Blades*	*Leaf Sheaths*	*Rhizomes*	*Total*
Sept.	0.67	0.36	0.12	1.15
Dec.	0.75	0.39	0.27	1.41
Mar.	0.93	0.42	0.24	1.59
June	0.51	0.27	0.18	0.96
Mean	0.71	0.36	0.20	1.28

down to 40 mm depth (Table 2). There was considerable spatial variability in bacterial biomass and production; e.g., the high values in May, which were probably due to a higher proportion of rhizosphere bacteria in the samples. Bacterial biomass was higher near seagrass roots than it was away from the roots. Doubling times for the bacterial population in the sediment were longer in winter (August) (Table 2). In the water column, bacterial productivity was a little lower in winter (August), and doubling times were also a little longer (Table 3). Bacterial productivity on leaf surfaces was low and variable, compared to that elsewhere in the seagrass bed (Table 3).

In calculating total bacterial productivity for the seagrass bed, an average water depth of 3 m was assumed. This is probably conservative, because seagrasses occur in deeper channels, but a much more detailed study would be needed to obtain more accurate results. Even with a 3-m deep water column, it can be seen that bacterial production in the water column is at least as great as that in the sediments (compare Tables 2 and 3). Total daily production in the water and sediments, measured with the thymidine technique, was about 76 mg C m^{-2} day^{-1}, to a depth of 50 mm in the sediments.

Results for bacterial production estimated with the phospholipid method agreed well with those of the thymidine technique, both in a mixed slurry of surface sediment and in cores (Table 4). In anaerobic sediment that was mixed into a slurry and

Table 2
Bacterial biomass and production in sediments

Date, 1982	Temperature (°C)	Surface Sediment[a]			Cores[b]
		Number (No. 10^{12} m^{-2})	Production (mg C m^{-2} day^{-1})	Doubling Time (days)	Production (mg C m^{-2} day^{-1})
Feb.	25	1.8 (0.5)[c]	3.3	1.1	n.d.[d]
Mar.	25	3.7 (0.3)	3.6	1.9	12 ± 3[c]
May	19	6.4 (0.7)	45.0	2.9	33 ± 5
Aug.	18	3.0 (0.2)	11	5.7	12 ± 1.8
Oct.	20	2.8 (0.3)	23	2.5	17 ± 2.5
Mean		3.5 (0.4)	15 ± 7[c]	2.8	18 ± 5

[a] Aerated zone of sediment, 0–3 mm depth.
[b] Core depths, 3–40 mm.
[c] Standard error, $n = 6$.
[d] Not determined.

Table 3
Bacterial numbers and production in water column and on leaves of seagrass. Temperatures are shown in Table 2

	Water Column			*Leaf Surface*		
Date, 1982	*Number (No. 10^{12} m^{-3})*	*Production[a] (mg C m^{-2} day^{-1})*	*Doubling Time (days)*	*Number (No. 10^{11} m^{-2})*	*Production (mg C m^{-2} day^{-1})*	*Doubling Time (days)*
Feb.	n.d.	27	–	3.7 (0.9)[b]	0.2	31
Mar.	1.2 (0.1)[b]	27	2.3	7.8 (0.7)	1.1	15
May	1.1 (0.1)	13	5.4	4.4 (0.4)	0.7	12
Aug.	1.1 (0.3)	16	4.2	1.2 (0.1)	1.6	1.5
Oct.	1.7 (0.1)	32	3.2	9.0 (1.1)	2.1	8
Mean	1.3 (0.2)	23 ± 4	3.8	5.2 (0.6)	1.1 ± 0.3	14

[a] Water column depth, 3m.
[b] Standard error, $n = 6$.

Table 4
Comparison of methods for estimating bacterial production using rate of incorporation of tritiated thymidine into DNA and ^{32}P into phospholipid[a]

	Thymidine Method ($mg\ C\ m^{-2}\ hr^{-1}$)	*Phospholipid Method* ($mg\ C\ m^{-2}\ hr^{-1}$)
Surface sediment	2.1	1.6
Cores (3–40 mm)	1.1 ± 0.2	1.6 ± 0.2[c]
Aerated slurry (3–40 mm)[b]	n.d.	24 ± 2.6

[a] Experiments were carried out simultaneously at midday in October 1982.
[b] Cores (3–40 mm depth) were combined and assayed in the same manner as aerated sediment with ^{32}P.
[c] Standard error, $n = 6$.

aerated, the rate of phospholipid synthesis was about 15 times faster than in undisturbed cores.

Sulfate Reduction

Although values for the rate of sulfate reduction in the sediments were rather variable, there was a noticeably higher rate of sulfate reduction around midday. Sulfide values were also higher around midday (Table 5). Integrated daily values for sulfate reduction were about 320 mg S m^{-2} day^{-1}, to a depth of 50 mm. Rates of sulfate reduction were very low below 50 mm.

Nitrogen Compounds

Ammonia concentration in interstitial water was lower in the presence of seagrass than in bare areas of the seagrass bed (Table 6). Concentrations did not vary significantly with time of day. Values for nitrate were less than 5 μM.

Primary amines were present in higher concentrations in the interstitial water surrounding the rhizomes (20–40 mm) than they were deeper in the sediment (Table 6). Concentrations were lowest at midnight. The C:N ratio of *Zostera* leaves varied little with

age, being in the range of 19 to 23 (Table 7). Values for C and N content of *Zostera* are given in Table 7.

Methanogenesis

Rates of methanogenesis were highest in the top 50 mm of sediment (Fig. 1). Time courses of *in vitro* methane production show an initial lag period that is longer in the deeper layers of sediment. Addition of H_2/CO_2 and acetate generally stimulated methane production. On an areal basis, the rate of methane pro-

Table 5
Sulfate reduction rates in Moreton Bay sediments[a]

A. As a Function of Depth

Depth (cm)	*Sulfate Reduction Rate ($mg/S\ m^{-2}\ day^{-1}$)*	
	Core 1	*Core 2*
0–5	266[b]	1123
5–10	10	32
10–15	30	6
15–20	3	3

B. As a Function of the Time of Day

Time of Day	*Sulfate Reduction Rate ($mg/S\ m^{-2}\ day^{-1}$)*
0600 to noon	67 ± 19[c]
Noon to 1800	128 ± 67
1830 to 0300	81 ± 36
0300 to 0900	80 ± 16
Total for day	336 (or 10.5 mmol $m^{-2}\ day^{-1}$)

[a] The sulfate concentration in the seawater and porewater was 28.0 mM and the temperature of all sediment depths varied from 25.5 to 27.0°C only. The pH of the seawater was 7.84, and the salinity was 36×10^{-3}. The sulfide concentration was very patchy and varied from 64 to 512 μg S g^{-1}; the average concentration was 256 μg S g^{-1}.

[b] The average of three samples.

[c] The SD for five replicates.

Table 6
Concentrations (μM) of primary amines and ammonia in interstitial water[a]

A. *Variation of Primary Amine Concentration with Time*

Depth (mm)	*Dawn*	*Midday*	*Dusk*	*Midnight*
20	116 ± 26[b]	70 ± 18	98 ± 29	56 ± 12
80	29 ± 4	24 ± 2	43 ± 5	43 ± 9
120	23 ± 5	20 ± 1	25 ± 5	24 ± 5
200	14 ± 2	18 ± 2	15 ± 1	20 ± 2

B. *Ammonia Concentration in Different Zones*

Depth	*Intertidal Zostera*	*Subtidal Zostera*	*Bare Sand*
20	27 ± 7[b]	35 ± 6	41 ± 8
80	23 ± 3	28 ± 8	71 ± 17
120	34 ± 8	34 ± 6	57 ± 13
200	63 ± 15	29 ± 1	62 ± 21

[a] Measured with fluorescamine, using glycine as a standard.
[b] Standard error, $n = 3$.

Table 7
Carbon and nitrogen content of *Zostera capricorni*[a]

Plant Components	*N*	*C*	*C:N*
Leaf and sheath, young	18.5 ± 0.6	355 ± 12	19
Leaf and sheath, mature	16.3 ± 0.9	358 ± 11	22
Leaf and sheath, senescent	14.0 ± 0.7	325 ± 5	23
Leaf and sheath, dead[b]	13.6 ± 1.1	284 ± 4	21
Rhizome and roots	7.1 ± 0.2	286 ± 23	40

[a] Values are mg g^{-1} dry weight, mean ± standard error, $n = 3$.
[b] Leaf brown but still attached to root.

duction calculated for the top 20 cm of sediment was 5.6 mg CH_4 m^{-2} day^{-1}.

Gas bubbles were observed emanating from the sediment. These were found to be mainly N_2 and O_2. Methane comprised a maximum of 0.03% of the gases; the highest values occurred in the early morning.

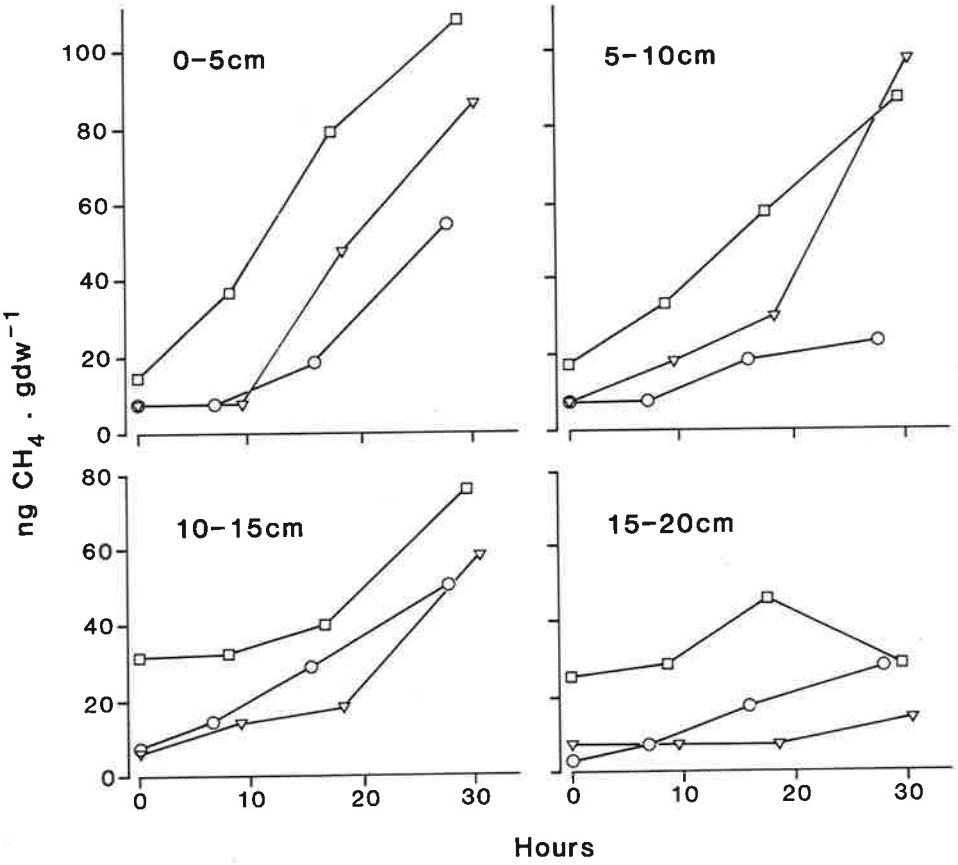

Fig. 1. Time courses of methane production at four depth intervals in seagrass sediments. Symbols: ○ = samples incubated without added substrate; ▽ = with 5 mM acetate, and □ = with H_2/CO_2.

Table 8
Extractable phospholipid ester-linked acyl and monohydroxy fatty acid methyl esters from Moreton Bay sediments

Fatty Acids	*Content (p moles g^{-1} dry wt)*	*OH–Fatty Acids*	*Content (p moles g^{-1} dry wt)*
i 14:0[a]	45 (44)[b]	A OH 15:0	8 (7)
i 15:0	250 (132)	B OH 15:0	36 (19)
a 15:0	287 (172)	A	24 (30)
15:0	450 (185)	2 OH 15:0	6 (2)
16:4w1*	30 (31)	B	15 (20)
i 16:0	121 (53)	2 OH 16:0	6 (2)
16:1w10*	28 (28)	3 OH 16:0	14 (20)
16:1w7	931 (392)	D	33 (26)
16:1w7 t	41 (29)	E	30 (20)
16:1w5*	36 (41)	F	28 (19)
16:1w13 t*	87 (43)	2 OH 17:0	40 (16)
16:0	2840 (965)	G	13 (7)
i 17:0*	85 (26)	H	8 (5)
17:1w6*	157 (129)	I	15 (12)
a 17:0	72 (19)	3 OH 18:0	2 (10)
17:1w6	46 (44)	12 OH 18:0	4 (4)
Cyc 17:0	24 (9)	3 OH 19:0	2 (2)
18:3w6	25 (24)	2 OH 21:0	47 (18)
18:2w6	166 (182)	A OH 23:0	4 (3)
18:3w3	33 (41)	3 OH 24:0	4 (4)
18:1w9	248 (194)	3 OH 25:0	3 (1)
18:1w7	468 (373)		
18:1w7 t	30 (18)		
18:0	448 (70)		
Cyc 19:0	30 (11)		
20:4w6	206 (306)		
20:5w3	114 (176)		
20:3w6	23 (20)		
22:6w3	25 (19)		
22:4w6	20 (15)		
22:0	100 (25)		
24:0	175 (46)		
Total	7640		420

[a] Fatty acids are designated as number of carbon atoms:number of double bonds with the position of the double bond nearest the methyl end of the molecule indicated. The prefixes anteiso (a) and iso (i) stand for a w terminal branching ethyl and methyl group, respectively, cyc indicates a cyclopropane ring, the number preceding the OH indicates the position of the hydroxyl from the carbonyl end of the molecule, a letter indicates the position is unknown; the

Analysis of Microbial Biomass and Community Structure

The aryl and monohydroxy fatty acids that are esterified to phospholipids in the sediment (after removal of plant material) are listed in Table 8. Much of the phospholipid in this sediment was present in bacteria, as shown by the larger amounts of fatty acids characteristic of bacteria and lower amounts of fatty acids found mainly in eukaryotes. The branched-chain 15 carbon acids, cis-vaccenic acid (18:1w7), 17 and 19 C cyclopropane acids, and the trans 16 monenoic branched and straight chain acids are all characteristic of bacteria. Long chain polyenoic acids are characteristic of eukaryotes and some blue-green algae. The hydroxy fatty acids are characteristic of bacteria (see Discussion). Palmitic acid (16:0) is found in all organisms. Values for muramic acid, nematode numbers, and various lipids used to measure biomass are given in Table 9. Plasmalogens, which are characteristic of anaerobic bacteria, constitute about 12% of the phospholipids in Moreton Bay.

Discussion

Seagrass Productivity

Most of the experimental work on bacterial production was carried out around *Zostera,* which predominated in the Pelican Banks area. In some parts of the same locality, two other species were present, *Halodule uninervis* and *Halophila ovalis,* comprising up to 20–30% of the seagrass biomass. We do not have any data on their productivity.

The estimates for production of *Zostera* agree well with productivity values for other species in other localities that were measured with marking techniques (see Zieman and Wetzel, 1980). These are minimum values, because root production and excretion of soluble organic matter are not included. Epiphyte

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suffix t indicates a trans configuration of the double bonds. * indicates fatty acids and hydroxy fatty acids for which no authentic standards were utilized in the identification. Letters indicate positions of hydroxy fatty acids of unknown structures. They are most likely to be unsaturated or branched structures.

[b] Data are given as the mean (standard deviation), $n = 7$.

Table 9
Comparisons of the measures of biomass and those of nematodes of the Moreton Bay sediments (27°34.95′S, 153°24.5′E) to those of Apalachee Bay, Florida (29°54′N, 84°27.5′W)[a]

Component	*Moreton Bay Queensland, Australia*	*Apalachee Bay*[b] *Florida, USA*
Biomass measures (nmoles/g dry wt)		
Extractable Phospholipid	15 (6)[b]	26 (7)
Plasmalogen Phospholipid	1.6 (0.7)[c]	0.49 (0.08)[c,d]
Muramic Acid	10 (5)[e]	22 (18)
Meiofauna		
Nematodes per 10 cm^2	1030 (330)[d]	1280 (386)
Ratios of fatty acids		
i + a 15:0/16:0	0.19 (0.08)[f]	0.11 (0.04)
cyc 17:0/16:0	0.01 (0.005)	0.006 (0.002)
18:1w7/18:1w9	2.0 (0.5)	1.81 (0.12)
Total polyenoic > 20/16:0	0.12 (0.17)	0.09 (0.03)

[a] Values are mean (standard deviation), $n = 20$, except where noted.
[b] Data taken from Findlay and White, 1983.
[c] Significant difference by analysis of variance ($P > 0.012$).
[d] Unpublished data.
[e] Data taken from Moriarty, 1980.
[f] Values are mean (standard deviation), $n = 7$.

production for the seagrass bed has not been measured, but should be included for comparisons with bacterial production. Penhale (1977) has estimated epiphyte production to be 18% of that of *Z. marina*. If we assume this applies to *Z. capricorni* and if we also use a value of 2% of net production being excreted as soluble organic matter (Penhale and Smith, 1977), the primary productivity of the seagrass bed would be about 1.6 g C m^{-2} day^{-1}. We may estimate, therefore, that about 30 mg C m^{-2} day^{-1} was excreted by the seagrasses and was available for use by the bacteria. This amount of C has to support both bacterial production and respiration (aerobic and anaerobic) or fermentation. Before comparing these values for carbon used by bacteria, we need to examine the methodology used to obtain them.

Bacterial Productivity Measured by Rates of DNA and Phospholipid Synthesis

The method for measuring bacterial growth rates using tritiated thymidine gives minimum values (Pollard and Moriarty, 1984). It gives values in agreement with other methods for growth rates in seawater (Fuhrman and Azam, 1982). All bacteria, however, need to use phosphate. In anaerobic sediments, the measurement of phospholipid synthesis may be a useful alternative method, but further work is needed to establish the accuracy of conversion factors. In particular, the rates of phospholipid synthesis are prone to disturbance (Table 4), and the effect of membrane turnover needs to be assessed, although it can probably be neglected in a 20-min experiment. Furthermore, the measurement of the specific activity of phosphate needs improvement. Our discussion on the comparison of the various rates measured here is not meant to be definitive, but a guide for developing hypotheses for future work.

Small cores were taken for the measurement of bacterial production, and as it was easier to sample sediment that was not covered with seagrass leaves or rhizomes, there was a tendency to avoid sediment close to plants. Recent progress now shows that isotope dilution of thymidine within bacterial cells can be prevented, and thus identical replicate subsamples would not be needed (Pollard and Moriarty, 1984). The value of 33 mg C m^{-2} day^{-1} for bacterial production in the sediment obtained with the thymidine method is, therefore, a conservative one. The results presented here show that growth rates (or production) of bacteria, measured with the thymidine method in the anaerobic sediment, are about 70% of the rates measured using ^{32}P (Table 4). For comparison with other measurements, a round figure of 60 mg C m^{-2} day^{-1} will be assumed. (Note that values for May were 78 [45 + 33] and October were 40 [23 + 17] [Table 2]).

In order to estimate the total carbon processed by the bacteria, the growth efficiency must be known. The growth efficiency of bacteria in aerobic culture may be over 50%, whereas anaerobic bacteria have lower efficiencies (Payne, 1970; Middleton and Lawrence, 1977). The average growth

efficiency of a natural community of heterotrophic bacteria may be lower than 50% (Koop et al., 1982). For the discussion here, we have assumed that a round figure for growth efficiency is 30%; thus the production of 60 mg C means that 200 mg C m^{-2} day^{-1} is required to support growth and respiration of bacteria in the sediment. Some of this organic matter would be oxidized to CO_2, and some would be fermented and eventually used by sulfate-reducing bacteria.

Methanogenesis

Methanogenesis was not a quantitatively significant process in these seagrass sediments; the value of 4.2 mg C m^{-2} day^{-1} represents no more than 2% of the total carbon flow through the microbial population. These *in vitro* rates for methanogenesis were somewhat higher than *in situ* rates in Florida seagrass sediments (Oremland, 1975). Rates of 0.7 mg CH_4 m^{-2} day^{-1} and 0.06–0.13 mg CH_4 m^{-2} day^{-1} were found in *Thalassia* and *Syringodium* beds, respectively. Rates of *in situ* methane production would be higher than *in vitro* rates if aerobic methane oxidation occurred.

Sulfate Reduction

As methanogenesis is not significant in these sediments, the sulfate-reducing bacteria are the final sink for carbon. Any carbon that is not respired by other groups of bacteria (e.g., aerobes and denitrifiers) is converted to CO_2 by the sulfate reducers. In many marine sediments, this is an important pathway (Jorgensen, 1980). Thus by measuring the rate of sulfate reduction, we can estimate the amount of carbon processed by anaerobic bacteria and compare that with the estimates obtained with the thymidine technique.

The rate of sulfate reduction was 320 mg S m^{-2} day^{-1}. If a stoichiometry of 1:2 is assumed for moles of $SO_4^=$ reduced to C oxidized, then the value of 320 mg S indicates that 240 mg C m^{-2} day^{-1} was oxidized. Acetate is probably the major electron donor in marine sediments and acetate-utilizing sulfate-reducing

bacteria are probably widespread (Widdel and Pfennig, 1981; Winfrey and Ward, 1983). The growth efficiency of these bacteria using acetate is about 12–16% (Middleton and Lawrence, 1977; Widdel and Pfennig, 1981). Thus about 30 mg C m^{-2} day^{-1} of new sulfate-reducing bacterial biomass would be formed during the oxidation of 240 mg C. From the results of the thymidine and phospholipid methods it was estimated that about 200 mg C was processed by the bacteria in the sediment. This is a good agreement between the two quite different procedures. The high value for the amount of carbon needed to support sulfate reduction shows that anaerobic processes are dominant in the sediment. More detailed studies are needed to determine the effects of spatial variability in the bacterial communities. A summary of carbon budget for the seagrass bed is given in Table 10.

Spatial variability in bacterial production was large and tended to mask seasonal variation. Growth rates were variable in cores of the anaerobic sediment (3–40 mm). Standard errors were generally around 10% (Table 1), but the range of variation was sometimes fivefold. There are two possible reasons for this: (1) patchiness in the distribution of active bacteria, and (2) variable distribution of label in the cores during the incubation. This level of variability is greater than that occurring seasonally (Table 2). Seasonal variation in the growth of *Zostera* was also low, and this was reflected in the bacterial growth rates. The doubling times of the bacteria give a much clearer indication of seasonal variation, as they are independent of spatial variation in biomass. Doubling times were 5 times longer in winter than in summer (Table 2).

Table 10
Summary of bacterial production

Zone	*Method*	*Total C Processed (mg C m^{-2} day^{-1})*	*New Bacterial Biomass (mg C m^{-2} day^{-1})*
Sediment	DNA synthesis	200	60
	Phospholipid synthesis	230	68
	Sulfate reduction	240	30
	Methanogenesis	4	?
Water column	DNA synthesis	77	23
Leaves	DNA synthesis	3	1

There is little net accumulation of organic matter in these sediments. Values for organic C and N are low and decrease from 2 mg C g^{-1} and 0.2 mg N g^{-1} in the surface sediment (0–10 mm) to about 1 mg C g^{-1} and 0.1 mg N g^{-1} at 200–210 mm depth (Moriarty, 1980). These values are consistent with the high rates of sulfate reduction and bacterial growth, which probably account for all the net primary production that is not exported from the seagrass bed. For comparison with other sulfate reduction data see Skyring et al. (1983).

Bacterial Production in the Water Column

In the water column, bacterial production was high over the seagrass beds (Table 3). Earlier work has shown that there is a diel cycle in bacterial growth rates, apparently coupled to release of organic matter during photosynthesis by the seagrasses (Moriarty and Pollard, 1982). There is a loose, flocculent sediment that occurs on the sediment surface and leaf surfaces in calm conditions, but which is readily suspended into the water column by wind-induced turbulence. This material contains many aggregated bacteria. Dissolved organic compounds excreted by the leaves would be absorbed by these bacteria and so contribute to the high growth rates observed on the sediment surface. In turbulent conditions, suspended aggregates would be exported from the seagrass bed during tidal exchange. The average bacterial production of 24 mg C m^{-2} day^{-1} in the water and on the leaves (Table 3) would require about 80 mg C m^{-2} day^{-1}, assuming that the efficiency of growth is 30%. The very low values for bacterial growth rates on the leaves are puzzling. They may be caused by release of a growth inhibitor from the leaves during sample handling (as reported by Moriarty and Pollard, 1982).

Summary of Bacterial Production

A comparison of the values for new bacterial biomass formed and the amount of carbon needed to support that bacterial production is presented in Table 10. These values may be compared

with the estimated net production of *Z. capricorni* and its epiphytes of 1.6 g C m^{-2} day^{-1}.

Combining the values for bacterial productivity measured with the thymidine technique and sulfate reduction, we estimate that a minimum of about 7% of net daily production was converted into new bacterial biomass. About 32% of net production would be needed to maintain this bacterial production if the efficiency of bacterial growth is assumed to be 30%. This value of 32% of production being used by bacteria suggests that decomposition of roots and leaves, as well as direct excretion of organic matter, are supporting bacterial growth. As there are few direct grazers on seagrass, all the net production must be either exported or decomposed by microorganisms. The results reported here show that more than a quarter of the net production was utilized by bacteria in the seagrass bed. Further work is needed to determine the proportions that are used by fungi and bacterial decomposition not yet accounted for in the seagrass beds, as well as the proportions that are exported or consumed directly.

Biochemical Analysis of the Microbial Biomass and Community Structure

The studies of rates of bacterial growth and turnover of carbon suggest that bacteria, particularly anaerobic bacteria, predominate in these sediments. Support for this conclusion is given by studies of the microbial community structure using measures of biomass.

Approximately 99% of the extractable fatty acids in eubacteria are found in their phospholipids. A number of techniques have been utilized to provide an indication of particular marker compounds or "signatures" of various groups of microorganisms (see White, 1983, and references therein).

Comparison of a sandbar in Moreton Bay to one in North Florida shows similarity in the total biomass (estimated as lipid phosphate), the bacterial content (estimated as muramic acid), and the total numbers of the most numerous meiofauna, the nematodes (Table 9). The ratios of branched 15:0 fatty acid of

cyclopropane 17:0 to the ubiquitous palmitic acid 16:0, and the ratio of 18:1w7/18:1w9, indicative of bacteria using the anaerobic biosynthetic pathway, were all similar in the two sediments; this indicates that the bacterial community structures were similar (Table 9). The microeukaryotic biomass, estimated as the total polyenoic fatty acids 20 carbon atoms long or longer relative to the palmitic acid, was also identical.

The only significant difference was the increased proportion of the anaerobic fermentative bacteria containing the vinyl ether plasmalogen bond in the plasmalogen. In sediments these lipids are, for all practical purposes, restricted to some of the anaerobic fermenters (White, 1983). Plasmalogens generally comprise around 30% of the phospholipids in those anaerobic bacteria that contain them (Kamio et al., 1969). The value of 12% plasmalogens in the Moreton Bay sediments means, therefore, that about 40% of the total phospholipids are present in strict anaerobic fermenters. As these comprise only a portion of the anaerobes, it is clear that anaerobes predominate in these sediments.

Bacterial biomass in these sediments, estimated from muramic acid content, was about 200 $\mu g\ Cg^{-1}$ dry weight (Moriarty, 1980). A similar value was obtained from the phospholipid content (Table 9), using a conversion factor of 50 μmol g dry weight (White et al., 1979*c*). As phospholipids are present in all organisms, this similarity suggests that bacteria comprised most of the biomass in these sediments. The low ratio of polyenoic fatty acids to palmitic acid also indicates that eukaryotic organisms were not numerous. The ratios of the bacterial markers (i and a 15:0, 15:0 and cyclopropane 17:0, 19:0) to palmitic acid are typical of sediments where bacteria are the predominant components of the biomass. Although more work is needed on the identification of fatty acids and ratios of fatty acids typical of particular groups of bacteria, the results here suggest that most of the bacteria in the Moreton Bay sediment were anaerobes. Besides the plasmalogen content, the presence of the anaerobic biosynthetic pathway is probably indicative of all anaerobes, including facultative anaerobes, and the monohydroxy fatty acids in phospholipids are found particularly in sulfate reducers.

These conclusions from biomass studies agree with the mea-

surement of rate processes, as sulfate reduction was shown to account for a high proportion of organic matter decomposition.

Dependence of Plant Production on Ammonia

The release of amino acids by seagrasses has been reported by Jorgensen et al. (1981). The results reported here also indicate that amino acids may be excreted by the seagrasses in Moreton Bay, but we have not yet quantified this process. Studies are underway to determine rates of ammonia turnover in Moreton Bay seagrass beds. The values for ammonia concentration that are lower around the seagrass roots than in deeper sediments (20 cm), or in areas with no plants, indicate that *Z. capricorni* is taking up ammonia from the sediment.

Measurements of the net production of seagrass and its nitrogen content can be used to estimate the amount of nitrogen taken up from the sediment. The amount of ammonia needed by the *Zostera* to maintain a leaf and sheath production of 1.07 g C m^{-2} day^{-1} is 56 mg N m^{-2} day^{-1}. A further 5 mg N m^{-2} day^{-1} would be needed for the rhizome production. Thus a minimum of 61 mg N m^{-2} day^{-1} is needed to support the seagrass production; the actual values will be higher because excretion and root production are not included. The ammonia content of subtidal sediment is about 35 mg N m^{-2} in the 0–20 cm depth zone, where most roots occur. As the ammonia concentration does not vary, the turnover time for the ammonia pool would be twice a day. The turnover time could well be faster, because losses due to nitrification and diffusion into the water column also occur.

The results reported here show that at least 253 mg C m^{-2} day^{-1} is decomposed by bacteria. As the average C:N ratio in the sediment is about 10 (Moriarty, 1980), 25 mg N m^{-2} day^{-1} would be released. Even if all this amount became available to the seagrass (after the bacteria are recycled through a grazing food chain), it would not be sufficient to supply the needs of the seagrass. Although the shortfall could be made up partly by nitrogen fixation, it seems likely that the estimate of the amount of organic matter decomposed by bacteria is too low. Bacterial productivity may be limited by N. As shown above, the total net production of bacteria in the sediment is about 60 mg C m^{-2}

day^{-1}. At a C:N ratio of 5, they would require 12 mg N m^{-2} day^{-1}, which is only a little less than that produced as ammonia. There would be competition between seagrasses and both heterotrophic and autotrophic bacteria for ammonia.

Bacterial productivity in these sediments may be two or three times higher than that reported here. Further work will need to examine the bacterial production that is associated with decaying leaves and roots, and the interdependence of C and N cycles.

Acknowledgments

We are grateful to Ted Wassenberg for assistance with field work; to Janet S. Nickels, Glen A. Smith, and Jeffrey H. Parker for carrying out lipid analyses; and to the late Michael R. Reed for technical assistance. The Baas Becking Laboratory is supported by the Commonwealth Scientific and Industrial Research Organization, the Bureau of Mineral Resources, and the Australian Mineral Industries Research Association, Ltd. W. G. Hunt was supported with funds from a Marine Sciences and Technologies Grant. David C. White is grateful to the Division of Fisheries Research, CSIRO, for providing travel funds to Australia.

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J. Exp. Mar. Biol. Ecol., 1986, Vol. 96, pp. 115–126
Elsevier

JEM 639

EXUDATION OF ORGANIC CARBON BY THE SEAGRASS *HALODULE WRIGHTII* Aschers. AND ITS EFFECT ON BACTERIAL GROWTH IN THE SEDIMENT

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(Received 14 September 1985; revision received 31 December 1985; accepted 10 January 1986)

Abstract: Between 6 and 28% (mean: 14%) of ^{14}C fixed by the leaves of *Halodule wrightii* Aschers. was translocated to the rhizomes and roots within 6 h. In the same time period 6 to 17% (mean: 11%) of total fixed ^{14}C was exuded into the sediment. About 1% was excreted into the water column. Bacterial production was determined using two methods: the rate of tritiated thymidine incorporation into DNA and the rate of ^{32}P incorporation into phospholipid. Bacterial production was 180 to 190 mg $C \cdot m^{-2} \cdot day^{-1}$ and was probably dependent on root decomposition as well as exudation. From the results of lipid analyses and synthesis it was concluded that bacteria were utilizing all the exuded organic ^{14}C. Most bacterial production was in the top 20 mm of sediment, which was the zone with the greatest root and rhizome biomass. Most of the ^{14}C exudate was also found in this zone.

Key words: seagrass; exudates; bacteria; thymidine; phospholipid; *Halodule wrightii*

INTRODUCTION

Seagrasses are major primary producers in many shallow tropical and temperate coastal regions. Their productivity is high compared with other plant communities, yet only a few animals feed on them directly (Zieman & Wetzel, 1980; Zieman *et al.*, 1984). Most animals that include seagrass leaves in their diet are dependent on epiphytic microalgae (Kitting, 1984; Morgan & Kitting, 1984) or a detrital food chain based on seagrass production and mediated by microorganisms (Moriarty, 1976; Klug, 1980; Ogden, 1980). Bacterial productivities in the sediment and water column of seagrass beds are high and show diel variations that imply a link with excretion or exudation of organic matter by the seagrasses during photosynthesis (Moriarty & Pollard, 1982; Moriarty *et al.*, 1985a).

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Several investigations of organic carbon excretion by seagrasses have been reported (Brylinsky, 1977; Penhale & Smith, 1977; Wetzel & Penhale, 1979), but the fate of organic compounds released into sediments from seagrass root and rhizomes has not been reported. New techniques for estimating microbial growth rates in sediments allow comparison of carbon release by seagrasses with carbon demand by microbial growth in sediments (Moriarty & Pollard, 1981, 1982; Moriarty *et al.*, 1985b).

We report here experiments that quantify the amount of photosynthetically fixed carbon released from leaves, roots and rhizomes of the seagrass *Halodule wrightii* Aschers., and that relate the amount of carbon released from the plant to that incorporated into bacterial biomass in both the water column and the sediments.

Materials and Methods

SITE STUDIED AND SAMPLE COLLECTION

H. wrightii was collected from a sand bank near the Florida State University Marine Laboratory (29°54′N : 84°29′W) between July and September, 1983.

For the studies with ^{14}C, cores (20 cm diameter) of sediment containing the seagrass were taken in the field with a corer made of polyvinyl chloride plastic (PVC) tubing. Cores were stored in shaded tanks of running sea water. Dead leaves were removed in order to minimize the contribution of epiphyte primary production. A separate set of cores was collected for the study of bacterial production, which was measured immediately after the cores were collected. Small cores (5-ml plastic syringes with their bases cut off) were taken at random amongst the seagrass plants in a 20-cm core. Different depth intervals were sampled after the large core was sliced at the required depth and the upper portion removed.

Bacterial production was measured in water column samples that were collected over the seagrass bed.

MEASUREMENT OF CARBON FIXATION

Seagrass production was determined using the experimental apparatus shown in Fig. 1 and a method employing ^{14}C similar to that of Bittaker & Iverson (1976). A separate sub-core for each experiment was removed from a 20-cm core with the lower section of the incubation cylinder. The cylinder was filled by allowing water to enter the cylinder slowly through a side port, thus minimizing turbidity inside the cylinder. Air escaped through a hole cut in the top plate. The side arm was sealed with a serum cap and the top hole was sealed with a stopper. After sediment had settled inside the cylinder, [^{14}C]$NaHCO_3$ (0.1 mCi, carrier free) was added to the cylinder through the serum cap septum. The cylinder contents were stirred periodically throughout the incubation period. A small portion of water was removed with a syringe for determination of the initial ^{14}C concentration in the cylinder by the method of Iverson *et al.* (1976).

Sea water flowed through the aquarium during the incubation period. Irradiation was measured with a submarine quanta sensor (LI COR, Inc., model 192 SB). Irradiation was either natural, in which case the energy intensity was kept at $\approx 1000\ \mu E \cdot m^{-2} \cdot s^{-1}$ with shade cloth, or General Electric PAR lamps were used at the same intensity. Saturating light energy for photosynthesis in *H. wrightii* is $\approx 1000\ \mu E \cdot m^{-2} \cdot s^{-1}$ (Williams & McRoy, 1982; Morgan & Kitting, 1984).

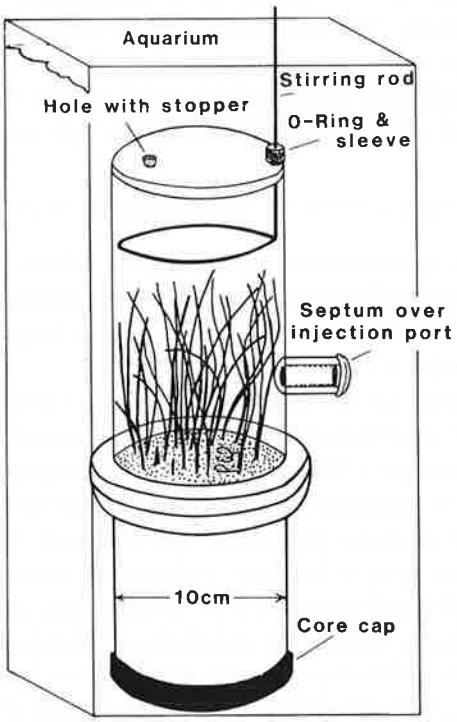

Fig. 1. Experimental apparatus, consisting of a clear acrylic incubation cylinder: the upper and lower sections were clamped together and sealed by an O-ring placed between the flanges.

Total carbon dioxide content of the water contained in the cylinder was determined by the method of Strickland & Parsons (1972).

A time course of the rate of labelling of leaves, roots and sediment was carried out with duplicate samples for each time period, using small cores in 25-mm diameter acrylic tubes.

DETERMINATION OF LEAF CARBON EXCRETION

The method of McKinley *et al.* (1977) was used with some modifications. Immediately after each incubation was stopped, 30 ml of water were taken from the

experimental cylinder with a 30-ml syringe equipped with a cannula. A filter holder with a 0.2-μm polycarbonate filter was placed on the end of the syringe and 15 ml of water were filtered into each of two plastic liquid scintillation vials. One ml of concentrated acetic acid was added to each vial after which the vial contents were frozen on dry ice and then freeze-dried. Water (2 ml) was added to dissolve the salts. Liquid scintillation cocktail (17 ml) was added and the radioactivity was determined with a liquid scintillation counter.

The filter was treated with several drops of concentrated acetic acid to remove inorganic ^{14}C. The organic ^{14}C was then dissolved with "Protosol" (New England Nuclear). Radioactivity was measured as described above.

Dark controls were prepared using aluminium foil to cover the incubation cylinders. Values for dark fixation of ^{14}C were subtracted from light values.

PROCESSING OF ^{14}C-LABELLED PLANT AND SEDIMENT MATERIAL

The water within the cylinder was decanted and its volume measured. The cylinder top was removed and the leaf material was cut at the sediment/water interface with a pair of stainless steel scissors and placed in a plastic bag. The leaf material was immediately frozen on dry ice.

The core cap was removed and then the experimental core was extruded from the cylinder. Only the top 5 cm of sediment was collected, as an earlier experiment on 20-cm deep sections had shown that $> 90\%$ of the radioactivity was in the top 5 cm. The plant material was removed by agitating it in sea water (≈ 200 ml) until sediment and root debris had fallen away from the living roots and rhizomes. Only a small amount of apparently live roots remained in the mass of dead root debris. The root and rhizome mass was washed in two changes of 100 ml of dilute sea water (1.5%). Roots and rhizomes were placed in one plastic bag and detritus in another. Both bags were frozen on dry ice. The sediment and wash water were acidified with 3 ml of concentrated acetic or *ortho*-phosphoric acid and transferred to large plastic bags and sealed. The acidified sediment and wash water were frozen on dry ice. Dead root matter was separated from the sediment by sieving through a 1-mm screen.

The radioactivity of ^{14}C was measured after the organic matter was dissolved with a tissue solubiliser. All materials were freeze-dried and weighed. Subsamples of leaves, roots and rhizomes, detritus, and sediment were pulverized, weighed and placed into glass liquid scintillation vials. About 2 mg of leaf, 5–10 mg of other plant material and ≈ 200 mg of sediment were used as subsample weights. One hundred μl of water were added to the plant material and 200 μl to the sediment. Five hundred μl of "Protosol" (New England Nuclear) were added to all samples, which were then placed in an oven at 45 °C for 24 h to accelerate solubilization.

After solubilization, plant material was bleached either under a GE 1000W PAR lamp for 30 min or in natural sunlight for several hours. Concentrated acetic acid (200 μl) and 10 ml of liquid scintillation cocktail were added to each sample. Sample ^{14}C activity was

measured with a liquid scintillation counter after particulate material had settled to the bottom of the LSC vials. Internal standardization indicated that quench due to the colour remaining in solution after processing was corrected with the external standard channels ratio calibration method.

Beer *et al.* (1982) have shown that tissue solubiliser, a quaternary ammonium compound, can be used to extract all fixed organic ^{14}C from plant tissue. In order to check that this was the case with *H. wrightii*, some radioactive leaf samples were combusted to yield $^{14}CO_2$, which was collected and the radioactivity determined as described by Bittaker & Iverson (1976). No significant difference was found between results with the combustion method and the one described above using the tissue solubiliser.

The incorporation of ^{14}C-labelled organic compounds into lipids in the sediment was examined. Radioactive lipids were extracted from freeze-dried sediment samples and the phospholipid, glycolipid, and neutral lipids were separated as described by King *et al.* (1977). The solvents were removed by evaporation at 40 °C and the ^{14}C radioactivity was measured in a liquid scintillation counter.

BACTERIAL PRODUCTION

Bacterial growth rates were determined using two methods. One method was based on the rate of tritiated thymidine incorporation into DNA (Moriarty & Pollard, 1981, 1982; Pollard & Moriarty, 1984). The other method, based on the measurement of rates of phospholipid synthesis with ^{32}P, was carried out as described by Moriarty *et al.* (1985b). Total phospholipid concentrations were determined as described by White *et al.* (1979).

RESULTS

Photosynthesis occurred at a linear rate from at least 30 min after the start of an incubation, indicating that the labelled and unlabelled pools of CO_2 in the leaf tissue rapidly reached equilibrium (Fig. 2). Translocation of the fixed carbon to the rhizomes and roots was particularly marked after 2 h. The lower rate of radioactive labelling in the first hour may be the result of fixed carbon moving into the leaf bases, which were included with the rhizomes. A similar lag period of ≈ 1.5 to 2 h occurred before fixed carbon was exuded into the sediment concurrently with translocation into the rhizomes and roots. The results were rather variable, but this was expected, because each sample was taken from naturally growing groups of plants of varying age and physiological condition.

The proportion of fixed radioactive carbon that was translocated to the rhizomes and roots within 6 h of photosynthesis varied between 6 and 28% of the total (Table I). A similar proportion was exuded into the sediment, although on average, a larger proportion was present in the rhizomes and roots (Table I). About half of the fixed ^{14}C

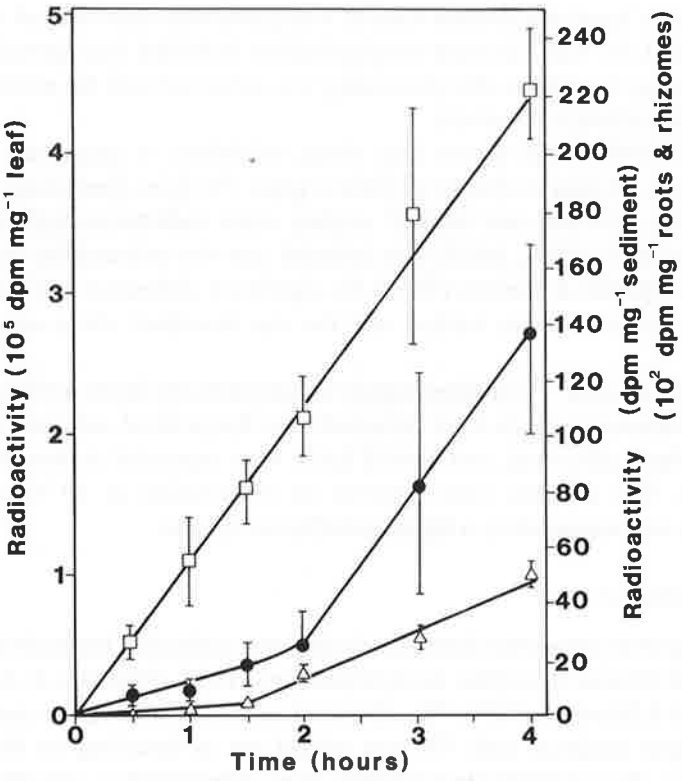

Fig. 2. Time course for fixation of ^{14}C in leaves and translocation to rhizomes and roots and exudation into sediment: values for dark controls were $<1\%$ of values in the light, and have been subtracted; □, leaf; ●, rhizomes and roots; △, sediment.

TABLE I

Translocation and release of fixed ^{14}C as a percentage of total fixed ^{14}C ($\pm$ range) in the top 5 cm of each core: DOC, dissolved organic carbon; POC, particulate organic carbon; ND, not determined.

Incubation time (h)	Number of samples	Roots and rhizomes	Sediment	Water	
				DOC	POC
3	2	6.3 ± 2.3	17.1 ± 10.1	0.5 ± 0.2	1.2 ± 0.8
3	1	26.0	8.0	0.3	0.3
3	3	13.0 ± 2.0	10.0 ± 3.0	ND	ND
4	2	7.6 ± 1.2	13.5 ± 1.5	0.3	ND
6	1	28.0	6.0	0.4	0.3
Mean of all samples ($\pm$ SD):		14 ± 11	11 ± 4	0.5 ± 0.2	0.08 ± 0.6

in the sediment was associated with the dead root material. Between 1 and 2% of the labelled fixed carbon was excreted into the water column, and about half of this was found in small particles (Table II).

Bacterial production varied from 5 to 12 mg $C \cdot m^{-2} \cdot h^{-1}$, with higher values occurring during the day (Fig. 3). At any one time, the values from different cores were variable, an indication that growing bacteria were patchily distributed in the sediment. For many of the time periods studied, there was little difference between the results of the phospholipid and thymidine methods for determining bacterial production (Fig. 3).

Most bacterial production occurred in the upper 20 mm of sediment (Table II). The greatest mass of rhizomes and roots was also in this zone. Over 85% of the plant

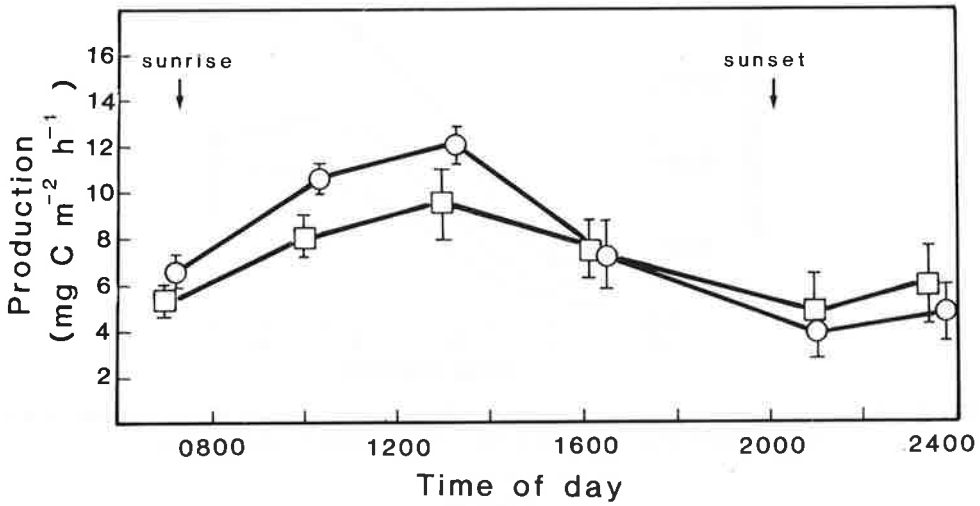

Fig. 3. Diel variation in bacterial production in sediment (0–40 mm) around *Halodule wrightii*, measured using two techniques: bars show standard errors for six replicate cores; ○, phospholipid method; □, thymidine method.

TABLE II

Depth distribution in sediment of root and rhizome biomass and exudates and of bacterial production measured with the phospholipid method: range of variation and number of samples are shown; exudation was measured at 40-mm intervals only.

Depth (mm)	Root and rhizome biomass (g dry wt · m^{-2}) $n = 2$	Total phospholipid (nmol · g dry wt^{-1}) $n = 2$	Bacterial production (mg $C \cdot m^{-2} \cdot day^{-1}$) $n = 5$	^{14}C exudate (% of total) $n = 4$
0– 20	365 ± 50	61 ± 3	8.4 ± 1.0	
20– 40	93 ± 38	52 ± 12	1.2 ± 0.1	90 ± 2
40– 60	60 ± 20	38 ± 2	0.1 ± 0.1	10 ± 2 (40–80 mm)
100–120	10 ± 5	21 ± 2	0.36 ± 0.2	<0.3 (80–120 mm)

material was within the upper 40 mm of sediment and most of the radioactive carbon was exuded in this zone (Table II). Thus, there was a good correlation between the depth distribution of bacterial production and exudation of organic matter by the seagrass. The rates at which lipids were labelled with ^{14}C in the sediment followed a similar pattern to the rate of exudation, with a lag of ≈ 1.5 to 2 h (Fig. 4). Of the radioactive organic matter exuded into the sediment $\approx 6.5\%$ was in lipids, including 1.5% in phospholipids.

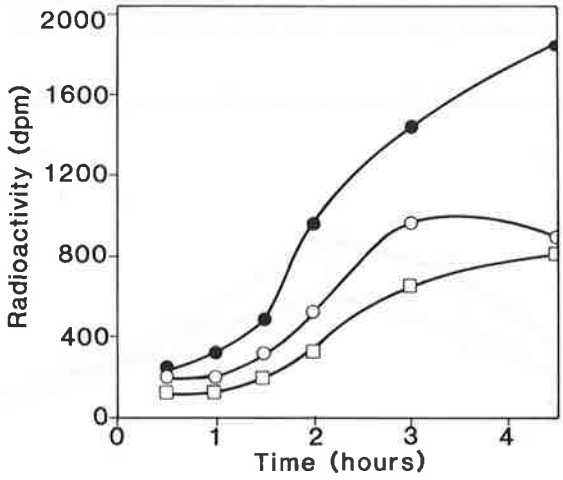

Fig. 4. Time course for labelling of lipids with organic ^{14}C in cores of sediment around *Halodule wrightii* during photosynthesis: ●, neutral lipid; ○, glycolipid; □, phospholipid.

TABLE III

Comparison of bacterial production and some components of seagrass production (± SE, *n*, number of samples): bacterial production in sediment was estimated by integrating values shown in Fig. 3.

Material	Production (mg C · m^{-2} · day^{-1})
Seagrass	
Total	1340 ± 300, *n* = 12
Rhizomes and roots	190 ± 40, *n* = 11
Exudate in sediment (0–40 mm)	150 ± 60, *n* = 11
Exudate in water	80 ± 10, *n* = 11
Bacteria	
Sediment (0–40 mm)	
Phospholipid method	180
Thymidine method	190
Water column	
(6 June)	20 ± 2, *n* = 9
(30 June)	12 ± 1, *n* = 3

Daily bacterial production in the upper 40 mm of sediment was $\approx 14\%$ of seagrass production (Table III). In the water, bacterial production was approximately equal to the production of carbon released into the water by *H. wrightii*. The extensive beds of other species of seagrasses in the same area would also have affected bacterial growth in the water column.

Discussion

Bacterial growth in the sediment and water around seagrasses seems to be dependent on organic matter released by them, but it is difficult to quantify the exact relationships. The work reported here shows that most organic matter was released into the surface (upper 20 mm) of sediment rather than to the water column. The correspondingly high production of bacteria in the surface sediment suggests that the bacteria were using organic compounds released from the seagrass.

The total bacterial production, estimated at 190 mg $C \cdot m^{-2} \cdot day^{-1}$ (Table III), would have required $\approx 45\%$ of the seagrass production if the bacteria are assumed to be 30% efficient in their utilization of the organic matter. The implications for other processes, such as nitrogen fixation and for food web dynamics, are important and deserve further study.

As only $\approx 10\%$ of the labelled fixed carbon was released, bacteria were obviously dependent on more than exudates and secretions released during photosynthesis. The diurnal increase in bacterial production (≈ 20 mg $C \cdot m^{-2} \cdot day^{-1}$; Fig. 3), would, however, account for the exudate production of 150 mg $C \cdot m^{-2} \cdot day^{-1}$ if the bacteria were 20 to 30% efficient.

The phospholipid data indicate that bacteria were utilizing the radioactive carbon soon after it was released. Phospholipids are major components of membranes, and their synthesis in these sediments is due primarily to bacterial growth (Moriarty *et al.*, 1985b). If we assume that 50 μmol phospholipid are equivalent to 1 g dry wt of bacteria (White *et al.*, 1979), that the average molecular weight of the phospholipids is 500, and that they are synthesized for 10 h at the rate shown in Fig. 4, we can estimate that 25 mg $C \cdot m^{-2} \cdot day^{-1}$ of bacterial biomass was formed from the radioactive carbon released by the seagrass. Thus it seems likely that much of the labelled organic carbon released into the sediment was used by bacteria for growth. One further assumption is that the specific radioactivity of exudates was the same as that of the CO_2 fixed in the leaves. (This would not be the case initially, but as it is too difficult to determine the actual specific activity, we have made the simplest assumption necessary for these calculations).

Other sources of organic matter for the bacteria would be derived from lysis and decomposition of old roots. The sediments contained detritus from old roots and rhizomes that was approximately equal in mass to the mass of living roots and rhizomes. It is likely that this plant material decomposes over more than one season, as winter

water temperatures are low. The studies reported here were carried out in summer, when the high water temperatures would promote decomposition of both the previous season's growth and new detritus.

There is a diurnal increase and nocturnal decrease in bacterial production around *Zostera capricorni* which may be a response to release of organic matter fixed during photosynthesis (Moriarty & Pollard, 1982). Diel variations in rates of nitrogen fixation in the root zone of a seagrass were probably related to photosynthesis and exudation of organic matter (Capone & Taylor, 1980). A similar pattern of changes in bacterial growth occurred in the sediment around *Halodule wrightii* and is probably a response to the exudation of organic matter (Fig. 3). The estimates of the total production of carbon exuded or secreted from the plants (Table III) may not be correct, if exudation continues throughout the night, and if the specific radioactivity is very different from that of the original CO_2. The diurnal increase in bacterial production implies, however, that less exudation occurs at night; further work on these processes is underway.

It has been suggested that recycling of CO_2 from the lacunae may dilute the $^{14}CO_2$ and alter the specific activity (Zieman & Wetzel, 1980). This is unlikely because gases are not stored in the lacunae, but flow down to and out from the roots continually during photosynthesis (Roberts *et al.*, 1984; Smith *et al.*, 1984; Thursby, 1984). The rate of incorporation of ^{14}C into leaf tissue was linear from the shortest time interval measured, and it extrapolated through zero (Fig. 2). If there were recycling of CO_2 and thus slow equilibration of $^{14}CO_2$ with pools in the leaves, a linear rate would not have been observed. Furthermore, Bittaker & Iverson (1976) showed that effects due to recycling of CO_2 were insignificant in the measurement of seagrass productivity.

If the specific activity of organic carbon translocated into roots and rhizomes is the same as that of the CO_2 fixed, the value of 190 mg $C \cdot m^{-2} \cdot day^{-1}$ (Table III) indicates that $\approx 15\%$ of primary production sustains the growth of roots and rhizomes, which have a correspondingly slower turnover than leaves. Longer term studies with pulse labelling and uniform labelling are needed for a complete study of these processes. Translocation and exudation of the radioactive fixed carbon appear to be closely linked, as the rates at which each occurred were similar (Fig. 2). The time period of 1.5–2 h for significant labelling of rhizomes, roots and exudates is similar to that reported for wheat seedlings (McDougall & Rovira, 1965; McDougall, 1970) and for translocation in *Zostera americana* (Harrison, 1978). The low rate of labelling of roots, rhizomes and sediment in the first hour could have been due to dark fixation of $^{14}CO_2$ transported in the lacunae.

The sites of exudation or secretion may be around the leaf bases or at the junction of roots with the rhizome, as well as near the root tips. This is a possible explanation for exudation and bacterial growth occurring mainly in the rhizome zone (upper 20 mm). It is unlikely that exudation is a wounding response, because a higher rate of exudation would have been expected in the small cores used for the time course (Fig. 2). Furthermore, in experiments with *Z. capricorni*, rates of exudation did not vary in cores of seagrass that were tested immediately or were left for 1 to 14 days (Roberts & Moriarty, unpubl. results).

The amount of carbon exuded or excreted into the water column through the leaves (or from algae on the leaves) was low in comparison with that exuded into the sediment. The values found here for excretion from leaves are similar to those reported for *Z. marina* and its epiphytes (0.9 to 2%) by Penhale & Smith (1977). The water column values are not total but net values, after absorption or uptake of organic matter by bacteria on leaf surfaces. Under the microscope, few bacteria were seen on the living portions of leaves, but there were many on the necrotic sections. Although bacterial production on leaves was not measured here, it is likely to be insignificant compared with that in the sediments, as was found with *Z. capricorni* (Moriarty *et al.*, 1985b).

Respiration by bacteria will contribute to under-estimates of the amount of carbon released into the water and sediment. Further work is needed to study the magnitude of this effect. If the generation times of bacteria in these sediments and water are similar to those reported for other seagrass beds (Moriarty *et al.*, 1985a), the time intervals of 2 to 4 h for these experiments are only a small fraction of the generation times. Thus, the loss of $^{14}CO_2$ by respiration would be small. Alternatively, if bacteria grow more rapidly in the rhizosphere than elsewhere, respiratory losses may be large. Further study is needed on this problem.

Acknowledgements

The authors thank the National Science Foundation of the United States for sponsoring portions of this work with grant numbers OCE-77-27052 and OCE-80-19757; the Florida State University Foundation for providing travel funds for D. J. W. Moriarty; and CSIRO Australia for awarding an overseas study grant to P. C. Pollard.

This work was carried out at Florida State University and we are grateful to Dr. D. C. White for helpful discussions and providing facilities in his laboratory. We thank J. S. Nickels for the phospholipid analyses.

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Marine Ecology - Progress Series (1990) in press

Primary and Bacterial Productivity of Tropical Seagrass Communities in the Gulf of Carpentaria, Australia

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ABSTRACT: Plant and bacterial productivities were compared at different seasons in 3 communities of seagrass in the Gulf of Carpentaria, Australia, in 1985. Seagrass biomass and shoot density were very variable, with no seasonal trend. The gross primary productivity of the seagrasses in each of the communities (Syringodium isoetifolium with Cymodocea serrulata in a bay, Halodule uninervis at a river mouth, and Thalassia hemprichii with Cymodocea rotundata on a reef flat) were determined seasonally from rates of lacunal gas production. Productivity varied seasonally, being lowest in winter (July). Values ranged from 0.6 to 1.0 g C m^{-2} day^{-1} for C. serrulata; 0.3 to 8.1 for S. isoetifolium; 0.9 to 3.5 for H. uninervis; 0.2 to 0.4 for T. hemprichii and 0.1 to 1.0 for C. rotundata. Gross community primary productivity, measured from diurnal changes in oxygen concentration in the water column, ranged from 3.3 in winter to 9.3 g C m^{-2} day^{-1} in summer at the bay site and 2.7 to 4 at the river mouth and 3.3 to 8.4 on the reef flat. Bacterial productivity was determined using the rate of tritiated thymidine incorporation into DNA. Most (90 to 95%) bacterial biomass was produced in the sediment. Between winter and summer, total bacterial productivity (including water column) ranged from 1.0 to 4.8 g C m^{-2} day^{-1} in the bay and from 0.6 to 2.5 at the river mouth and 0.6 to 3.7 on the reef flat. Bacterial productivity averaged 43% (range 10% to 90%) of gross primary productivity, and thus would account for about half of the primary production if their growth efficiency were 50%. Animals appeared to have an impact on bacteria at the sediment surface in summer, when specific growth rates and productivity were high, but numbers were low.

INTRODUCTION

In pelagic systems, zooplankton may graze at least 50% and up to 100% of primary production (Valiela, 1984). In benthic coastal ecosystems with vascular plants, however, bacteria are the dominant consumers of primary production by the macrophytes, because most animals cannot digest the fibrous plant material, which also has a low protein content (Mann, 1988). Thus where seagrasses are major primary producers, they may be important in marine food chains, but indirectly. Most animals that utilise seagrass production are dependent on decomposition and a detrital food chain or on epiphytic and benthic microalgae in seagrass beds (Klug, 1980; Moriarty, 1976; Morgan and Kitting, 1984). Quantitative studies on the cycling of carbon from primary producers through bacteria to animals have now become possible with the development of methods for quantifying bacterial production, based on the incorporation thymidine into DNA (Moriarty, 1986). Bacterial productivity in temperate seagrass beds is linked to seagrass productivity (Moriarty and Pollard, 1982; Moriarty et al., 1985). It is difficult to determine the contribution of bacteria to carbon cycling and respiration in sediment in the presence of other organisms. With the thymidine method, one can estimate bacterial productivity, i.e. the rate at which new bacterial biomass is formed, and then calculate the rate of carbon cycling by bacteria if their growth efficiency is known.

Few studies have been made on productivity of seagrasses in the tropical Indo-Pacific region. Recently, however, the seagrass beds in Papua New Guinea have been studied by Brouns (1985; 1987a, b) and Brouns and Heijs (1986). Production averaged 6.4 g ash-free dry weight m^{-2} day^{-1} over a year, with little significant seasonal variation in a mixed seagrass bed of six species including the five that were studied in this report (Brouns, 1987b). In Indonesia, Lindeboom and Sandee (1989) reported that gross productivity of a Halodule uninervis community ranged from 2.5 to 4.1 g C m^{-2} day^{-1} and that of a Thalassia hemprichii community ranged from 1.2 to g C m^{-2} day^{-1} in October. Net productivity ranged from 0.06 to 1.1 g C m^{-2} day^{-1}. Thus plant productivity in these tropical communities is high and we would expect bacterial productivity to be high also.

We selected three communities in the Gulf of Carpentaria, Australia, for studies on the relationships between primary and bacterial productivity. The productivity of each community, and of seagrasses and bacteria in small sections of the communities, were studied to provide some insight into carbon cycling during each season, especially in the sediment.

MATERIALS AND METHODS

Study sites

The study sites were at Groote Eylandt, which is a continental island with similar geomorphology to the mainland nearby; the climate is monsoonal with rain occurring mostly between December and April (Fig. 1). The first sampling site was near a river mouth. It was a monospecific stand of Halodule uninervis (Forssk.) Aschers. on a sandy sediment in a community of H. uninervis and Halophila ovalis inshore and Syringodium isoetifolium (Aschers.) Dandy and Cymodocea serrulata (R.Br.) Aschers. et Magnus offshore (site group E described by Poiner et al., (1987). The second sampling site was a bay with mostly S. isoetifolium and some C. serrulata on a silty clay sediment (site group F descibed by Poiner et al., (1987)). There were no macroalgae present at that site. The third sampling site was a reef flat fringed on the landward side by mangroves and on the seaward side by a coral reef; macroalgae comprised a substantial proportion of plant biomass there. The seagrasses Thalassia hemprichii (Ehrenb.) Aschers. and Cymodocea rotundata Ehrenb. et Hempr. ex Ashers. were present (described by Poiner et al., (1987) as site group C). The sediment was a muddy silt with some coral rubble, 4 to 7 cm deep on a hard calcareous substrate. At each study site a net or cage 4 to 6 m^2, with a mesh of 80 to 100 mm width, was used to protect research workers from crocodiles and sharks.

Studies were carried out in January, May, July and October, 1985. Water temperatures were: January - mean: 32°C, maximum: 34°C (38°C on the reef flat); May - mean: 30°C, maximum: 31°C; July - mean: 23°C, maximum: 24°C; October - mean: 29°C, maximum: 33°C. The highest temperatures occurred over the reef flat. The light intensity in the seagrass beds was measured with a submersible integrating light meter (Biospherical Instruments Inc., Model QSI-140). Maxima were 1500 $\mu E\ m^{-2}\ s^{-1}$ at the river mouth and bay sites and 2000 at the reef flat in January; 1200 in May; 900 in July and 2400 in October. Day lengths (light intensity of > 200 $\mu E\ m^{-2}\ s^{-1}$) were 11 h in January, 10 h in May, 9.7 h in July and 10.5 h in October.

All temperatures and oxygen concentrations in the water column and bottles were measured with a submersible electrode that produces oxygen at the same rate at which it is consumed and, therefore, the water does not require stirring (Leeds and Northrup, Model 8500).

Biomass and Shoot Density

Six replicate cores (10 cm diameter) of seagrass shoots, rhizomes, roots and sediments were collected to a depth of 12 cm at the first two sites and to the full depth of the reef flat (5 to 7 cm). Shoots were counted and then the above-ground plant material was removed and separated into seagrass species. Plant material (rhizomes and roots) was sieved out from sediment

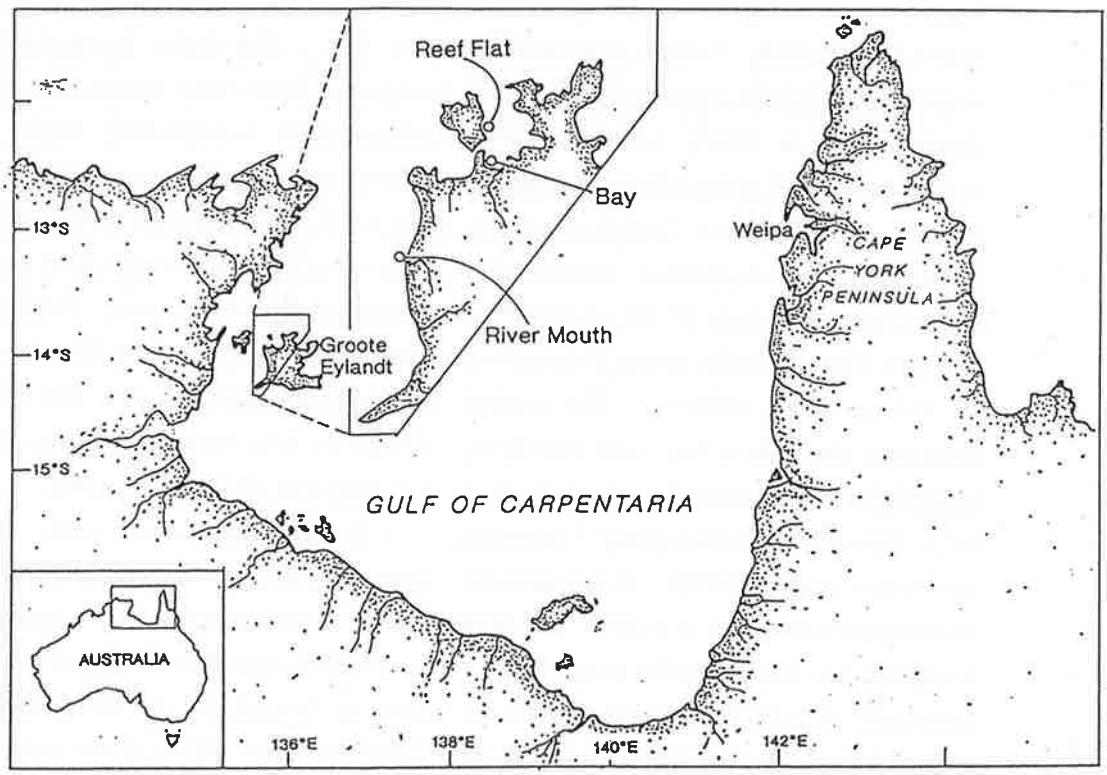

Figure 1. Location of study sites at Groote Eylandt, Gulf of Carpentaria.

and separated into species. All plant material was dried at 105°C and weighed.

Primary Productivity

The diel curve procedure described by Odum and Hoskin (1958) was used to determine gross community productivity and respiration from changes in oxygen concentration in the water flowing over the seagrass bed. Techniques for determining productivity that are based on measurements of changes in oxygen concentration in the water around seagrasses give good estimates of the contribution of algae as well as the seagrasses (see Discussion and Lindeboom and Sandee, 1989).

To determine the relative contributions of benthic and pelagic organisms to the uptake of oxygen from the water column, oxygen changes were measured in enclosed duplicate samples of the water column together with and separate from the benthos. Samples of the water column were enclosed in 4.5 litre clear glass bottles and incubated in situ. Cores (100 mm diameter) containing sediment, macroalgae and seagrass were collected and clear acrylic chambers (2 l capacity) placed over them. They were incubated underwater in the seagrass bed. The water in each enclosure was stirred before oxygen concentrations were measured.

The photosynthetic rates of the seagrasses were determined with the lacunal gas technique (Roberts and Moriarty, 1987). Measurements were made on shoots of seagrasses with pipettes attached in place of the rhizome. Four shoots were pooled for each sample. The shoots were returned to the seagrass bed and thus were exposed to natural conditions of light, temperature and water movement. Depth was recorded each time the gas volume was measured, and the volume was corrected to that at atmospheric pressure. Calibration of the lacunal gas method for S. isoetifolium and C. serrulata has been reported elsewhere (Roberts & Moriarty, 1987). For this work, it was assumed that the composition of the gas in the lacunae of the other species was the mean of that in Zostera capricornii, S. isoetifolium and C. serrulata, viz. 33.6% oxygen (range 32.2 to 34.3) (Roberts and Moriarty, 1987). Gross productivity in terms of carbon was calculated from the values for oxygen production, assuming a 1:1 molar ratio (Roberts & Moriarty, 1987). An analysis of variance was used to examine differences between seasons.

Bacterial Productivity

Bacterial growth rates were determined from rates of tritiated thymidine incorporation into DNA (Moriarty and Pollard, 1981; 1982 and Pollard and Moriarty, 1984).

At each sampling occasion, 4 replicate cores of sediment (8 mm diameter, 4 cm deep) were collected for incubation with thymidine, 2 cores each were collected for determining blank values and numbers of bacteria. For each sample to be incubated with tritiated thymidine, the upper 2 mm was extruded and dropped into a plastic

tube with 50 µl of filtered seawater; the next 8 mm was kept for determining dry weight of the sediment. At each 1 cm depth interval, a further 2 mm section was sampled. Tritiated thymidine was added (25 µCi, 2.5 nmol [methyl-^{3}H]thymidine) and samples were incubated for 10 minutes. Ethanol (80% v/v, containing 100 mg/l thymidine) was added and samples were transported to the laboratory. The ethanol was removed after centrifuging by aspirating off the fluid. Two ml of NaOH (0.3 m), containing 10 mM thymidine, was added. Samples were heated at 100° C for 30 min., cooled, centrifuged, washed and then dialysed as described by Pollard (1987).

Water samples (25 ml each) were incubated with 20 µCi tritiated thymidine (18 nM final concentration) for 10 minutes in summer and 20 minutes at other seasons. Formaldehyde (0.5% v/v final concentration) was added to preserve the samples, which were then filtered at the laboratory, and washed with trichloroacetic acid as described by Pollard and Moriarty (1984).

Bacterial productivity was determined in the fine, flocculent material that settles onto the seagrasses and sediment in calm conditions, but is easily resuspended by disturbance. Covers (100 mm diameter), with 2 litre chambers attached, were carefully placed over the seagrass. The flocculent material was then resuspended by stirring and 5 ml samples of the 2 l volume were taken by syringe through ports in the chamber. Incubations with tritiated thymidine (90 nM) were treated as described above for water samples.

Numbers of bacteria were counted in samples of water and sediment that were preserved in formaldehyde (0.5% v/v). Direct microscopic counts and size measurements of bacteria were made using epifluorescence microscopy and acridine orange (Hobbie et al.,1977). The average carbon content of bacteria was 40 fg per cell, based on the factor of 220 fg μm^{-3} (Bratbak and Dundas, 1984).

The growth rates and productivity of the bacteria were calculated as described by Moriarty (1986). A conversion factor of 5×10^{17} cells produced per mol of thymidine incorporated was used (Moriarty, 1988). Analyses of variance were used on log-transformed values of radioactivity (disintegrations per minute) in DNA (*i.e.* bacterial productivity), numbers of bacteria per core and specific growth rates (productivity divided by numbers) to examine differences due to time of day, depth in the sediment and season.

RESULTS

Primary Production

The shoot density and biomass of the seagrasses was variable within the study sites (Tables 1, 2). Root and rhizome biomass was greatest in the top 4 cm of sediment and decreased rapidly with depth at all 3 sites (Tables 1, 2, 3). An exception was Halodule uninervis at the river mouth after January (Table 2). Halodule uninervis in the study area was covered by sand during a cyclone in

Table 1. Biomass of seagrass at the bay site in 1985 (see Figure 1). Means and standard deviation are shown; n = 6 (except July, n = 4).

Species / Month	Shoot density No. m^{-2}	S.D.	Leaf biomass g m^{-2}	S.D.	Root biomass Depth (cm)	g m^{-2}	S.D.
Syringodium isoetifolium							
January	6500	1300	130	25	0-4	650	280
					4-8	130	130
					8-12	25	45
May	3360	2400	160	110	0-4	345	100
					4-8	85	45
					8-12	40	20
July	9300	390	22	7	0-4	170	75
					4-8	160	150
					8-12	50	40
October	2900	2000	270	190	0-4	380	100
					4-8	170	70
Cymodocea serrulata							
January	74	160	5	12		nd	
May	350	300	50	40	0-4	70	25
					4-8	12	20
July	770	280	120	17	0-4	115	80
					4-8	25	30
October	270	200	80	60	0-4	50	30
					4-8	20	20

Table 2. Biomass of Halodule uninervis at the river mouth site (Figure 1). Means and standard deviation are shown.

	Shoot density			Leaf biomass			Root biomass			
Month (1985)	No. m^{-2}	S.D.	n	g m^{-2}	S.D.	n	depth (cm)	g m^{-2}	S.D.	n
January	16000	7000	5	185	95	5	0-4	590	244	5
							4-8	170	76	5
							8-12	120	110	5
May	9600	5000	4	60	32	4	0-4	82	68	4
							4-8	130	35	4
							8-12	160	60	4
July	13000	3000	4	78	15	4	0-4	83	36	4
							4-8	63	26	4
							8-12	150	70	4
October	9200	2000	5	35	9	5	0-4	163	55	5
							4-8	104	34	5
							8-12	150	75	5

Table3. Plant biomass on the reef flat site (Figure 1). Sediment was about 5 cm deep, on a hard carbonate substrate. Means and standard deviation are shown (n = 6)

Species	Shoot density		Leaf biomass		Root biomass	
Month (1985)	No. m^{-2}	S.D.	g m^{-2}	S.D.	g m^{-2}	S.D.
Cymodocea rotundata						
January	450	370	52	43	80	75
May	430	480	50	60	120	110
July	78	140	5	10	nd*	nd
October	430	280	30	20	160	220
Thalassia hemprichii						
January	700	240	70	25	490	200
May	650	300	65	31	930	610
July	700	100	75	20	630	450
October	350	200	34	22	650	110
Algae						
January			250	240		
May			190	110		
July			210	240		
October			115	90		

* Not determined (biomass too low to separate from *T. hemprichii).*

February, 1985. Biomass and shoot density were variable, due to a patchy distribution of seagrasses (Table 3). Macroalgae were the dominant components of the above-ground biomass on the reef flat, ranging from 63% to 72% of the total (Table 3).

The photosynthetic rate per unit leaf weight was significantly greater in summer than in winter in Cymodocea rotundata ($P < 0.5$) and Syringodium isoetifolium ($P < 0.005$) (Fig. 2). Seasonal variation was apparent, with fastest growth in spring, in Thalassia hemprichii ($P = 0.01$). No significant seasonal variation in productivity of Cymodocea serrulata and H. uninervis (was observed Fig. 2).

The productivity of the seagrasses per shoot varied significantly between seasons for all species. It was 6 times smaller in autumn and winter than in summer for S. isoetifolium ($P < 0.001$) and 3 times in H. uninervis ($P < 0.01$); C. rotundata was twice as productive in summer as in the other seasons ($P < 0.05$) (Tables 4, 6). There were not enough data to determine whether C. serrulata was more productive in summer, but it was significantly more productive in spring ($P < 0.01$) (Table 4). Thalassia hemprichii was more productive in spring than at other seasons ($P < 0.01$) (Table 5). The differences in seasonal variation between photosynthetic rate per unit leaf weight and per shoot, especially for H. uninervis, occurred because shoots were larger in spring and summer than in winter. C. serrulata was more productive (per shoot) than S. isoetifolium in all seasons (Table 4).

The values of shoot productivity were used with the shoot density data to calculate productivity per m^2 of the study areas (Tables 4, 5, 6). The highest values for gross productivity on an areal basis (9.2 ± 1.7 g C m^{-2} day^{-1}) were for S. isoetifolium in summer (Table 4). Although the photosynthetic rate of S. isoetifolium was lower than C. serrulata its shoot density was much greater. Similarly, the productivity of H. uninervis was high because its shoot density was high, although its photosynthetic rate per shoot was an order of magnitude lower than that of the other seagrasses (Table 6). The chosen site at the river mouth had a particularly high density of seagrass compared to the surrounding community, in order to facilitate studies of the effect of seagrass production on bacterial production.

At the bay study site, gross productivity of the community was generally similar to that of the seagrasses (Table 4). In contrast, on the reef flat gross productivity of the community was greater at all seasons than the corresponding productivity of seagrasses; thus the algae were the dominant primary producers (Table 5). This is consistent with the data in Table 3, showing macroalgal biomass to be more than the leaf biomass of seagrasses. At the river mouth, there was little correspondence between community and seagrass productivities, but as pointed out above

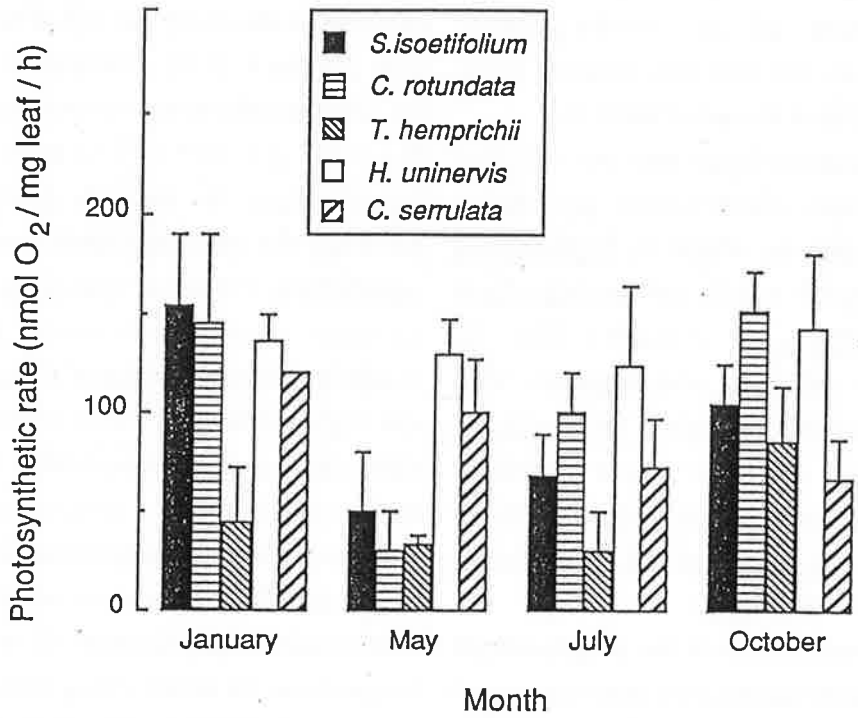

Figure 2. Photosynthetic rates of seagrasses at sites 1 and 3, expressed as total oxygen produced per hour, per mg dry weight of leaves. Standard deviations (n = 4) are shown.

Table 4. Primary productivity of seagrasses and the whole community at the bay site. Values for seagrasses are compared on a per shoot and areal basis. Community productivity (P) and respiration (R) were determined by diurnal changes in oxygen concentration in water, for which the ranges are shown. Values are mean ± standard deviation (n = 4).

Month	*Syringodium isoetifolium*	*Cymodocea serrulata*	*S. isoetifolium*	*C. serrulata*	Total seagrass	Community P	Community R	Oxygen Max.	Oxygen Min.
	mg C shoot^{-1} day^{-1}		g C m^{-2} day^{-1}					mg O_2 l^{-1}	
January	1.4 ± 0.3	2.0 [a]	9.2 ± 1.7	0.15 ± 0.3	9.3	9.3	7.6	10.2	6.7
May	0.42 ± 0.25	1.7 ± 0.4	1.4 ± 1.7	0.6 ± 0.5	2.0	5.7	5.4	7.9	4.2
July	0.23 ± 0.07	1.3 ± 0.4	2.1 ± 0.1	1.0 ± 0.4	3.1	3.3	3.5	8.7	5.2
October	1.2 ± 0.03	3.0 ± 0.2	3.5 ± 3.3	0.8 ± 0.7	4.3	6.0	5.1	10.5	5.4

[a] replicates lost during storm.

Table 5. Primary productivity of seagrasses and whole community on the reef flat (± standard deviation, n = 4). See legend to Table 4 for further details.

Month	*Cymodocea rotundata*	*Thalassia hemprichii*	*C. rotundata*	*T. hemprichii*	Total seagrass	Community P	Community R	Oxygen max.	Oxygen min.
	mg C shoot^{-1} day^{-1}		g C m^{-2} day^{-1}					mg l^{-1}	
Jan	2.2 ± 0.7	0.6± 0.4	1.0 ± 0.8	0.4 ± 0.3	1.4	8.4	8.0	12.4	3.9
May	0.4 ± 0.3	0.4 ± 0.1	0.2 ± 0.2	0.3 ± 0.1	0.5	6.3	4.6	9.0	2.8
Jul	0.8 ± 0.2	0.4 ± 0.2	0.1 ± 0.2	0.1 ± 0.03	0.2	3.3	2.9	9.8	5.6
Oct	1.3 ± 0.2	1.1 ± 0.4	0.6 ± 0.4	0.4 ± 0.2	1.0	5.2	4.4	5.3	10.6

Table 6. Primary productivity of the seagrass *Halodule uninervis* and whole community at the river mouth. See legend to Table 4 for further details. Values are mean ± standard deviation; n = 4.

Month	Seagrass Productivity		Community P	Community R	Oxygen Max.	Oxygen Min.
	mg C shoot^{-1} day^{-1}	g C m^{-2} day^{-1}	g C m^{-2} day^{-1}		mg O_2 l^{-1}	
Jan	0.22 ± 0.02	3.5 ± 0.1	2.7	1.2	6.1	4.7
May	0.09 ± 0.01	0.9 ± 0.06	3.4	3.0	9.1	6.2
Jul	0.08 ± 0.03	1.1 ± 0.1	3.4	2.9	10.2	3.5
Oct	0.11 ± 0.03	1.0 ± 0.06	4.0	4.0	12.9	6.2

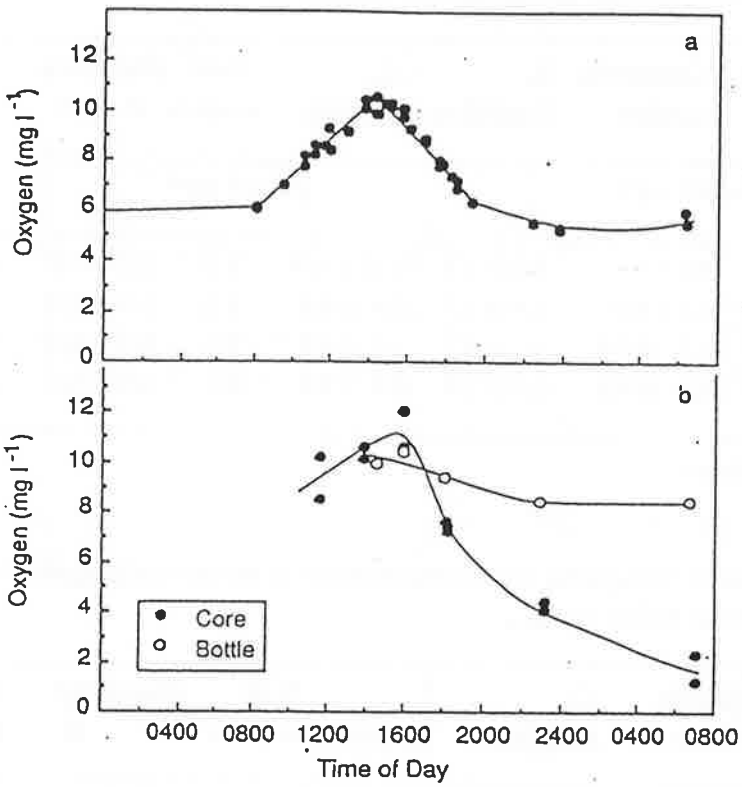

Figure 3. Example of diel changes during October in oxygen concentration in the water. (a) water over the reef flat; (b) in chambers containing cores of sediment with algae and seagrasss and in bottles with water only; where separate points are not shown at each time interval, values for duplicates were within the size of the plot symbols.

the species composition and shoot density at the study site was different from that of the surrounding community (Table 6). Variability due to environmental factors could not be estimated, due to logistical problems that prevented repeating measurements of diel changes in oxygen concentration over a series of days or weeks.

The marked diel variation in oxygen concentration in the water was reproduced when cores of sediment were enclosed in chambers, but not when water alone was enclosed, indicating that benthic organisms were responsible for much of the respiration (Fig. 3).

Bacterial Production

In the water column over the reef flat, specific growth rates of bacteria varied significantly, both diurnally and seasonally ($P < 0.01$). The most rapid growth rates were observed in summer and the slowest in winter (Fig. 4). The diel variation was linked to the tides; growth rates were slower at high tide than at low tide (Fig. 4). Both growth rates and productivity of bacteria were significantly greater over the seagrass bed than further offshore at both the reef flat ($P < 0.01$) and bay sites ($P < 0.001$) (Fig. 5). Bacterial numbers were nearly constant across the transect (range: ± 5% of the mean), so specific growth rates varied in a similar manner to productivity (Fig. 5). The average daily productivity and specific growth rates of bacteria in the water column were greater in summer than at other seasons, but the absolute areal values are small compared to sediment values (Tables 7, 8). Productivity was lowest in winter.

Growth rates of bacteria attached to the flocculent material were similar to those free in the water column, but with two exceptions, productivities were greater (Table 7). Like those in the water column, these values were very low compared to sediment on an areal basis. Productivity of bacteria in the flocculent material on the Syringodium isoetifolium was very variable, reflecting variability in the amount of flocculent material on the seagrass (Table 7).

Most of the bacterial biomass in both seagrass beds was produced in the sediment with greatest productivity in summer (Tables 7, 8; Fig. 6). Spatial variability was large (± 20% of mean) and tended to obscure patterns in diel variation. A three-way analysis of variance of productivity at different times of the day with season and depth in the sediment showed that there was no significant interaction between time of day and the other factors, and that there was a significant difference between the early morning values and other times of the day ($P = 0.0001$; Student-Newman-Keuls test at $P = 0.05$). The early morning values were, therefore, deleted from further analyses to show seasonal and depth effects. Specific growth rates were calculated only from midday values in order to minimise effects of diel variation.

Table 7. Bacterial productivity (P) and specific growth rates (μ) at the bay and reef flat sites. Specific growth rates are average values for the day; the productivity values were integrated over a day; sediment values were integrated down to 12 cm depth in the bay and 7 cm on the reef flat. Standard errors of bacterial numbers were between 5% and 10% of the mean. Total bacterial productivity is shown as a rounded percentage of total community gross productivity.

Month	Seawater			Flocculent Material		Sediment	Total	
	Number 10^9 l^{-1}	μ h^{-1}	P mg C m^{-2} day^{-1}	μ h^{-1}	P mg C m^{-2} day^{-1}	P g C m^{-2} day^{-1}	P g C m^{-2} day^{-1}	%PP
Bay site:								
January	1.3	0.08	100	0.05	110	4.6	4.8	50
May	1.8	0.06	90	0.03	10	0.9	1.0	20
July	1.2	0.05	40	0.08	290	1.7	2.0	60
October	1.4	0.07	80	0.08	100	2.0	2.2	40
Reef flat:								
January	7.5	0.12	48	0.05	31	3.6	3.7	45
May	8.5	0.07	32		70	0.5	0.6	10
July	5.0	0.04	11	0.02	72	0.7	0.8	30
October	7.3	0.04	17	0.12	144	3.5	3.6	70

Table 8. Bacterial productivity (P) and specific growth rates (μ) at the river mouth (Halodule uninervis community). Specific growth rates are average values for the day; the productivity values were integrated over a day; sediment values were integrated down to 12 cm depth. Standard errors of bacterial numbers were between 5% and 10% of the mean. Total bacterial productivity is shown as a percentage of total community gross productivity (%PP).

Month	Seawater			Sediment	Total	
	Number 10^9 l^{-1}	μ h^{-1}	P mg C m^{-2} day^{-1}	P g C m^{-2} day^{-1}	P g C m^{-2} day^{-1}	%PP
January	1.1	0.1	90	2.4	2.5	90
May	1.0	0.04	30	0.8	0.9	25
July	0.7	0.03	10	0.6	0.6	20
October	1.2	0.05	30	1.2	1.2	30

Table 9. Analyses of variance in bacterial productivity, numbers and specific growth rates in sediment due to season and depth in the sediment at the 3 sites. Abbreviations: n = number of samples for each site; df = degrees of freedom.

Site	Source	Degrees of freedom	Bacterial Productivity n	F value	Probability	Numbers of bacteria n	F value	Probability	Specific growth rates n	F value	Probability
Bay	depth	4	280	2.8	0.026	65	3.7	0.01	59	1.7	0.16
	season	3		46.1	0.0001		26.5	0.0001		3.1	0.036
	depth x season	12		2.5	0.004		13.3	0.0001		3.97	0.0005
River mouth	depth	4	370	27.8	0.0001	65	3.5	0.01	61	1.3	0.27
	season	3		44.2	0.0001		11.9	0.0001		11.3	0.0001
	depth x season	12		2.6	0.002		5.0	0.002		1.8	0.08
Reef flat	depth	4	360	12.1	0.0001	65	1.5	0.21	58	0.96	0.44
	season	3		145	0.0001		189	0.0001		23.6	0.0001
	depth x season	12		4.4	0.0001		30.0	0.0001		2.8	0.007

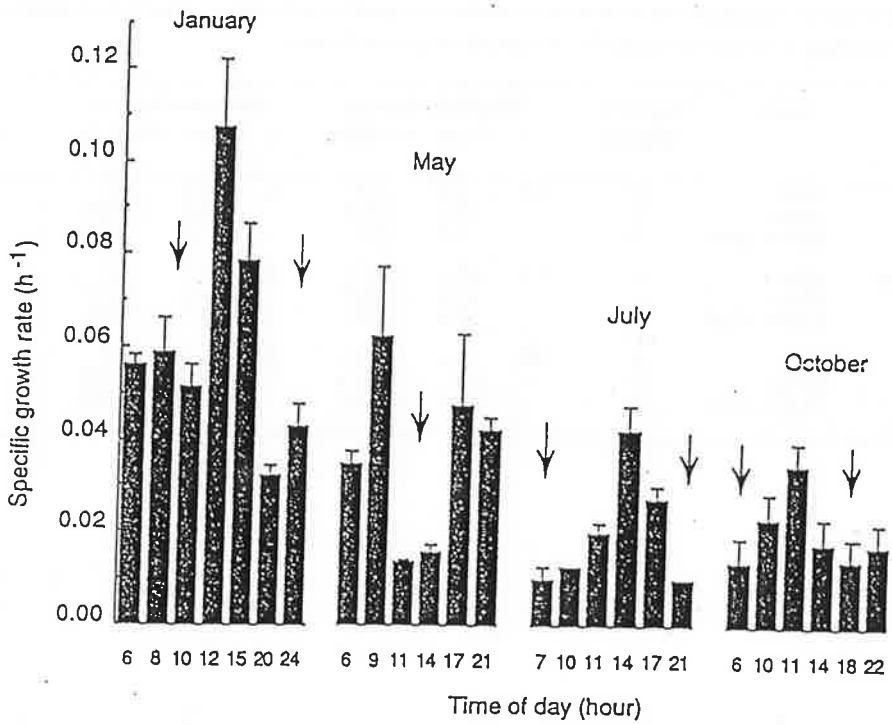

Figure 4. Specific growth rates of bacteria in the water column over the reef flat at different seasons and times of the day. Times of high tide are indicated by arrows.

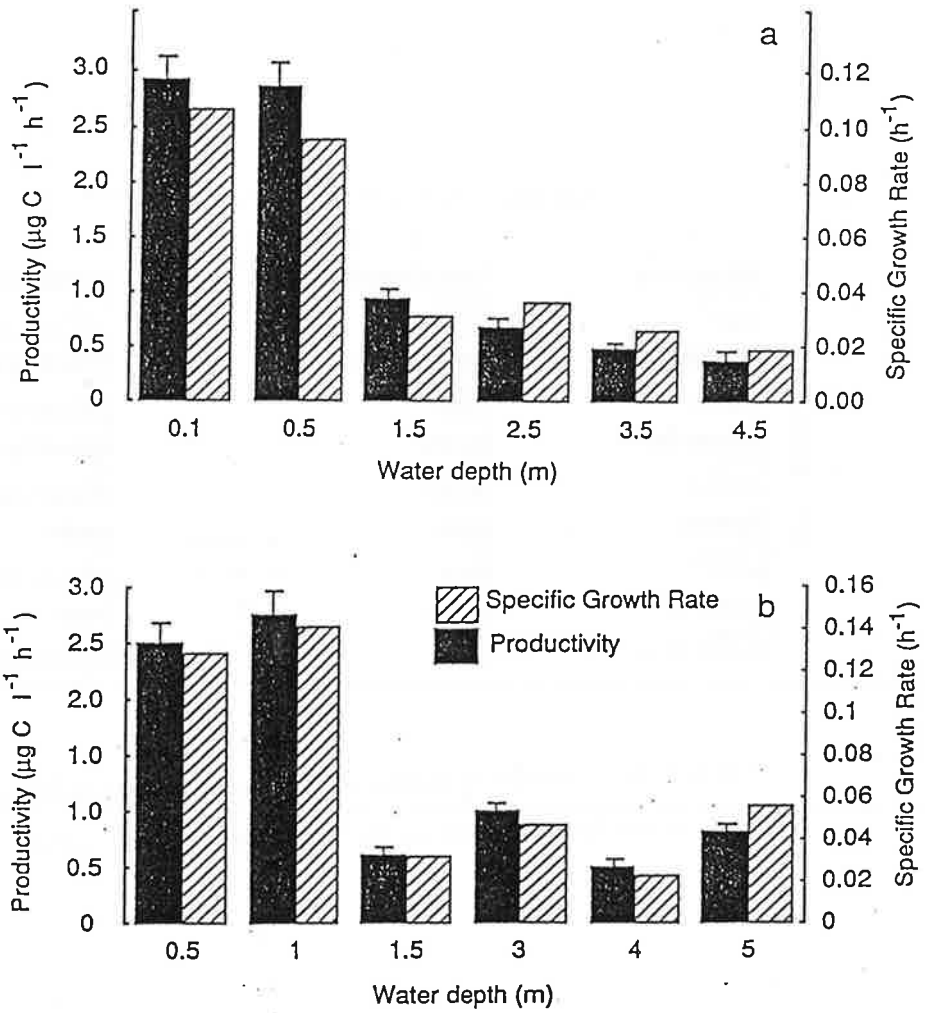

Figure 5. Productivity and specific growth rates of bacteria in the water column on a rising tide at mid tide height in transects from near shore to 1 km offshore in autumn; water temperature 29°C. Water depths at low tide are indicated. Duplicate determinations were made of both numbers and productivity; the range of variation is indicated for productivity. (a) Bay; seagrass shoot density decreased with increasing water depth to 2.5 m; no seagrass was present below 3.5 m. (b) Reef flat. The reef crest was at 2 m depth. No seagrasses or macroalgae were present inside the reef crest at 1.5 m.

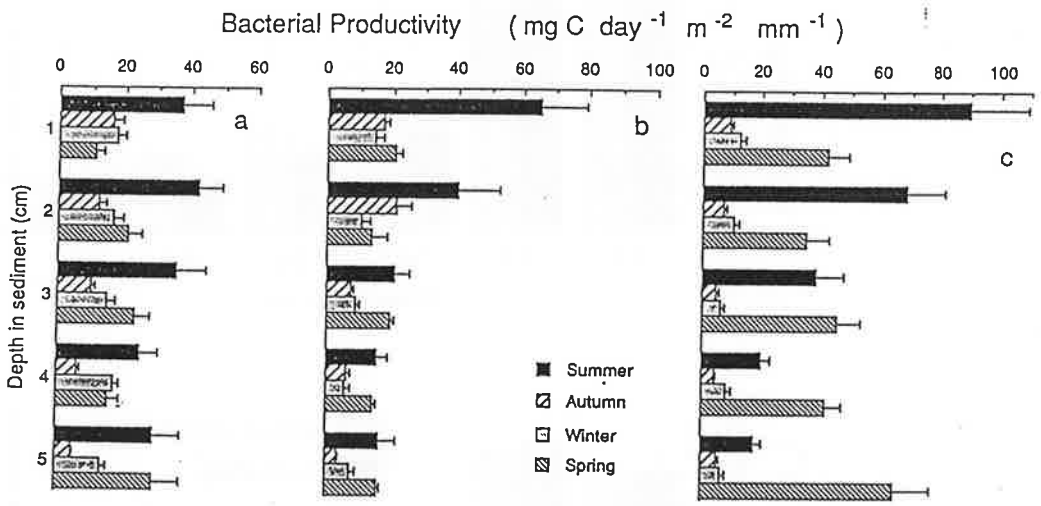

Figure 6. Depth profiles of bacterial productivity in sediment in the bay (a), river mouth (b) and reef flat (c). See Table 9 for analyses of variance.

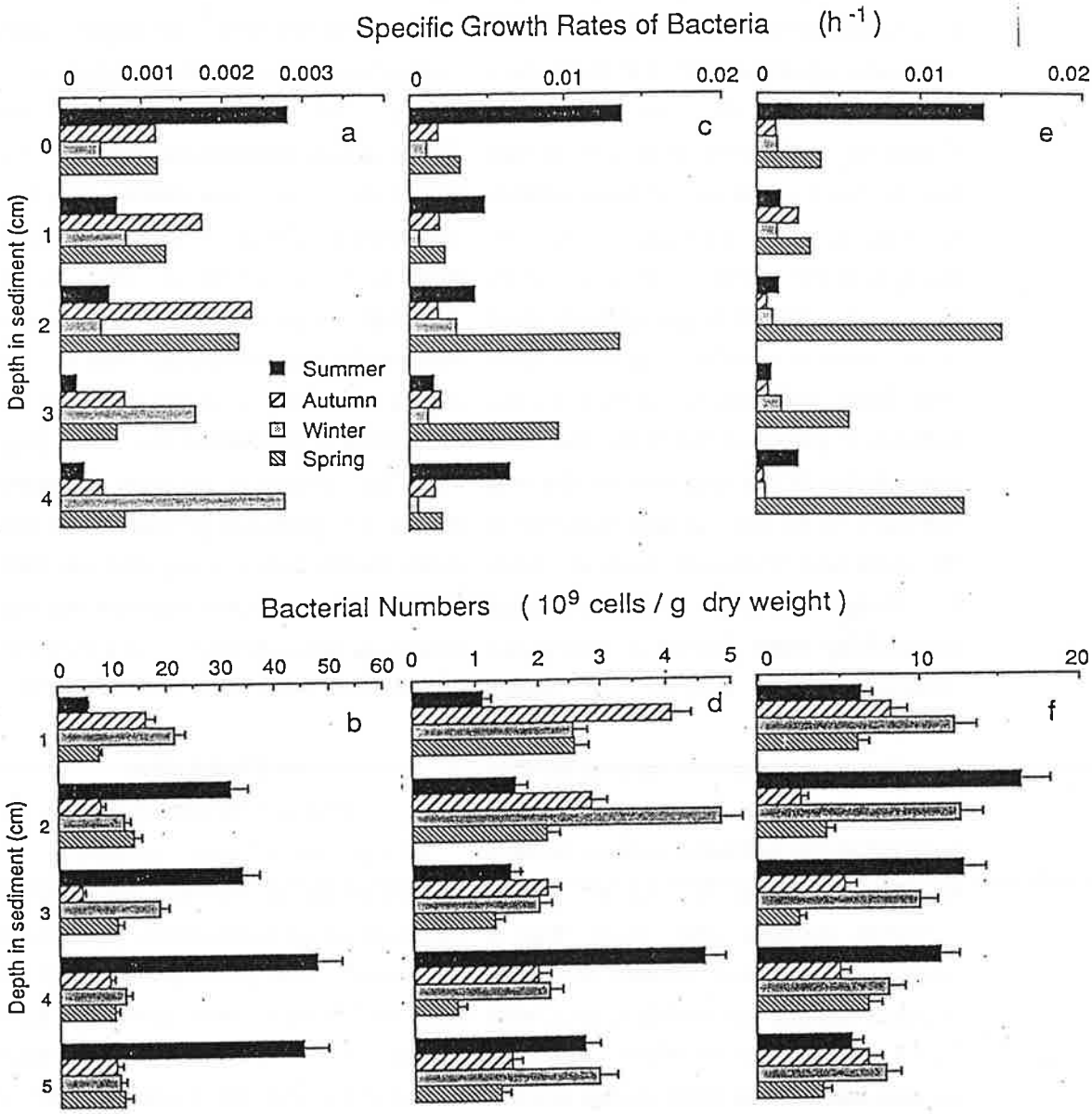

Figure 7. Specific growth rates of bacteria and numbers of bacteria in the upper 4 cm of sediment in the bay (a, b), at the river mouth (c, d) and on the reef flat (e, f). See Table 9 for analyses of variance.

Bacterial productivities were particularly high at the surface of the sediment in summer at the river mouth and on the reef flat, and decreased towards the bottom of the sediment profile (Fig. 6). An analysis of variance showed that these differences were very significant and that there was a significant depth interaction with season (Table 9). In spring, productivity was high at 3 to 4 cm in the sediment profile; in winter, productivities were low throughout the sediment (Fig. 6). At the bay site, i.e. around S. isoetifolium, most of the variation was due to seasonal rather than depth differences (shown by the different F values in Table 9). Bacterial productivity in the sediment on the reef flat was 5 to 45 times greater than that in the water and flocculent material (Table 7). Total values for integrated daily productivity were greater in spring and summer than in autumn and winter (Tables 7, 8).

The specific growth rates of bacteria varied with season, being most rapid in summer at the sediment surface in all 3 seagrass beds (Fig. 7). An analysis of variance showed that there was a significant interaction between depth and season at the bay and reef flat sites (Table 9). This was due to rapid growth of bacteria below 2 cm depth during seasons other than summer (Fig. 7a,e). At the river mouth, there was not a significant effect due to depth (Table 9), although the data in figure 7c suggest that there should be because growth rates were rapid in spring at 2 to 4 cm. Bacterial productivities were found to be high in only one of the replicate cores collected at midday; in other words, the effect was caused by spatial variability and was not significant.

Bacterial numbers were about 1 order of magnitude lower in the Halodule uninervis bed (Fig. 7d) than in the Syringodium isoetifolium bed (Fig. 7b), but productivities were similar, thus specific growth rates of bacteria were 5 to 10 times faster in the former (Fig. 7a, c). Although the growth rate of bacteria was faster at the sediment surface than at other depths in January at all sites, the number of bacteria at the surface was lower (Fig. 7). The number of bacteria at deeper depths was generally greater in summer at the muddy sites (the bay and reef flat). Much of the variance in numbers was due to season, although there was a very significant interaction with depth (Table 9).

DISCUSSION

Primary Productivity

Temperature was probably an important factor causing the seasonal variation in photosynthetic rates of the seagrasses. The photosynthetic rate per unit leaf weight was lower in some species in winter when the mean temperature was 10° lower. Although light intensity was lower in winter than in summer at the top of the leaf canopy, on sunny days it was above 1000 $\mu E\ m^{-2}\ s^{-1}$, which is saturating for at least some of these species (Roberts and Moriarty, 1987). In other species of seagrass,

temperature probably has a controlling influence on growth, provided light intensity is high (Barber and Behrens, 1985; Brouns and Heijs, 1986; Bulthuiis, 1983; Kirkman et al. 1982).

The photosynthetic rate per unit leaf weight of Halodule uninervis did not vary markedly with season, but its daily productivity (per shoot or unit area) was lower in winter, due to the smaller size of the shoots, and the shorter days. The values for gross productivity of Thalassia hemprichii are low for January, possibly because the water was too hot. The measurements were made during a period when low tide occurred around noon, and water temperature on the reef flat reached 38°C. I.R. Poiner and C. Thorogood (personal communication) noted that growth rates of Thalassia hemprichii, determined by the plastochrone method at the same site, were depressed at water temperatures of about 40°C.

Seagrass productivity on an areal basis was considerably greater in summer than in winter for all seagrass beds. The seasonal differences were influenced by various factors, especially photosynthetic rate and shoot density. For example in the bay study area, S. isoetifolium accounted for 98% of the production in summer and 68% in the winter because its shoot density was an order of magnitude greater than that of C. serrulata. Day length was 10% to 15% shorter in July, so this contributed to lower daily productivity values in winter. Brouns (1987c) found that the production of S. isoetifolium, C. rotundata, C. serrulata and Halodule uninervis was greatest in February and March in Papua New Guinea. Where shoot densities were similar, his values for production were not very different from those that we recorded. For example, the shoot density of S. isoetifolium at Broun's station 6 was about 7000 m^{-2} and the productivity was between 9.2 g ash free dry weight m^{-2} day^{-1}, i.e. about 3.7 g C m^{-2} day^{-1} in August (Brouns, 1987c). Our values for July (the nearest corresponding period) were 9000 shoots m^{-2} and 3.5 g C m^{-2} day^{-1} (Tables 1, 4).

The values we obtained for gross productivity of a community of Halodule uninervis agreed with those of Lindeboom and Sandee (1989). Their values ranged from 3.3 to 4.7 g C m^{-2} day^{-1} for the whole community in October in Indonesia and ours were 4 g C m^{-2} day^{-1}, of which the seagrass contributed 25%. They estimated epiphyte production to be about 36% of the total primary production. Benthic microalgae must also be important contributers to primary production at thia site. We found, as they did, that the net production of the community was generally low, i.e. the difference between community respiration and production was low (see Tables 4, 5 and 6). This indicates that much of the primary production was utilised within the community. Where substantial net production was apparent, e.g. at the river mouth and bay sites in January (see Tables 4 and 6), we may conclude that

export and/or deposition of organic matter occurred.

Methodology

The results of the two methods used here to determine primary productivity were similar. The seagrasses, especially S. isoetifolium, were the dominant plants at the bay site and thus were the major primary producers. Epiphytes were not abundant on the S. isoetifolium and its dense cover would have shaded benthic microalgae. Thus agreement in values obtained by the two different methods could be expected at the bay site, in contrast to the values for productivity on the reef flat, where macroalgae were abundant.

The diel curve method for estimating the whole community productivity has limitations, as discussed by Odum and Hoskin (1958). The factors that substantially affect the accuracy of the results are respiration during the day, diffusion of oxygen to or from the atmosphere, and variation in water depth across the seagrass bed, relative to the measurement station. Diffusion is difficult to measure accurately, yet the values for gross community productivity in the Syringodium bed in January and October (where algae were insignificant) agreed well with the values for seagrasses themselves determined by the lacunal gas method (Table 4). Most oxygen exchange in the water column over a seagrass bed is due to the benthic community (Lindeboom and Sandee, 1989; Odum and Hoskin, 1958).

The diel curve method in the open water column provides an estimate of gross primary productivity and respiration of the whole community. More detailed studies are needed to determine the individual contributions of the major components: seagrasses, macroalgae, epiphytic and epibenthic microalgae. The ratio of net photosynthesis to respiration in seagrasses and green algae is about 5 to 6, so their contribution to the respiratory lossses was not a substantial proportion (Buesa, 1977). In other words, net productivity in the macrophytes is about 20% smaller than gross productivity.

Methods for determining productivity based on oxygen measurements have been criticised (Zieman and Wetzel, 1980). This criticism had been advanced partly because it was not known then how much oxygen passed to the roots via the lacunae, nor whether it was stored. Only about 15% of oxygen produced during photosynthesis is transported via lacunae to the sediment and would not be accounted for over short term measurements (Roberts and Moriarty, 1987). In other words, only a little oxygen is stored and thus this is not a serious problem with the method. Lindeboom and Sandee (1989) have discussed methods for determining productivity that depend on oxygen measurements, and conclude that they are satisfactory. As they point out, no better, simple methods for determining productivity of a whole community are available.

Bacterial Productivity

Relationship to Primary Productivity and Temperature

Bacterial production is dependent on seagrass production on at least two time scales. Firstly, bacterial growth rates change over a period of hours in response to exudation of organic matter during photosynthesis (Moriarty and Pollard, 1982; Moriarty et al., 1985). Secondly, over a period of weeks or months, bacteria grow while decomposing dead plant material (Harrison and Mann, 1975). It seems from our data that bacterial production is coupled to primary production on a seasonal basis, with most activity in summer and least in winter or autumn, even though this is a tropical location, with the lowest temperature that we recorded being 21° C. The noticeable seasonal variation in bacterial productivity was more pronounced than that in primary productivity, which suggests that the seaonal change of 10° C in mean temperature had more impact on bacterial activity than on the plants. Alongi (1988) found that bacterial productivity was lower in mangrove sediment in winter. Furthermore, he found that when intertidal mud banks below mangroves were exposed to air that was cooler than the water, bacterial growth rates decreased (Alongi, 1988). Temperature is a very important factor controlling the productivity of bacteria, through its effect on the rate of hydrolysis of organic polymers, especially cellulose and protein. This first step in the decomposition of particulate organic matter, derived from dead leaves, rhizomes and roots of seagrasses, seems to be the rate limiting one, but few studies have been made of this process (Godshalk and Wetzel, 1977).

On the reef flat and in the bay, both seagrass and bacterial productivity were 5 times lower in winter (July or May) than in summer (Tables 4, 7). Similarly, at the river mouth seagrass and bacterial productivity were 3 to 4 times lower in winter than in summer (Tables 6, 8). This suggests that the bacterial productivity was limited by the rate of organic matter supply from the seagrasses and that temperature had a controlling influence on metabolic rates. In spring, specific growth rates of bacteria were faster at depths of 2 to 4 cm, which is the main root zone of the seagrasses S. isoetifolium, T. hemprichii and C. rotundata . This is likely to be due to stimulation of bacterial growth by organic matter exuded from the roots when the seagrasses (see Fig. 7a, c, e).

The proportion of primary productivity that is utilised by bacteria is variable and not easily determined. It depends on the average growth efficiency for the whole bacterial community and the proportion of biomass to extracellular products formed by the bacteria. The total amount of organic carbon from primary producers needed to support the observed rates of production by the heterotrophic bacteria would be equal to

the bacterial productivity if the bacteria were 50% efficient, and steady state conditions applied, i.e. if rates of respiration and input of organic matter were the same (Strayer, 1988; Moriarty, in press). Bacteria in the sediment use not only organic matter supplied directly from the primary producers, but also recycled from animals and previous bacterial activities. Values for bacterial productivity that are equal to or greater than primary productivity are possible when carbon cycling is tightly coupled, and bacterial growth efficiencies are high (see Tables 7, 8). More frequent sampling would be required to determine how close coupling was temporally.

If the bacteria were 50% efficient in their use of organic matter, the values reported here indicate that they utilised from 20% to 60% of primary production at the bay site and 10% to 70% on the reef flat (Table 7). At the river mouth, the corresponding values for the whole community was 90% in January, but it is not valid to compare bacterial growth in this study site with the whole community. The seagrass productivity at this site was greater than that of the whole community because the shoot density was much higher than nearby. Bacterial productivity was 70% of seagrass productivity. If most of the organic matter entering the sediment is decomposed by bacteria, the growth efficiency of the bacteria can be estimated (Moriarty, in press). The calculated efficiencies are between 20% and 40% for the bay site, which are within the ranges quoted for natural systems (Findlay *et al.* 1986; Robinson *et al.* 1982). Thus it seems likely that the main fate of primary production in these seagrass beds is recycling to carbon dioxideby bacteria in the sediment (Moriarty, in press).

The higher specific growth rates of bacteria on the sediment surface in summer, as well as the high productivity, indicate that leaf and algal decomposition was more rapid in summer (Figs. 6, 7a, c, e). On the reef flat, seagrass root and rhizome biomass was high compared to leaf and algal biomass, which suggests that its decomposition after death would support more bacterial growth than aboveground biomass (Table 3). Most bacteria were probably utilising decaying seagrass tissue rather than exudates of organic matter from living seagrasses. Diel trends were sometimes apparent, but were not as marked as those reported elsewhere because they were masked by spatial variability (Moriarty and Pollard, 1982; Moriarty et al., 1986). The sampling strategy was designed to show trends on a broad areal and seasonal basis, rather than individual diel variation.

More information is needed on the rate of root turnover compared to leaf turnover and more frequent measurements of bacterial and primary production are needed to establish how closely production of organic matter is coupled in time to decomposition. There is substantial spatial, seasonal and interannual variation in seagrass biomass

in these seagrass beds (I. R. Poiner, personal communication).

Trophic Implications

A factor contributing to control of bacterial productivity is the effect of grazing on them by animals. We would expect the bacterial population density (numbers) to be highest at the sediment surface when productivity and specific growth rates were rapid. In January, however, although specific growth rates were rapid, bacterial numbers were lower at the surface than deeper in the sediment at all 3 sites, which is a good indication that grazing by animals was intense (Fig. 7). The sediments were anoxic below about 2 mm and thus most meiofauna and protozoans would be concentrated in the surface layer (Coull and Bell, 1979). Protozoans are probably the most important grazers on bacteria in sediment, but more information is needed on their interactions with bacteria (Fenchel and Jorgensen, 1977).

The bacterial biomass production each day in summer (4.8 g C m^{-2} day^{-1} at the bay site, 3.7 g C m^{-2} on the reef flat, and 2.5 g C m^{-2} day^{-1} at the river mouth), is a substantial food resource for animals. Even though this is a tropical environment, this resource was much less in winter. It seems, however, that only the bacteria in the surface sediment were grazed to a noticeable extent, and then only in summer.

The grazing activity would decrease the proportion of organic matter that is recycled in the sediment, making it difficult to assess the proportion of seagrass production that is decomposed by bacteria each day. At this stage we cannot determine what proportion of bacterial production is grazed by animals and what is recycled via cell lysis and exudation of organic compounds in the sediment. The lower specific growth rates in the deeper sediment layers, especially in the bay and reef flat sites, suggest that the main fate of organic carbon was recycling within the bacterial community.

Acknowledgement. This work was supported by a Marine Sciences and Technologies grant (No. 81/0319).

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RELATIONSHIPS OF BACTERIAL BIOMASS AND PRODUCTION TO PRIMARY PRODUCTION IN MARINE SEDIMENTS

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I. INTRODUCTION

In pelagic ecosystems, bacterial population density does not vary greatly and may be controlled as much by animal grazing as by availability of organic substrates (1, 2). The sediment environment is more complex. Organic nutrients and oxygen enter from the surface (and roots of plants in shallow water) and are rapidly depleted with depth. Animals are mostly confined to the surficial oxic layers, which may be 1 or 2 mm in muddy sediments and 1 or 2 cm in coastal sandy sediments (3). Burrowing macrofauna increase the effective surface area of oxic sediment for protozoans and meiofauna. Animals stimulate biogeochemical cycling of organic carbon and nutrients in many sediments (4,5).

The interactions of animals, bacteria and organic matter are complex, which means it is difficult to draw conclusions that apply generally. Bacteria have an important trophic function in sedimentary foodwebs, and therefore animals can control bacterial population densities and production, although this does not seem to be common (see Alongi, this volume). In general, bacterial populations seem to be regulated primarily by the supply of organic matter. In this paper, I will discuss evidence for this conclusion and possible mechanisms by which bacterial biomass and growth rates are controlled in sediments.

Oxygen and sulphate reduction are the two most important terminal processes in the oxidation of organic carbon in marine sediments. It is difficult to determine the relative contributions of the various major groups of bacteria and animals to oxygen reduction and thus the importance of bacteria in mineralising organic carbon. Sulphate reduction, however, is solely a bacterial process and thus gives an estimate of carbon cycled through anaerobic bacteria. This is a minimum estimate because CO_2 is released by the fermentative bacteria that provide substrates to the sulphate–reducers. In some coastal sediments, 50 to 70% of organic carbon is respired by sulphate–reducers (6). Most animals cannot live in sediments where anaerobic processes predominate, and so it is not likely that animals graze bacteria significantly in anoxic sediments. Thus animals would not be important as controllers of bacteria in those sediments.

In order to determine whether bacteria are the main decomposers of primary production in sediments, *i.e.* that the various functional groups recycle all the carbon entering the sediment to CO_2, the proportion of organic carbon input that is required to support the observed rate of bacterial metabolism has to be measured. Bacterial metabolism will result in carbon being used for maintenance (respiration), production of new biomass and production of extracellular polymers. Extracellular polymers are probably important products of benthic bacteria, but no practicable methods for determining their rate of synthesis are available. In a complex sediment community, only biomass production and respiration can be measured with some accuracy. The flux of carbon through the major heterotrophic bacteria can be calculated from values of bacterial productivity determined from the rate of tritiated thymidine incorporation into DNA. Carbon flux through sulphate-reducing bacteria can be determined separately, because they do not take up thymidine (7).

Carbon flow through the sediment can be modelled; to do so completely, one would need to know the biomass, growth rates, and growth efficiencies of all the major groups of bacteria, but this is not yet feasible. We need to know the relationship between bacterial production and input of organic carbon, *e.g.* from primary production, in order to discuss controls on bacterial production and biomass. It is obvious that if bacterial growth efficiency is 0.5, then the total amount of organic carbon needed by the bacteria would be twice their production. It is not obvious, however, that at the same time in a closed system at steady state, bacterial productivity (i.e. rate of production) would be equal to the rate of input, not half the rate of input. A simple model is described here that demonstrates this relationship between bacterial productivity and the rate of organic carbon input.

II. MODEL RELATING PRIMARY TO BACTERIAL PRODUCTIVITY

Bacteria in the sediment use not only organic matter cycled directly from the primary producers,

but also recycled from animals and previous bacterial activities. Thus the actual proportion of primary production entering a sediment that is utilised by bacteria cannot be readily determined. It depends on the average growth efficiency of the bacterial community and the sources of organic matter.

Strayer (8), in discussing secondary production in a lake, pointed out that secondary production was the sum of production in all consumer groups and could be greater than input from primary production when recycling occurred. In the case of the seagrass beds, I consider this concept from slightly a different point of view, *viz.* where there is a continuous input of primary production, how are the rate measures of carbon flow related, i.e. how is bacterial productivity related to primary productivity?

The simplest way to examine this concept is with a model system having a daily input of CO_2 that is converted to organic carbon by primary producers and the only output being CO_2 via bacterial respiration (*i.e.* a closed system with respect to organic carbon). On the first day of this system's operation:

$$P_{b1} = PE \qquad (1)$$

and

$$R = P(1\text{-}E) \qquad (2)$$

where P_b is bacterial productivity, P is primary productivity, R is bacterial respiration and E is efficiency. Some typical values for productivity at different growth efficiencies are shown in Table I as initial values.

Table I. Bacterial productivity (P) as percentage of input of organic carbon in a simple model with no losses. Values at steady state are shown for bacterial productivity, cumulative input to the bacteria (i.e. including recycling) and respiration (R) in relation to a constant external input of 100 units of carbon for different growth efficiencies.

State	*Efficiency*	*Input*	*P*	*R*
Initial	*0.3*	*100*	*30*	*70*
Second	*0.3*	*130*	*39*	*91*
Final	*0.3*	*143*	*43*	*100*
Initial	*0.5*	*100*	*50*	*50*
Second	*0.5*	*150*	*75*	*75*
Final	*0.5*	*200*	*100*	*100*
Initial	*0.7*	*100*	*70*	*30*
Second	*0.7*	*170*	*119*	*51*
Final	*0.7*	*333*	*233*	*100*

On subsequent days, the bacterial production from the first day is recycled and must be added to the daily input of primary production. Thus:

$$P_{b2} = (P + P_{b1})E \qquad (3)$$

and

$$R = (P + P_{b1})(1\text{-}E) \qquad (4)$$

and P_{b2} is greater than P_{b1} (see Table I). After a period of time, bacterial productivity increases in proportion to primary productivity until a steady state is reached at which time input equals output, *i.e.* primary productivity equals the rate of respiration. Some values for bacterial productivity as percentages of primary productivity at steady state are given in Table I. It is clear that when the growth efficiency is equal to or greater than 0.5, bacterial productivity is equal to or greater than primary productivity.

Bacterial productivity at steady state may be calculated or found by iteration. At steady state, respiration equals input, thus equation (*4*) above for respiration may be written as:

$$P = (P + P_b)(1\text{-}E) \qquad (5)$$

After rearrangement, this becomes:

$$P_b = \frac{PE}{(1 - E)} \qquad (6)$$

This is the same as the equation given by Strayer (8), but derived using a somewhat different approach and assuming that there are no losses. In order to calculate growth efficiency this equation may be rearranged to give:

$$E = \frac{P_b}{(P + P_b)} \qquad (7)$$

This method for calculating growth efficiency in the natural environment gives minimum values for bacterial efficiency because animals eat some of the bacteria.

When bacteria are removed from the system by grazing or export, the proportion of bacterial to primary productivity is lower. This may expressed as shown in equation (*8*), where losses (L) are subtracted from inputs, including inputs from recycling of bacterial production.

$$P_b = (P + P_b - L)E \qquad (8)$$

Thus at a growth efficiency of 0.5, bacterial productivity would be 50% of primary productivity if all the bacterial production were removed from the system each day; *i.e.* the initial conditions shown in Table I would apply each day.

III. FACTORS LIMITING BACTERIAL PRODUCTION

A. Organic Carbon Supply

Primary productivity in three tropical seagrass communites in summer ranged from 4 to 9.3 g C m^{-2} day^{-1} and bacterial productivity was 2.5 to 4.8. In winter, primary productivity ranged from 2.7 to 3.3 g C m^{-2} day^{-1} and bacterial productivity from 0.6 to 1.0 (9). An example of seasonal variation in productivities is given in Table II. Bacterial production was 20% to 60% of the estimated net primary production (Table II). If all the primary production were recycled through bacteria only (*i.e.* if grazing were negligible) the growth efficiencies would be between 0.2 and 0.4 (Table II). These values are within the ranges quoted for natural systems and for cultures of anaerobic bacteria (10 – 12). Individual anaerobic processes are less efficient than aerobic processes, but the overall efficiency of an anaerobic community could be as high as an aerobic one (10).

Table II. Comparison of gross primary (PP) and bacterial productivity (BP) in a tropical seagrass bed (15). Values are g C m^{-2} day^{-1}. Bacterial productivities are shown as percentages of net primary productivities (%PP; estimated as 20% of gross productivity). Growth efficiencies of the bacteria, calculated from equation 5, are shown.

Month	*PP*	*Bacteria*		*Efficiency*
		BP	*%PP*	
January	*9.3*	*4.8*	*50*	*0.4*
May	*5.7*	*1.0*	*20*	*0.2*
July	*3.3*	*2.0*	*60*	*0.4*
October	*6.0*	*2.2*	*40*	*0.3*

The conclusion from our data, therefore, is that most bacterial productivity was coupled on a seasonal basis to primary productivity, *i.e.* all or most organic matter produced by the seagrasses and algae was decomposed in the same season, and it was mineralised predominantly by bacteria. An independent measurement of growth efficiency is needed to validate this conclusion. If the composite growth efficiency of the bacterial community was found to be substantially greater (perhaps 0.5 or 0.6), then animal control would be significant. The importance of values for growth efficiency of natural communities of bacteria has been discussed by Williams (13) in relation to carbon cycling in pelagic systems.

Bacterial numbers in surface sediment vary considerably between environments, and are not a reliable indicator in sediments of trophic status or rates of organic matter input. In the seagrass beds, it is bacterial production, rather than biomass, that is controlled by the supply of organic matter. Primary and bacterial productivities were closely correlated in the seagrass beds, but bacterial numbers varied by up to an order of magnitude (9). Other examples showing bacterial population density to be a less sensitive indicator than productivity of organic carbon input are seen in coral reefs and on the continental slope (14,15). On the east Australian continental slope, just below the shelf break at about 300 to 400 m water depth, there is evidence of a higher rate of input of organic matter to the sediment than on the shelf, or deeper down the slope. Bacterial numbers ranged from 1 x 10^8 cells g^{-1} dry weight of sediment to 5 x 10^8 at 400 m, and growth rates were about 0.01 h^{-1}. This bacterial population density is 1 to 2 orders of magnitude lower than that on the seagrass beds, and growth rates were similar, which would suggest that the input of organic matter was also 1 to 2 orders of magnitude lower. At 1000 m, however, bacterial densities were an order of magnitude higher (about 2 x 10^9 cells g^{-1}), but growth rates were 1 to 2 orders of magnitude lower (15). One possible reason for the difference in population density is that grazing was greater at the 400 m site than at the 1000 m site. Other factors may also be important, *e.g.* the temperature was lower and the composition of the organic matter may have been less favourable for promoting bacterial growth.

Deep within the sediment, below the zone of bioturbation, the organic matter that is available to the bacteria is that which was buried with the sediment or diffuses down through the pore water. As the latter is a very slow process, the bacteria use up their supply of organic matter and numbers and productivities decrease (15). In this part of the sediment, where there are no animals, bacterial population density as well as productivity are correlated to the supply of organic matter.

In general, heterotrophic bacterial productivity in sediments is a function of the rate of input of organic matter and temperature (see below). Specific growth rates are also directly related to these factors and to the population density of bacteria. A question to be answered is: to what extent is the population density controlled by death and lysis of bacterial cells? In surface sediment, where organic matter enters the sediment and animals are concentrated, productivity and growth rates are greater than deeper in the sediment, but removal of bacteria by grazing animals complicates study of this question. Presumably, in the deeper anoxic sediment, death and lysis is the main control on numbers and a higher proportion of dead cells should be observed. This has indeed been reported: a higher proportion of dead cells and cell

"ghosts" was found at 20 cm depth in a seagrass sediment than at the surface (16).

The natural mortality rate of bacteria, as distinct from that due to grazing, is an important variable governing the population density in sediment. In a sediment with a constant input of organic matter, the population density is regulated by the substrate concentration, and with no grazing the mortality rate would equal the specific growth rate (2). The specific growth rate that is determined with the thymidine method (see below) is an average for all populations, but not all bacteria would be growing at one time. One population of bacteria will continue to grow and divide until conditions become unfavourable, *e.g.* a nutrient source is exhausted. Another population will then start to grow on the metabolic products of the first. Does the first population become dormant or die? Presumably, most bacteria must die because otherwise population density in the sediment below the zone of bioturbation would remain high, but this was not observed (15).

B. Nitrogen

The overall growth efficiency of a community or consortium of bacteria is influenced by the C:N ratio and nature of the organic matter. Plant material generally has a high C:N ratio relative to bacterial cells, so an input of nitrogen from other sources (*e.g.* nitrogen fixation) would enhance growth efficiency, and result in high standing stocks of bacteria. Nitrogen has been shown to have a controlling influence on bacterial biomass and growth in an experimental system (17).

C. Temperature

Temperature is important in controlling rates of metabolic activity and bacterial growth. Bacterial productivity was 5 times lower in winter than in summer in tropical seagrass beds, and it was suggested that this was due mainly to lower temperatures of water in winter: 23°C compared to 32°C or more in summer (15). Alongi (18) found that bacterial productivity was lower in mangrove sediment in winter. Furthermore, he found that when intertidal mud banks below mangroves were exposed to air that was cooler than the water, bacterial growth rates decreased (18). Temperature is an important factor controlling the productivity of bacteria, through its effect on the rate of hydrolysis of organic polymers, especially cellulose and protein. This first step in the decomposition of particulate organic matter, derived from dead leaves, rhizomes and roots of seagrasses and mangroves, seems to be the rate limiting one, but few studies have been made of this process (19,20).

The response of bacteria to organic matter input occurs over a number of time scales, from diel to seasonal. A greater response to a given temperature change, say 10°C, in the tropics than at higher latitudes is likely because rates of mineralisation vary exponentially with temperature (21). In other words, the higher the mean temperature, the greater the response variations.

IV. METHODOLOGY

As Wright (2) has pointed out, the development of qualitative concepts in microbial ecology has been stimulated by the development of quantitative methods, but we still deal largely with qualitative phenomena. In studying carbon cycling in sediments, methods are important, because growth rates of bacteria need to be determined accurately, but direct methods (counting bacteria under the microscope) are not readily applicable. The best method currently available for determining bacterial growth rates in sediment depends on the principle that the rate of tritiated thymidine incorporation into DNA is directly related to the rate of DNA synthesis, which in turn is related to growth rate (7). Estimates of bacterial biomass productivity, obtained from the thymidine method and estimates of cell volume made from microscopical observations, usually agree within a factor of 2 or 3 with values obtained by other methods (15, 22). Furthermore, the values for bacterial productivity are generally within the range of 20% to 70% of primary productivity, and thus are not excessive compared to results obtained when adenine is used instead of thymidine (7). Animals could be important as controllers of bacterial production and population density in sediments where much of the primary production is readily digestible without bacterial decomposition, *e.g.* benthic mats of diatoms or cyanobacteria on coral reefs (14).

The microbial ecologist now has the tools for determining numbers, biomass, growth rates and productivity of heterotrophic bacteria and primary producers. More detailed qualitative questions can now be answered about the role of bacteria in sedimentary biogeochemical processes and foodwebs. Perhaps it is now appropriate to combine these with autecological studies and determine which bacteria are active at any particular time or location. Metabolic methods, like the thymidine method, give results that apply only to the most active bacteria at the time they are used.

SUMMARY

A simple model is described relating bacterial productivity to the rate of organic carbon input. These two processes will be equal in a closed system when bacterial growth efficiency is 0.5.

The population density of bacteria in surface sediments is controlled by mortality, both natural and grazing, and input of organic matter and thus varies considerably between sediments. Deep within the sediment, where animals are absent, bacterial productivity and biomass is controlled by the availability of organic matter. Bacterial productivities, which are very high in tropical seagrass beds and mangrove swamps, are controlled primarily by the input of organic matter. Although there is some evidence for control of growth rates and numbers of bacteria by grazing animals at the sediment surface, they also are affected to some extent by the supply of organic matter from primary producers. Most organic carbon is fully recycled to CO_2 through the bacterial community itself.

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E. Role of Bacteria in Coral Reef Ecosystems

Marine Biology 53, 193-200 (1979)

MARINE BIOLOGY

CSIRO, Division of Fisheries and Oceanography, Reprint No. 1059

Biomass of Suspended Bacteria over Coral Reefs

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Abstract

The biomass of bacteria suspended in water flowing over coral reefs at Lizard Island and Yonge Reef (Northern Great Barrier Reef) was estimated by measurement of muramic acid. Values ranged from 20 mg C m^{-3} in the open water up to about 60 mg C m^{-3} over the reef flat. Direct counts of total numbers of free bacteria were made for comparison. Values of around 2.0 x 10^9 cells μg^{-1} muramic acid showed that there was a good agreement between direct counts and muramic acid content of free bacteria in the open water. In samples containing suspended particulate matter, ratios of direct counts to muramic acid concentration were lower, because bacteria on particles could not be counted. Thus, these ratios were used to indicate the proportions of bacteria attached to particles. Changes in the biomass and numbers of bacteria were determined in water masses identified either by a drogue or fluorescein, as they moved across the reefs. In the zone on the outside of the reef, the number of free bacteria decreased compared to open sea water, but total biomass increased, showing that particulate matter containing bacteria was thrown up into suspension. About 50% of bacteria were attached to particles. Water flowing over the reef flats contained much particulate material with bacteria attached. Bacteria constituted between about 5 and 20% of particulate organic carbon.

Introduction

Bacteria are important in coral reefs both in the recycling of nutrients and as a food source for many species of an animals (DiSalvo, 1974; Sorokin, 1974). Quantitative studies on the biomass of bacteria have been difficult, because techniques based on counting and measuring the sizes of cells (e.g. Sorokin and Kadota, 1972) are difficult or impossible to apply accurately when many bacteria are attached to particles. Coral reef waters are noted for the large numbers of aggregates of organic matter, which contain bacteria, and which are important as food for many animals (Johannes, 1967; Coles and Strathman, 1973; Gerber and Marshall, 1974). As the quantitative importance of bacteria in coral reef systems is not well established, a study was made of the variations that occurred in bacterial numbers and biomass in water flowing over reefs at Lizard Island and Yonge Reef (Northern Great Barrier Reef). A secondary objective of this work was to determine whether there was a good correlation between muramic acid in sea water and direct counts of bacteria, because in the water column most bacteria are Gram-negative and thus their muramic acid content is closely correlated with biomass (Moriarty, 1977).

Materials and Methods

Locality and Water Sampling

Transects were marked out across the windward and leeward reefs of the Lizard Island lagoon (Fig. 1). Water masses, followed either with a drogue or a fluorescein marker, were sampled between Stations A and G on the windward reef and Stations H and F on the leeward reef. Wind speed and direction were noted. (The prevailing winds were from the east.) In addition, a sample was taken 1 km seaward of the windward reef (Station H) each time a transect was sampled. Water was also collected over Yonge Reef on a line following the direction of the

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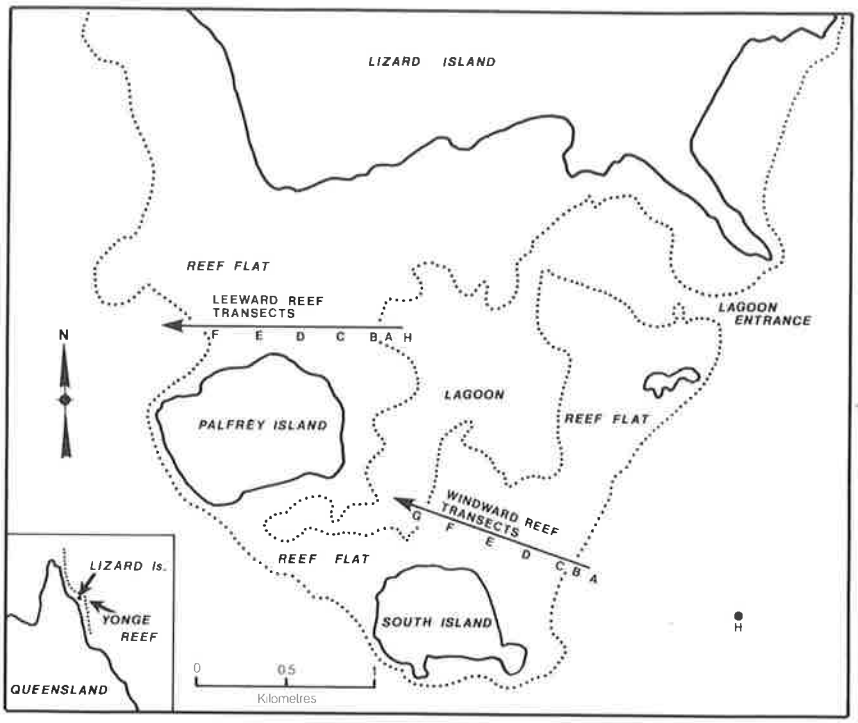

Fig. 1. Map of sampling localities at Lizard Island. Approximate positions of transects are indicated; actual stations varied with wind direction and speed. Arrows indicate predominant direction of waterflow. Open-water windward reef station, H, was 1 km seaward. Water samples only were also taken over Yonge Reef (inset)

prevailing wind (from the south-east), but a water mass was not followed. Transects were sampled between September 2 and 9, 1977. They are labelled in this paper with the date and hour of the day at which they were commenced; for example, Transect 4.1400 was sampled on September 4 commencing at 14.00 hrs. Water, collected in large plastic bins, was brought to the laboratory and well mixed before subsamples were removed. Delays of up to 2 h occurred between collection of water and treatment in the laboratory. Preliminary experiments were conducted which showed that there was no significant change in the number of bacteria during this time period. Details of the transects and the methods used to mark and sample the water masses will be published elsewhere (D. Barnes, personal communication).

Enuermation of Bacteria

Samples containing 10 ml of sea water were stained with acridine orange and filtered through 0.2 µm polycarbonate filters. Direct counts were made using a Leitz Dialux microscope fitted with a Ploem illuminator and a Ploemopak filter System K (Daley and Hobbie, 1975; Hobbie *et al.*, 1977). Between 12 and 20 fields of view were counted for each filter and the results were subjected to an analysis of variance.

Muramic Acid Assays

The methods for assaying muramic acid in sediments have been described elsewhere (Moriarty, 1975, 1977, 1978). The modifications described below give greater sensitivity. One litre samples of water were filtered through 47 mm diameter polycarbonate filters (pore size 0.2 µm). The filters were rinsed with 2 ml of distilled water and fixed with 2 ml of 5% formalin for 2 min. Excess fluid was removed under vacuum and the filters were stored frozen.

The filters were hydrolysed with 4M HCl in sealed tubes at 100°C for 6 h and then acid was removed *in vacuo*. The filters were washed 3 times with distilled water, the washings were combined and 20 µl of Na_2HPO_4 (0.5M) were added. The pH was adjusted to between 7.6 and 8.0 with NaOH (1M). After centrifuging to remove calcium, the pH was lowered to about 2.0 with HCl and the solutions

were then freeze-dried. The dry residue was extracted with about 10 ml of diethyl ether acidified with concentrated HCl (0.1% v/v). The ether was discarded and the residue, dried under a stream of air, was taken up in 0.5 ml distilled H_2O, adjusted to pH 8.8-9.0 with NaOH (1M) and made up to 0.6 ml. A sample (150 µl) was removed and frozen until the assays were carried out. The remaining volume was adjusted to pH 12.5 with 5 to 10 µl of 5M NaOH, incubated at 35°C for 2 h, and then the pH was adjusted to 8.8-9.0 with 3M HCl. Having noted the volume required for pH adjustment, the final volume was adjusted to 0.51 ml, and 170 µl were taken for assay of D-lactate released during alkali hydrolysis. A further 170 µl were assayed using 50 µl of D-lactate (1 ng μl^{-1}) as an internal standard. All sample volumes were adjusted to 0.220 ml for assay of D-lactate. Thus the three samples that were assayed contained the same amount (¼) of the original sample mixture and would have been affected to the same extent by any inhibitory component. Another modification to the procedures published previously was that 200 µl (instead of 100 µl) of the lactate assay mixture was assayed for NADH. The precision of the assays for muramic acid is about ±10%.

Relationship between Counts and Muramic Acid Values

Earlier work has shown that a marine Gram-negative pseudomonad contained about 5×10^{-10} µg muramic acid (MA) $cell^{-1}$ (Moriarty, 1977). This is equivalent to 2×10^9 cells μg^{-1}MA. Because many other marine Gram-negative bacteria had a similar proportion of muramic acid per unit biomass (8 ± 3 µg MA mg^{-1} C) as the pseudomonad (Moriarty, 1977), and most bacteria in sea water are Gram-negative (Watson *et al.*, 1977), about 2×10^9 cells:1 µg MA would be expected in sea water. Muramic acid content of a bacterial cell is more closely correlated with surface area than biomass, and bacteria vary in the thickness of the peptidoglycan wall layers that contain muramic acid (Moriarty, 1977). This variation is more marked in the Gram-positive bacteria, many of which have a much thicker peptidoglycan layer than the Gram-negative bacteria. To calculate biomass, therefore, it is necessary to know the relative proportions of these types of bacteria. Because Gram-negative bacteria occur in sediments in unknown proportions, and no other reliable methods have been available to estimate bacterial biomass in sediments, it has not been possible to check the accuracy of biomass estimated from muramic acid content. In the water column, accurate counts can be made of the number of cells provided they are free (i.e., not attached to particles). It should be possible to obtain a reasonable correlation of counts with muramic acid content, if it is assumed that the average biomass of individual bacterial cells in water samples from similar localities does not vary much. The ratios of direct counts to total muramic acid and to muramic acid passing through 10 µm filters were used to check the interpretation of ratios calculated for samples from the various transects.

Results

Windward Reef

There was a decrease in the number of free bacteria and an increase in muramic acid concentration as the water approached or crossed the reef crest. When the wind speed was less than 10 knots, muramic acid concentration increased over the crest (Fig. 2), but when the speed was in excess of 15 knots, muramic acid concentration increased in front of the reef crest, and decreased as the water flowed over the crest (Fig. 3). Beyond the reef crest, muramic acid concentration and the number of free bacteria either remained constant or increased slightly on most occasions. Rates of change per m^{-2} of bacterial biomass in the water masses as they moved across the reef flat (Stations C to G) were mostly negligible.

Leeward Reef

As water flowed from the lagoon onto the leeward reef, the number of free bacteria decreased and then remained constant or increased slightly. Muramic acid concentrations were rather variable, but tended to increase (Fig. 4).

Yonge Reef

The number of free bacteria was about 3 times higher in water windward of the reef than over the reef or leeward of the reef (Fig. 5). Muramic acid concentrations were a little higher over the reef. Because a water mass was not followed, the values shown here may not accurately represent the changes that occurred as water flowed over the reef, but they do indicate the general trend.

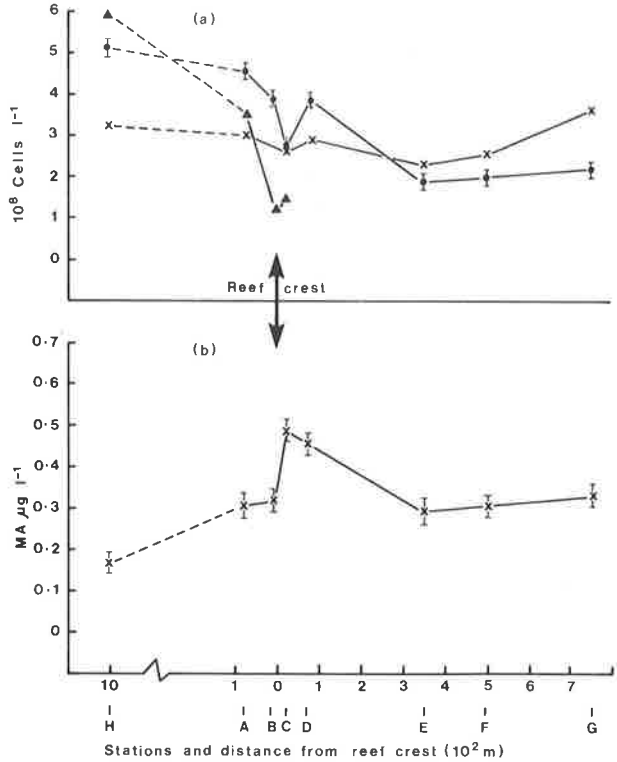

Fig. 2. Total number of free bacteria (a) and muramic acid (MA) values (b) over windward reef; wind speed <10 knots. Transects were 2.1600 (circles), 3.2000 (triangles), and 4.1400 (crosses). Continuous lines indicate that a water mass was followed between stations. Standard errors of bacterial counts and approximate precision of muramic acid analyses are for 1 transect each; the other values displayed a similar variation

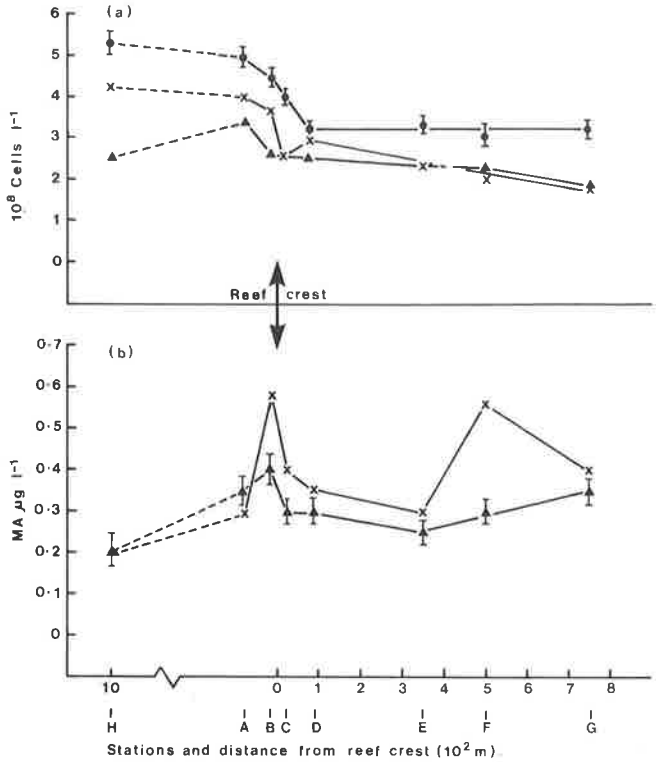

Fig. 3. Total number of free bacteria (a) and muramic acid values (b) over windward reef; wind speed >15 knots. Transects were 2.1000 (circles), 6.0000 (crosses), and 6.0900 (triangles)

Relationship between Counts and Muramic Acid Values

The values of 1.2 x 10^9 to 2.1 x 10^9, obtained for the ratio of bacteria counts to muramic acid in the open water (Station H, windward reef and Yonge Reef, Table 1) agree well with those expected (see "Materials and Methods"). In many samples, particulate matter with embedded bacteria was observed, but it was not possible to count these bacteria. Muramic acid analyses did include particulate matter. Thus the lower ratios (0.4 to 1.0) of counts to muramic acid in water flowing over the reef flats indicate that many bacteria were attached to suspended particles. Confirmation of this interpretation is given by the data presented in Table 2. Between 43 and 55% of muramic acid from 3 stations occurred on particles greater than 10 µm in diameter. The ratios of direct counts to muramic acid in particles less than

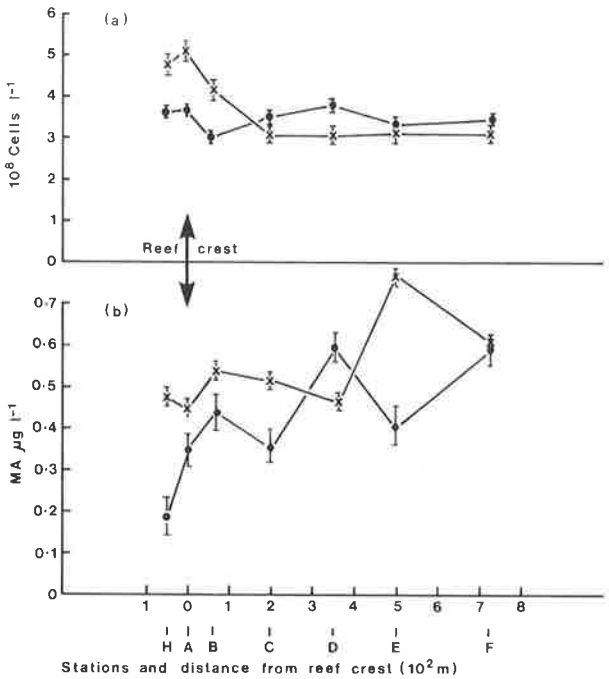

Fig. 4. Total number of free bacteria (a) and muramic acid values (b) over leeward reef; wind speed >13 knots. Transects were 9.0900 (circles), and 9.2100 (crosses)

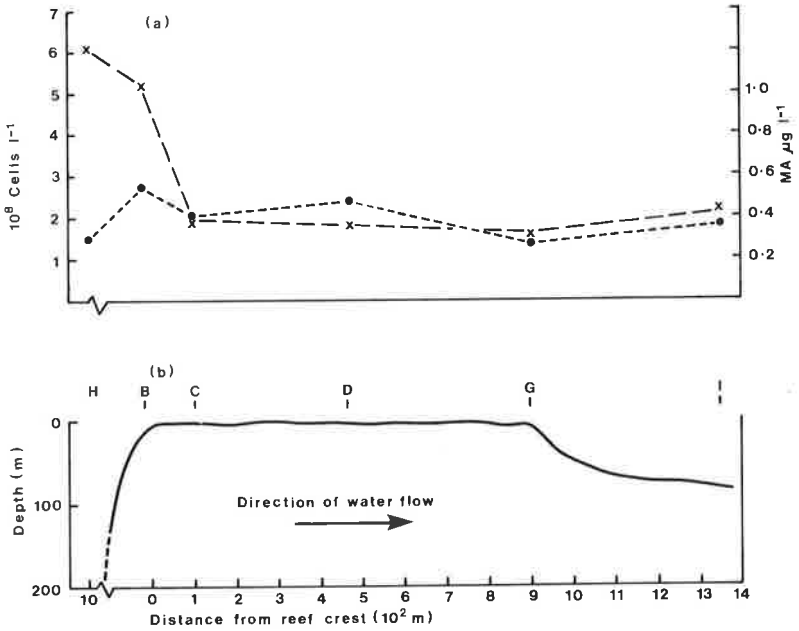

Fig. 5. (a) Total number of free bacteria (crosses) and muramic acid values (circles) over Yonge Reef, Outer Barrier; wind speed <10 knots; dashed lines indicate water mass was not followed; water was collected on 4 September, between 11.00 and 12.00 hrs. (b) Approximate depth profile across sampling transect; station labels indicate positions over the reef equivalent to those on windward reef, Lizard Island; in addition a station (I) was sampled about 500 m west of the reef to show the differences in open water on each side

Table 1. Ratio of direct counts of bacteria to muramic acid (MA). Values are 10^9 cells μg^{-1} MA. Transect codes represent date (September, 1977) and hour of day collection commenced; e.g. 4.1400 = 4 September, 14.00 hrs. See Fig. 5 for station positions on Yonge Reef. Station I was about 500 m west of Yonge Reef. -: no collection made tion made

Station	Windward reef transects			Leeward reef transects			Yonge Reef
	4.1400	6.0000	6.0900	9.0900	9.1400	9.2100	
H	1.9	2.1	1.2	2.1	1.1	1.0	2.0
A	1.0	1.3	1.0	1.0	0.8	1.1	-
B	0.9	0.6	0.7	0.7	0.5	0.7	0.9
C	0.5	0.6	0.8	1.0	0.7	0.6	-
D	0.6	0.8	0.8	0.6	0.7	0.6	0.5
E	0.8	0.8	0.9	0.8	0.9	0.4	0.4
F	0.8	0.4	0.8	0.6	0.4	0.5	-
G	1.1	0.4	0.5	-	-	-	0.6
I	-	-	-	-	-	-	0.6

Table 2. Bacteria attached to particles suspended in water over the windward reef, Lizard Island. Total muramic acid was measured on 1 l samples collected on 0.2 µm filters. Muramic acid was measured on particles greater than 10 µm in diameter that were collected by filtering 3 l samples of water through a 10 µm screen. Muramic acid on smaller particles was calculated by difference. Free bacteria were counted in the complete samples

Station	Bacteria (No. cells x 10^9 l^{-1})	Muramic acid ($\mu g\ l^{-1}$)			Ratio cells:MA (No. x 10^9:1 µg)	
		Total	>10 µm	<10 µm	Total	<10 µm
H	0.45 ± 0.02	0.35	0.15	0.2	1.3	2.2
C	0.28 ± 0.01	0.5	0.25	0.25	0.6	1.1
G	0.40 ± 0.02	0.45	0.25	0.20	0.9	2.0

10 µm were between 1.1 and 2.2, which are similar to the ratios obtained at the open-water stations. The lower ratios (e.g. 0.6 at Station C) in the complete water samples result from particulate material containing prokaryotic organisms in suspension. Most of these organisms were identified, under the fluorescence microscope, as bacteria; very few blue-green algae were observed.

Discussion and Conclusions

Relationship between Direct Counts and Muramic Acid

At the open-water stations, the ratios of the number of free bacteria to muramic acid were close to the expected values. An exact correspondence would not be expected, because the results would be affected by many factors, including variations in size of cells, composition of cell walls and sampling errors. These results demonstrate that there is no serious error in using muramic acid to estimate biomass in the water column. As some particles were observed in the open-water samples, the ratios that were measured would be minimal estimates. The values for Station H (Table 2) show that the ratio of cells to muramic acid for free bacteria is about 2.2. The lower value of 1.1 for Station C (Table 2) may mean that some Gram-positive bacteria were present. They are probably more numerous in the sediment and are likely to have been suspended in the water by turbulence in the shallow zone immediately behind the reef crest. Further work on the nature of the bacteria in the sediments is necessary before more accurate calculations can be made of biomass in sediments or in particles thrown up into suspension.

If bacteria are aggregated or attached to particles, direct counts will underestimate the actual numbers present. Thus, over these reefs, where 50% or more of bacteria in the water column were attached to particles, counting of cells not only underestimated the biomass of bacteria, but gave a false indication of the changes occurring in bacterial biomass as water flowed over or past the reefs. The combination of direct counting and muramic acid measurements provided information about the proportion of bacteria on particles in the water. For example, a ratio of about 0.6 was obtained when half the bacteria were attached to particles greater than 10 µm in diameter (Table 2). Muramic acid assays are time-consuming, so this technique of combining them with direct counting halves the number of assays necessary to determine whether a substantial number of bacteria are attached to particles.

Effects of Water Movement

When the wind speed was less than about 10 knots, there was little change in muramic acid concentration in the zone to the windward side of the reef, but a large increase occurred as water crossed the reef crest, presumably because it was only in this shallow area that turbulence was great enough to suspend particles from the sediment. When the wind speed was greater than 15 knots, water at the windward edge of the reef (Fig. 1, Station B), was very turbulent and contained much suspended particulate matter. The increase in muramic acid concentration and the low values for the ratio of free bacteria to muramic acid (0.5 to 1.0) show that the suspended particles contained bacteria. The values of around 0.5 show that over half the bacteria were attached to particles. The decrease in muramic acid between Stations B and C (Fig. 3b) suggests that many particles either fell out of suspension or were trapped by animals. The water slowed down as it approached the crest, but speeded up again after passing the crest. The evidence suggests that at lower wind speeds water remained longer in the zone outside the reef, moving along the front reef, before crossing the reef crest (B. Scott, personal communication). It was not possible, therefore, to calculate the mass transport of bacteria across the reef crest. As the water took less than 30 min to cross this zone, bacterial growth can be disregarded as a factor influencing the increase in muramic acid concentration. The location of Station B was variable, as it was difficult at night or in rough weather to sample close to the crest. This would explain why on some occasions large changes were observed between Stations A and B and on others, between B and C. Johannes (1967) reported that water flowing over an Eniwetok reef picked up particulate material containing bacteria. The observations of Westrum and Meyers (1978), that organic carbon concentrations increased at the reef crest and decreased behind the crest, agree with these findings for muramic acid concentrations.

Although muramic acid increased in the zone immediately outside the reef, the numbers of free bacteria decreased significantly ($P < 0.01$), except on Transect 4.1400. This effect was especially pronounced over Yonge Reef, where there

was a three-fold difference. Possibly turbulence caused aggregation of the bacteria or they may have been trapped by mucus released by corals. Coles and Strathman (1973) noticed that mucus contained bacteria and detritus. Johannes (1967) observed that aggregates, collected immediately inside the reef, contained established bacterial colonies and commented that there would not have been sufficient time for turbulence to have caused their formation. He suggested that bacteria already embedded in mucus were thrown up into suspension by the water. The increase in muramic acid at Station B (Fig. 3) and Station C (Fig. 2) support his argument that bacteria attached to particles are brought up into suspension. The significant reduction in the numbers of free bacteria on most occasions as water approached or crossed the reef crest suggests, however, that turbulence also influences the formation of aggregates from bacteria already present in the water column.

Water leaving the reef flats (Stations F or G) generally had a low ratio of counts to muramic acid when the wind speed was greater than 15 knots, indicating that suspended particulate matter with attached bacteria was carried away. This effect was not noticed at lower wind speeds; for example a ratio of 1.1 was found at Station G on Transect 4.1400. All ratios measured for Station H on the leeward reef were over 1.0, indicating that particulate matter was not present in large proportions (Table 1). This suggests either that water entering the lagoon over the reef flats lost much suspended particulate matter or that much of the water in the lagoon flowed in through the deep entrance (Fig. 1).

Table 3. Comparison of bacterial biomass (BC) and particulate organic carbon (POC). Bacterial biomass was calculated from muramic acid values (see "Biomass of Suspended Bacteria"). Particulate organic carbon (POC) values are taken from C. Crossland (personal communication); POC was collected on Whatman GF/C filters and measured by the dichromate method (Strickland and Parsons, 1972). BC (%): Values for bacterial carbon, as a percentage of POC, calculated on assumption that 50% of bacteria passed through the GF/C filters. -: no collection made

Station	Transect 4.1400			Transect 6.0000			Transect 9.2100		
	BC	POC	BC	BC	POC	BC	BC	POC	BC
	(mg m^{-3})		(%)	(mg m^{-3})		(%)	(mg m^{-3})		(%)
H	17	243	3	20	185	5	40	242	8
A	25	281	4	24	152	8	36	205	9
B	26	184	7	48	165	14	44	147	15
C	40	259	8	32	168	9	42	234	9
D	37	154	8	28	171	8	38	354	5
E	24	500	2	24	172	7	62	374	8
F	24	116	10	45	120	18	48	259	9
G	27	176	7	32	82	20	-	-	-

Biomass of Suspended Bacteria

To convert muramic acid values to biomass, a formula has been proposed which takes into account the relative proportions of Gram-negative and Gram-positive bacteria in the population (Moriarty, 1977). Using this formula, a round value of 100 x MA can be used to estimate bacterial carbon in the open water, and 80 x MA can be used for water over the reefs, where some Gram-positive bacteria are present. Bacterial carbon represented between about 2 and 20% of particulate organic carbon (POC; Table 3). A strict comparison is not possible, because POC was collected on glass-fibre filters (pore size about 2 to 5 µm) and bacteria were collected on filters with a pore size of 0.2 µm. If less than 50% of the bacteria passed through GF/C filters, which could happen if free bacteria were trapped in parts of the filter clogged by organic matter, bacterial biomass would constitute an even higher proportion of the total organic matter. In round values, bacteria comprised on average about 10 to 15% of organic matter in the water column. A method for measuring POC retained by 0.2 µm polycarbonate filters is needed before more precise comparisons can be made.

Sorokin (1974) measured the biomass of bacteria in lagoon water at Heron Island (Great Barrier Reef) and found between 30 and 70 mg C m^{-3}, similar to the values described here for Lizard Island (Table 3). His quite different technique was based on measuring the sizes of bacteria counted on filters. He found that about 20 to 30% of bacteria over reefs occurred in aggregates.

Organic Aggregates and Trophic Role of Bacteria

The formation and occurrence of aggregates of organic matter as water flows over coral reefs have been studied by many people. Johannes (1967) suggested that the aggregates, composed of coral mucus with embedded detritus and microorganisms, were an important source of food for zooplankton and some species of fish. Coles and Strathman (1973) measured organic C and N in aggregates and found that coral mucus became enriched in nitrogen with age. In other words, the aggregates probably pick up microorganisms and/or contain actively growing microbial populations. The data presented here for the decrease in ratio of bacterial numbers to muramic acid as water flowed over the reef support these conclusions and, further, show that bac-

teria are a major component of the microbial population. Although blue-green algae were observed under the microscope, they were not abundant and their numbers were too low for statistical analysis. As many animals feed on organic aggregates, the data on their muramic acid content is in agreement with the views of Sorokin (1974) that bacteria have an important trophic role in reef environments.

Acknowledgements. This work is a part of the studies undertaken by the LIMER Expedition Team, 1977. I wish to thank all members of the team for their help, especially Dr. D. Barnes who organised the collecting of water samples. I am grateful to the Australian Institute of Marine Science for logistic support and to the staff of the Lizard Island Research Station for their cooperation and provision of facilities.

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Date of final manuscript acceptance: April 20, 1979. Communicated by G.F. Humphrey, Sydney

Aust. J. Mar. Freshwater Res., 1982, **33**, 255–63

Feeding of *Holothuria atra* and *Stichopus chloronotus* on Bacteria, Organic Carbon and Organic Nitrogen in Sediments of the Great Barrier Reef

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Abstract

Organic carbon and nitrogen and bacterial biomass were measured in the sediments and gut contents of *H. atra* and *S. chloronotus* on the Great Barrier Reef. Organic carbon averaged from 3·4 to 4·7 mg g^{-1}, organic nitrogen from 0·20 to 0·31 mg g^{-1} and muramic acid from 1·4 to 3·3 $\mu g\ g^{-1}$ dry weight of surface sandy sediments. Bacterial biomass, determined by muramic acid measurements, averaged 3–8% of organic carbon in the sediments; blue-green algae accounted for 3–7% of muramic acid. Significantly higher values of organic carbon and nitrogen and muramic acid were found in foregut contents of the holothurians, indicating selective feeding on organically rich components of the sediment. Carbon values were 16–34% higher in the foregut than in the sediment, nitrogen values 35–111% higher and muramic acid values 33–300% higher. These values indicate that bacteria and nitrogenous components of the organic matter were selectively eaten. Values for organic carbon and nitrogen and muramic acid were generally lower in the hindgut than in the foregut, due to digestion and assimilation. Assimilation efficiencies averaged 30% for organic carbon, 40% for organic nitrogen and 30–40% for muramic acid (bacteria). Detritus (non-living matter) probably constituted 60–80% of the organic matter in the sediment and thus the food of the holothurians.

Introduction

Bacteria are believed to be important in coral reef processes such as organic matter decomposition and nutrient regeneration and in reef food chains, but few quantitative studies on bacterial populations have been made, especially in sediments. Sorokin (1974) has reported a value for bacterial biomass of 42 $\mu g\ C\ g^{-1}$ dry weight of sediment at Heron Island on the Great Barrier Reef. He pointed out that the biomass of bacteria in coral reef sediments was high (2–4% of organic matter) and that the bacteria were important as food for many animals. There have been no detailed quantitative studies, however, on the trophic role of bacteria in coral reef sediments because it has been difficult to measure bacterial biomass. The development of the muramic acid method for measuring bacterial biomass now makes it possible to quantify bacterial populations in sediments and to determine how much bacterial biomass is eaten by animals (Moriarty 1975, 1980). Good correlations have been obtained between biomass estimates made by direct counts of bacteria and by the muramic acid method (Moriarty 1979, 1980). Holothurians are widespread and conspicuous deposit-feeding animals on coral reef sediments. They have some commercial importance as a bêche-de-mer fishery. It has long been supposed that bacteria were important as food for them, and Bakus (1973), in his review on holothurians, hypothesised that they assimilate primarily bacteria and detritus. In this paper the hypothesis that bacteria are important dietary components is tested with muramic acid measurements which measure prokaryotic biomass only and not total

microbial biomass. Two preliminary surveys were carried out on pooled samples and in one of these the method was compared with direct microscopy by Dr Y. Sorokin. Improvements in the methods as a result of these early analyses permitted detailed studies on individual samples, combined with an analysis of total organic carbon and nitrogen to determine the relative importance of bacteria in the food of holothurians. The results of these analyses are reported here.

Materials and Methods

Sediments

Samples were collected on the reef flat near the research station at Heron Island, Great Barrier Reef Province, and at sites south and west of the research station at Lizard Island, Great Barrier Reef. Sites 1, 2 and 4 had a sandy substrate with small coral outcrops; water depth 1 m. Most animals at site 2 were feeding on the sandy substrate and some were feeding on a hard rocky surface. Samples of animals were taken from each substrate. Site 3 had a fine sandy layer a few millimetres thick on a rocky coral outcrop. Site 5 had a sandy substrate, 10 m depth, near a coral outcrop. The holothurians were not abundant in areas with no coral.

For the preliminary surveys (Tables 1 and 2), sediments to a depth of 15 mm were collected in corers (diameter 24 mm) and six samples were pooled for analysis. *Holothuria atra* Jaeger and *Stichopus chloronotus* Brandt were dissected within 1 h of capture. Sediment was taken from the first 5 cm and last 5 cm of the gut. Samples were pooled from three animals at each locality. For direct counts 1-g subsamples of the wet sediments were weighed out. Samples for counting were treated immediately with 6 M HCl. Samples for muramic acid analysis were frozen and taken to Brisbane where they were freeze-dried.

For the subsequent detailed analyses (Tables 3 and 4), undisturbed sediment to a depth of 10 mm in front of each animal was collected (corer diameter 85 mm), frozen on dry ice and then freeze-dried. *H. atra* and *S. chloronotus* (25–30 cm in length) were collected, brought to the laboratory on ice and dissected within 1 h of capture. Sediment was taken from the first 5 cm and the last 5 cm of the gut, frozen on dry ice and then freeze-dried. Each sample was kept individually for comparison with sediment from the same location. A glass pH electrode was used to measure pH values in the intestinal fluid of the holothurians.

Sediments were milled to lessen variation in analyses due to the large range in particle size and composition.

Muramic Acid Analysis

For the first survey, carbonate was removed by treatment with 6 M HCl and samples were analysed as described by Moriarty (1975). Subsequent work showed that this technique overestimated muramic acid; a correction factor was found empirically with which values from the early survey could be corrected. All subsequent analyses were carried out as described by Moriarty (1980). Sample sizes for analysis were 2 g of surface sediments and 200 mg of gut contents.

Organic Carbon and Nitrogen

A modified Perkin–Elmer model 240 elemental CHN analyser was used to measure organic carbon and nitrogen in the presence of carbonate (Moriarty and Barclay 1980). Sample sizes for analysis were 150–300 mg of sediment or gut contents.

Direct Counts

For counting bacteria, carbonate was removed by adding 6 M HCl. Samples were then diluted with 10 ml distilled water, and bacteria in 0·1-ml subsamples were collected on 25-mm membrane filters (pore size 0·2 μm). Filters were stained with erythrosin and bacteria counted and measured as described by Sorokin and Kadota (1972). Bacterial biomass was estimated from the counts and measurement of cell dimensions.

Blue-green algae were counted using acridine orange and epifluorescence. Samples were first treated with acetic acid (2 ml g^{-1} sediment, diluted to 10% v/v) to remove carbonate. A water-immersion 25 × objective and 12·5 × eyepiece were used. Other details were as described by Moriarty (1980). Blue-green algae were counted in three size categories, from which biomass was calculated. To estimate muramic acid content, it was assumed that all blue-green algae had a muramic acid content of 1×10^{-7} μg per 80 μm^3 cell volume (Moriarty 1977). As the biomass of blue-green algae, and hence their muramic acid content, was low compared to bacteria in these sediments (see Results), errors due to different thicknesses of peptidoglycan in different blue-green algae have no effect on the conclusions drawn.

Results

Preliminary studies on the ingestion of bacteria by holothurians indicated that not only were bacteria selectively ingested but they were also digested (Tables 1 and 2). The results obtained with the muramic acid procedure compared well with bacterial biomass estimated by direct microscopy. There were at least three times more bacteria

Table 1. Bacterial biomass in sediments and gut contents of *H. atra* at Heron Island
The muramic acid (MA) method for estimating biomass was compared with a direct counting procedure by Dr Y. Sorokin (see Materials and Methods). Samples were collected in January 1976. Gut contents of three animals were pooled. Sediments were taken with small corers to a depth of 15 mm. All values are per gram dry weight of sediment

Sample	MA concn ($\mu g\ g^{-1}$)	10^{-9} × No. of bacteria per gram	Bacterial biomass From MA (mg C g^{-1})	Bacterial biomass From counts (g^{-1})
Sediment	1·8	3·2	0·14	0·2
Sediment	1·6	2·3	0·13	0·1
H. atra foregut	6·8	17	0·54	0·7
H. atra hindgut	4·4	6	0·35	0·3

in the foregut contents than in the sediment. The lower bacterial biomass and muramic acid values in the hindgut indicate that digestion and assimilation had occurred. Blue-green algae were present in these sediments, but their biomass was low compared to the bacteria; at most no more than 10% of the muramic acid was present in the blue-green algae. As a large amount of sediment was required for analysis, gut contents were

Table 2. Bacterial biomass in sediments and gut contents of holothurians at Lizard Island
Biomass was estimated from muramic acid values. Samples were collected in June 1977. Gut contents from three individuals were pooled for each sample. Sediments were collected with small corers to depth of 15 mm. S, sediment; F, foregut contents; H, hindgut contents

Site	Species	Muramic acid concn ($\mu g\ g^{-1}$)			Bacterial biomass (mg C g^{-1})	
		S	F	H	S	F
1	*H. atra*	1·0	3·4	0·9	0·08	0·27
	S. chloronotus	1·0	3·6	1·2	0·08	0·29
5	*H. atra*	2·0	6·8	2·8	0·16	0·54
3	*S. chloronotus*	1·6	3·0	1·6	0·13	0·24

pooled. Recent improvements in the muramic acid procedure, making it much more sensitive, enabled studies to be carried out on individual animals, with some statistical analysis.

The concentrations of organic carbon and nitrogen were generally higher in the foregut contents than in the sediment or hindgut contents (Table 3). These differences were highly significant in the case of nitrogen in all samples, but some samples did not show very significant differences in carbon content. The animals had selected organic matter with a higher nitrogen content than average, as shown by the lower C : N ratio in

the foregut contents. The differences in the C:N ratio were significant in all cases (Table 3). Of the 21 animals sampled from a sandy substrate, only one had less organic carbon in the foregut than the average for the sediment at that site (site 1). A direct comparison between sediment on the rock substrate at site 2 and the animals feeding there was not possible. Although a sediment sample was scraped from the rock surface, its composition was very variable and there was no way of knowing whether the

Table 3. Organic carbon and nitrogen in sediments and gut contents of holothurians at Lizard Island
Sites 1, 2 and 5 had a sandy substrate. At site 2, animals were also collected from a hard rock substrate. Individual animals and sediment cores to a depth of 10 mm were collected for analysis in March 1979. S, sediment; F, foregut contents; H, hindgut contents; s.e., standard error; *P*, level of significant difference between S and F or F and H with Mann–Whitney rank test. Values are per gram dry weight or per square metre of sediment

Site	*n*	Organic carbon ($mg\ g^{-1}$)			Organic carbon ($g\ m^{-2}$)	Organic nitrogen ($mg\ g^{-1}$)			Organic nitrogen ($g\ m^{-2}$)	C:N ratio		
		S	F	H	S	S	F	H	S	S	F	H
1[A]												
Mean	6	3·7	4·3	3·3	460	0·20	0·42	0·24	25	18	10	14
s.e.		0·3	0·3	0·1	37	0·01	0·03	0·02	1			
P(%)			10	1			1	1			1	
2												
H. atra	3	3·4	4·1	2·4		0·23	0·31	0·02		15	13	12
S. chloronotus	3	3·7	4·5	4·1		0·28	0·40	0·27		13	13	15
Mean		3·5	4·3	3·2	511	0·25	0·35	0·24	36	14	13	13
s.e.		0·2	0·2	0·4	29	0·02	0·02	0·02	3			
P(%)			5	10			1	1			5	
2												
H. atra	6	4·7	6·3	4·8		0·31	0·48	0·35		15	13	14
S. chloronotus	3	4·2	6·3	4·4		0·25	0·52	0·30		17	12	15
Mean		4·6	6·3	4·6	570	0·29	0·49	0·33	36	16	13	14
s.e.		0·2	0·3	0·3	25	0·02	0·03	0·02	2			
P(%)			1	1			1	1			1	
2 (rock surface)[A]	4[B]	6·0	5·7	3·0		0·41	0·46	0·22		15	12	14

[A] *H. atra* was only species analysed.
[B] Only one sediment sample was analysed.

animals had fed on that material. This sample indicates, however, that there was more organic matter in this sediment, which is reflected in the high values for organic carbon and nitrogen in the foregut (Table 3). On the sandy substrates, the animals appeared to be feeding on the sediment to a depth of about 10 mm, so sediment was collected to 10 mm for analysis. At site 2, sediment was also collected to a depth of 3 mm: there was no significant difference in carbon, nitrogen and muramic acid content between this sample and 10 mm samples. Results for the two species were combined at each site, as there was no apparent feeding difference between the species.

In 23 out of 25 animals, there was more muramic acid in the foregut than in the sediment, and less in the hindgut than in the foregut (Table 4). The differences between sediment and foregut were significant at two sites. At site 2 the differences were not significant because two animals had less muramic acid in their foregut than the average for the sediment. Some of the sediments had a thin layer of blue-green algae on the

Table 4. Bacteria and blue-green algae in sediments and gut contents of holothurians at Lizard Island
n.s., not significant. See Table 3 for explanation of abbreviations

Site	*n*	Muramic acid ($\mu g\ g^{-1}$)			Muramic acid ($mg\ m^{-2}$)	Bacterial carbon ($mg\ g^{-1}$)			Bacterial carbon ($g\ m^{-2}$)	Organic carbon (%)		10^{-7} × No. of cells of blue-green algae per gram			Muramic acid content of blue-green algae ($\mu g\ g^{-1}$)		
		S	F	H	S	S	F	H	S	S	F	S	F	H	S	F	H
1[A]																	
Mean	6	1·8	5·3	6·1	223	0·14	0·42	0·49	17	4	10		Few			Trace	
s.e.		0·1	0·5	0·8	12												
P(%)			1	n.s.													
2																	
H. atra	3	3·3	2·9	2·5		0·26	0·23	0·20		8	6						
S. chloronotus	3	2·6	5·1	2·9		0·21	0·41	0·23		6	9						
Mean	6	3·0	4·0	2·7	438	0·24	0·32	0·22	35	7	7	1·3	1·6	1·5	0·1	0·1	0·1
s.e.		0·2	0·5	0·2	22							0·2	0·2	0·2			
P(%)			10	10													
4																	
H. atra	6	1·8	6·6	4·0		0·12	0·53	0·32		3	9						
S. chloronotus	3	1·4	7·2	3·5		0·11	0·58	0·27		3	9	1·2			0·1		
Mean	9	1·7	6·8	3·8	211	0·13	0·54	0·30	0·16	3	9						
s.e.		0·3	0·7	0·4	37												
P(%)			1	1													
2 (rock surface)[A]	4[B]	5·2	7·4	4·4		0·42	0·59	0·35		7	10	1·6	1·5	1·1	0·15	0·1	0·1

[A] *H. atra* was only species analysed.
[B] Only one sediment sample was analysed.

surface, but these accounted for only about 0·1 g g^{-1} sediment, which was about 3–7% of total muramic acid. Bacterial biomass constituted about 3–8% of organic matter in the sediments, but was about 6–10% of organic matter in the foregut contents (Table 4).

About 25% of the animals that were collected contained little or no sediment. There was no difference between animals collected soon after dawn, or before sunset. In those that were feeding, sections of the intestine were distended with gas. The pH was 6·5–6·9 in the midgut region of both species.

Discussion

The results reported here indicate that *H. atra* and *S. chloronotus* not only selected organic matter from the sediment, but that they selected nitrogenous components. There is a greater concentration of nitrogen relative to carbon in the foregut contents (Table 5). In 21 out of 25 animals, the proportion of muramic acid in the foregut was

Table 5. Selectivity and assimilation of food components by holothurians

Selectivity is the percentage increase of the component in the foregut over that in the sediment. Assimilation efficiencies were calculated from the difference between the ratios of each organic component to inorganic weight in foregut and hindgut contents. The amount assimilated was calculated on the assumption that 80 g sediment was eaten each day by a *H. atra* 25 cm long (see text). MA, muramic acid. Bact. C, bacterial carbon

Site	Species	*n*	Selectivity (%)			Assimilation (efficiency, %)			Assimilation (amount, mg day^{-1})		
			C	N	MA	C	N	MA	C	N	Bact. C
1	*H. atra*	6	16	111	194	23	43	0	80	14	0
	S. chloronotus and *H. atra*	6	25	35	33	25	27	32	88	8	8
4	*S. chloronotus* and *H. atra*	9	34	55	300	27	33	44	136	13	21
2, rock surface	*H. atra*	4	—	—	—	47	52	40	216	21	21

two- to threefold greater than in the sediment, which shows that the animals were selecting sediment fractions that contained bacteria. Both Sorokin's results (Table 1) and direct microscopy with epifluorescence, which showed that blue-green algae were not more abundant in the gut contents (Table 4), confirmed that the higher muramic acid values were due to more bacteria in the gut contents and not to a selection of blue-green algae. Muramic acid has proved to be very useful in quantifying bacterial biomass in coral reef sediments, because most bacteria are firmly bound in large aggregations of slime and cannot be dispersed by vigorous blending. Thus, enumeration with direct microscopy is not only tedious but prone to error because of patchy distributions on the filter surface. Sorokin's results, however, agreed well with those of the muramic acid procedure, although a correction factor was needed for these early measurements of muramic acid. The relative values between sediment, foregut and hindgut are valid, although the absolute values may be in error. It is most unlikely that the higher values for bacterial biomass in foregut contents were due to bacterial growth in the foregut. The foregut is a simple tube through which food material passes. There would not be much time for bacteria to grow and if there was organic matter which supported rapid bacterial growth, it would be more likely that bacteria in the sediment would have utilized it first.

Some reports in the literature claim that holothurians feed selectively, particularly with respect to particle size (Yamanouti 1939, cited by Webb *et al.* 1977; Hauksson 1979; Roberts 1979), whereas others have found no selection based on particle size (Sloan and von Bodungen 1980; L. S. Hammond, personal communication). It is clear, however, that holothurians do feed selectively on organically rich components of sediments. Measurement of this selectivity is difficult as there is considerable variability in sediment composition and the levels of organic carbon and nitrogen are usually low in coral reef sediments. The results reported show that, on average, organic carbon levels were 16–34% more in the foregut than in the sediment (Table 5). Although some individuals had apparently not selected organic matter, it is possible that the carbon content of the sediment where they had fed was lower than average. Selective feeding by holothurians has been reported by Massin and Jangoux (1976) who noted that *H. tubulosa* selectively ingested sand with an organic coating and rejected cleaned sand. They commented that *H. tubulosa* recognized the organic matter with gustatory receptors on the peribuccal podia. L. S. Hammond (personal communication) found that organic carbon, organic nitrogen and chlorophyll *a* were more concentrated in the foregut contents of three species of holothurians in the West Indies. Webb *et al.* (1977) reported that *H. atra* fed selectively on organic matter, and Hauksson (1979) showed that *S. tremulus* (Gunnerus) selected organically rich components of sediment. Thus, this evidence suggests that chemosensory receptors control feeding selectivity, and that if any particle-size selection occurs, it is a result of non-uniform distribution of organic matter in sediments.

Bacteria were selectively ingested (Table 5), and their biomass in the foregut contents constituted up to 10% of the organic carbon and nitrogen. Thus, they are important food components, but what are the other components? The biomass of blue-green algae was only about 5–10% of the bacterial biomass, and thus no more than 1% of the total organic carbon. Although other micro-algae were not quantified, it was noted during microscopic counting of blue-green algae that no other group of algae was present in very much larger numbers. Thus, total algal biomass would not have been much more than about 10% of total organic carbon. Meiofauna constitute about 1–5% of organic carbon in these sediments (C. M. Moriarty, personal communication). The main living components including protozoa of the sediment thus make up perhaps 20–40% of the organic matter and detritus may constitute 60–80%.

From a calculation using a formula proposed by Trefz (1958), sediment takes about 11 h to pass through a *H. atra* 25 cm long. As the gut held a total of about 40 g of sediment, the amount of organic carbon and nitrogen and bacteria that were assimilated each day can be estimated. A *H. atra* 25 cm long assimilated 80–216 mg C day^{-1}, 8–21 mg N day^{-1} and 8–21 mg bacterial carbon day^{-1} (Table 5). These data show that bacterial carbon accounts for about 10% of the carbon assimilated. All other living organisms would comprise another 10–20% of organic carbon. Thus, it may be concluded that about 50–150 mg C day^{-1} of detritus, or non-living matter, is assimilated from the sediment. L. S. Hammond (personal communication) also concluded that detritus *per se* was a more important food source than micro-organisms for holothurians in the West Indies. Similar conclusions were reached by Cammen (1980) for food components of the polychaete *Nereis succinea* (Frey and Leuckart).

Transmission electron microscopy has shown that many of the bacteria are embedded in slime layers and mucus (unpublished observations). Extracellular polymers excreted by bacteria and other organisms are considered part of detritus, along with decomposing organic matter, and these may well be important food items

for deposit feeders. There has been much discussion in the literature on whether detritus or micro-organisms are more important as food for deposit feeders. Detritus is a very broad term, and in cases where higher plant material is the main source it may not be readily digestible by animals and thus micro-organisms would be important. In these reef sediments, however, mucus from animals and slime from bacteria are probably the main sources of detritus, and some of this material may be readily digestible as indicated by the results reported here (Table 5).

Assimilation efficiencies of animals in the field cannot be measured precisely because it is not possible to provide the animals with diets of known composition and then to collect fresh faeces. Approximate efficiencies were calculated here using the composition of the contents of the foregut and hindgut, on the assumption that no change occurred in the inorganic matter (Table 5). In fact, some dissolution of $CaCO_3$ would have occurred because the midgut fluid was acidic and gas was observed in the gut in many animals, but the amount was insignificant. No statistically significant differences in carbonate content could be determined between foregut and hindgut with the CHN analyser. Dissolution of carbonate in the gut of holothurians has been quantified by Hammond (1981) who used alkalinity measurements to show that less than 0·25% of ingested sediment was dissolved.

Most animals assimilated proportionally more nitrogen than carbon (Table 5), and the differences between foregut and hindgut contents were all significant (Table 3). Yingst (1976) reported an assimilation efficiency of 22% for organic carbon by *Parastichopus parvimensis* (H. L. Clark). *S. tremulus* assimilated 6–51% of organic carbon from sediments, with a mean of 27% (Hauksson 1979). Bakus (1973) cites references which report assimilation efficiencies of about 50% for organic carbon and nitrogen by tropical holothurians. There are difficulties in measuring low levels of organic carbon and nitrogen in carbonate sediments, which may be why some authors (e.g. Webb *et al.* 1977) found little or no assimilation of organic carbon or nitrogen in some cases. Modifications to the Perkin–Elmer CHN analyser allowed larger quantities of sediment to be combusted and thus accuracy was improved (Moriarty and Barclay 1980).

As 23 out of the 25 animals that were sampled had less muramic acid in the hindgut than in the foregut, it may be concluded that bacteria are digested and assimilated by the holothurians. The actual assimilation efficiency of bacteria may be higher than the values of 32–44% for muramic acid, because muramic acid is a component of peptidoglycan in the bacterial cell wall. Lysis of bacteria and digestion of cell contents could occur, without digestion of muramic acid. In other words, the assimilation efficiencies of muramic acid are minimum values for the assimilation efficiency of bacteria. Yingst (1976) reported an assimilation efficiency of 47% for bacteria in an experimental study of *P. parvimensis*. Trefz (1958) noted that amoebocytes from the gut fluid of *H. atra* phagocytized *Bacillus subtilis* and she suggested this as the mechanism for digestion of bacteria. This mechanism is consistent with the decrease in muramic acid between foregut and hindgut. If lysis only had occurred, bacterial cell walls and hence muramic acid may have remained and large differences would not have been observed.

Acknowledgments

I am grateful to Dr Y. Sorokin, Institute of Oceanology, Gelendjyk, Krasnodar District, U.S.S.R., for his enthusiastic participation in the early part of this work and

for agreeing to use his direct microscopic method for measuring bacterial biomass. I am also grateful to Ewen Wynne for assistance with carbon and nitrogen analyses; to Gerald Miles for assistance in counting blue-green algae; and to Margaret C. Barclay for help in collecting and preparing the sediments for analysis. Dr L. S. Hammond provided information from his Ph.D. thesis and offered helpful comments on the manuscript. I am grateful to the staff at Lizard Island Research Station for their assistance and provision of facilities.

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Manuscript received 11 June 1981, accepted 21 October 1981

Marine Biology 85, 285–292 (1985)

Marine Biology
International Journal on Life in Oceans and Coastal Waters

Temporal and spatial variation in bacterial production in the water column over a coral reef

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Abstract

Production and doubling times of the bacterial populations in the water around and over the reefs at Lizard Island, Great Barrier Reef were measured during summer and winter, 1982 and 1983. Bacterial productivity, determined from the rate of tritiated thymidine incorporation into DNA, was high over the reef flats and a *Thalassia hemprichii* sand flat (28 to 58 μg C l^{-1} d^{-1}). Bacterial growth rates increased during the day and fell at night over the reef flats and seagrass bed. Growth rates were slower over the reef front and in open water. Doubling times ranged from about 2 d in the open water to about 3 h over the reef flat in summer. As numbers did not increase, grazing was probably intense on the reef flats. Growth rates were much slower in winter. The main source of organic nutrient used by the bacteria was probably mucus released following photosynthesis in the corals. The cyanobacterium *Synechococcus* sp. was sometimes very numerous, especially in summer when 2×10^8 cells l^{-1} were recorded in one water mass. The number of bacteria was also very high in summer, with values ranging from 1×10^9 to 2.5×10^9 l^{-1}.

Introduction

Coral reefs are areas of high productivity (Lewis, 1977). Thus, turnover of organic matter and nutrient cycling must be rapid on reefs, and yet it is only within the last decade that the importance of bacteria in coral reef ecosystems (and indeed in marine ecosystems in general) has been documented. Studies by Sorokin (1973, 1974, 1978) showed that bacteria were very numerous, and that their productivity was high compared to open oceanic waters. He used a technique involving dark fixation of ^{14}C to measure production, but the accuracy of this method has been questioned (van Es and Meyer-Reil, 1983) and, furthermore, it is not specific to bacteria because all organisms fix small amounts of CO_2 in the dark. A better technique for measuring bacterial growth rates and hence production is based on the rate of tritiated thymidine incorporation into DNA; it is specific to heterotrophic bacteria (Fuhrman and Azam, 1980, 1982; Moriarty and Pollard, 1981, 1982; Moriarty, in press). We have applied the thymidine method to determine whether bacterial production was indeed high in coral reef waters around Lizard Island, northern Great Barrier Reef.

Changes in the biomass of bacteria and particulate organic carbon have been measured in water masses flowing over the reefs of Lizard Island (Moriarty, 1979; Crossland and Barnes, 1983). Although individual water masses were followed, the total flux of bacteria or organic matter between water and sediments, or even within the water, cannot be inferred from changes in biomass or concentration. Bacterial growth rates must be measured in order to study the role of bacteria in transforming organic matter, and their contribution to food chains in the coral reef ecosystem. The source of bacterial productivity may be mucus released from a large number of corals in the reef front region, where marked changes in bacteria and particulate organic carbon have been observed (Westrum and Meyers, 1978; Moriarty, 1979). Preliminary studies at Lizard Island had shown that bacterial production was sometimes ten times higher in water flowing off the back reef than over the reef front. In this paper we report the results of a survey to locate the main areas of bacterial production on the reefs around the Lizard Island lagoon.

Materials and methods

Study site

Lizard Island lies in the northern region of the Great Barrier Reef (Latitude 14°38′S). It is a continental island surrounded by fringing reefs, which join it to smaller islands to the south. Prevailing winds blow from the south-east for most of the year, and thus the main reef front zone in the study area extended between the south-east tip of

Lizard Island and South Island (Fig. 1). Besides extensive sandy reef flats with small coral outcrops, there was a large sand flat with the seagrass *Thalassia hemprichii* (Site M, Fig. 1). A detailed description of the reef flat communities has been given by Pichon and Morrissey (1982). The study was carried out in spring 1980, summer and winter 1982, and summer 1983.

Bacterial enumeration

Samples of the whole water column, or to a depth of 10 m if the water was deeper, were collected by lowering a weighted polypropylene bottle and allowing it to fill while descending. Water samples for bacterial counts were well mixed to disperse particles, and were preserved at 4 °C in formaldehyde (3% v/v). Bacteria were counted using epifluorescence microscopy and acridine orange staining (Hobbie *et al.*, 1977); details of the system used here have been described by Moriarty (1979). *Synechococcus* sp. was counted using the same system, but acridine orange was omitted; this was done within a day after collecting the samples, as it was found that their autofluorescence faded with time.

Growth rates

Growth rate measurements were started within 10 to 20 min after water collection, either at the research station or on beaches near the study sites. Isotope-dilution experiments were carried out as described by Moriarty and Pollard (1982), to check that the tritiated thymidine was incorporated into DNA without a decrease in specific activity [see Pollard and Moriarty (1984) for further details].

Routine measurements of bacterial growth rates in January 1983 were made as follows. Water samples (50 ml) were dispensed into two or three polypropylene bottles. To each sample, 0.92 MBq (25 μCi) of [^{3}H-methyl]thymidine [specific activity: 1.85 MBq (50 Ci mmol^{-1})] were added and the bottles were incubated in a water bath at ambient water temperature for 20 min. The total concentration of thymidine was 10 n*M*. The incubations were stopped by filtering the samples through cellulose acetate membrane filters (25 mm; 0.2 μm pore size). Each filter was immediately washed five times with 2 ml of ice-cold 3% (w/v) trichloroacetic acid (TCA). Blanks were prepared by filtering immediately after adding isotope. Filters were transferred to polypropylene centrifuge tubes, 2 ml of 5% TCA were added, and the tubes were heated at 100 °C for 30 min. After cooling, 0.5 ml portions were transferred to small liquid-scintillation vials and the radioactivity was counted. Growth rates were calculated as described by Moriarty and Pollard (1982), but using a conversion factor of 2×10^{18} cells dividing per mole thymidine incorporated (Moriarty, in press).

Growth rate measurements made during field trips before January 1983 were carried out as described by Moriarty and Pollard (1982), using a conversion factor of 2×10^{18}. This method involved filtering samples onto cellulose nitrate filters, and extracting the DNA with 0.3 *M* NaOH. Time-course and isotope-dilution analysis was carried out as described by Moriarty and Pollard (1981, 1982). As adenine has been suggested as a substrate for measuring DNA synthesis (Karl, 1981), an experiment was conducted to compare it with thymidine. A time-course experiment using adenine was conducted, using experimental conditions similar to those described for thymidine, except that 1.48 MBq of [2-^{3}H]adenine (specific activity 0.85 MBq mmol^{-1}) were added to each 50 ml of seawater. The DNA was extracted with 0.3 *M* NaOH.

Two different methods for measuring the rate at which tritiated thymidine was incorporated into DNA were compared in time-course experiments. One method was to extract the filtered samples with cold TCA, which removes only low molecular weight compounds (Fuhrman and Azam, 1980). It is assumed that DNA is the only, or at least predominant, macromolecule labelled. The other method was to extract DNA with NaOH, and separate it from RNA and protein (Moriarty and Pollard, 1982).

To measure bacterial growth on particles, incubations with tritiated thymidine were carried out as described above, with care being taken to mix the samples gently. Water samples were filtered through 3 μm pore-size cellulose acetate filters. Separate samples were filtered through 0.2 μm pore-size filters for comparison. As some free, unattached bacteria were retained by the 3 μm pore-size filters, a correction factor was obtained by counting bacteria in water samples before and after filtration through the 3 μm pore-size filters.

Results

Numbers of bacteria and *Synechococcus* sp.

The number of bacteria in the water was different over various parts of the reef, as shown in Fig. 1. The values are an average for several different sampling periods on 19 and 20 January 1983, during most of which time the weather was calm. Over the *Thalassia hemprichii* bed, bacteria were almost twice as abundant as elsewhere and also numbers were high on the ebb tide in a channel (Site N) through which water flowed after passing over the seagrass bed. There were no marked differences in numbers of bacteria elsewhere, although there were generally lower numbers over the reef flats or in water that had flowed over the reef flats into the lagoon or reef front with tidal currents. A cyanobacterium (*Synechococcus* sp.) was sometimes very abundant, accounting for up to 20% of the total bacterial population in one instance. In the open water, a threefold variation was seen over 4 h (2 July, Table 1) and a variation of two orders of magnitude over 3 d (29 June–2 July, Table 1).

Methodology for growth-rate measurement

The two different methods for measuring the rate at which tritiated thymidine was incorporated into DNA compared

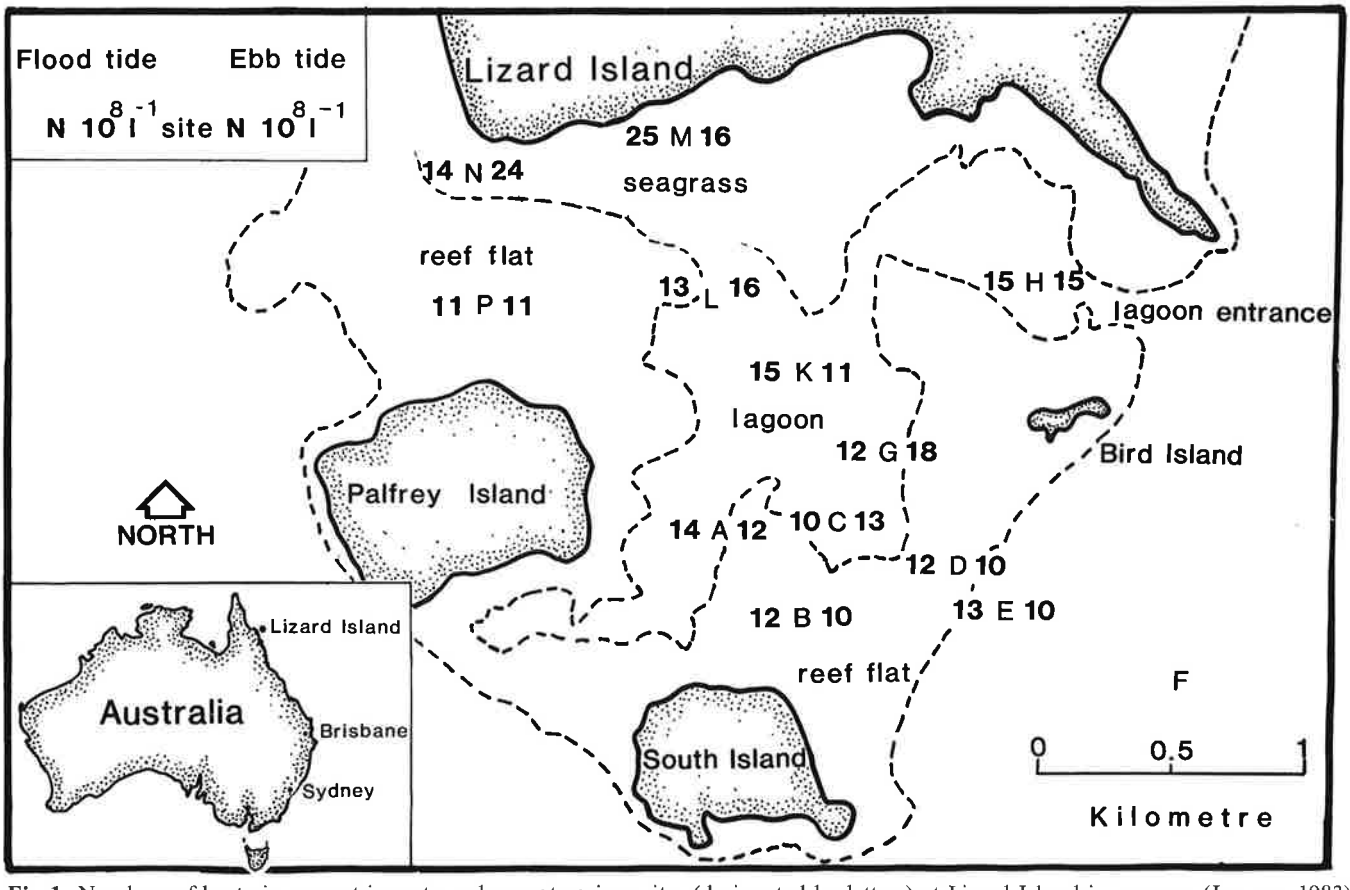
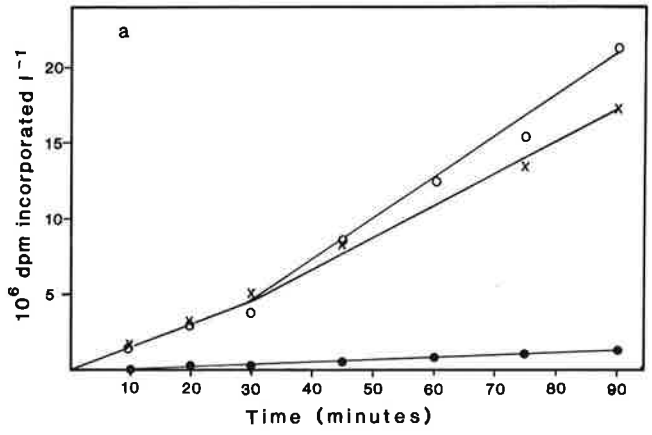


Fig. 1. Numbers of bacteria present in water column at various sites (designated by letters) at Lizard Island in summer (January 1983). Values found on flood tide are shown to the left of each site symbol, values during ebb tide to the right



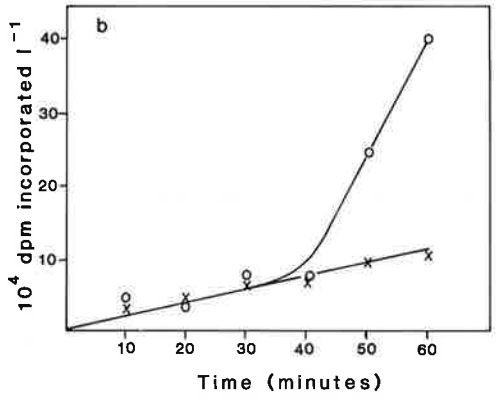


Table 1. *Synechococcus* sp. Seasonal and daily variation in numbers. Values shown are means ($n = 12$); standard errors were between 5 and 8% of the mean for all samples. ND: not determined

Date	Time of day (hrs)	*Synechococcus* sp. (10^7 l^{-1})		
		Openwater	Reef front	Reef flat
29 June 1982	09.00	11.0	ND	ND
2 July 1982	12.00	0.5	0.7	0.2
2 July 1982	16.00	0.1	0.3	0.1
18 Jan. 1983	08.30	11.8	12.6	5.5
19 Jan. 1983	22.00	20.0	14.6	15.7

Fig. 2. Two examples of time-courses for incorporation of tritiated thymidine into DNA in two water masses (a, b). A time-course for adenine (●) (40 μCi in 50 ml water samples) incorporation into purified DNA was also carried out (a). ×: thymidine (20 μCi in 50 ml water) incorporation into purified DNA; ○: thymidine incorporation into cold TCA-insoluble matter

Table 2. Seasonal and diurnal variation in bacterial growth rates (h^{-1}). Means for triplicate determinations of bacterial cell production rates (10^7 nos. l^{-1} h^{-1}) are also shown; range of variation was within 10% of the mean. Sites are shown in Fig. 1

Season Time of day	Temperature (°C)	Open water (Site F)		Reef front (Site E)		Reef flat (Site D)	
		Production rate	Specific growth rate	Production rate	Specific growth rate	Production rate	Specific growth rate
Winter (1982)							
09.00 hrs	22	0.35	0.005	0.11	0.002	0.20	0.005
noon	23	0.37	0.005	0.16	0.002	0.15	0.004
16.00 hrs	23	0.22	0.004	0.77	0.012	0.92	0.032
Spring (1980)							
09.00 hrs	27	1.3	0.042	0.71	0.019	1.04	0.016
Summer (1982)							
noon	28	4.9	0.061	7.5	0.107	11.7	0.285

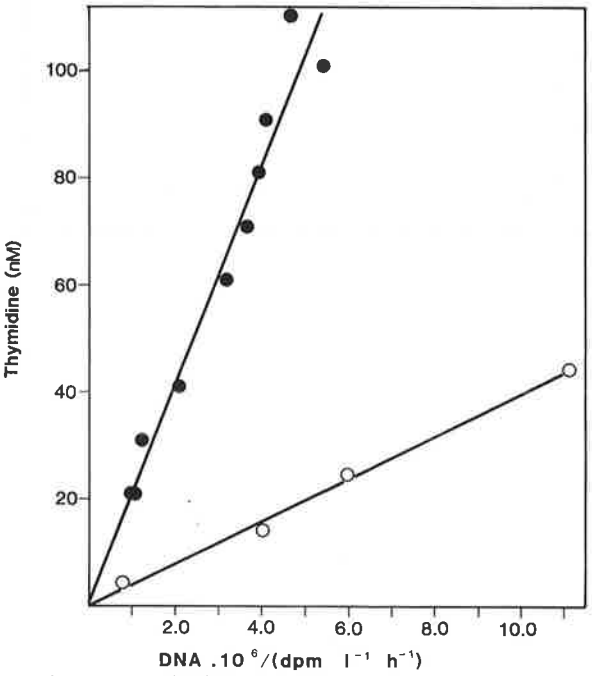


Fig. 3. Isotope-dilution plots for incorporation of tritiated thymidine into DNA. ○: 10 μCi added to 50 ml water samples; ●: 50 μCi added to 50 ml water. Incubation time 15 min

well for the first 20 or 30 min in time-course experiments (Fig. 2). For the first 30 min of incubation, there was no significant difference between the radioactivity incorporated into DNA that was extracted into NaOH, and radioactivity incorporated into all macromolecules precipitated by cold trichloroacetic acid. After 30 min, there was generally more radioactivity recovered with TCA than was present in DNA purified after NaOH extraction. In some cases, the differences were small (Fig. 2 a), whereas with other experiments, large differences occurred (Fig. 2 b). In both experiments shown in Fig. 2, the rate of incorporation of radioactivity into TCA-insoluble material increased with time. The rate of thymidine incorporation into DNA also increased after about 30 to 40 min in one experiment (Fig. 2 a). Tritiated adenine was much less effective as a precursor for labelling DNA than thymidine, as the rate of adenine incorporation was much slower (Fig. 2 a).

No isotope dilution occurred during incorporation of tritiated thymidine into DNA, starting with a concentration as low as 5 n*M* (Fig. 3). There was no change in slope of the isotope-dilution plots at high concentrations of thymidine, an indication that thymidine kinase was not a rate-limiting step.

Growth rates of bacteria

The fastest bacterial growth occurred in water over the reef flats and seagrass bed. Bacterial growth rates were generally lower in the winter than in the summer. There was, however, considerable patchiness in the distribution of rapidly growing bacteria during the summer (Table 2; Fig. 4). Growth rates varied with time of day, tidal currents and proximity to reefs. There was a marked increase in growth rates over the reef flats in the winter afternoon following a low tide (Table 2), when the water mass had spent some time over the reef flats. High productivity and fast growth rates were found in summer over the reef flats (Table 2).

Bacterial doubling times in various parts of the Lizard Island reefs in summer are shown in Fig. 4. In general, doubling times for the whole bacterial community were very fast over the reef flats, or in water that had flowed from the flats into the lagoon or over the crest to the reef front. The bacterial doubling time on the reef front during ebb tide was 28 h when south-east winds drove water onto the reef, in contrast to 6 h when no wind was blowing and current flow reversed, bringing water off the reef flat (Fig. 4).

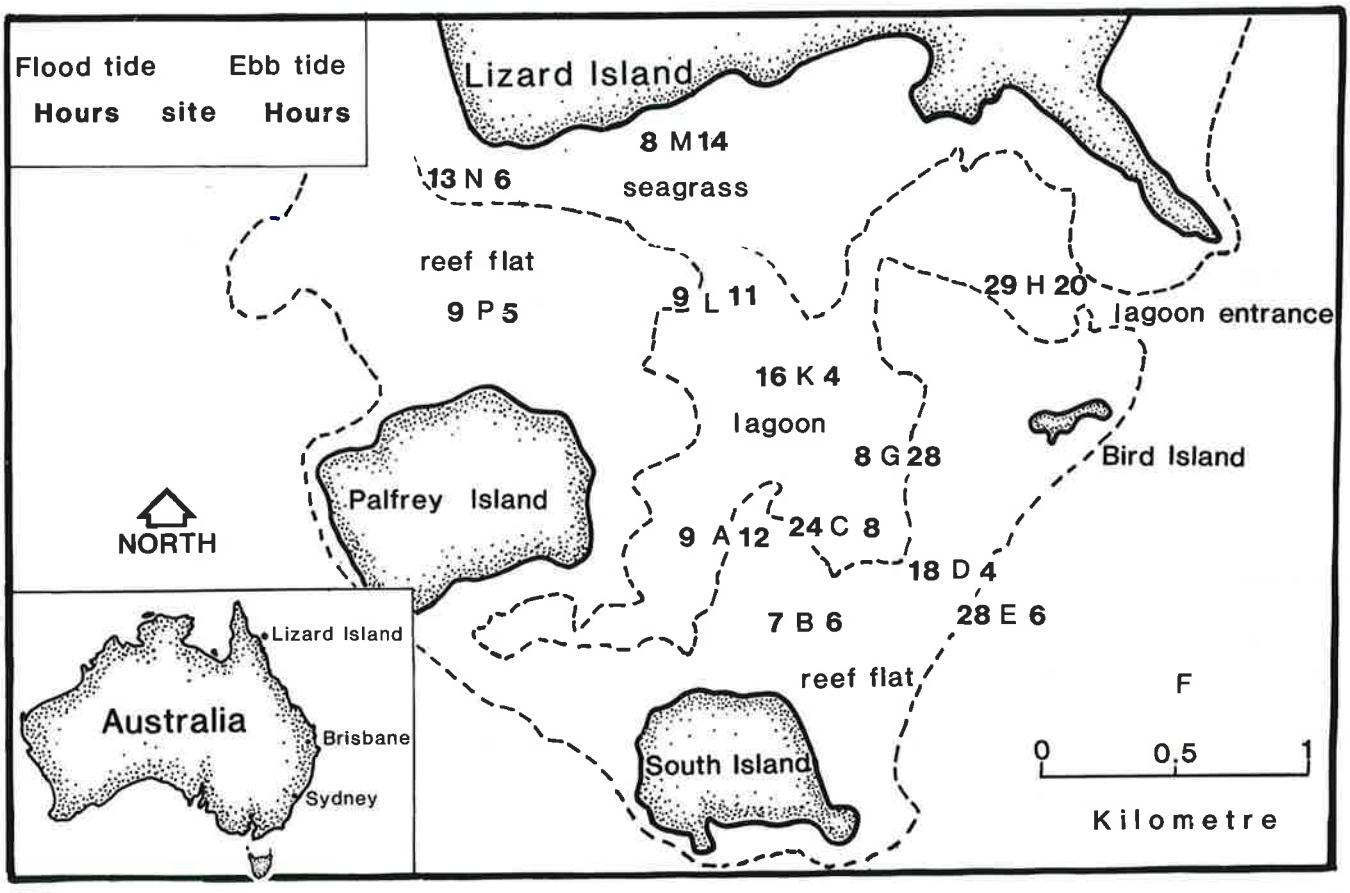


Fig. 4. Doubling times of bacteria in water column at various sites in summer (January 1983). Values are doubling times in hours – for flood tide to the left of each site symbol, for ebb tide to the right

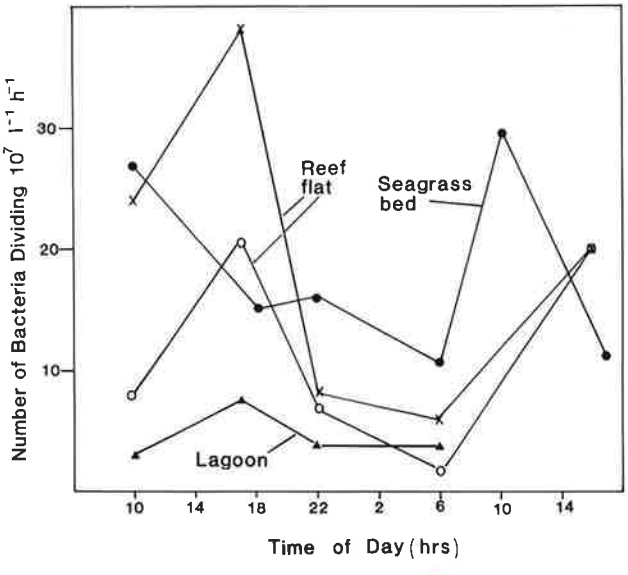


Fig. 5. Diel variation in bacterial ~~doubling times~~ production in the water over various sites. Duplicate determinations were made for each point; duplicate values usually differed by less than 10%

The values for doubling times in Fig. 4 are averages for four or five measurements over a diel period, and show a clearer diurnal variation than tidal. Bacterial growth rates were consistently slower at night and early morning than during the day. A very pronounced increase in growth rates occurred over the reef flats in the late afternoon, and over the seagrass bed in the late morning (Fig. 5). Lower diurnal variations were observed in lagoonal water and outside the reef.

As particular water masses were not followed during the diel studies, detailed variations in growth rate and number with time cannot be interpreted. It is clear, however, that there was a consistent and marked increase in rates of bacterial production in water masses over the reef and seagrass flats during the day which was not related to the tidal cycle.

In the open water and reef front area, over 30% of the bacterial production was associated with particles (Table 3). In the lagoon, and in the water over the seagrass bed, up to 50% of bacterial production occurred on particles. The water was visibly more turbid in these areas than outside the reef.

Table 3. Bacterial production rates on particles in the water column. Means of duplicate determinations are shown; variation was mostly less than 10% from the mean

Site	Cell production rates (No. dividing 10^7 l^{-1} h^{-1})		Production on particles as % of total
	0.2 μm filter	3 μm filter[a]	
Open water	2.4	0.9	31
Reef front	1.8	0.8	37
Reef crest	0.8	0.3	31
Lagoon entrance	1.6	1.0	52
Lagoon	11.2	5.7	43
Seagrass bed	15.2	9.0	49

[a] Corrected for 14% free bacteria retained on 3 μm filter

Discussion

The phototrophic *Synechococcus* sp. does not incorporate thymidine into DNA, and thus its activity is not included in studies on the heterotrophic bacteria (Pollard and Moriarty, 1984). The fast doubling times of heterotrophic bacteria in water over the reefs, especially in summer, show that the bacterial populations were in a very dynamic state. Growth rates were faster in the middle of the flats than over the reef front, except when water in the reef front area had come from the reef flats. It is likely, therefore, that bacterial growth rates were influenced primarily by processes occurring on the flats. Dissolved organic matter, released into the water column, was probably the main source of nutrients that stimulated growth; resuspension of particles with attached bacteria was unlikely as the weather was calm. The many outcrops of coral on the reef flats would support bacterial growth by exuding mucus and other organic compounds. This exudation was probably linked to photosynthesis, as growth rates reached a maximum in the afternoon. The diurnal stimulation of bacterial growth over the reef flats lagged behind that over the seagrass bed. The response of bacteria in the water column over the *Thalassia hemprichii* bed was similar to that recorded for bacteria in water over a *Zostera capricorni* bed in Moreton Bay (Moriarty and Pollard, 1982). The seagrasses were presumably excreting organic matter immediately after fixing CO_2, whereas the zooxanthellae excrete organic carbon into their coral host, which subsequently releases mucus (Crossland *et al.*, 1980). The delay in mucus release, which was at a maximum between noon and 16.00 hrs (Crossland *et al.*), means that an increase in bacterial growth due to utilization of the mucus would not occur until the afternoon. The diel variation in bacterial growth on the reef flats (Fig. 5) is consistent with the diel pattern of mucus release by corals.

The production of bacteria in coral reef waters in summer was high compared to other oceanic systems (Table 4). A large amount of organic matter is needed to support this bacterial production. If the bacteria have a growth efficiency of 50%, the production values of 28 to 56 μg C l^{-1} d^{-1} on the reef flat convert to 56 to 112 μg C l^{-1} d^{-1}, which is the total amount of carbon needed for bacterial respiration and growth. The average water depth over the flats was about 1.5 m, so about 84 to 168 mg C m^{-2} d^{-1} had to be excreted or suspended into the water column. This is a conservative estimate; if the growth

Table 4. Bacterial production in the water column. Daily rates for the coral reefs were calculated by integrating diel curves in production, assuming each cell contained 1.5×10^{-14} g C. Range in hourly rates for different times of the day is shown. nd: no data

Environment	Season	Production		Source
		μg C l^{-1} d^{-1}	μg C l^{-1} h^{-1}	
Reef flat (Site B)	Summer	56	0.9 –5.7	Present study
Reef flat (Site D)	Summer	28	0.3 –3.1	
Reef flat (Site D)	Winter	4	0.12–0.48	
Reef front (Site E)	Summer	26	0.3 –2.1	
Reef front (Site E)	Winter	5	0.06–0.48	
Lagoon water (Site K)	Summer	18	0.9 –3.0	
Open water (Site F)	Summer	11	0.1 –0.7	
Open water (Site F)	Winter	3	0.03–0.24	
Thalassia hemprichii bed (Site M)	Winter	58	1.6 –4.3	
Zostera capricorni bed, Queensland	Autumn	3	0.1 –0.3	Moriarty and Pollard (1982)
Californian coast	Spring	nd	0.08–0.8	Fuhrman and Azam (1982)
Chesapeake Bay	Spring	nd	0.3 –3.1	Ducklow (1982)
Georgia coast	Summer	nd	0.2 –2.0	Newell and Fallon (1982)

efficiency was lower, even more would be needed. It is unlikely that such a large amount of organic matter could be supplied by benthic algae in this system during photosynthesis, as they would have to excrete up to 100% or more of their net production, and furthermore, benthic bacteria would compete for any organic matter that was supplied by the algae (see following paper: Moriarty *et al.*, 1985). Mucus derived from corals is a more likely source of the organic nutrients, as corals do secrete large amounts (Richman *et al.*, 1975; Crossland *et al.*, 1980; Rublee *et al.*, 1980), and bacterial populations develop very rapidly on coral mucus (Ducklow and Mitchell, 1979 b). Further work is needed to determine the relative amounts of organic matter that are derived from different sources such as benthic algae and corals and used by bacteria. Slower bacterial growth rates would be expected on the reef front because the volume of water over the corals is greater than on the reef flat, and thus the concentration of exuded compounds would be lower. The water was four to ten times deeper over the reef front, so the total bacterial production per square meter was very much greater than that over the reef flats.

About half the bacterial growth occurred on particles in the lagoon (Table 3). This indicates that dissolved organic matter was converted into particulate matter, perhaps as a result of bacterial activity, as water passed over the flats and into the lagoon. Sorokin (1978) showed that bacteria aggregate on particles in the presence of mucus. This may result from alteration of the coral mucus (Ducklow and Mitchell, 1979 a) as well as from slime layers excreted by the bacteria. Earlier work has shown that up to half of the bacteria over the reefs were aggregated in particles, and that bacteria comprised about 20% of particulate organic matter (Moriarty, 1979). The distribution of bacterial numbers in the water column was generally similar to that observed previously, where numbers were lower over the reef flat than in water outside the reef (Moriarty, 1979). There was, however, considerable variability, which is probably due to interactions on a small scale between water masses and the reefs. This variation is the end result of the balance between factors leading to increases in numbers (growth and suspension from benthic substrates) and factors governing loss (predation, sinking, lysis). Bacterial densities may vary seasonally; in many of the summer samples, the numbers of bacteria were greater than in winter.

As the bacterial community was doubling at a fast rate (4 h in a summer afternoon), and numbers did not increase, or even decreased, as water moved over the reef flat, bacteria and particles must have been removed rapidly. Thus there was a large flux of bacteria, and presumably also dissolved and particulate organic matter, which was not apparent from the numbers or concentrations observed in the water masses as they flowed over the reef (Moriarty, 1979). Marshall *et al.* (1975) inferred that there was a flux of organic matter with losses being due to filter-feeding. Both grazing by filter-feeders as well as sinking of particulate matter are likely to have been mechanisms for removing bacteria. Bacterial productivity in winter was low, which suggests that exudation occurred much more slowly at the lower winter temperatures.

Bacterial numbers were high over the seagrass flat and in water that had come from the seagrass flat (Fig. 1: Sites M; and N, ebb tide). The seagrass flat was sandy, with few or no sessile filter-feeders, and thus growth of bacteria was apparent as an increase in number. Sinking of organic particles into the sediment may also remove bacteria from the water column. Bacterial production in the sediments is greater than can be accounted for by microalgal production, and thus requires an input from the water column (see following paper: Moriarty *et al.*, 1985).

The trophic role of bacteria in reef systems is certainly very important, with many different benthic filter-feeders utilizing them (Sorokin, 1973, 1978), as well as zooplankton that eat mucus aggregates containing attached bacteria (Gerber and Marshall, 1974; Gottfried and Roman, 1983). Grazing is probably the main mechanism for removal of bacteria, although more quantitative studies are needed. The role of Protozoa, for example, in reef systems is not known.

Acknowledgements. We are grateful to T. V. de Kluyver for technical assistance. We thank Dr. and Mrs. B. Goldman, of the Lizard Island Research Station, for their helpful assistance. This work was carried out with the support of funds from a Marine Sciences and Technologies grant.

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Date of final manuscript acceptance: January 5, 1985.
Communicated by G. F. Humphrey, Sydney

Marine Biology 85, 293–300 (1985)

Marine Biology

Productivity of bacteria and microalgae and the effect of grazing by holothurians in sediments on a coral reef flat

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Abstract

Bacterial productivity in sandy sediments on reef flats at Lizard Island, Great Barrier Reef was determined from the rate of incorporation of tritiated thymidine into DNA. The study was conducted during January 1982 and July 1983. A small diurnal increase occurred in sediments having a dense population of microalgae. Bacterial production was 120 to 370 mg C m^{-2} d^{-1} in summer on reef flats, which was equivalent to 30–40% of primary production by benthic microalgae. In winter, rates of primary production by benthic microalgae and secondary production by bacteria were about one-half to one-fifth of those in summer. There was much variation in production, due to patchiness in the distribution of benthic microbes, especially microalgae. Doubling times for the bacteria in surface sediment were 1 to 2 d in summer and 4 to 16 d in winter on the reef flats. These high productivity values for bacteria indicated that a net input of organic matter to the sediment was needed to support the growth of bacteria. Sediment bacteria thus have a very important role in transforming organic matter on the reef flats. Grazing by *Holothuria atra* depressed both primary production and bacterial production. It was estimated that these holothurians ate about 10 to 40% of bacterial carbon produced each day in summer, and thus have an important role in the carbon cycle. Harpacticoid copepods were numerically important components of the benthic meiofaunal community and probably had a significant impact on bacterial density as grazers.

Introduction

Sandy flats occur on large areas of many coral reefs, and yet little attention has been given to their role in reef productivity and nutrient cycling. Few studies have been made of the function of bacteria in these processes in sediments of reef flats, or indeed elsewhere on coral reefs. Sorokin (1974, 1978, 1981) has shown that bacteria in reef flat sediments are numerous and productive compared to other marine environments. Bacteria have been shown to comprise 3 to 8% of organic matter in the reef flat sediments of Lizard Island, Great Barrier Reef (Moriarty, 1982). In order to assess the importance of bacteria in processes such as the carbon cycle and food webs, measurements of productivity as well as biomass are needed, but the measurement of bacterial growth rates, and thus productivity in sediments, has been hampered by lack of suitable techniques. The accuracy of the method used by Sorokin (1978), *viz.* the fixation of $^{14}CO_2$ in the dark, is doubtful (van Es and Meyer-Reil, 1983). With the development of the tritiated thymidine method, growth rates of most heterotrophic bacteria in sediments can now be measured with reasonable accuracy (Moriarty and Pollard, 1981, 1982; Moriarty, in press). We have used the thymidine technique to measure bacterial production in the sediments on reef flats at Lizard Island, and show in this paper that bacteria have a very significant role in secondary production.

Earlier work has shown that holothurians feed on bacteria and detritus (Bakus, 1973; Moriarty, 1982), but these studies could not show whether holothurians had a significant effect on bacterial productivity. We report here the results of studies carried out with cages to determine the effect of grazing on bacterial production in the sediments. Renaud-Mornant and Helléouet (1977) studied interaction between *Holothuria atra* (Jaeger) and the meiofauna; holothurians did not eat meiofauna, but competed with meiofauna for micro-organisms as food. We carried out experiments to determine whether this effect was significant in the Lizard Island sediments. As bacterial numbers in sediments are probably regulated by Protozoa and meiofauna (Lee, 1980), an investigation of meiofauna density in reef sediments is reported here.

Materials and methods

Study site

Lizard Island lies in the northern region of the Great Barrier Reef (Latitude 14°38′S). It is a continental island surrounded by fringing reefs which join it to smaller islands to the south. Extensive areas of sandy flats occur between the islands, some with small coral outcrops (Sites A, C, F, Fig. 1), other areas are bare sand (Sites B, G), and one area (Site D) contains a seagrass bed (*Thalassia hemprichii*). The intertidal zone of a sandy beach (Site E) was studied. Details of the benthic communities have been described by Pichon and Morrissey (1982). The sediment-feeding holothurian *Holothuria atra* occurs commonly in the sandy areas among the coral outcrops on the reef flats (density about 0.5 to 1 individuals m^{-2}; it does not occur in the bare sandy or seagrass flats. Prevailing winds blow from the south east for much of the year, causing turbulence and stirring of sediment on the reef flats. In summer, however, the weather is often calm, with no prevailing wind, and the sediments are less disturbed. Water temperatures were 29 °C in summer and 23 °C in winter during the course of our study, which was carried out in 1982 and 1983.

Sediment analyses

Two methods were used to collect sediment. Firstly, a petri dish was pressed into the sediment to a depth of 4 mm, and a flat plastic sheet was pushed underneath to retain the surface layer. This method was not very satisfactory in coral sediments with a variety of grain sizes. A second method was adopted, in which a cylinder (50 mm diam) was pressed into the sediment and a spoon was used to scoop sediment from the upper 4 to 5 mm into a container. As the grain size was variable (up to 5 mm in size) on the reef flats, samples varied in their composition and depth of sediment. On the beach, the upper 2 to 3 mm of fine-grained sand was collected. Growth rates of bacteria were determined within 5 to 10 min after collecting the sediment. Sediment was preserved by freeze-drying for analysis of chlorophyll *a*, muramic acid and organic carbon and nitrogen composition.

Muramic acid was determined using high-performance liquid chromatography, as described by Moriarty (1983). Organic carbon and nitrogen were determined with a Perkin Elmer CHN analyser, modified as described by Moriarty and Barclay (1980). Algal pigments were extracted with acetone 90% v/v in water and the absorbance was measured at 665 nm. Values are reported in extinction

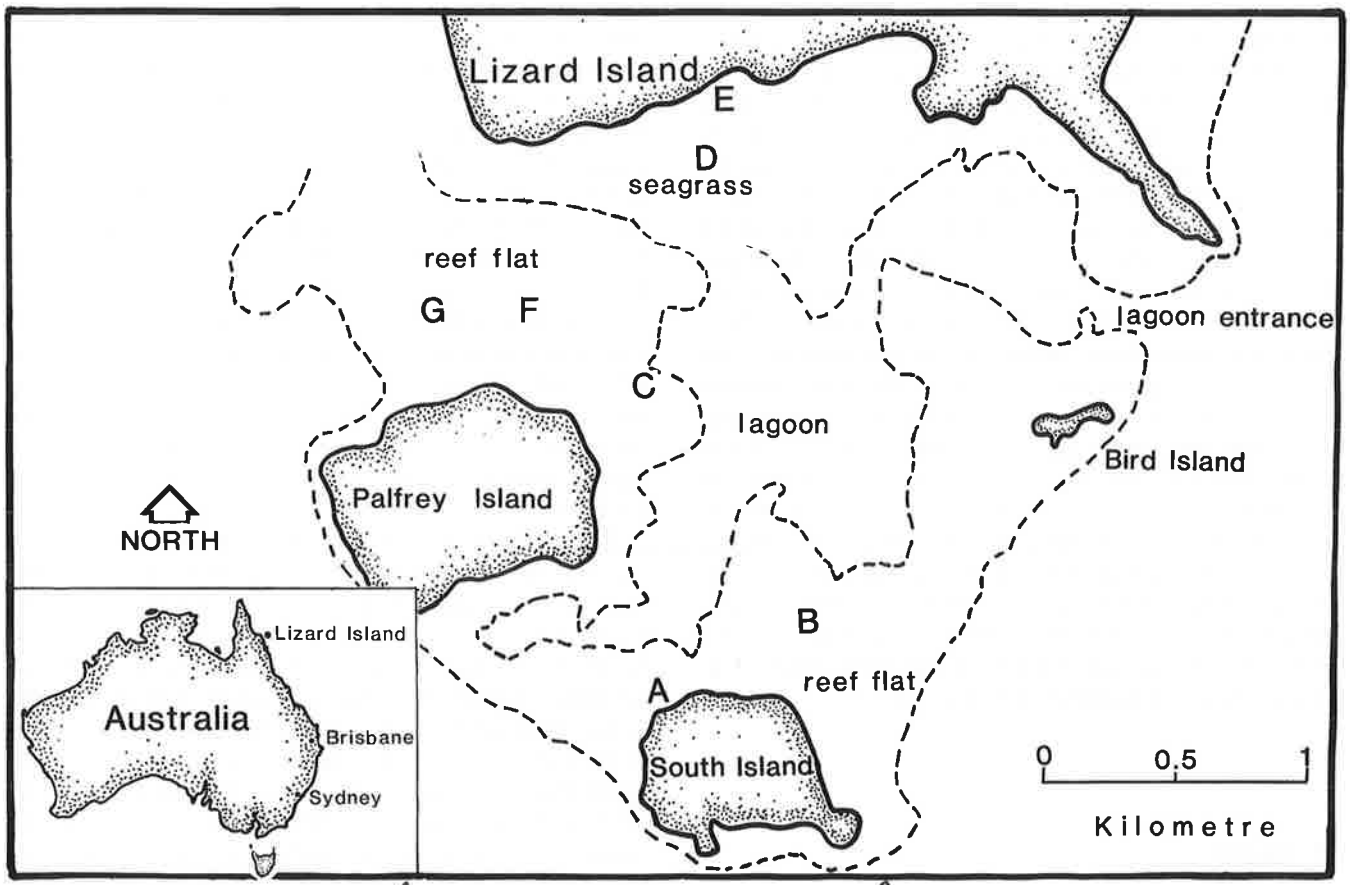

Fig. 1. Location of sampling sites. Site A: sand surrounded by small coral outcrops; Site B: bare sand, near a beach, with the nearest coral at least 200 m away; Site C: amongst dense coral near the edge of the leeward reef; Site D: a sandy flat with seagrass (*Thalassia hemprichii*) and no coral; Site E: a sandy beach, with no coral nearby; Site F: site with light coral cover, and large patches of bare sand; Site G: bare sand

terms only, because many degradation products of chlorophyll are present in the sediment, precluding conversion to weight of chlorophyll or biomass of algae. The values are useful for showing general trends. Chlorophyll concentration was calculated using a specific absorption coefficient of 72 (Strickland and Parsons, 1972).

Bacterial growth rates were calculated from the rate of tritiated thymidine incorporation into DNA, as described by Moriarty and Pollard (1981, 1982). Preliminary experiments were carried out to check that the rates of incorporation were linear and that no isotope dilution occurred. Incubation times were 15 min in summer and 20 min in winter. Tritiated thymidine in DNA was diluted to a small extent when sediment sample sizes of about 0.5 to 1.0 g dry weight were used with 1.85 MBq (50 μCi) of tritiated thymidine (specific activity: 1.85 MBq nmol^{-1}). No dilution was found when sediment samples of 0.2 to 0.5 g were used. The main source of dilution is *de novo* synthesis of thymidine nucleotides, but this can be prevented by using a sufficiently high concentration of thymidine (Pollard and Moriarty, 1984).

Primary production was determined by measuring changes in oxygen concentration and the amount of $^{14}CO_2$ fixed during incubations in light and dark chambers. Chambers used for the oxygen method were 90 mm in diameter and 50 mm deep. Water samples (20 to 50 ml) were withdrawn through tubing for measurement of oxygen concentration with a Winkler technique (Strickland and Parsons, 1972) at 1 h intervals. Chambers used for $^{14}CO_2$ fixation were 50 mm in diameter and of 50 ml volume. The ^{14}C-bicarbonate (37 kBq) was injected into the chamber and left for 2 h. Preliminary experiments with a time-course showed that $^{14}CO_2$ fixation was linear from 15 min to over 3 h after starting the incubation. The upper 20 mm of sediment was removed, the water was drained off, and total wet weight was determined. Portions (2 g wet weight) were extracted with 2 ml NCS (a quaternary amine from Amersham, UK) overnight. Portions of the NCS (0.4 ml) were placed in scintillation vials, 80 μl of glacial acetic acid were added, and vials were left open for 6 h to remove $^{14}CO_2$. Control experiments showed that all $^{14}CO_2$ exchanged to the atmosphere with this treatment. Bubbling was not necessary. Vials were then capped and left in sunlight for 2 to 4 h to bleach pigments. The results obtained by this simple method agreed to within 5% of values obtained by combusting sediment at 500 °C in the CHN analyser (Moriarty and Barclay, 1980) and collecting $^{14}CO_2$ from organic matter as described by Taft (1976).

Primary production was measured at 1 h intervals (O_2 method) or 2 h intervals ($^{14}CO_2$ method). Problems were experienced with the oxygen method after mid-morning, as oxygen bubbles formed when the water was supersaturated with oxygen. In order to measure the specific activity of ^{14}C-bicarbonate, the total dissolved inorganic carbon concentration was determined from alkalinity titration in some seawater samples (Strickland and Parsons, 1972). The water was found to be saturated with dissolved inorganic carbon. Daily primary production was calculated by integrating diurnal curves of production. Alternatively, approximate estimates were obtained by multiplying the productivity between 09.00 and 10.00 hrs by ten. Results obtained using this calculation were within the range of variation of diurnal measurements. The range was large, because the microalgae were patchily distributed in the sediment.

Holothurian experiments

Cages of 1 m^2 area and 0.3 m high were constructed from wire mesh (10 mm square holes). Up to 10 *Holothuria atra* were placed in some cages; other cages were left empty. Control sites nearby with no treatment were also analysed. Holothurians that escaped from the cages were replaced each day during the experimental period (1 to 2 wk). Smaller mesh was not used, because we wanted to minimise alterations in sediment composition that might be caused by lowered water flow. The holothurians were able to contract and pass through quite small openings. As only short field trips were possible, it was necessary to use a high density of *H. atra* in order to obtain a measurable effect in that time period. Natural densities of about 20 m^{-2} have been reported on other Pacific reefs (Renaud-Mornant and Helléouet, 1977), but on Lizard Island reef flats, the natural density did not exceed 2 individuals m^{-2}. Their distribution was patchy. The holothurians were found among low coral outcrops. Densities varied from zero on wide expanses of bare sand to an average of about 0.5 individuals m^{-2} on sand patches no more than 1 to 2 m wide between coral outcrops.

Meiofauna were collected to a depth of 20 mm in corers of 16 mm diameter. Three replicates were taken for each site or cage. The meiofaunal animals were fixed in formaldehyde (4% v/v) and stained with rose bengal. They were separated from sediment with three extractions in 30% Ludox (colloidal silica), as described by De Jonge and Bouwman (1977). Almost all meiofauna except forams were removed by this treatment. Forams were counted in the remaining sediment.

Results

Bacterial growth rates varied diurnally; the amount of increase during the day depended on whether the sediment was disturbed or grazed. On sediment that was disturbed by grazing animals, a slight diurnal increase in production rates was noticeable (Fig. 2). Where a cage was used to exclude large animals, a thin algal mat (diatoms and cyanobacteria) developed, and bacterial production increased about two-fold during the day (Fig. 2). The diurnal increase in bacterial growth rates suggests that some of the bacterial growth was dependent on excretion of organic matter by the algae. This effect was noticeable because algal production was high where grazers were excluded.

Bacterial production was often a high proportion of primary production. It was 30 to 40% of microalgal production at Site C on the reef flat in spring (Fig. 3). Both

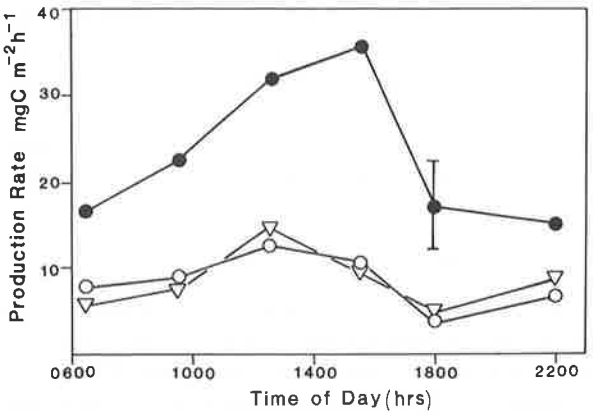

Fig. 2. Diel variation in rates of bacterial production in surface sediment, measured with tritiated thymidine method. ●: Undisturbed area (cage with no holothurians), a microalgal mat was noticeable; ○: cage with 10 *Holothuria atra* present; ▽: control area, grazed or disturbed by *H. atra* and fish. Triplicate determinations were made for each point shown; range of variation was within the size of points, except where bar is shown

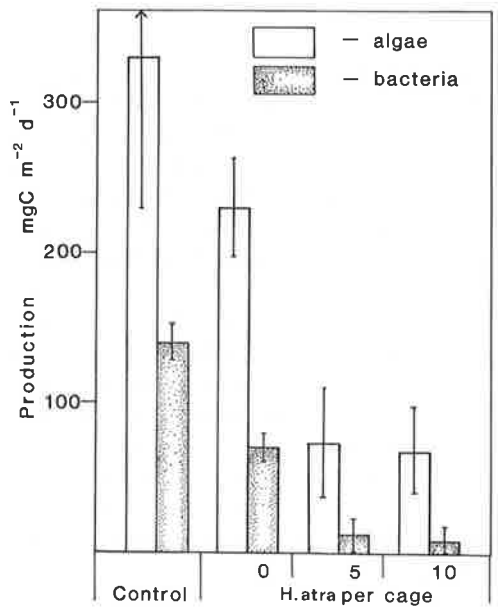

Fig. 3. Comparisons of primary and bacterial production in surface sediment at Site C. Primary production: range of variation for 3 replicates; bacterial production: standard deviation, 4 replicates. Four treatments were analysed: control area, open sand, no grazing by holothurians; 0, cage with no *Holothuria atra*; 5, cage with 5 *H. atra;* 10, cage with 10 *H. atra*

algal and bacterial production were markedly depressed by a high density of holothurians (5 to 10 individuals m^{-2}) in cages (Fig. 3). The average density of *Holothuria atra* in the area where the cages were placed was about 0.5 individuals m^{-2}, although around coral outcrops the density was often 1 to 2 m^{-2}. The values for productivity were variable, a result of the heterogeneous composition of the sediment and disturbance by animals. The control area used here was ungrazed, and thus had a higher productivity than the area under the cage with no *H. atra,* which was a normal grazed area.

The biomass of microalgae and bacteria and total organic carbon and nitrogen decreased in sediments of a reef flat (Site B) on which *Holothuria atra* were feeding (Table 1). In contrast, when *H. atra* were caged in a bare sandy region (Site A) well away from their normal feeding area, they had little effect on the sediment, presumably because they did not feed. The total organic matter content and the biomass of microalgae and bacteria was lower in the bare sandy area (Site A) than on the reef flat among coral outcrops (Site B) (Table 1).

Algal and bacterial productivities were lower in winter than in summer (cf. Tables 1 and 2). The estimated bacterial doubling times were considerably longer in winter (Table 2). These doubling times are approximate, because they are based on muramic acid values and not direct counts (which are not possible in the sediment). The presence of an anaerobic layer with H_2S production only 2 to 5 mm below the surface sediment was noticed in sheltered areas in summer. This is an indication that the flux of organic matter into the sediment and the rate of its decomposition were high.

Bacterial production in the sediment on the seagrass (*Thalassia hemprichii*) flat was higher than that on the reef flat. On the beach, however, the bacterial biomass and production, as well as organic matter content, were much lower than the reef flat and nearby seagrass areas (Table 2).

Oxygen uptake was measured in dark chambers at the same time as algal production. Respiration varied between 300 and 600 mg C m^{-2} d^{-1} (assuming an RQ of 1.0) in chambers placed at dawn and left in the same place during the day with water being changed every hour. When chambers were moved to a new area of sediment each hour, respiration increased throughout the morning from about 10 to 20 mg C m^{-2} h^{-1} to over 100 mg C m^{-2} h^{-1}.

The effect of grazing by *Holothuria atra* on microalgae and bacteria is shown clearly in the results of an experiment where cages were placed for a period of 2 wk (Table 3). In the absence of grazing, the biomass and production of bacteria and microalgae rose about twofold. Grazing had slightly more effect on the microalgae than the bacteria. The control area was also grazed with a natural density of about one to two *H. atra* m^{-2}. Fish also disturbed the sediment in the control area. For the first week, many of the holothurians remained in the cage; an average density of about 5 to 7 individuals m^{-2} was maintained by replacing any missing holothurians. In the second week, however, most holothurians escaped and had to be returned to the cage each day.

The numbers of harpacticoid copepods varied inversely with the intensity of grazing by holothurians (Fig. 4). In one case (Site F, Cage 3) little change occurred, but here the initial density of copepods was low. This was a preliminary experiment, and only three replicates were counted for each cage on each site, in order to see whether meiofauna density varied in different areas. There were not enough replicates for a statistical analysis. No notice-

Table 1. Comparison of microbial biomass and production in sediment from two areas of reef flat, January, 1983. See Fig. 1 for location of sites. Cages with 10 *Holothuria atra* were placed at each site and left for 1 wk before surface sediment (0 to 4 mm depth) was analysed. Values are means, with standard errors in parentheses, for 4 replicates. E_{665} = extinction at 665 nm

Site	Production (mg C m^{-2} d^{-1})		Doubling time	Algal pigment	Muramic acid	Organic carbon	Organic nitrogen
	Algal	Bacterial	(d)	(E_{665} g^{-1})	(μg g^{-1})	(mg g^{-1})	(mg g^{-1})
B							
control	207 (40)	120 (36)	2.1	0.37 (0.11)	1.5 (0.2)	4.3 (0.8)	0.33 (0.09)
cage	130 (25)	48 (12)	2.5	0.24 (0.03)	0.8 (0.2)	3.8 (0.3)	0.28 (0.02)
A							
control	180 (40)	120 (41)	1.2	0.07 (0.01)	0.9 (0.1)	2.0 (0.3)	0.13 (0.01)
cage	140 (16)	144 (48)	1.2	0.06 (0.01)	1.0 (0.2)	2.2 (0.1)	0.12 (0.01)

Table 2. Microbial biomass and production at different sites on the reef flats in winter (July). See Fig. 1 for location of sites. Surface sediment (0 to 4 mm) was analysed. Standard errors are shown in parentheses. Algal production was measured using both ^{14}C and O_2 methods; there was no significant difference in results

Site	No. of replicates	Production (mg C m^{-2} d^{-1})		Doubling time	Muramic acid	Organic carbon	Organic nitrogen
		Algal	Bacterial	(d)	(μg g^{-1})	(mg g^{-1})	(mg g^{-1})
B Reef flat	8	66 (20)	20 (10)	16	1.8 (0.1)	2.9 (0.1)	0.18 (0.05)
D Seagrass flat	4	ND	78 (54)	4	1.8 (0.1)	1.9 (0.03)	0.15 (0.01)
E Beach (low intertidal)	4	ND	1 (0.5)	82	0.46 (0.02)	0.33 (0.007)	0.03 (0.005)

Table 3. Effect of grazing by *Holothuria atra* on microbial biomass and productivity in surface sediment (0 to 4 mm), January 1982. The site was on the leeward reef, Site C (Fig. 1). Cage 1 contained no *H. atra;* Cage 2 contained 10 *H. atra*; cages were left for 2 wk before sampling

Site	Production (mg C m^{-2} d^{-1})		Doubling time	Algal pigment[c]	Muramic acid[d]	Organic carbon[e]	Organic nitrogen[e]
	Algal[a]	Bacterial[b]	(d)	(E_{665} g^{-1})	(μg g^{-1})	(mg g^{-1})	(mg g^{-1})
Control	350 (40)	190	1.5	0.25 (0.002)	1.8 (0.3)	3.1	0.40
Cage 1	670 (100)	370	2	0.5 (0.01)	4.2 (0.3)	4.8	0.60
Cage 2	230 (25)	190	1.3	0.15 (0.01)	1.5 (0.2)	3.0	0.24

[a] Mean (SE, $n=4$)
[b] Integrated from diel measurements (Fig. 2); range about $\pm$ 10%
[c] Mean (range for duplicates)
[d] Mean (range for triplicates)
[e] Means of duplicates; range $\pm$ 10%

Table 4. Meiofauna numbers in sediment from three sites. Means and standard deviation for number per 200 mm² area in the top 20 mm depth of sediment are given

Site	Nematodes	Copepods	Polychaetes	Ostracods	Forams	Nauplii	Others
Site G							
mean	89	120	24	10	23	54	6
SD, $n=6$	33	58	6	12	9	59	4
Site F							
mean	96	68	39	2	20	34	6
SD, $n=9$	33	37	16	2	27	26	6
Site C							
mean	85	86	24	3	13	17	7
SD, $n=12$	114	41	14	2	10	10	6

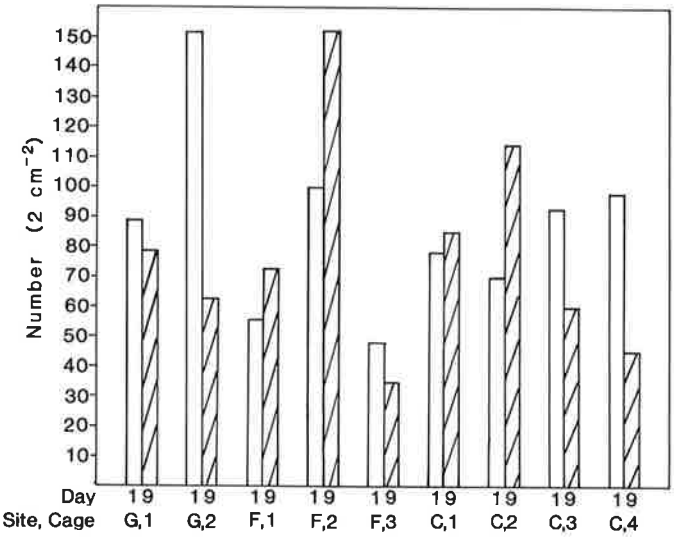

Fig. 4. Effect of *Holothuria atra* density on numbers of copepods in sediment at Sites C, F, and G. Numbers per 200 mm² to a depth of 20 mm are shown. 1: control area, grazed normally; 2: cage with *H. atra* excluded; 3: cage with 5 *H. atra;* 4: cage with 10 *H. atra.* Open bars: numbers on Day 1; shaded bars: numbers on Day 9

able changes were observed in the numbers within other groups of animals in the meiofauna. The average density of meiofauna is shown in Table 4. The distribution of all groups at any one site was very patchy.

Discussion

The work reported here shows that bacteria are far more important in transforming organic matter and in food chains on the reef than could be inferred from their biomass alone. The fast doubling times of 1 to 2 d, especially in summer, indicate that much organic matter is being produced and thus is promoting bacterial growth. Growth rates were measured in the aerobic zone only, so when anaerobic bacterial production is considered also, it is clear that the flux of organic matter through bacterial decomposition processes must be large. As bacterial production was high in proportion to algal production, it is unlikely that sufficient organic nutrients could be supplied by microalgal excretion or detritus in the sandy sediment.

An estimate of the organic carbon required to support the sediment bacteria can be derived from the production rates. If it is assumed that the growth efficiency in the sediment is about 50%, the organic carbon requirement would be two times the production rate. In summer, 250 to 500 mg C m^{-2} d^{-1} would be needed in grazed sediment. In winter, about 40 to 150 mg C m^{-2} d^{-1} would be needed. As anaerobic processes also occur, the growth efficiency is probably lower, so these are minimum values. Particulate organic carbon (POC) concentrations of about 200 mg C m^{-3} have been found in spring (Crossland and Barnes, 1983). If this is similar in summer, and POC is the only source of carbon for sediment bacteria, about 10% of the POC would be needed each hour (from a 1 m-deep water column).

Another source of organic matter used by the heterotrophic bacteria could be organic compounds such as mucus exuded by corals and other animals (Johannes, 1967; Ducklow and Mitchell, 1979). Mucus aggregates from corals are derived from excreted mucus, cellular debris and zooxanthellae. They contain protein, amino acids and amino sugars, as well as lipid and polysaccharide, and thus are nutritionally valuable (Ducklow and Mitchell, 1979; Krupp, 1981). Encrusting algae on hard surfaces may also produce detritus. This organic matter is a likely source of organic nutrients for the bacterial community in the surface sediment. A question to be resolved is how far excreted mucus is transported in the water column before it settles or is removed. The high bacterial biomass, organic carbon and nitrogen content and the high rates of bacterial production in sediment on reef flats surrounded by coral outcrops compared to beach or bare sand flats suggest that such material is not transported far. Furthermore, bacterial growth rates in the water column are very rapid over the reef flat, and thus organic matter in the water is metabolised rapidly (see preceding paper: Moriarty *et al.*, 1985). The fast growth of bacteria in the water and sediment during summer indicates that substantial quantities of organic matter must be cycling between water, sediment and benthic organisms. Measures of organic carbon concentrations or bacterial biomass would not

show this. Release of inorganic nitrogen by growing bacteria, or by grazers on bacteria is an important component of the nitrogen cycle on reefs. Rapid rates of denitrification and nitrification have been found in these Lizard Island sediments (P. I. Boon, personal communication).

Because there are problems in determining algal respiration, we have not attempted to calculate net daily production of microalgae by subtracting algal respiration integrated over a 24 h period. Instead, we report net production for the day time only. Both the ^{14}C and O_2 methods gave similar results (assuming photosynthetic and respiratory quotient values of 1.0). The main point that we wish to make is that the bacterial production rates required as much or more carbon than was fixed by the microalgae in many cases, and thus other sources of carbon were required. An estimate of the bacterial component in benthic respiration cannot be made, because the proportion of respiration due to the aerobic bacteria cannot be obtained with the thymidine method, which also measures anaerobic bacterial growth (Pollard and Moriarty, 1984).

The doubling times in summer of 1 to 2 d for bacteria in the sediment indicate that not only must there be a very rapid flux of organic matter into the sediment, but also grazing on the bacteria must be intense. Bacterial biomass remained low in the sediment, which means that the bacteria must have been removed rapidly. Of the many types of animals that feed on bacteria, ranging from Protozoa to fish, only holothurians, and to a small extent, meiofauna, were examined here. The cage experiments demonstrated that holothurians do limit bacterial biomass, as it increased when they were excluded. Each *Holothuria atra* eats about 20 to 50 mg bacterial carbon d^{-1} (Moriarty, 1982). At a density of one *H. atra* m^{-2}, these holothurians would utilize about 10 to 40% of the bacterial carbon produced each day in summer. As Sorokin (1974) has pointed out, these holothurians are important as consumers of bacteria. Presumably their feeding rate must be lower in winter, or they rely more on microalgae.

Holothuria atra feeds on detritus as well as microalgae and bacteria, (Moriarty, 1982). An estimate of the amount of detritus in the reef sediment compared to the biomass of microorganisms and meiofauna may be obtained from the values given here. If all the pigment absorbing at 665 nm were chlorophyll *a*, a maximum estimate of microalgal biomass would be given by using a conversion factor of 30 mg C mg^{-1} chlorophyll *a* (Banse, 1977). For the control area on the leeward reef (Site C, Table 3) algal biomass would be about 0.6 mg C g^{-1}, which is about 20% of organic carbon. Bacterial biomass (assuming a conversion factor of 80 mg C mg^{-1} muramic acid) is 0.14 mg C g^{-1}, or 5% of organic carbon. Meiofaunal biomass is low, being about 50 μg C g^{-1} (based on values of 0.75 μg C for nematodes, 0.45 μg C for copepods, 1.82 μg C for polychaetes and 1 μg C for others, taken from unpublished data of Keliher and Moriarty). Thus, detrital mass probably accounts for 60 to 70% of organic carbon as suggested previously (Moriarty, 1982).

The movement of the holothurians away from bare sand flats or from the cages after a week was presumably a response to the absence or low concentration of food in those areas. As discussed elsewhere, they are probably selective feeders, with chemosensory ability (Moriarty, 1982). Because the biggest difference in microbial biomass between the bare sand and heavily grazed cages was due to microalgae, it may have been these to which *Holothuria atra* was responding. Sournia (1976) has reported that *H. atra* grazed on patches of blue-green algae.

The inverse correlation between harpacticoid copepods and holothurian density could be due to competition for food. As Renaud-Mornant and Helléouet (1977) point out, it is unlikely that *Holothuria atra* actually fed on the meiofauna. Our results show that, as in many other marine sediments (Hicks and Coull, 1983), harpacticoid copepods are numerically important components of the meiofauna. Many of them feed preferentially on bacteria, and thus their effect on biomass, and probably growth rates also, is likely to be significant. Copepods have been shown to need between 1 and 3.5 μg carbon each per day of bacterial carbon (Hicks and Coull). Using these values we can estimate that, at Site C, the copepods would eat 0.4 to 1.5 g C m^{-2} d^{-1} if bacteria were the only food source. The lower value is within the range of values for bacterial production at Site C (Table 3), so it is clear that the numbers of meiofauna present in these reef sediments are sufficient to have a considerable impact on bacterial population density. In fact, these data suggest that bacteria alone cannot supply all the food for copepods. Copepods also eat microalgae and detritus (Hicks and Coull, 1983), so the estimated amount of bacteria needed by copepods in these coral reef sediments is too high. Extending this discussion to other meiofauna, we conclude that it is likely that much of the new bacterial biomass produced each day would be grazed by meiofauna, and thus the fast doubling times in sediment are realistic. A more detailed study on changes in the species composition as well as total numbers of copepods in response to changes in algal and bacterial biomass would be rewarding.

Acknowledgements. We are grateful to Dr. P. D. Nicholls, Dr. D. W. Klumpp, and Dr. T. V. Verheyen for assistance with field work and oxygen measurements at Lizard Island. We thank Dr. and Mrs. B. Goldman and staff at the Lizard Island Research Station for their assistance. This work was carried out with the support of funds from a Marine Sciences and Technologies grant.

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Date of final manuscript acceptance: January 5, 1985.
Communicated by G. F. Humphrey, Sydney

Proceedings of the Fifth International Coral Reef Congress, Tahiti, 1985, Vol. 3

BACTERIAL PRODUCTIVITY AND TROPHIC RELATIONSHIPS WITH CONSUMERS ON A CORAL REEF (MECOR I)

LA PRODUCTIVITE BACTERIENNE ET LES RELATIONS TROPHIQUES AVEC LES CONSOMMATEURS SUR UN RECIF CORALLIEN (MECOR I)

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ABSTRACT

The productivity and trophic role of bacteria on coral reefs is reviewed and reassessed in light of new observations on the effect of coral mucus and meibenthos on bacterial growth rates. Bacterial biomass and productivity are greater in sediments than in the water column. There is considerable seasonal variation with bacterial numbers and growth rates several times greater in summer than winter.

Incubation experiments with and without mucus from Acropora spp. and Porites spp. show that bacterial production in mucus-enriched seawater was significantly greater than in untreated seawater.

In laboratory culture experiments, the presence of meiofauna enhanced bacterial growth only when twice the natural density of meiofauna at Davies Reef, Great Barrier Reef, were used. However, higher concentrations of NH_4 and NO_2 in culture dishes with meiofauna than in control cultures suggests that meiofauna enhance the regeneration of N.

The importance of protozoans, benthic copepods, holothuroids and filter feeders as major consumers of bacteria on coral reefs is supported by the information available to date.

RESUME

La productivité et le rôle trophique des bactéries dans les récifs coralliens sont revus et réévalués à la lumière de nouvelles observations sur les effets induits par la présence des mucus coralliens et de la méiofaune sur les vitesses de croissance bactérienne. La biomasse et la productivité bactériennes sont plus importantes dans les sédiments que dans l'eau. En plus, il y a des variations saisonières considérables dans le nombre des bactéries et leurs taux de croissance sont plusieurs fois plus important en été qu'en hiver.

Les incubations expérimentales avec et sans les mucus issus d'Acropora spp. et Porites spp. montrent que la productivité bactérienne est beaucoup plus grande dans l'eau de mer contenant du mucus que dans l'eau de mer, n'en contenant pas.

Durant des expériences en laboratoire, la productivité bactérienne a augmenté seulement lorsque la population de la méiofaune a atteint le double de la population d'origine dans le Davies Reef, Grande Barrière. Cependant, les concentrations plus élevées de NH_4 et NO_2 dans les boîtes de culture contenant de la méiofaune laissent penser que la méiofaune favorise la régénération de l'azote.

Dans l'état actuel des connaissances, l'importance des Protozoaires, des Copépodes benthiques, des Holothuries et des filtreurs, en tant que consommateurs de bactéries dans l'écosystème récifal, est ici confirmée.

INTRODUCTION

Pioneering studies of the productivity, biomass and trophic role of microbes, particularly bacteria, on coral reefs were conducted by Sorokin (1973, 1978) and DiSalvo (1973). These studies constituted a watershed in that prior to their publications the accepted notion amongst many coral reef workers was that microbes played an unimportant role in reef metabolic processes and trophic exchanges (Odum and Odum, 1955).

Recent research by Ducklow and Mitchell (1979) and Moriarty (1979, 1982) have demonstrated that bacterial biomass and productivity constitute a considerable proportion of reefal organic matter and metabolic activity and that bacteria are important in the degradation of coral mucus and algal detritus.

In this paper we attempt to summarize what is known about microbial biomass and productivity on coral reefs and to present some new data on the role of bacteria in trophic networks with particular emphasis on benthos. This synthesis grew out of the collaborative workshop, Microbial Ecology on a Coral Reef (MECOR) which investigated the ecological significance of bacteria on one reef of the Great Barrier Reef during winter 1984.

Microbial Biomass and Productivity

Microbial biomass in water over different coral reefs is equivalent (Table 1). There is, however, considerable seasonal variation with numbers in winter being lower than in summer (Moriarty et al., 1985a).

Table 1. Biomass and productivity of bacteria and photosynthetic production in water over coral reef flats.
? - not stated; n.d. - not determined.

Locality	Season	Bacteria		Algal	Reference
		Biomass $\mu g\ C\ l^{-1}$	Prodn $\mu g\ C\ l^{-1}d^{-1}$	Prodn	
Majura Atoll Marshall Is.	?	19	7.5	4.1	Sorokin, 1973
Kaneohe Bay Hawaii	?	43	28	3.6	Sorokin, 1973
Heron Is. G.B.R.	?	41	20	6.7	Sorokin, 1973
Lizard Is. G.B.R.	winter	50	4	n.d.	Moriarty et al., 1985
	spring	97	37	n.d.	
	summer	150	56	n.d.	
Davies Reef G.B.R.	winter	45	3	n.d.	Moriarty unpubl.

Estimations of microbial productivity show expected variations because markedly different techniques were employed. Sorokin (1973, 1978) used dark incorporation of $^{14}CO_2$ to measure productivity. This technique is inaccurate, however, because dark incorporation is not directly coupled to microbial growth. By contrast, tritiated thymidine incorporation into DNA (Moriarty et al., 1985a,b) is directly proportional to cell division and specific for heterotrophic bacteria (Moriarty, in press). The rates in Table 1 are comparable, nevertheless, and may reflect seasonal variations and patchiness.

Short term fluctuations in nutrients also influence bacterial growth rates. In summer, day-time growth rates may be four times greater than those at night; rates in water over reef flats are much higher than in lagoon water or water outside the reef (Moriarty et al., 1985a). Such variations probably result from the release of photosynthate from algae and of mucus from corals during the day. Further evidence is the higher bacterial productivity over reef fronts than elsewhere on reefs (Moriarty et al., 1985a). Phytoplankton production is low and does not contribute substantially to water column bacterial productivity (Table 1).

Bacterial production in reef sediments is high and comparable to rates of microalgal production (Moriarty et al., 1985b). The different techniques employed by Sorokin (1973, 1978) and Moriarty et al. (1985b) make direct comparisons difficult as the former reports bacterial production (to a depth of 2.5 cm) as 1.4 g C $m^{-2}d^{-1}$ compared to 0.62 g C $m^{-2}d^{-1}$ for primary production at Majuro Atoll. Moriarty et al. (1985b) report productivity in the top 4 mm as 0.12 to 0.19 g C $m^{-2}d^{-1}$ (summer) and as 0.01 to 0.12 g C $m^{-2}d^{-1}$ (winter) for bacteria and 0.18 to 0.40 g C $m^{-2}d^{-1}$ (summer) and 0.04 to 0.08 g C $m^{-2}d^{-1}$ (winter) for primary production on Lizard Island.

Like the water column, there are marked seasonal and diel variations in bacterial and algal production in coral reef sediments. At Lizard Island, bacterial doubling times in reef flat sediments are 1 to 2 days in summer and 16 days in winter (Moriarty et al., 1985b). These differences, like those in the water column, are more likely related to a greater supply of organic matter in summer than higher temperatures.

Microbial Nutrition

Particulate matter derived from material eroded from the shallow zones of a reef, fragments of turf algae and macroalgae, exudates from algae, and coral mucus are some of the sources of nutrients for microbes on coral reefs (DiSalvo, 1973). Much of this organic matter (ca 80%) is, however, refractory and not readily available for microbial consumption (Meyers, 1980).

Of particular interest is the role of coral mucus as a substrate for microbial growth. Coral mucus that is shed by corals naturally or during stress such as exposure or tactile stimulation, is an important substrate for bacterial growth (Ducklow and Mitchell, 1979). We performed similar studies with Acropora spp. and Porites spp. mucus in winter. Bacterial growth in mucus-enriched seawater was several times greater than growth of bacteria in untreated seawater (Fig. 1). These findings of rapid nutritional stimulation are contradictory to those of Pascal and Vacelet (1982) who reported that mucus is a poor substrate for bacterial growth when measured over several days.

Consumers of Pelagic Bacteria

The major consumers of bacteria in the plankton are probably flagellate and ciliate protozoans which are abundant in reefal waters (Landry et al., 1984; B. Sherr, pers. comm.). Pelagic flagellates and ciliates range in

numbers from 180-193 ind.ml^{-1} and 1.0-1.2 ind. ml^{-1}, respectively. Using literature values on their ingestion rates of bacteria (ciliates: Curds and Cockburn, 1968; flagellates: Fenchel, 1982), protozoa may, depending upon their abundances and patch sizes, consume from 10 to 100% of bacterial production per unit volume in the water column.

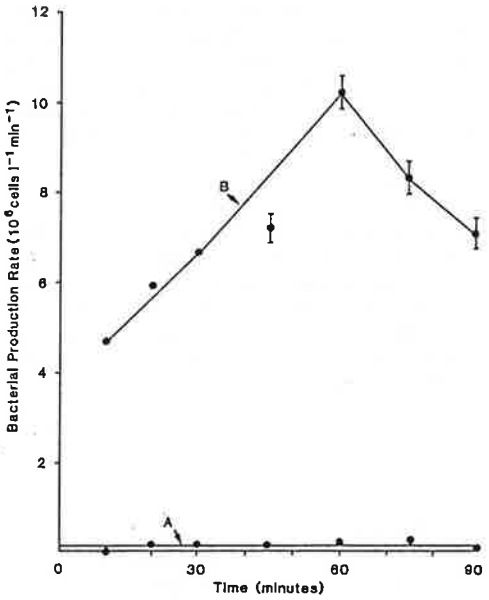

Figure 1. Stimulation of bacterial production in a seawater-mucus mixture was measured as per Moriarty et al. (1985a). (A) A time course for production in seawater without mucus (B) Bacterial production with mucus present.

Many pelagic bacteria are associated with particles (Moriarty, 1979). It was estimated that up to 50% of bacterial biomass was on particles in water over the Lizard Island reef flats (Moriarty, 1979); 30-50% of bacterial production occurred on particles (Moriarty et al., 1985a). Bacteria associated with suspended particulates should be more accessible to pelagic (e.g., pomacentrid and caesiid fishes) and benthic (e.g. sponges) filter feeders than unattached microbes. In fact, recent estimates of pelagic protozoans at Davies Reef suggest that protozoans may be consumed by benthic filter feeders as they pass across the reef flat (B. Sherr, pers. comm.).

Sessile benthos may be the major invertebrate consumers of pelagic bacteria in light of adjunct studies conducted during MECOR which showed that macrozooplankters, such as schooling mysids, consume only a tiny proportion of bacteria produced in waters over Davies Reef (Mullin and Roman, in press). Whether any other groups, particularly larval and gelatinous zooplankton such as salps and doliolids, have a greater impact on reef bacteria remains to be investigated; their trophic potential should not be underestimated.

Benthic suspension and filter feeders remove an unknown proportion of the microbial biomass as water passes across a reef (Reiswig, 1971; Wilkinson, 1978). These consumers include sponges, corals, bivalve molluscs, crinoids and ascidians. Of these, only sponges and corals have been studied, but not to an extent necessary to estimate the proportion of microbial production that is consumed. Considering that sponges filter bacteria with efficiencies between 95% and 99% (Reiswig, 1971; Wilkinson, 1978) it is probable that sponge populations in deeper water can remove much of the microbial biomass produced in shallow water.

Hard and soft corals also have the potential to consume microorganisms, particularly those attached to particles through entrapment on mucus surfaces (DiSalvo, 1973). Sorokin (1978) showed that a wide variety of corals consume bacteria with assimilation rates of between 5 and 80% of respiration rates. Considering the large populations of corals in some zones of coral reefs, it is conceivable that corals themselves could remove much of the microbial production.

Consumers of Sediment Bacteria

Protozoans, nematodes, copepods, turbellarians, polychaetes, crustaceans, echinoderms and molluscs live within reefal sands to form a network of sedimentary food webs (Grassle, 1973). Some of these benthic groups, in particular protozoans and meiofauna, have been largely ignored although they are, with the exception of bacteria and microalgae, the numerically dominant organisms in reef ecosystems (DiSalvo, 1973; Alongi, submitted).

To date, the only published description of protozoans (excluding foraminiferans) in reefal sediments has been provided by DiSalvo (1973). He found a rich fauna of ciliates, flagellates and meiobenthos in a variety of reefs with protozoans attaining densities of 10^6 per m^2. Cryptomonad flagellates and dinoflagellates were almost equally common and were outnumbered by colorless nanoflagellates. At Davies Reef, we investigated protozoan densities in different zones of the reef (Moriarty et al., unpubl. data). Ciliates and flagellates approached numbers equal to those found by DiSalvo (1973) ranging from 10^4 to 10^6 m^{-2} for ciliates and from 10^5 to 10^7 m^{-2} for flagellates. Highest densities of flagellates (primarily cryptomonads and colorless euglenoids) occurred in the very fine sands of the lagoon among callianassid burrows and mounds. Lowest protozoan densities were found at the most physically-disturbed zones on the reef flat.

The amount of bacterial production consumed by benthic protozoans may be roughly estimated by multiplying standing stocks by literature values on ingestion rates. In Davies Reef sands, protozoans are estimated to consume only a small proportion of bacterial production (generally 5%), although this estimate can be as high as 30% where protozoans are aggregated into small patches on the reef flat (Moriarty et al., unpubl. data).

Meiofaunal communities on coral reefs have been investigated, but nearly all prior studies have focused on taxonomy rather than on community structure and function (Renaud-Mornant and Helleouet, 1977; Alongi, submitted). Numbers of meiobenthos vary greatly among reefal biotopes

with total densities ranging from less than 10^2 to more than 10^6 individuals per m^2. An examination of nematodes inhabiting Davies Reef indicates a dominance of epistrate feeders and omnivore/predator groups rather than bacteriovores (Alongi, submitted). Epistrate feeders are dominant from reef crest to shallow lagoon sands; bacteria-detritus feeders are abundant only in the deep (15 m) lagoon. Epistrate feeders consume proportionately more algae (diatoms and chlorophytes) than bacteria (Alongi and Tietjen, 1980). In fact, we estimate that nematodes in Davies Reef consume only a small (1-10%) portion of bacterial production (Moriarty et al., unpubl. data).

Among the meiofauna, harpacticoid copepods may be of greater trophic importance based on their numerical dominance and high ingestion rates of bacteria (Hicks and Coull, 1983). In Davies Reef sands, ingestion of bacteria by copepods may account for much of bacterial production (Moriarty et al., unpubl. data). Moriarty et al. (1985b) came to a similar conclusion regarding the trophic significance of copepods in coral sands of Lizard Island.

Consequently, a laboratory study was undertaken during MECOR to assess the effect of meiofauna on bacteria and to see if bacterial growth rates were enhanced by addition of ammonium to simulate ammonia excretion by meiobenthos. Several workers have postulated that meiofauna stimulate bacterial productivity through grazing and excretion of phosphorus and nitrogenous compounds (see review of Tietjen, 1980).

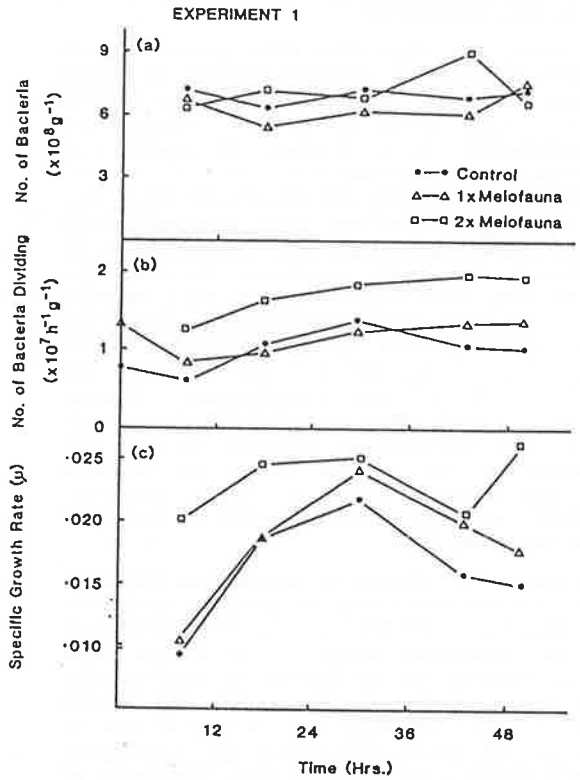

Figure 2. Effect of meiofauna on (a) bacterial numbers, (b) production and (c) specific growth rates. Bacterial production was estimated using methodology of Moriarty et al. (1985b). Bacterial numbers were estimated by epifluorescence microscopy (Hobbie et al.,1977).

Our laboratory experiments were conducted in glass dishes at constant temperature (24°C) and salinity (35°/oo) using organisms obtained from Davies Reef. The first experiment consisted of three sets of replicate dishes that received the natural density of meiofauna, approximately twice the natural density, and no meiofauna as the control. The second experiment had the same design but included a fourth set of dishes without meiofauna, but enriched with ammonium chloride to simulate NH_3 excretion.

After 30 hours, bacterial production in the treatment with double the natural density of meiofauna was significantly greater (one-way analysis of variance; least significant differences test; p 0.05) than the other treatments in both experiments (Figs. 2 and 3). Specific growth rates were not significantly different between treatments, although in the first experiment there was a trend for faster rates in the presence of meiofauna. When the data points for the first experiment were summed over time, there was a significant relationship between specific growth rate and meiofaunal density (p 0.05, linear regression; $r^2 = 0.96$). Bacterial numbers were not significantly (p 0.05) different between treatments.

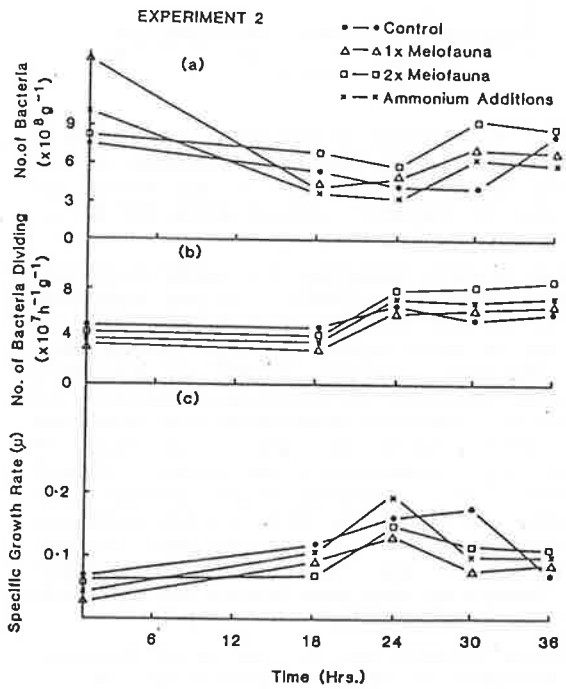

Figure 3. Effect of meiofauna and ammonium additions on (a) bacterial numbers, (b) production and (c) specific growth rates. Bacterial numbers and production were estimated as in Experiment 1. The amount of ammonium chloride added (as 3 mg N per dish) is equivalent to the amount produced by natural densities of meiofauna at Davies Reef (Gray, in press).

Bacterial production in the presence of ammonia was greater than in the control after 30 h (Fig. 3). Ammonia concentrations were

higher in the presence of meiofauna than in the control, which suggests that ammonia was excreted by the meiofauna (Table 2).

Table 2. Concentrations of inorganic P and N compounds (ug at.l^{-1}) in culture media after 36 hours (Experiment 2). Each value represents total overlying water from each replicate dish. Nutrients were measured using the methods described in Strickland and Parsons (1972).

Treatment	PO_4	NH_4	NO_2	NO_3
Control	1.44,0.80	8.45, 3.01	0.08,0.06	1.27,0.19
1x Meiofauna	1.19,1.10	11.14,21.12	0.10,0.15	0.12,0.07
2x Meiofauna	1.39,1.10	25.42,26.88	0.20,0.31	0.18,0.15
NH_4 addition	1.08,1.18	30.72,29.76	0.15,0.17	0.19,0.26

These experiments indicate that meiofauna may increase bacterial production in marine sediments. There may, however, be a threshold density of meiofauna below which no significant changes in bacterial production occur. The failure of a natural density of meiofauna to affect greatly the dynamics of bacteria suggest that these animals included only a small fraction of bacterial production in their diets. The meiofauna was taken from sands rich in benthic algae (diatoms, chlorophytes, turf algae, Halimeda debris, etc.) and was composed primarily of algal feeders (Alongi, submitted). Thus, the proportion of bacterial production consumed by meiobenthos will depend greatly upon the dominant feeding types (e.g. algal vs. bacterial feeder) within different reef zones.

Larger, motile benthos such as polychaetes, callianassid shrimps and tellinid bivalves are abundant residents on coral reefs (Grassle, 1973), but their ingestion rates of bacteria are virtually unknown. The studies of Sorokin (1973) do suggest that many of these macroconsumers assimilate bacteria with high (90%) efficiency.

The feeding biology of holothuroids has been well-studied as they are conspicuous consumers of microbes on coral reefs. Holothuroids feed on microalgae, bacteria and particulate detritus (Renaud-Mornant and Helleouet, 1977; Moriarty, 1982; Moriarty et al., 1985b). Biomass and growth rates of bacteria at Lizard Island were depressed by the intense grazing of holothuroids (Moriarty et al., 1985b). It was estimated that Holothuria atra consumed 10 to 40% of the daily bacterial production in summer (Moriarty et al., 1985b).

Synthesis and Questions

As a heuristic exercise we have attempted to depict the equation balancing bacterial production and consumption (Fig. 4). The source of these data are from the literature, unpublished data from the MECOR Workshop and our own experiments. Available knowledge is meager and those values we do present are approximate and based on a few valid experiments.

Consumption of bacteria in the water column may not balance production as protozoa and copepods generally do not consume more than 10 to 30%. Larvaceans and salps may consume a similar or lesser proportion but details of their populations are lacking. As much as 50% of the microbial community in coral reef waters is associated with particles. Thus consumption by fish and sessile filter feeders, or sedimentation into lagoons, may account for the remainder of water column bacterial production.

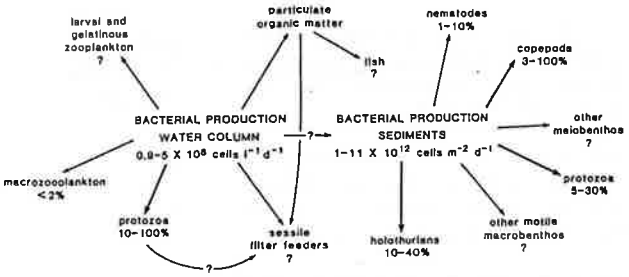

Figure 4. Estimates of consumption of bacterial production using ingestion rates and consumption estimates of Fenchel, 1982 (flagellates), Curds and Cockburn, 1968 (ciliates), Hicks and Coull, 1983 (benthic copepods), Tietjen, 1980 (nematodes), Mullin and Roman, in press (mysids) and Moriarty et al., 1985b (holothurians).

Similarly, consumption of bacteria in sediments may not balance production, although protozoans and copepods have the potential to consume a large proportion of benthic bacterial productivity. Like their planktonic counterparts, some less abundant benthic invertebrates such as turbellarians are voracious bacterial feeders and they may ingest a disproportionate fraction of bacterial production (Staarup, 1970).

A dilemma is posed by the order of magnitude difference in bacterial productivity between summer and winter. Do consumers necessarily reduce their bacterial consumption and as a consequence, basic metabolism in winter or do the consumer populations show an order of magnitude reduction in numbers? Alternatively is there a switch in food preference from the more abundant bacteria in summer to microalgae and detritus in winter?

These questions and those concerning the role of bacteria in coral reef ecology can only be answered by more detailed studies conducted at different seasons over scales large enough to allow an integration of all parts of a coral reef.

ACKNOWLEDGMENTS

This study was supported by the Australian Institute of Marine Science (Contribution No. 295) and by National Science Foundation Grant INT-8312117 (U.S.-Australia Cooperative Science Program). Contribution no. 1 from the Microbial Ecology on a Coral Reef Workshop, held at the Australian Institute of Marine Science, July-August 1984.

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Coral Reefs (1987) 6:63–70

Coral Reefs

The dynamics of benthic microbial communities at Davies Reef, central Great Barrier Reef

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Accepted 19 February 1987

Abstract. The dynamics of benthic microbial communities were examined within different functional zones (reef crest, reef flat, lagoon) of Davies Reef, central Great Barrier Reef, in winter. Bacterial numbers did not change significantly across the reef with a mean abundance ($\bar{x} \pm 1$ SE) of 1.3 (± 0.6) $\times 10^9$ cells g^{-1} DW of sediment. Bacterial production, measured as thymidine incorporation into DNA, ranged from 1.2 (± 0.2) to 11.6 (± 1.5) mg C $m^{-2}h^{-1}$ across the reef and was significantly lower in a reef crest basin than in the other zones. Bacterial growth rates (μ) across the reef (0.05 to 0.33 d^{-1}) correlated only with sediment organic carbon and nitrogen. Protozoan and meiofaunal densities varied by an order of magnitude across the reef and correlated with one or more sediment variables but not with bacterial numbers or growth rates. Nutrient flux rates were similar to those found at other reefs in the central and southern Great Barrier Reef, and are significantly lower than rates measured in temperate sand communities. In the front lagoon, bioturbation and feeding acitivity by thalassinid shrimps (*Callianassa* spp.) negatively influenced microbial and meiofaunal communities with a net import of organic matter necessary to support the estimated rates of bacterial productivity. In lagoonal areas not colonized by shrimps, primary productivity (400–1 100 mg C $m^{-2} d^{-1}$) from algal mats was sufficient to support bacterial growth. It is suggested that deposit-feeding macrobenthos such as thalassinid crustaceans play a major role in the structuring and functioning of lower trophic groups (bacteria, microalgae, protozoa, meiofauna) in coral reef sediments, particularly in lagoons.

Introduction

Coral reef sediments, particularly in lagoons, receive large amounts of particulate detritus derived from primary production on the reef crest and reef flat zones. Bacteria play an essential role as decomposers of this detrital matter (Di Salvo 1973; Sorokin 1981; Moriarty et al. 1985b) and their production can be equivalent to about 30% to 40% of benthic microalgal production (Moriarty et al. 1985a) in reef sediments. Although bacterial activity has been determined previously in many reef environments, simultaneous measurements of the biomass of microbial trophic groups, community metabolic rates and nutrient fluxes have not been reported.

It has been suggested that several trophic groups (e.g. protozoans and meiofauna) have the potential to consume a significant fraction of the bacteria in reef sediments and to stimulate bacterial growth rates by their grazing activities (Moriarty et al. 1985b). However, the actual amounts of bacteria and microalgae consumed by reef benthos remain to be determined empirically. Moreover, macrofauna may also be important in controlling microbial distribution and rates of activity. For example, thalassinid shrimps are abundant in the lagoon at Davies Reef where they alter sediment bed structure by selectively sorting sands near and within their burrows for food (Tudhope and Scoffin 1984).

Because coral reefs include a variety of sedimentary habitats ranging from sand patches on reef flats to lagoonal beds characterized by algal mats or thalassinid shrimp mounds, it is possible that microbial biomass and rates of primary and microheterotrophic production may be regulated by different factors across different coral reef zones (reef crest, reef flat, lagoon). In this paper we examine: (1) the abundance and productivity of benthic microbial and meiofaunal communities and their relationship with sediment characteristics and thalassinid shrimps within different reef habitats and (2) benthic community metabolism and nutrient fluxes within the lagoon at Davies Reef in the central Great Barrier Reef.

Material and methods

Davies Reef is an open lagoonal reef (6.2 km long, 3.2 km wide) located at 18° 50'S, 147° 39'E in the central Great Barrier Reef (Fig. 1). The wind-

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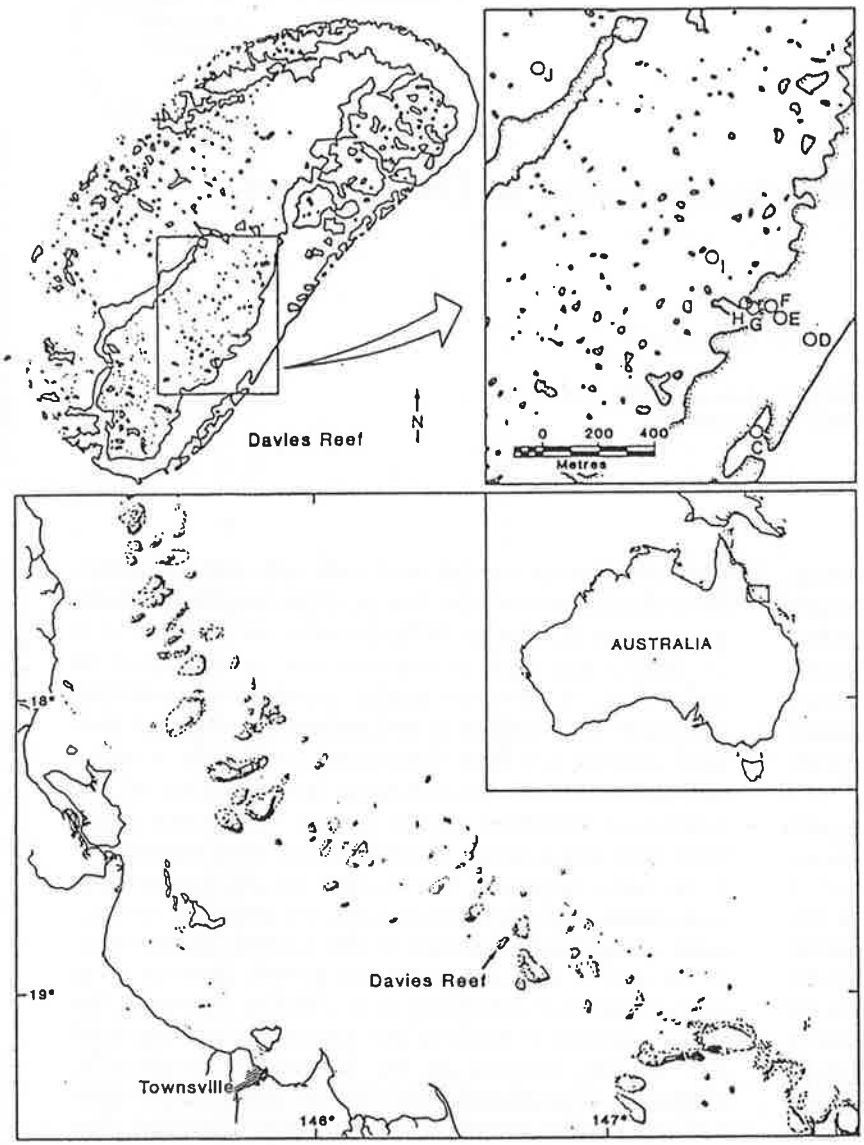

Fig. 1. Chart of Davies Reef and location of stations in relation to the Great Barrier Reef and the Australian coast

ward flat is oriented towards the predominant southeast trade winds. The lagoon, comprising approximately 50% of the planimetric area of the reef (Tudhope and Scoffin 1984), is largely open to the leeward side. The reef is subject to flushing by semi-diurnal tides; tidal currents run in a north-south direction (Pickard 1986).

Seven sampling stations were established on a transect across the reef with an additional station located in the deep back lagoon (26 m) where algal mats were present (Fig. 1). Essential features of the transect stations and their sediment characteristics are provided in Table 1. The sediments at all of the stations were carbonate sand consisting of coral, mollusc and crustacean shell, coralline algae and *Halimeda* debris as well as foraminiferan and radiolarian tests (Tudhope and Scoffin 1984). At station I, samples were collected at the base of intersecting thalassinid shrimp mounds as well as on the top and sides of mounds.

Transect survey and analytical methods

At each station, bacterial growth rates were calculated from the rate of tritiated thymidine incorporation into DNA as described by Moriarty et al. (1985a). Separate experiments were carried out to determine that the incubation time (15 min) was within the linear portion of the incorporation curve. Isotope dilution curves showed that addition of 7.5 nmol of thymidine per sample was sufficient to overcome any dilution effects (Pollard and Moriarty 1984). Sediment cores were taken to a depth of 1 cm (0.5 to 1.0 g of sediment). 1.11 MBq (30 μCi) [methyl-^{3}H] thymidine (specific activity: 40 Ci mmol^{-1}) was added to each sample and incubated at in situ temperatures (24–26 °C). Bacterial production was calculated using a conversion factor of 2.0×10^{18} cells dividing per mol thymidine incorporated (Moriarty 1986).

Bacterial numbers and biovolumes were estimated from replicate cores [0.5 to 1.0 g DW (dry weight) sediment core^{-1}, $n = 3$] using epifluorescence microscopy (Hobbie et al. 1977). The sediment was treated with 10% (v/v) acetic acid in distilled water overnight to remove carbonates, and then was homogenized for 5 min at 20,000 rpm with an Ultra-Turrax blender (Janke and Kunkel KG, Freiburg/Breisgau, FRG). Cell bioyolumes were determined microscopically. The average size of bacteria in these sediments was 0.2 μm^3. A value of 1.25×10^{-13} g C μm^{-3} was used to convert cell volume to carbon. This value falls within the range of values found by other workers (e.g. Ferguson and Rublee 1976).

Protozoan numbers were determined from 3 replicate cores (5 cm deep, 1.1 cm inner diameter) per station using the cold $MgCl_2$ flushing method of Alongi and Hanson (1985). Ciliates and large flagellates in the supernatant were counted in a Petri dish with the glass bottom lined into 1 cm^2 grids. A fixed number of grids were counted and results are expressed as number of individuals per 10 cm^2 of sediment. Small (<20 μm) flagellates were enumerated from a separate 1 ml portion of the filtrate using the method of Sherr et al. (1983). Briefly, flagellates were preserved in acid Lugol's solution and counted on a haemocyto-

Table 1. Summary of sediment granulometry and other physical characteristics of transect stations at Davies Reef. Each value in Part I represents a pooled mean of replicate samples. Organic carbon, nitrogen and pigment concentrations in Part II represent mean ±1 SD

Variable	Station								
	C	D	E	F	G	H	I(t)	I(s)	I(b)
I. *Granulometry*									
Median grain size (mm)	0.38	1.14	0.73	0.37	0.29	0.24	0.12	0.12	0.12
Inclusive graphic standard deviation (σ_1)	0.85	1.79	1.34	0.92	0.98	0.64	1.18	1.47	1.13
Percent sand (by weight)	96.9	94.2	93.9	96.2	93.4	85.9	77.9	76.7	75.8
Percent silt-clay (by weight)	2.6	1.3	1.8	2.9	2.6	13.6	21.4	21.7	23.2
Total organic matter (% by weight)	0.3	0.5	1.4	0.6	1.2	0.9	2.2	2.1	2.8
II. *Other sediment and physical characteristics*									
Organic carbon (mg g^{-1} DW)	3.4±0.3	3.9±0.3	3.2±0.4	4.2±0.2	4.4±0.9	6.0±1.5	3.2±0.3	4.1±0.3	4.5±0.3
Organic nitrogen (mg g^{-1} DW)	0.2±0.002	0.3±0.04	0.2±0.02	0.3±0.04	0.3±0.05	0.4±0.04	0.2±0.01	0.3±0.04	0.4±0.04
Chlorophyll *a* (μg g^{-1} DW)	0.9±0.3	6.2±1.4	3.3±1.1	3.6±1.3	2.7±0.5	2.8±0.7	0.9±0.4	1.7±0.2	1.0±0.4
Phaeopigments (μg $^{-1}$ DW)	1.1±0.4	0.3±0.1	0.2±0.0	0.3±0.3	0.3±0.4	0.4±0.4	0.2±0.3	0.3±0.1	0.4±0.2
Mean water depth (m)	12	2	2	3	5	6	16	16	16
Reef zone	Open basin; reef front	Sandy hollow; reef flat	Sandy hollow; reef flat	Sandy hollow; reef flat	Disturbed algal mat; shallow lagoon	Undisturbed shallow lagoon	Front lagoon; top (t), side (s) and between (b) shrimp mounds		

meter. The method recovers 68±8% ($\bar{x}$+1 SE) of the protozoa (Alongi and Hanson 1985).

Meiofaunal densities were estimated from 3 replicate cores (5 cm deep, 2.5 cm inner diameter) per station. Samples were fixed with a mixture of 1:500 (v/v) Rose Bengal in buffered seawater with formaldehyde (5% v/v). In the laboratory, sediments were passed through a set of two sieves made of plankton netting, the top one with a mesh opening of 500 μm and the bottom screen with a mesh size of 45 μm. Organisms retained on the finer mesh were considered as meiofauna and counted under a dissecting microscope (Alongi 1986).

Chlorophyll *a* and phaeopigments were estimated from 3 replicate cores per station by extraction with acetone (90% v/v with water) using the method of Lorenzen (1967) Absorbance was measured at 665 nm on a Turner LC-50 fluorometer. Total organic matter and grain size were estimated from 3 sediment cores (2.5 cm inner diameter) per station. Grain size analysis of the sediments followed methods described by Folk (1974). Total organic matter was determined by weight loss after treatment with 27% hydrogen peroxide (Buchanan 1984). Organic carbon and nitrogen were determined with a Perkin Elmer CHN analyzer modified as described by Moriarty and Barclay (1981).

Community metabolism and nutrient fluxes in the lagoon

Benthic community metabolism and nutrient fluxes were measured at lagoon stations G, I and J using 2 hemispherical clear acrylic chambers with a circular area of 0.9 m^2 and a volume of 315 l. The chambers were inserted 10 cm into the sediment with a flange preventing further settling. To maintain water movement over the sediment surface, water was recirculated through the chambers by an impeller pump.

Oxygen was measured using a temperature-compensated polarographic electrode system (gold cathode) with a polyethylene membrane. A battery-operated impeller mounted under the oxygen electrode maintained water flow over the membrane. The electrode was calibrated prior to use using oxygen-free nitrogen gas and oxygen-saturated seawater as described by Kinsey (1979). Temperature was measured using a thermistor probe. Oxygen concentrations and temperature were recorded at 10 min intervals over 24 h on microprocessor-controlled data recorders.

Water samples for nutrient analysis were withdrawn by divers at approximately 4 h intervals from a port in the water circulation system. The samples were filtered through 0.4 μm membrane filters and analyzed immediately on an Autoanalyzer or frozen for later analysis within 3 weeks.

Total oxygen changes in the chambers (sediment and water) were corrected for oxygen changes in the water overlying the sediments by concurrent in situ incubation of light and dark bottles (Strickland and Parsons 1972). Oxygen concentrations in the 500 ml bottles were measured initially and after using a temperature-oxygen probe.

Salinity at each site was determined on water samples collected when the chambers were inserted. Salinity values were needed to convert oxygen saturation readings to concentration values. Oxygen concentrations, diel production and respiration were calculated as in Kinsey (1979) but adapted for an enclosed water mass. In order to calculate production in terms of carbon from oxygen values, photosynthetic and respiratory quotients (PQ and RQ) were assumed to be 1 for Davies Reef lagoon sediments. In shallow lagoon environments where PQ and RQ have been measured previously, quotients are close to 1 (Atkinson and Grigg 1984; Kinsey 1985a, b). In conjunction with the dome studies, bacterial numbers and production in the sediments were determined at approximately 3 h intervals at stations G and I, and at 6 h intervals (because of depth-restricted diving time) at station J. The samples were collected within 3 m of the domes and analyzed as described above.

Results

Transect survey

Bacterial numbers did not change significantly across the transect [Student-Newman-Keuls (SNK) test, $P>0.05$; Sokal and Rohlf 1969) with a mean abundance (±1 SE) of 1.3 (±0.6)×10^9 cells g^{-1} DW of sediment (Table 2). Bacterial production and specific growth rates were significantly lower at station C (reef crest) (SNK test, $P<0.05$) than at the other sites. Bacterial production rates (±1 SE) ranged from 1.2 (±0.2) mg C m^{-2} h^{-1} to 11.6 (±1.5) mg C $m^{-1}h^{-1}$ and specific growth rates ranged from 0.05 to 0.33 d^{-1}.

Ciliate numbers ($\bar{x}$±1 SE) ranged from 72 (±26) to 742 (±461) individuals · 10 cm^{-2} across the transect

Table 2. Mean (±1 SE) bacterial numbers, production and specific growth rates (μ) in sediments (0–1 cm) across the transect

Station	Numbers $\cdot g^{-1}$ DW $\times 10^9$	Production* (mg C m^{-2} h^{-1})	μ (d^{-1})
C	0.9±1.2	1.2±0.2	0.05
D	1.5±0.7	6.1±0.7	0.14
E	1.4±0.7	9.0±4.0	0.20
F	1.5±0.6	8.4±3.0	0.18
G	1.2±0.6	11.2±4.0	0.33
H	1.2±0.5	11.6±1.5	0.33
I (top)	1.2±0.4	2.4±2.0	0.07
I (side)	2.0±0.8	6.5±1.0	0.11
I (between mounds)	1.8±0.6	10.0±4.0	0.18

* Values for bacterial production were calculated from the thymidine incorporation rate (pmol $\cdot g^{-1} \cdot h^{-1}$) and conversion factors of 2×10^{18} cells produced per mol of thymidine incorporated and 0.25×10^{-13} g C $\cdot$ cell^{-1} (see Methods)

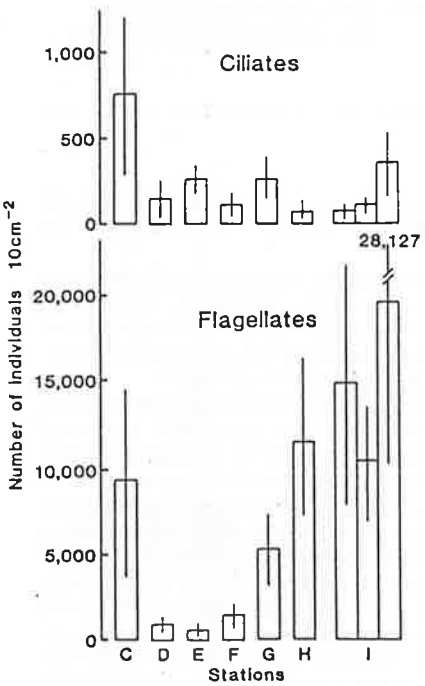

Fig. 2. The distribution and abundances of benthic ciliates (upper graph) and flagellates (lower graph) across transect sites C to I at Davies Reef. Bars depict means and vertical lines represent one standard error of replicate cores per station. At site I, bars depict from left to right, top, side and between mound sites

(Fig. 2). Numbers at stations C, E, G and I (between mounds) were not significantly different (SNK test, $P > 0.05$) but were significantly greater than at the other stations. Flagellate numbers ($\bar{x} \pm 1$ SE) ranged from 554 (±72) to 19,039 (±8565) individuals $\cdot$ 10 cm^{-2} across the stations (Fig. 2). Flagellate densities at coarse sand sites (stations D, E and F) were significantly lower than at stations C, G, H and I (between mounds) SNK test, $P < 0.05$).

Harpacticoid copepods were the most abundant meiobenthic taxon at the reef crest (station C), reef flat (stations D to F) and shallow lagoon sites (stations G and

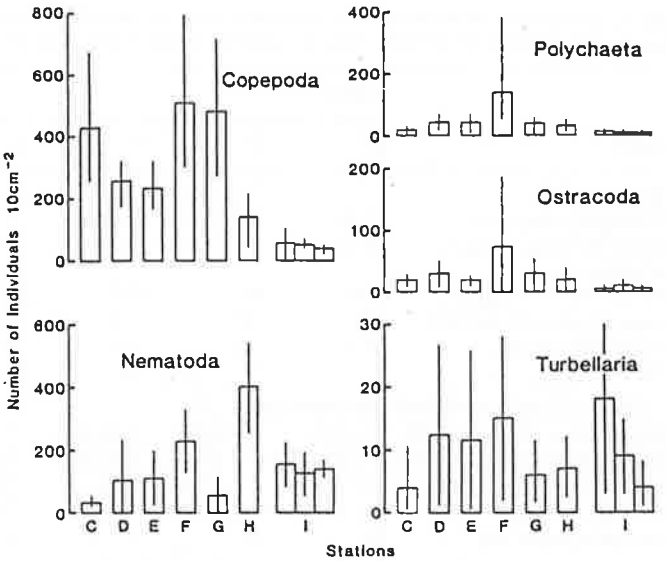

Fig. 3. The distribution and abundances of the major meiobenthic taxa across transect sites C to I at Davies Reef. Bars depict means and vertical lines represent one standard error of replicate cores per station. At Site I, bars depict, from left to right, top, side and between mound sites

H) (Fig. 3). Copepod densities at stations C, F and G were significantly greater than at stations H and I (SNK test; $P < 0.05$) where nematodes were the most abundant taxon and copepods least abundant. Densities of polychaetes, ostracods and turbellarians did not differ significantly (SNK test, $P > 0.05$) among the stations (Fig. 3).

Across the transect, bacterial growth rates were correlated significantly ($P < 0.05$) with total organic carbon (Kendall's coefficient of rank correlation, tau (τ) = + 0.70) and nitrogen ($\tau = +0.82$). Bacterial numbers were not correlated with any variables. Ciliate densities were correlated significantly only with phaeopigments ($\tau = +$ 0.88; $P < 0.01$), whereas numbers of flagellates were inversely correlated with chlorophyll $\cdot a$ ($\tau = -0.78$; $P < 0.01$) and positively correlated ($P < 0.05$) with percent sand ($\tau = +0.86$), total organic matter ($\tau = +0.70$) and percent silt and clay ($\tau = +0.87$). Nematode densities were significantly correlated only with organic carbon ($\tau = +0.75$; $P < 0.05$). Copepod densities were positively correlated with percent sand ($\tau = +0.88$; $P < 0.01$), but negatively correlated to total organic matter ($\tau = -0.75$; $P < 0.05$) and percent silt and clay ($\tau = -0.85$; $P < 0.05$). Numbers of other meiobenthic groups (e.g. turbellarians, ostracods, polychaetes) were not correlated with any of the variables examined.

Microbial communities on thalassinid shrimp mounds

Rates of bacterial productivity between the shrimp mounds and on their sides were not different, but were greater than rates of production at the top of the mounds (SNK test, $P < 0.05$) (Table 2). Ciliate abundances were not significantly different on the top and sides of shrimp mounds, but were less than in sediments between mounds (Fig. 2). Organic carbon concentrations were signifi-

Table 3. Mean community metabolic rates (mg C m^{-2} d^{-1}) in lagoon sediments measured as oxygen fluxes in domes over 24 h

Site	Gross primary production	Community respiration	P/R
Lagoon slope (G)	400 (±40)	520 (±80)	0.8
Front lagoon (I)	270	230	1.2
Deep lagoon (J)	1100	920	1.2

cantly lower at the top of mounds than on the sides and between mounds (SNK test, $P<0.05$). Organic nitrogen concentrations decreased significantly from the top to the bottom of the mounds. Neither chlorophyll *a* concentrations nor bacterial numbers differed significantly between different areas of the mounds.

On shrimp mounds, bacterial growth rates correlated significantly ($P<0.01$) with organic carbon ($\tau=+0.93$) and nitrogen ($\tau=+0.94$), phaeopigments ($\tau=+0.87$), silt-clay ($\tau=+0.78$; $P<0.05$) and ciliates ($\tau=+0.96$; $P<0.001$). Ciliate densities correlated with percent silt-clay ($\tau=+0.99$; $P<0.001$) and with phaeopigments ($\tau=+0.87$; $P<0.01$), organic carbon ($\tau=+0.78$; $P<0.05$) and organic nitrogen ($\tau=+0.87$; $P<0.01$).

Community metabolism and nutrient fluxes

Gross production rates were 400, 270, and 1 100 mg C m^{-2} d^{-1} in sediments at stations G, I and J, respectively (Table 3). In the shallow lagoon (station G) there was no net community production; the mean gross production to respiration (P/R) ratio was 0.8. In both of the deeper lagoon sites (stations I and J), the P/R ratio was 1.2. Net community production at station I (40 mg C m^{-2} d^{-1}) was lower than than at station J, the deepest site (180 mg C m^{-2} day^{-1}).

There were 2 to 4 fold changes in thymidine incorporation rates at each of the three lagoon sites during a diel period (Fig. 4). At stations G and I, bacterial growth rates changes significantly over the 24 h period (one-way ANOVA, $P<0.001$) but there was no distinct diel pattern (SNK test). Bacterial growth rates did not change significantly at Station J during the diel period (ANOVA, $P>0.05$). At all three stations, bacterial numbers changed significantly over the 24 h sampling period (one-way ANOVA $P<0.001$) but there were no obvious diel patterns to the changes. Bacterial production at stations G, I and J over this 24 h period were 134, 270 and 247 mg C m^{-2} d^{-1}.

Initial nutrient concentrations in the domes were comparable at all three sites (Table 4). There was a net release of ammonium and silicate from sediments at all three stations with extremely low and variable phosphorus fluxes (Table 4). There were no significant correlations of the rates of nutrient flux (ammonium, phosphate or silicate) with bacterial growth rates, numbers or oxygen flux rates (Kendall's rank correlation analysis).

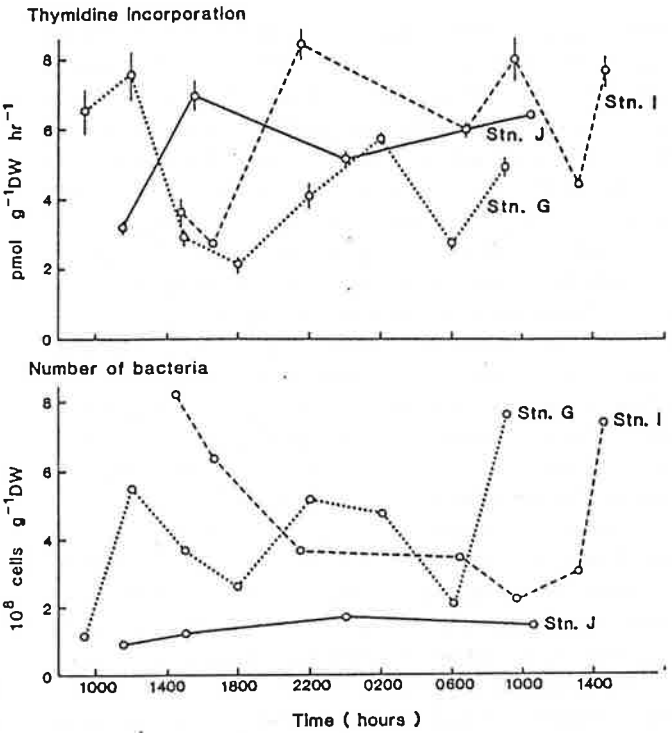

Fig. 4. Thymidine incorporation rates and bacterial numbers per g dry weight (mean ±1 SE) during a diel period at three stations in Davies Reef lagoon

Table 4. Initial concentrations (μmol^{-1}) and mean flux ($\mu mol \cdot m^{-2} \cdot d^{-1}$) rates (in parentheses) of dissolved nutrients in benthic enclosures. Flux rates were calculated by summing the fluxes over 4 h intervals from the 24 h dome incubations. Each value represents mean ±1 SE. ND = no data

Site	PO_4^{-3}	SiO_2^{-3}	NH_4^+	$NO_2^- + NO_3^-$
Lagoon slope (G)	0.29±0.03 (−28)	1.39±0.75 (+393)	0.25±0.23 (+212)	0.13±0.01 (+10)
Front lagoon (I)	0.21±0.07 (+1)	1.64±0.66 (+57)	0.04±0.02 (+143)	0.16±0.01 (ND)
Deep lagoon (J)	0.56±0.01 (−7)	1.16±0.18 (+806)	0.10±0.09 (+544)	ND (ND)

Discussion

An important question in microbial ecology is whether nutrient limitation or grazing by consumers is more important in controlling bacterial numbers and growth. In this study, bacterial growth rates correlated only with total organic carbon and nitrogen suggesting that sediment bacteria are predominantly nutrient limited during winter at Davies Reef. Only in the front lagoon where abundant thalassinid shrimps sort and process enormous quantities of reef sediments (Tudhope and Scoffin 1984) are bacterial communities obviously affected by larger organisms.

Strong correlations between the growth of bacteria and their food supply have been observed in a variety of

different marine systems such as in regions of upwelling (Gocke et al. 1983) and in areas where sedimentation of phytoplankton blooms (Meyer-Reil 1983) and faecal matter (Tenore et al. 1982) frequently occur. In microcosms, Hanson (1982) and Alongi and Hanson (1985) found that bacterial biomass and productivity increased up to detrital nitrogen rates greater than 200 mg N m^{-2} d^{-1} – a rate indicative of organically enriched systems and certainly greater than rates of detrital nitrogen supply within coral reef ecosystems (Hatcher 1983).

Lack of correlation of bacterial numbers and growth rates with protozoan and meiofaunal densities may have been due to lag periods in the predator-prey relationship. However, the possibility exists that protozoans and meiofauna may not always control bacterial populations in sediments. Recent studies in the coastal ocean (Ducklow et al. 1986), in reef waters (Landry et al. 1984), in microcosms (Alongi and Hanson 1985; Moriarty et al. 1985 b) and in tropical mangrove sediments (Alongi 1987) have indicated that consumers are not always capable of controlling bacterial populations and that most of these bacteriovores consume a variety of other foods in order to maintain a balanced diet (Di Salvo 1973). In laboratory experiments, Moriarty et al. (1985 b) found that the presence of protozoans and meiofauna enhanced bacterial growth only when twice the natural density of these organisms from Davies Reef were used. Protozoa and meiofauna may not graze down bacterial populations if rates of bacterial production are several orders of magnitude higher than estimated ingestion rates of consumers (Alongi 1987).

The numbers of bacteria were not high and did not vary much across the transect even though growth rates varied. There may well be a limiting population density of bacteria below which changes in density are slight as growth slows or stops and bacteria enter a resting phase. Presumably, there must be a bacterial density in sediment below which it is energetically inefficient for protozoa to feed. Such a mechanism would explain why growth rates but not numbers vary across the transect.

However, we found that bacterial growth rates, organic carbon and nitrogen concentrations and ciliate numbers were lowest at the top of thalassinid shrimp mounds suggesting that organic matter is removed from sediments by the shrimps, thus slowing bacterial growth rates. Although meiofaunal densities did not differ with mound structure, the low population densities (<200 individuals·10 cm^{-2}) and very low numbers of nematode species (Alongi 1986) compared to other tropical sands of identical grain size (McIntyre 1968), indicate that they are also negatively effected by ghost shrimps. In sandy beaches in South Africa, Dye and Furstenberg (1978) similarly found that *Callianassa kraussi* altered sediment bed structure and growth of meiofaunal communities. Microbial communities were not examined in their study.

Earlier reef work has shown that deposit-feeding macrobenthos on other reefs significantly affect microbial and meiofaunal populations. For example, the holothurian *Holothuria atra* does not eat meiofauna but competes with meiofauna for microbes as food (Renaud-Mornant and Helleouet 1977; Moriarty 1982) and can depress benthic microalgal and bacterial productivity on reef flats (Moriarty et al. 1985 a). Ghost shrimps appear to have an identical trophic role in lagoon sands at Davies Reef.

Primary and microheterotrophic production and nutrient fluxes

The P/R ratio of Davies lagoon sediments was 1.2 except at the lagoon edge, indicating that there is net community production of organic matter over much of the lagoon bottom. Although reef lagoons have generally been regarded as net sinks for organic matter (Kinsey 1979; Harrison 1983), our study and other recent work (Atkinson and Grigg 1984; Kinsey 1985 a, b) has shown that net community production in some lagoons does occur. In our study, the rate of gross primary production was highest at the deepest lagoon station probably due to the presence of diatom and cyanobacterial mats (primarily *Oscillatoria* spp.) (G. Hallegraaf, personal communication). Algal mats were present at all of the lagoon stations, but the bioturbation activities of thalassinid shrimps at station I disrupted the mat, and tidal currents and wave action appeared to continually disrupt the sediment surface at Station G. Rates of gross primary production in Davies Reef lagoon were similar to those measured in Enewetok lagoon (Harrison 1983), although in Harrison's study there was no net community production and rates of metabolism decreased with increasing depth into the lagoon.

Assuming a bacterial growth efficiency of 50% (Ducklow 1983), bacteria in the lagoon sediments produced 268, 570 and 494 mg C m^{-2} d^{-1} at stations G, I and J, respectively, or 67%, 200% and 45% of gross primary production. This indicates that, at least at Station I, benthic primary production was not sufficient to maintain the observed bacterial growth rates and suggests that an import of organic matter would be necessary. Likely sources of organic matter are coral mucus (Moriarty et al. 1985 b) or particulate organic material from reef flat turf algal communities (Hatcher 1983). If the growth yield of bacteria is lower (Newell 1984) or the carbon content of bacteria higher (Bratbak 1985) then the carbon flow through bacterial pathways may be even higher than our calculated values.

The patterns of nutrient flux measured in Davies Reef lagoon were similar to those found at other reefs in the central and southern Great Barrier Reef (Hatcher and Frith 1985). In previous measurements of benthic nitrogen flux at Davies Reef (H. Iizumi, personal communication), ammonium release was of the same order of magnitude as measured during this study (ca. 290 μ mol m^{-2} d^{-1}). Rates of nutrient flux in reef lagoon sediments are several orders of magnitude lower than rates mea-

sured in coastal temperate sand communities (Nixon 1981). Of particular interest is that microbial densities and bacterial growth rates were not correlated with the nutrient fluxes in Davies lagoon suggesting that other organisms such as thalassinid ghost shrimps play a role in influencing the regeneration of nutrients from lagoonal reef sediments.

Conclusions

The dynamics of microbial and meiobenthic communities are apparently governed by a variety of abiotic factors at the reef crest and across the reef flat. In the front lagoon, bioturbation and feeding by thalassinid shrimps negatively affect organic carbon and nitrogen concentrations, and bacterial and ciliate densities. In these lagoonal sediments, a net import of organic matter appears to be necessary to support the estimated rates of bacterial productivity. In lagoonal areas not colonized by ghost shrimps, the sediments were heavily carpeted with algal mats, and bacterial productivity equalled only 22% and 33% of primary productivity. Coupled with low nutrient efflux rates, this suggests that in non-bioturbated lagoon sediments significant utilization of dissolved nutrients and organic production by autotrophs occur sufficient to support bacterial growth.

Acknowledgements. We thank H. Streiner, K. Navin and C. M. Moriarty for their assistance. J. A. Hansen was supported by a postdoctoral Research Fellowship at the Australian Institute of Marine Science. D. M. Alongi was supported by NSF Grant no. INT-8312117/U.S.-Australia Cooperative Science Program) and by a Postdoctoral Research Fellowship at the Australian Institute of Marine Science. Contribution no. 7 from the Microbial Ecology on a Coral Reef Workshop (MECOR), held at the Australian Institute of Marine Science (July–August 1984). Contribution no. 375 from the Australian Institute of Marine Science.

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F. Role of Bacteria in Aquaculture Ponds

Microb Ecol (1986) 12:259–269

MICROBIAL ECOLOGY

Bacterial Productivity in Ponds Used for Culture of Penaeid Prawns

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Abstract. The quantitative role of bacteria in the carbon cycle of ponds used for culture of penaeid prawns has been studied. Bacterial biomass was measured using epifluorescence microscopy and muramic acid determinations. Bacterial growth rates were estimated from the rate of tritiated thymidine incorporation into DNA. In the water column, bacterial numbers ranged from 8.3×10^9 l^{-1} to 2.57×10^{10} l^{-1} and production ranged from 0.43 to 2.10 mg C l^{-1} d^{-1}. In the 0–10 mm zone in sediments, bacterial biomass was 1.4 to 5.8 g C m^{-2} and production was 250 to 500 mg C m^{-2} d^{-1}. The results suggested that most organic matter being supplied to the ponds as feed for the prawns was actually being utilized by the bacteria. When the density of meiofauna increased after chicken manure was added, bacterial biomass decreased and growth rates increased.

Introduction

Detrital food chains, based on the use of manures and compost, have been used in aquaculture for centuries, especially in Southeast Asia [4]. Heterotrophic bacteria convert organic detritus into protein and presumably constitute an important food source for fish in ponds [23]. In concert with protozoa and meiofauna, aerobic bacteria mineralize organic matter, providing inorganic nutrients such as N and P for algal growth [7, 11]. The mineralization process may be detrimental in ponds where primary production is not desired, e.g., in high intensity farming where feed pellets are supplied, because not only will feed pellets be decomposed by the microbes, but oxygen will be depleted in the water column.

The role of bacteria in aquatic ecosystems can now be quantified with modern techniques to measure their numbers, biomass, and production [26]. A particularly valuable approach is the estimation of production rates, using the rate of tritiated thymidine incorporation into DNA, because there is a direct correlation between DNA synthesis and bacterial division rate [9, 10, 15, 18, 19]. This method specifically measures the growth of heterotrophic bacteria; it does not measure the growth of cyanobacteria (blue-green algae), eukaryotic algae, or fungi.

In the work reported in this paper, production of bacteria was determined in aquaculture ponds to which a pelleted food was fed to penaeid prawns. The

influence of chicken manure on bacterial production was also studied. From these results it was possible to estimate how much of the organic matter was decomposed by bacteria. The effect of meiofauna on bacterial biomass and production was also investigated. Only a short time was available for the studies reported here, but it was sufficient to assess the usefulness of the methodology for studies of bacterial production in ponds.

Materials and Methods

Site

The ponds were constructed at Gelang Patah, near Johore Bahru, Malaysia, on acid-sulfate mangrove soil. Due to problems with alkalinity and pH, about half the water in each pond was exchanged daily on a tidal cycle [25]. Penaeid prawn species were cultured in the ponds. Salinity was between 26 and 28 parts per thousand, and water temperature ranged from 28–32°C.

The ponds had a peripheral canal about 3 m wide and 1.5 m deep, with a layer of soft sediment. The center of the ponds was 1 m deep, with a thin (20–30 mm) layer of soft sediment over a heavy clay substrate. Water depth was lowered to depth of about 50 cm at low tide once per day, and then refilled on the next high tide from a river fringed by extensive mangrove forests.

Pond Treatments

Ponds Stocked with Prawns. At the time of this study, pond 11 (0.5 ha) was supplied with pelleted food at a rate of 10 kg dry weight d^{-1} (1 g C m^{-2} d^{-1}). Pond 29 (0.25 ha) was supplied with pelleted food at a rate of 16 kg d^{-1} (3.2 g C m^{-2} d^{-1}). Pond 23 (0.25 ha) contained a nursery pen (10 m × 20 m) which was supplied with a food mash at a rate of 5 g dry weight m^{-2} d^{-1} initially, and then 8 g m^{-2} d^{-1} a day before the measurements reported here were made. Pond 26 (0.15 ha) was untreated, except for water exchange, and was used as a control.

Pen Experiments with Chicken Manure. Pond 32 (1.0 ha) contained 3 pens, each 3 m in diameter, constructed with plastic mesh (5 mm square holes) in the central area of the pond. Pen A was supplied with chicken manure at a rate of 0.6 g dry weight m^{-2} d^{-1}, for 1 week prior to sampling for bacterial biomass, organic C, and N determinations and meiofauna numbers. Pen B was treated with chicken manure for 2 weeks, and pen C was treated with manure for 3 weeks at the same rate as pen A.

A sample of the complete water column was obtained by using a long plastic tube which was pushed down to about 5 cm from the bottom; it was then stoppered and removed. Water was brought back to the laboratory for analysis within 10 min after collection. All analyses were carried out on pond water collected between 8 a.m. and 2 p.m. before the daily tidal exchange. Samples for microscopy were preserved with formaldehyde (3% v/v final volume). Bacteria were counted using epifluorescence microscopy after staining with acridine orange [28, 29] with modifications described by Moriarty [13]. Cell volume was determined from the sizes of bacteria measured on photographs, and biomass was calculated with a conversion factor of 0.22 g C cm^{-3} [2]. For muramic acid measurements, 50 ml of water were filtered through Whatman GF/F filters and then polycarbonate membrane filters (0.2 μm pore size). The filters were combined and dried at 100°C. Muramic acid was extracted and measured on a high pressure liquid chromatograph after precolumn derivation with *o*-phthaldialdehyde [14].

Bacterial production measurements were carried out according to the principles described by Moriarty and Pollard [18, 19]. The following procedure was used for the pond survey. Before collecting the water, 30 μl (30 μCi) [5-methyl-^{3}H]thymidine (51 Ci $mmol^{-1}$) were added to each of a series of polypropylene tubes. The final concentration of thymidine was 60 nM, after pond water (10 ml) was added. Tubes were capped, shaken, and then incubated at *in situ* temperature for 15

min. Each sample was filtered through cellulose acetate filters (0.2 μm, 25 mm diameter) to stop the incubation. Each filter was washed five times with 2 ml of 3% (w/v) ice-cold trichloroacetic acid (TCA). Filters were stored, and later 2 ml of 5% TCA were added, and the filters were heated at 100°C for 5 min. One milliliter was removed for liquid scintillation counting. Zero time controls were used for blanks, i.e., the samples were filtered immediately after adding pond water.

To check whether macromolecules other than DNA were being labeled, two time courses were carried out. One was stopped and treated with TCA as described above; this method does not separate DNA from other macromolecules. In the other experiment the DNA was extracted and separated from RNA and protein. The incubation was stopped with cellulose nitrate filters, and each filter was washed twice with ice-cold tap water and transferred to polypropylene tubes containing 2 ml of 0.3 M NaOH. The DNA was extracted as described by Moriarty and Pollard [19]. Recovery was found to be 100%, using ^{14}C-DNA as a standard [19]. Isotope dilution experiments were also conducted to show that the concentration of thymidine was high enough to inhibit *de novo* synthesis, i.e., dilution did not occur (see [19, 21]).

Sediment Measurements

Sediment was cored using 50 ml plastic syringes with their bases cut off. The top 10 mm was collected for analysis of organic C and N and muramic acid. Cores from canal and center areas were analyzed separately; four cores were pooled for each zone. Sediments were sun-dried then weighed and stored. Muramic acid was measured as described by Moriarty [14].

For the measurement of thymidine incorporation rates, preliminary experiments were carried out using a sediment slurry to measure isotope dilution and to do a time course on the rate at which tritiated thymidine was incorporated into DNA. The top 10 mm from three cores (50 ml) were combined and a small plastic spoon was used to dispense about 1.0 g into each of a series of polypropylene tubes containing 50 μCi of tritiated thymidine (see [19]). Incubations were carried out at *in situ* temperature for 15 min (or various intervals for the time-course experiment) in a water bath on the pond bank, out of direct sunlight, as soon as the samples were collected. The incubations were stopped by adding 2 ml 0.6 M NaOH. The DNA was extracted by heating the samples in a pressure cooker for 30 min and proceeding as described by Moriarty and Pollard [19].

Subsequent measurements of thymidine incorporation rates were made using the top 10 mm of sediment taken with small corers (5 ml syringes) and treating each core as an individual sample. This treatment minimized oxygenation of anaerobic sediment. Duplicate samples were analyzed for both center and canal zones in each pond. Because the preliminary experiments showed only a small amount of isotope dilution, 100 μCi (100 μl) of [methyl-^{3}H]thymidine (51 Ci mmol^{-1}) were used for later experiments. No significant isotope dilution was observed with this amount of tritiated thymidine (see [21]). Incubations were carried out as described above and were stopped with 2 ml of 0.1 M NaOH. This lower concentration (0.1 M) of NaOH was used to minimize losses of DNA during storage. The tubes were capped and stored for 1 week, and the DNA was extracted after 2 ml of 0.6 M NaOH was added. Recovery of DNA, measured with ^{14}C-DNA, was 80%. See Moriarty and Pollard [19] for further details of the technique for extracting DNA and determining rates of DNA synthesis.

For enumeration of the meiofauna, three cores 30 mm in diameter and 20 mm deep were taken and combined for each sample. The cores were extruded into beakers containing formaldehyde (3% v/v) and rosebengal. Clay was washed out through a fine sieve (0.5 mm) and 30% colloidal silica (Ludox, Dupont) was used to separate the meiofauna from the remaining sediment and detritus [3]. Two or three treatments with Ludox were used. Very few, or no, animals were found to be left in the sediment when it was checked after the Ludox treatment.

Results

The rate of incorporation of tritiated thymidine into DNA in sediments was linear for 20 min, and in the water column the rate was linear for 1 hour, but

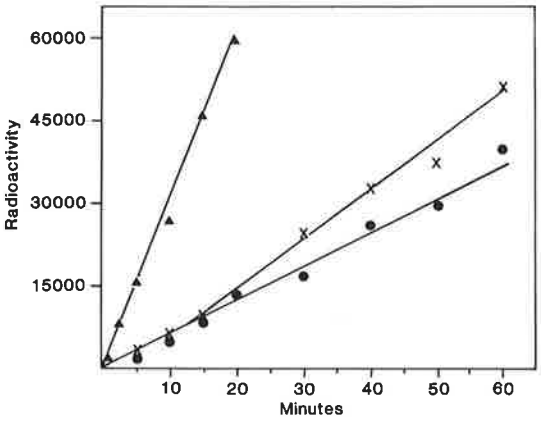

Fig. 1. Time course for tritiated thymidine incorporation into DNA. Radioactivity units are disintegrations per min per 10 ml. Symbols: ▲ incorporation into extracted DNA in sediment samples; ● incorporation into extracted DNA in water column samples; × incorporation into macromolecules insoluble in trichloracetic acid in water column.

the rate of incorporation into TCA-insoluble compounds increased after about 20 min (Fig. 1). The time periods chosen for the pond survey experiments were less than these limits for linear rates of DNA synthesis.

Bacterial numbers in the water column were higher in most ponds receiving organic matter than in control ponds (Table 1). The presence of particles with attached bacteria, which could not be counted accurately, contributed to the variability. Most bacteria were between 0.4 and 0.8 μm in diameter and 0.5 and 1.5 μm in length. The average cell volume was 0.14 μm^3, and thus biomass was 30 fg (a total of 546 bacteria was measured). Biomass values were also calculated from the muramic acid content of bacteria in the water. With the factor of 30 fg C $cell^{-1}$, the mean ratio of biomass calculated from both methods for six ponds was 1.1 with a 9% coefficient of variation. Thus, there was a good agreement between the two methods. The same conversion factor was used to calculate bacterial production rates in terms of carbon.

Bacterial production was higher in the water column of all ponds receiving organic matter than in the control ponds (Table 1). Bacterial production was significantly lower in the water of the control ponds than in the river water ($P < 5\%$). Production in the three ponds receiving pellets was significantly higher than in the control ponds ($P < 1\%$). Doubling times were fastest in pond 29, which was the only pond that was being aerated. Bacterial biomass and production were generally higher in the sediments of ponds with an organic matter input, but the effects of organic matter on either biomass or production were not as marked as in the water column (Table 2). Where large amounts of food were being added (ponds 29 and 23) there was a significantly higher rate of bacterial production than in control pond sediments ($P = 1\%$). Production rates were similar in the mangrove sediment and the control pond, but the doubling time was slower in the mangrove sediments (Table 2).

Chicken manure had a marked effect on bacteria in pond sediment. One week after adding manure, bacterial biomass had increased, and then over the next 2 weeks it fell 5-fold (Table 3). Bacterial growth rates (average for the whole community) increased over the same period. The number of animals in the meiofauna increased markedly after manure was added (Table 4). As it was

Table 1. Effect of pelleted food on bacterial numbers and production in the water column

Pond	Treatment	Food input g C m^{-2} d^{-1}	Number of bacteria 10^9 l^{-1}	Production* g C m^{-2} d^{-1}	Doubling time (h)
River:		0	8.8 ± 0.8	0.76 ± 0.2[a]	6
26	Control	0	8.3 ± 0.6	0.43 ± 0.04[b]	10
32	Control	0	8.0 ± 1.0	0.45 ± 0.01[b]	9
11	Pellets	1.0	8.8 ± 0.7	1.07 ± 0.09[c]	4
29	Pellets	3.2	11.3 ± 1.4	1.32 ± 0.11[c]	4
23	Pellets	4.0	25.7 ± 4.3	2.10 ± 0.19[c]	8

* Analysis of variance: [a] > [b] significant at 5% level; [b] < [c] significant at 1% level
The water depth was 1 m, thus 1 mg C l^{-1} = 1 g C m^{-2}. Values shown are mean ± SE with n = 9 for number and n = 5 for production

not possible to count the meiofauna before manure was added, an example of normal density is taken from data for another pond [16]. Growth rates ranged from 0.03–0.17 for nematodes; 0.03–0.15 for copepods, and 0.11–0.3 for polychaetes.

Discussion

Bacterial Contribution to Pond Carbon Budgets

Bacterial production in the water column was due partly to input from the river, because half the water in the ponds was exchanged daily. The river water drained a large area of mangroves, and so the value of 0.76 mg C l^{-1} d^{-1} (Table 1), although large, is not surprising. In pond 26, which received no organic matter except that in the river water, the bacterial productivity in the water column was about half that in river water, as expected when half the water was exchanged daily.

Much of the added organic matter in feed pellets was supporting bacterial growth, particularly in the water column. Productivity of bacteria was closely correlated with input of organic matter. Approximate estimates of the amounts of organic matter needed to support both bacterial production and respiration would be twice the production values in water and at least 3 times the production values in sediment. These estimates are based on average conversion efficiencies of 50% in the water column and 30% in sediments for the utilization of organic matter by bacteria. Aerobic bacteria in culture are often more than 50% efficient [20]. Anaerobic bacteria, such as sulfate reducers, are much less efficient (around 12–15%) [12, 27]. As bacteria in the pondwater were utilizing pellets comprised of protein and readily digestible carbohydrate, a conversion efficiency of 50% is not unreasonable. In fact, it fits well with the data, because with this factor, utilization of carbon by bacteria balances the input. If the conversion efficiency were much lower, bacterial production and respiration would be in excess of input. It is possible, however, that the average carbon

Table 2. Effect of pelleted food on bacterial biomass and production in sediment

Pond	Treatment	Food input g C m^{-2} d^{-1}	Biomass g C m^{-2}	Production* g C m^{-2} d^{-1}	Doubling time (d)
Mangrove		0	3.6 (3.1–4.2)	0.25 (0.20–0.27)	10
26	Control	0	1.5 (1.4–1.7)	0.26 (0.20–0.40)[a]	4
11	Pellets	1.0	2.7 (2.1–3.4)	0.24 (0.11–0.35)[a]	9
29	Pellets	3.2	2.6 (2.3–3.0)	0.41 (0.36–0.47)[b]	4
23	Pellets	4.0	5.8 (5.2–6.4)	0.50 (0.47–0.52)[b]	8

* Analysis of variance: [a] < [b] significant at 1% level
Values for a mangrove sediment on the river bank away from the ponds are also given. Mean and range are given for 4 determinations of production and for duplicates of biomass

Table 3. Effect of chicken manure on bacterial biomass, growth rates, and meiofauna in sediment

		Bacteria			
Pen	Treatment	Biomass g C m^{-2}	Production mg C m^{-2} d^{-1}	Specific growth rates* d^{-1}	Meiofauna number per 10 cm^2
	Open pond	3.4 ± 0.00	440 (340–520)	0.13 ± 0.03	144
A	Manure, 1 week	4.3 ± 0.20	350 (280–420)	0.08 ± 0.01[a]	300
B	Manure, 2 weeks	1.9 ± 0.20	180 (140–220)	0.10 ± 0.03[b]	1,500
C	Manure, 3 weeks	0.8 ± 0.07	230 (170–290)	0.25 ± 0.04[c]	1,500

* Analysis of variance: [a] < [c] and [b] < [c] significant at 5% level
The experiment was carried out in 3 pens as described in Materials and Methods. Values are mean ± range; n = 2 for biomass and number. See Table 4 for details of meiofauna numbers

content of the bacteria was lower than the value used here (see [2]), and thus a lower growth efficiency could apply.

To obtain an estimate of organic matter from added food pellets utilized by bacteria in the water, an average value of 0.44 mg C m^{-2} d^{-1} for the control ponds was deducted from the production values for ponds 11, 29, and 23, and the result was doubled to allow for respiration. For ponds 11, 29, and 23, these estimates are 1.26, 1.76, and 3.32 respectively. In pond 11, the estimate is close to the amount of added food pellets, and indicates that bacteria were utilizing most of the added food. In pond 29, the estimate is only about half the value for added food, but values for bacterial production in sediment are significantly higher in pond 29 than 11. In the nursery area of pond 23, the very high level of bacterial production (Table 1) required most of the added food mash supplied to the young prawns to sustain it. Thus, the food material was dissolving or remaining as small suspended particles in the water column

Table 4. Effect of manure treatment on numbers of meiofauna

Pen	Sample	Nema-todes	Harpac-ticoid copepods	Poly-chaetes	Others	Total
Open pond[a]		132	8	0	4	144
A	1	104	40	112	136	392
	2	96	24	16	64	200
B	1	200	480	256	320	1,256
	2	328	560	520	464	1,872
C	1	160	640	440	208	1,448
	2	368	652	304	200	1,524

[a] An example of meiofauna density in a stocked pond taken from Moriarty et al. [16]
Pen A: manure treatment for 1 week prior to Feb. 6; Pen B: manure treatment for 2 weeks prior to Feb. 6; Pen C: manure treatment for 3 weeks prior to Feb. 6. Prawns were stocked on Feb. 6
Values are number 10 cm^{-2}, 2 cm depth

and providing a substrate for bacterial growth. The very high productivity of bacteria in the sediment would account for the remainder of added mash in the nursery area. The greater bacterial activity in the sediment of pond 29 than in pond 11 is indicated by the faster doubling times (Table 2). It is clear, therefore, that most of the added feed material was not being used by the prawns, but rather was supporting bacterial growth. Net primary production did not contribute significantly to bacterial production in these ponds [16].

Because the time available for this work was limited, it was not possible to study these problems in more detail. The work does demonstrate, however, that aquaculture ponds are useful as experimental sites in microbial ecology because inputs and outputs to the system can be measured. It is possible, for example, to study problems such as the growth efficiency of bacteria in the aquatic environment with different types of carbon source. More research is needed on this topic, in order to improve estimates of bacterial production and carbon cycling.

Effect of Bacteria on Oxygen Concentration

Values for bacterial production in the water column may be used to estimate consumption of oxygen by the bacteria in the planktonic community. Taking pond 23 as an example, at 50% efficiency for utilization of organic C, the bacteria in the water column of the nursery would consume about 5.6 mg O_2 l^{-1} d^{-1}. More oxygen would be used by aerobic bacteria at the sediment surface. Deoxygenation would occur if aeration was not provided, and, in fact, aeration was needed in this pond. Pond 29 was being aerated and the fast doubling times for the bacterial populations in this pond compared to other ponds are probably a result of the aeration. Bacteria in the water and sediment required about 4.0 mg O_2 l^{-1} d^{-1} in pond 29, which is quite high and shows that aeration was desirable. Oxygen supply by exchange from the atmosphere, primary produc-

Table 5. Comparison of bacterial numbers and production in the ponds with some other environments

Site	Number No. l^{-1}, or No. m^{-2}[a]	Production[a] $\mu g\ C\ l^{-1}\ h^{-1}$ or $mg\ C\ m^{-2}\ h^{-1}$[a]	Reference
Water			
Pellet-fed ponds	8.8×10^9–2.6×10^{10}	39–87	This study
Manured ponds	1.2×10^{10}–1.3×10^{10}	37	This study
Seagrass beds	2.8×10^9–6.8×10^9	0.1–0.3	[19]
York River estuary, USA	1×10^9–8×10^9	0.3–3	[5]
Eutrophic lake	4×10^8–2.3×10^9	0.2–7.1	[1]
Open ocean	5×10^8–2.5×10^9	0.08–0.8	[10]
Sediment			
Pellet-fed ponds	7×10^{13}–2.1×10^{14}	6–21	This study
Manured ponds	5.3×10^{13}–8.3×10^{13}	12–17	This study
Seagrass beds	4.3×10^{13}–1.7×10^{14}	2–7	[13, 19]
Coastal sediment	9.7×10^{12}–4.1×10^{13}	0.7–1.7	[6]

[a] Units: for water l^{-1}; for sediment m^{-2} and 10 mm depth

tion, and flushing with river water are unlikely to be sufficient for maintaining a level high enough to avoid stress for animals when bacterial production is so high.

Methodology

It is unlikely that the productivity in sediments has been overestimated due to disturbance or aeration during experimental treatment. DNA synthesis is closely regulated in bacteria, and changes in external conditions do not immediately affect the rate of thymidine incorporation into DNA [15]. The only macromolecule that was labeled significantly during the first 15 min was DNA (Fig. 1). Other compounds, possibly proteins, were labeled after 15 min, so all incubations were kept shorter than this to minimize errors.

The production values have been expressed in terms of 1 day, although measurements were made during only part of the day. Major diel cycles in bacterial production are unlikely to have occurred here as there was not a close link with excretion of organic matter during photosynthesis by a macrophyte such as seagrass [19]. There may have been small variations linked to water exchange or daily food pellet addition. Riemann and Sondergaard [22] found that diel variations in bacterial growth rates were minor in seawater and lakes. As the bacterial production in these ponds was dependent mainly on organic matter already present in the sediment, or from an excess supply of feed pellets, diel variations are likely to have been small and thus any errors in calculating daily carbon budgets would be small. Further work is needed to check this assumption.

Role of Meiofauna

Grazing by meiofauna, and perhaps protozoa, is apparently an important factor limiting bacterial densities and production in the pond sediments. The inverse correlation between bacterial density changes and meiofaunal density in the pen experiment supports the concept that meiofauna can limit bacterial biomass [11] (Table 3). Stimulation of bacterial growth rates by the grazing pressure also occurred. Total bacterial productivity fell because a large proportion of the bacteria were removed. The effect of protozoans was not measured, but presumably was also substantial. Protozoa are probably the main grazers on bacteria in sediments [8]. Further work is needed to quantify the food chain dynamics at trophic levels occupied by protozoa and meiofauna in ponds such as these where the higher consumers are predominantly carnivores. Bacteria were not significant components in the prawns' diet in these ponds [16], so growth efficiencies at the lower trophic levels could control the productivity of these ponds.

Comparison with Natural Environments

These aquaculture ponds support greater numbers and productivity of bacteria than do the ocean or lakes (Table 5). The productivity of bacteria in sediments associated with seagrasses is similar to that in pond sediment. Bacterial biomass and productivity in mangrove sediments was also similar to that in the ponds (Table 2), and indicates that bacterial activities are important in mangrove ecosystems.

Schroeder [23] reported values for bacterial numbers that are 2–3 orders lower than those found for this study in the water column and up to 7 orders of magnitude lower in the sediments. The difference is due to technique. Schroeder [23] counted colonies of bacteria on agar plates, but plate counts considerably underestimate true numbers [26]. Direct counts using epifluorescence microscopy are simple to make in the water column, but more difficult in sediments where many bacteria cannot easily be separated from particles. Muramic acid determinations are, therefore, more useful in sediments and give reasonably accurate results [13, 17]. As shown in the results here, there was a good agreement between direct counts and muramic acid values in the water column.

Conclusions

The work reported here was a preliminary survey of bacterial productivity in the prawn ponds; there was insufficient time for detailed analyses with many replicates. The work does show, however, that bacterial activities accounted for most of the organic carbon added to the ponds. The prawns needed only a small amount of the pelleted food [16]. Schroeder [24], using ratios of stable carbon isotopes, reached similar conclusions concerning ponds used for culture of common carp and the prawn, *Macrobrachium rosenbergii.* These animals were found to have fed more on natural foods rather than pelleted food.

The techniques for bacterial production, when combined with analysis of nutrient turnover and primary production, can be used to study quantitatively the carbon cycle in ponds, and show what happens to organic matter inputs. If protozoa and meiofauna are studied as well as the animals of commercial interest, a detailed analysis can be made of food chain dynamics. From these types of studies, predictions can be made about the effect of supplemental feeding or how much pelleted food can be substituted by manuring. Such studies would be particularly valuable in ponds where microphagous fish such as mullet or tilapia are cultured.

Acknowledgments. I am grateful to Harry L. Cook, Team Leader, Coastal Aquaculture Development Project, Gelang Patah, Malaysia, for organizing my visit and for much help while I was there. I thank Mr. Ti Teow Loon, Director of the Research Station, for his help and hospitality. I am grateful also to Mr. Rosly bin Hassan, Mr. Ismail bin Abu Hassan, Miss Chu Siu Chiam, and Mr. M. Thanabal for their assistance at Gelang Patah. Dr. Abdullah Sipat, Department of Biochemistry and Microbiology, University Pertanian Malaysia, kindly made facilities available in his laboratory for some of this work. I also thank Mr. P. C. Pollard and Mr. T. V. de Kluyver for assistance at Cleveland. The work was carried out with funds supplied by the United Nation's Food and Agriculture Organization. I am grateful to Dr. H. W. Ducklow for critically reviewing the manuscript.

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MALAY. AG.J. 54 (1983) 37-51.

PRIMARY PRODUCTION AND MEIOFAUNA IN SOME PENAEID PRAWN AQUACULTURE PONDS AT GELANG PATAH

by

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ABSTRACT

As part of a study to determine whether natural food chains were important in ponds used for culture of penaeid prawns, measurements were made of primary production and meiofauna numbers at Gelang Patah, Malaysia. The results of a preliminary survey are reported here. Net primary production was measurable in the upper part of the water column, but in the complete water column, daily respiration exceeded gross primary production in all ponds. Respiration ranged from 7.6 to 12 mg O_2 $l^{-1}d^{-1}$ in all ponds, and gross primary production from 2.6 to 5.2 mg O_2 $l^{-1}d^{-1}$. The excess respiration was due to a large bacterial community. When chicken manure was added to pond sediment, bacterial biomass increased, followed 1 to 2 weeks later by an increase in meiofauna density. A survey of meiofauna in ponds showed that numbers of meiofauna animals, particularly polychaetes, were low in ponds where prawns were stocked. It was concluded that the meiofauna was a component in the food of the prawns in these ponds.

Introduction

Penaeid prawns are being grown to a marketable size in ponds at the Coastal Aquaculture Development Project at Gelang Patah, Malaysia. In an attempt to lower production costs, experiments are underway to determine whether part or even all of an expensive pelleted feed can be substituted by chicken manure. Schroeder (1978) estimated that microorganisms comprised about half of the food supply for fish in intensely-manured fish ponds. He pointed out that the manure itself was not a useful food source, but that it supplied nutrients, particularly N and P, which promoted a high production of bacteria on the organic matter present (mainly straw particles). Bacteria comprise a small part of the food of penaeid prawns; meiofauna and small macrofauna are the major items of their diet in their natural habitat (Hall, 1962; Chong and Sasekuumar, 1981; Moriary and Barclay, 1981). In manured ponds the food chain would be manure-bacteria-meiofauna-prawns and also might include protozoa.

The work reported in this paper was carried out as part of a preliminary study on the role of microbial food chains in aquaculture ponds. It is aimed at showing the direction more detailed studies should take. Studies on the density and distribution of meiofauna and the effects of prawn predation are reported here. This is part of a preliminary study on the role of bacteria and meiofauna in the prawn ponds. Other work has shown that in these ponds there is a high biomass and production of bacteria which is stimulated by adding chicken manure (Moriarty, 1986). Most feed pellets that were supplied to prawns were being utilized directly by the bacteria. The work indicated that bacterial respiration was intense. In this paper, data on respiration and primary production are presented. Nutrient concentrations were also measured.

Materials and Methods

The ponds were constructed on acid-sulfate mangrove soil. Due to problems with low alkalinity and low pH, about half the water in each pond was exchanged daily on a tidal cycle. Salinity was between 26 x 10^{-3} and 28 x 10^{-3} and water temperature ranged from 28° to 32°C.

All experiments were carried out in 1983.

Pond Treatments

The ponds had a peripheral canal about 3 m wide and 1.5 m deep, with a layer of soft sediment. The centre of the ponds was 1 m deep, with a thin (20–30 mm) layer of soft sediment over a heavy clay substrate. Water depth was about 1 m in the centre and was lowered to a depth of about 50 cm at low tide once per day, and then refilled on the next high tide from a river fringed by extensive mangrove forests.

Ponds stocked with prawns. Pond 11 (0.5 ha) was stocked with 6,500 postlaraval *Penaeus merguiensis* 3 months before this study. During this study, it was supplied with pelleted food at a rate of 10 kg d^{-1}. Pond 29 (0.25 ha) was stocked with 26,400 larval *P. monodon* 3 months before this study. During this study, it was supplied with pelleted food at a rate of 16 kg d^{-1}. Pond 23 (0.25 ha) contained a nursery pen (10 m x 20 m), which was stocked with 100,000 larval *P. merguiensis* and supplied with a food mash at a rate of 5 g dry weight $m^{-2} d^{-1}$ initially, and then 8 g $m^{-2} d^{-1}$ a day before the measurements reported here were made.

Effect of chicken manure on ponds. Pond 22 (0.15 ha) was unstocked and had chicken manure added to it for 2 weeks at the rate of 0.6 g dry weight $m^{-2} d^{-1}$. The manure contained 400 mgC g^{-1} and 27 mg organic N g^{-1}. Pond 21 (0.15 ha) was also unstocked and had chicken manure supplied to it at the same rate as pond 22 for 3 weeks. Ponds 21 and 22 were stocked with 30,000 and 43,000 *P. merguiensis* after the studies on chicken manure were completed. At the time the oxygen and primary production measurements were made (see below) a water-stable pellet was being supplied.

Pen experiments with chicken manure. Pond 32 (1.0 ha) contained 3 pens, each 3 m diameter, constructed with plastic mesh (5 mm square holes) in the central area of the pond. Pen A was supplied with chicken manure at a rate of 0.6 g dry weight $m^{-1} d^{-1}$, for 1 week prior to sampling for bacterial biomass, organic C and N determinations and meiofauna numbers. Pen B was treated with chicken manure for 2 weeks and pen C was treated with manure for 3 weeks at the same rate as pen A.

Water Column Measurements

A column of water was removed using a long plastic tube (2.5 cm diameter), which was pushed down to about 5 cm from the bottom, and then it was stoppered and removed. Water was brought back to the laboratory and processed immediately for analysis of nutrients.

Primary production and respiration were measured using the light and dark bottle technique and changes in oxygen concentration were determined using a Winkler titration procedure (Strickland and Parsons, 1968). Water was sampled at 3 depths (surface, midwater (40–60 cm) and within 20 cm of the bottom (80–90 cm); 300 ml glass bottles were incubated *in situ* at each depth for two hours. A simple water sampling method was used (Fig. 1). When the sampler was pushed down to the required depth, water flowed with minimum turbulence into the bottles through the tubing. Air escaped through small holes in the lid. The volume of the plastic container was sufficient to allow about 3 volumes of water to pass through each bottle.

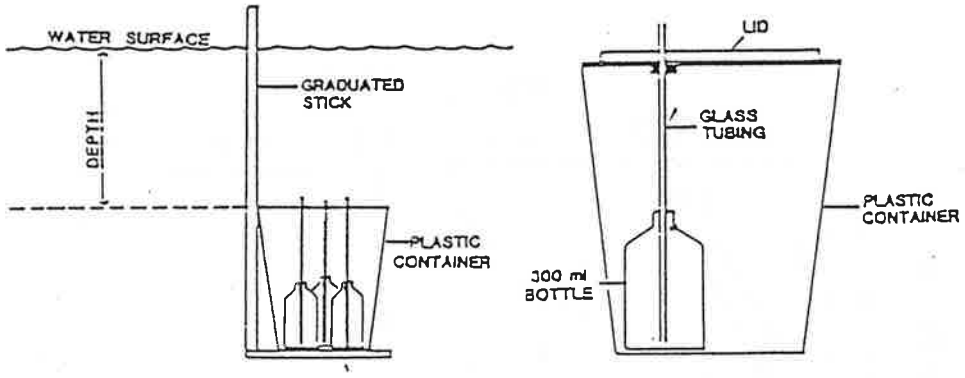

Fig. 1: Diagram of apparatus for collecting water for oxygen and primary production measurements.

Ammonia and phosphate were measured immediately after water was collected and filtered through Whatman GF/F filters, according to procedures in Strickland and Parsons (1968). Nitrate and nitrite were measured about 1 week later in water that had been filtered through Whatman GF/F filters, and preserved with mercuric chloride. Organic C and N were measured with a Perkin Elmer model 240 CHN analyser. Particulate matter in water samples (50 ml) was collected on Whatman GF/F filters.

For meiofauna enumeration 3 cores, each 20 mm diameter and 20 mm deep were taken and combined for each sample. The cores were extruded into beakers containing formalin (3% v/v) and rose bengal. Clay was washed out through a fine sieve (50m) and 30% colloidal silica (Ludox, Dupont) was used to separate the meiofauna from the remaining sediment and detritus (de Jonge and Bouwman, 1977). Two or three treatments with Ludox were used. Very few, or no, animals were found to be left in the sediment when it was checked after the Ludox treatment.

Results

The average depth of the euphotic zone in the ponds was less than 70 cm (Figure 2a). There was considerable variation between ponds in the rate of net primary production at the surface (Figure 2a). Primary production in the ponds increased from dawn until noon and then decreased (Figure 2b).

More carbon was required to support respiration in the total water column than was generated by gross primary production (Table 1). In other words, more oxygen was consumed than was produced by photosynthesis. In terms of oxygen, gross production ranged from 2.6 m.g. O_2 $l^{-1}d^{-1}$ in pond 26 to 5.2 mg O_2 $l^{-1}d^{-1}$ in pond 22 and respiration was 7.6 mg O_2 $l^{-1}d^{-1}$ in pond 26 and 12 mg O_2 $l^{-1}d^{-1}$ in pond 22. At the surface, respiration was less than gross production and thus there was a measurable net production, but in the lower part of the water column, respiration was higher (Table 2). Oxygen concentration was quite low at the bottom of the water column in two on the ponds and could have been detrimental or even lethal to prawns (Table 2).

Polychaetes and copepods were present at only a low density in ponds containing

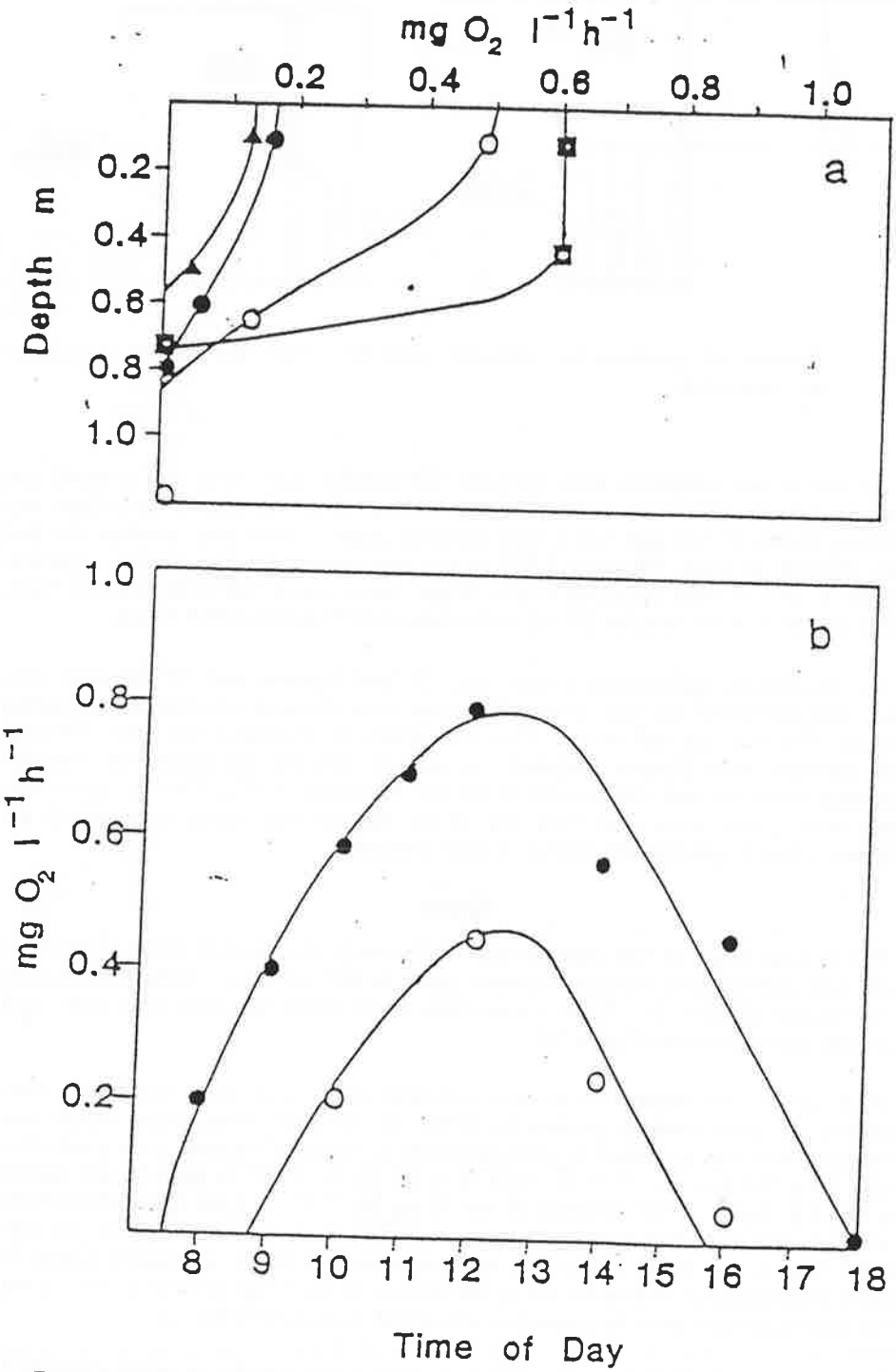

Figure 2. Variations with depth and time of day in net primary production.

(a) Effect of depth on net production in 4 ponds; ● = pond 29; ○ = pond 21; ■ = pond 22; ▲ = pond 26.

(b) diurnal variation in net production in pond 11; ● = production near surface (10 cm); ○ = production at 60 cm.

Table 1

Primary production and respiration in pond water.
All values in g C m^{-2} d^{-1} .

Pond	Date	Gross Primary Production	Total Respiration	Bacterial Respiration[2]
29	16 February	1.20	2.5	1.3
29	24 April	0.81	2.4	
20	24 February	1.40	2.30	
21	26 February	1.53	2.9	0.7
21	24 April	1.28	3.6	
22	23 April	1.56	3.47	
26	26 April	0.77	2.27	

Data from Moriarty (1986) assuming 50% growth efficiency. Only two values were determined on the same occasion as oxygen measurements were made.

Table 2

Oxygen production and consumption in pond water. These measurements were made two months after the studies using chicken manure in ponds 21 and 22; all ponds were stocked with prawns and pelleted food was supplied.

Pond	Depth cm	Gross Primary Production mg O_2 l^{-1} h^{-1}	Respiration mg O_2 l^{-1} h^{-1}	Oxygen Concentration mg O_2 l^{-1}
29	10	0.49	0.34	4.61
	60	0.43	0.39	3.38
	80	0.08	0.20	0.92
22	10	0.95	0.36	7.63
	44	1.1	0.49	6.50
	75	0.28	0.54	1.84
21	10	0.69	0.21	7.17
	65	0.69	0.49	7.63
	120	0.23	0.36	3.17

adult prawns. The numbers of meiofauna increased with respect to the control pond (26) in the ponds that were receiving chicken manure. Nematodes were particularly abundant after 2 weeks, and harpacticoid copepods after 3 weeks of manure treatment (Table 3). Very large numbers of nematodes were found in the sediment of the nursery area in pond 23, although they were patchily distributed. A comparison of meiofauna numbers in various ponds with the meiofauna in a natural mangrove habitat is given in Table 3. Polychaetes, and to a lesser extent harpacticoid copepods, were more common in the mangrove sediment on a creek bank, then in the ponds.

Bacteria were insignificant in the food of the prawns in the pens (Table 4). The C:N ratios of the gut contents were low (5.4—6.3), an indication that protein was a large part of their food, which suggests that animals (meiofauna) were being eaten. These prawns were not fed pellets.

Concentrations of inorganic nitrogen and phosphate were low in the water column, even in ponds that were being fertilised with chicken manure (Table 5). Organic N concentration was higher, particularly in ponds 11, 29, 23 (receiving pelleted food) and ponds 21 and 22 (chicken manure for 2 or 3 weeks). Ponds 26, the control pond, and pond 20, which had been treated with chicken manure for only 1 week, had low concentrations of organic N compared to other ponds or river water. Pond 5, which was not treated in any way, had a high concentration of N, but the water level was low and thus exchange with the sediment would be facilitated.

Table 3

Meiofauna in pond sediments. All values are means for number $10cm^{-2}$; standard errors for 4 samples are shown in parentheses.

Pond	Treatment	Nematodes		Harpacticoid Copepods		Polychaetes		Others	Total
11	pellets, adult prawns	622	(185)	134	(52)	4	(2)	52 (26)	812
29	pellets, adult prawns	132	(45)	8	(8)	0		4 (4)	144
23	nursery[a], mash larvae	1388	(564)	624	(48)	0		184 (96)	2196
20	manure, 1 week	91	(33)	198	(132)	6	(6)	42 (16)	337
22	manure, 2 weeks	892	(251)	624	(157)	0		186 (64)	1702
21	manure, 3 weeks	338	(122)	978	(179)	4	(4)	142 (64)	1462
26	none	28	(7)	412	(158)	0		4 (4)	444
Mangrove, intertidal creek bank		252		516		96		0	864

[a] Mean and range for duplicates only.

Table 4

Analysis of foregut contents of prawns in pens in pond 32.
Values are mg g^{-1} of dry weight.

Pen	Species	Organic C	Organic N	C:N	Bacteria
A	*Penaeus monodon*	177	29	6.1	1.3
	P. merguiensis	185	34	5.4	0.7
B	*P. monodon*	198	35	5.7	1.2
	P. merguiensis	181	28	6.6	0
C	*P. monodon*	223	33	6.8	0.7
	P. merguiensis	143	25	5.6	0

Table 5

Particulate organic C and N in water column retained on Whatman GF/F filters and total inorganic N[a].

Pond	NO_3^- μM	NH_4^+ μM	Organic C mg l^{-1}	or g m^{-2} Organic N	C:N
11	10	< 0.2	4.0	0.58	6.9
29	3	< 0.2	5.5	0.72	7.6
20	3	< 0.2	3.9	0.25	15.6
22	6	< 0.2	4.3	0.64	6.7
21	nd[b]	0.5	3.7	0.44	8.4
26	nd	< 0.2	3.4	0.19	17.9
23 nursery	3	1	5.4	0.80	6.7
23 open water	nd	< 0.2	5.4	0.46	11.7
River	3	< 0.2	4.6	0.58	7.9

[a] NO_2^- was below the level of detection (< 2μM).

[b] nd = not detectable (< 2μM).

Discussion

Primary and Bacterial Production

Bacterial productivity was high in the water column and sediments of these ponds, and it seemed that much of this productivity resulted from the food pellets thrown into the ponds (Moriarty, 1986). One question which arose was how much algal production contributed to the pond carbon budget. It was possible to carry out only two measurements of primary production at the same time as bacterial production was measured. From the measurements made, it seems that primary production is high (Table 1). As half of the water was exchanged each day, only half the production would contribute to pond production over a longer term. Primary production in the river water was not measured. For future work, chlorophyll *a* concentration should be measured or algal cells counted, so that comparisons of primary production can be made on a biomass rather than volumetric basis.

There are problems in determining what proportion of primary production is available for eventual use by bacteria or other organisms. True values for net production are difficult to measure, especially with the oxygen technique. Algal respiration depends on previous light history; also, photorespiration may occur. These points have been discussed by Gant (1974), who reported that there were quite large diurnal fluctuations in respiration rates in a shallow tropical lake. The dark bottle measures total community respiration, which is primarily due to algae and bacteria. An independent estimate of bacterial respiration can be obtained from the thymidine technique for measuring bacterial growth rates, by assuming that if the growth efficiency is 50%, bacterial respiration is equal to production. (Although the estimate of 50% can be used for approximate calculations, it is possible that 30% may be more usual for planktonic bacteria if the organic matter is not readily degradable (Koop *et al.*, 1982).

From the data for the whole water column (Table 1), it is obvious that respiration considerably exceeded photosynthesis and thus there could be no net production in the pond as a whole. For example, in pond 29 on 16 February, bacterial production was 1.3 g C m^{-2} d^{-1} (Moriarty, 1986), and thus bacterial respiration had the same value. Subtracting this from total community respiration, the remaining community respiration was 1.2 g C m^{-2} d^{-1}, which was the same as the gross primary production. Thus after removing the bacterial component, respiration in the whole water column throughout 24 hours would have used up all the gross primary production. The euphotic zone was limited to the upper part of the water column by turbidity and brown colour in the water.

In pond 21 on 26 February, bacterial respiration was 0.76 mg C $m^{-2}d^{-1}$. Subtracting this from total respiration, there is a deficit of about 0.7 g C $m^{-2}d^{-1}$ in respiration which is not made up by gross primary production. As pointed out above, bacterial growth efficiencies may be closer to 30% than 50%, in which case bacterial respiration would be 1.6 g C $m^{-2}d^{-1}$ and net production for the whole water column would be 0.17 g C $m^{-2}d^{-1}$.

The conclusion from this discussion is that in general, there was no overall net production by the algal community itself and primary production did not contribute significantly to other levels of production in ponds where pelleted food was supplied. Bacterial production must be dependent on other sources of organic matter. These sources are the pelleted food supplied to prawns in ponds 11, 29 and 23; chicken manure in ponds 20, 21 and 22; and organic matter in river water in all ponds. A comparison of inputs of organic matter and bacterial requirements for the water column shows some correspondence (Table 6). In ponds 29 and 23, receiving large amounts of pellets or mash,

Table 6

Comparison of organic C input to ponds with organic C required to support bacterial production and respiration.
All values are g C $m^{-2} d^{-1}$.

Pond	Input	Bacterial requirement[a]	
	Feed or Manure	Water	Sediment
11	1.0	1.84	0.8
29	3.2	2.64	1.36
23 (nursery)	4.0	4.2	1.67
21	0.24	1.4	1.07
22	0.24	1.78	1.2

[a] Calculated from production values (Moriarty, 1986), using a growth efficiency of 50% for water column and 30% for sediments.

the bacteria in the water column did not use all carbon supplied, but the bacterial production in the sediments in those two ponds was high. This indicates that most of the organic matter being supplied to these ponds was being utilized directly by bacteria in both the water and sediments.

Some bacterial growth in the sediments may have been at the expense of organic matter already present in the sediment. In ponds being fertilised with manure this is most likely. The manure contained sawdust, which would not be utilized immediately by bacteria. As it seems likely that nitrogen (and perhaps phosphorus) were limiting in the ponds (see below), the organic N and P in the manure would stimulate bacterial decomposition of organic matter in the sediment. Some evidence for this is seen in the experiment with pens in pond 32, in which the C:N increased due to a decrease in organic N content, even though manure was added (Table 7). An explanation for this is that bacteria were decomposing a part of the organic matter with a consequent loss of N, probably due to denitrification. The results suggest that the high levels of organic matter in the pond sediments were due in part to a low rate of bacterial decomposition controlled by low availability of N. Further evidence for a limiting role for N and P is seen in the low concentrations of inorganic N and P in the water column. The high rates of bacterial production and primary production indicate that the nutrients were rapidly recycled and thus control the rates of production. Most nitrogen in the water column was present as organic N. Simpson *et al.* (1983) also found that phosphate concentration was very low; their values for NH_4^+ were higher (10–50 μM) in these ponds.

Table 7

Effect of chicken manure on organic C and N content and bacterial production in sediment (the top 10 mm)

Pond (d)	Treatment	C:N	Bacteria		
			Biomass[c] g C m^{-2}	Production[b] mg C m^{-2} d^{-1}	Doubling time
32	Outside pens[a]	13.1 ± 1.5	3.4 ± 0.0	444 ± 54	6
Pen A	Manure, 1 week	14.0 ± 0.8	4.3 ± 0.20	346 ± 69	8
Pen B	Manure, 2 weeks	18.6 ± 1.6	1.9 ± 0.20	182 ± 39	6
Pen C	Manure, 3 weeks	20.7 ± 0.8	0.8 ± 0.07	230 ± 57	2

[a] The experiment was carried out in 3 pens in Pond 32 (see Table 3).
All values are mean ± standard error, n = 4, except as noted (from Moriarty, 1986).

[b] Values are mean ± S.E., n = 3.

[c] Values are mean ± range of duplicate determinations.

A build-up of organic matter in the pond sediments is generally regarded as undesirable because it promotes anaerobic processes, particularly sulfate reduction. Sulfate reduction is the chief terminal process of carbon mineralization in marine systems, including brackish water ponds, because sulfate is present at high concentrations. Its end product, H_2S, is toxic. In these ponds, iron pyrite also forms, and there are considerable problems with water quality due to acid production during oxidation of iron pyrite (Simpson *et al.*, 1983). Anaerobic conditions keep iron pyrite in the reduced state and thus lessen problems due to low pH and alkalinity. From this point of view, maintenance of anaerobic conditions in the pond sediment may be beneficial where acid-sulfate soils are a problem. Prawns, however, cannot tolerate low oxygen concentrations and it may be that some of the instances of mortality in these ponds were due to lack of oxygen and not low pH.

Meiofauna as Food for the Prawns

The feed pellets were primarily the base of a microbial food chain, rather than being used as food for the prawns. Bacteria comprised a minor part of the prawns' diet (Table 4). Under the microscope fragments of animals, benthic microalgae and unrecognizable material were seen in the foreguts. The productivity studies were not refined enough to show whether a small proportion of the feed pellets was being used directly. Studies with labelled food (e.g. by using a dye or polystyrene beads) would be needed to show this.

Meiofauna as well as small macrofauna are eaten by penaeid prawns (Hall, 1962; Chong and Sasekumar, 1981; Moriarty and Barclay, 1981). The work reported here demonstrates that the meiofauna is a link between bacteria and the prawns in these ponds. In the pen experiments, the number of meiofaunal animals decreased after the prawns were added (Moriarty, 1986; see Table 8). Polychaetes and copepods were particularly preferred, as almost all of them were eaten. A few nematodes remained (Table 8). There were marked differences in the meiofauna community structures in the various ponds. These differences were probably due mainly to different intensities of predation. Bell and Coull (1978) showed that *Palaemonetes pugio* fed on meiofauna, causing a marked reduction in numbers in a salt marsh habitat. Polychaetes were almost absent from ponds 11 and 29 where prawns were stocked; a small population of wild prawns was present in other ponds and presumably was preying on polychaetes. As polychaetes are larger than nematodes and copepods, the prawns would gain more

Table 8

Effect of manure treatment and prawn predation on meiofauna in Pond 32[a].

Values are number 10 cm^{-2}, 2 cm depth.

Pen	Date	Sample	Nematodes	Harpacticoid Copepods	Polychaetes	Others	Total
A	6 Feb[a]	1	104	40	112	136	392
		2	96	24	16	64	200
A	19 Feb	1	32	0	0	8	40
		2	40	0	0	0	40
B	6 Feb	1	200	480	256	320	1256
		2	328	560	520	464	1872
B	19 Feb	1	48	8	0	8	64
		2	48	8	0	8	64
C	6 Feb	1	160	640	440	208	1448
		2	368	652	304	200	1524
C	19 Feb	1	10	0	0	0	16
		2	0	0	0	16	16

[a] Pen A: manure treatment for 1 week prior to 6 Feb;
Pen B: manure treatment for 2 weeks prior to 6 Feb;
Pen C: manure treatment for 3 weeks prior to 6 Feb.
Prawns were stocked on 6 Feb.
Data from Moriarty (1986).

energy per unit effort expended by feeding on polychaetes. When small areas of pond 32 were enclosed in pens, and prawns excluded, the number of polychaetes quickly increased (Table 8). Harpacticoid copepods and nematodes were much less numerous in ponds 11 and 29 than in pond 23 or the ponds receiving manure. Bacterial productivity was high in ponds 11 and 29, and thus food was not scarce for the meiofauna. We conclude, therefore, that the prawns in the ponds were feeding on the meiofauna. It is probable that only a small proportion of their food consisted of meiofauna. The biomass and production of meiofauna in ponds containing prawns was low (see below: Tables 3, 9), and was not sufficient to support prawn production. The only small macrofauna observed were molluscs about 10–20 mm in size. Obviously, the prawns must have been eating some of the pelleted food also.

Table 9

Comparison of biomass and production of bacteria and meiofauna in some ponds.

	Pond				
	11	29	23 (nursery)	21	22
Biomass (mg C m^{-2})					
Bacteria[a]	2700	2600	6400	1800	2500
Nematodes[b]	46	10	104	25	67
Copepods[b]	6	0.4	28	44	28
Production (mg C $m^{-2} d^{-1}$)					
Bacteria[a]	240	410	500	320	360
Nematodes	7	1	16	4	10
Copepods	1	0	4	6	4

a From Moriarty (1986).

b Based on carbon content of 0.75 μgC nematode^{-1} and 0.45 μgC copepod^{-1} (unpublished work).

An estimate of the food requirements of the prawns may be obtained from the yield at harvest (Table 10). If we assume that the prawns utilized the food eaten with a 10% efficiency, which is within the range measured for *P. merguiensis* in culture (Sedgwick, 1977), the estimated feeding rate is 10 times the growth rate. Thus in pond 11, the prawns ate about 150 mg C $m^{-2} d^{-1}$, which is 15% of the pelleted food, but more than the meiofaunal production and less than the bacterial production. In pond 29, the prawns required an amount of carbon equivalent to about 7% of the pelleted food. It is not surprising, therefore, that bacterial production did account for most of the added food. Severe mortality of prawns sometimes occurred, due to acidity from the acid-sulfate soils on which these ponds are constructed and probably also to low oxygen concentration. It was difficult, therefore, to balance feeding levels with stocking rates, as mortality was difficult to measure before harvest time.

Table 10

Prawn production.

Pond	Area ha.	Number Harvested	Stocking size Total Length mm	Harvest size Total Length mm	Growth Rate mg C $m^{-2}d^{-1}$	Feeding Rate mg C $m^{-2}d^{-1}$
Penaeus merguiensis						
11	0.5	6120	20 – 25	120	15	150
Penaeus monodon						
29	0.25	6000	70	136	23	230

Relationships between Bacteria and Meiofauna

Interactions between manure additions, bacteria and meiofauna are best seen in the pen experiments, where prawns were excluded. One week after adding manure, bacterial biomass had increased (Table 9). Bacterial biomass decreased twofold after two weeks of manure treatment and was 5 times lower after 3 weeks. The marked decline in bacterial biomass occurred at the same time as a marked increase in meiofauna (Table 8). Bacterial growth rates were stimulated, presumably in response to grazing by the larger number of meiofauna. Protozoa were not examined in this study, but their role should be looked at in future work, as they also control bacterial populations. The greatest density of nematodes was found in the nursery area of pond 23, which also had the highest bacterial biomass. Thus, the meiofauna density appears to be directly correlated to bacterial biomass. If a readily degradable carbon source (e.g. straw) had been added with the manure, bacterial and meiofaunal biomass and productivities would have been much higher, and thus supported more prawns.

From the changes in number of meiofauna in the pens and ponds 20, 21 and 22 with time after manure addition, it is possible to estimate growth rates of meiofauna.

The relative or specific growth rate (r) was calculated using the following equation:

$$r = 1/t \ln (N_t/N_o)$$

where t is time (days); N_t is the final number and N_o the orginal number of animals. Growth rates were found to range from 0.06 to 0.19 d^{-1} for nematodes; from 0.10 to 0.17 d^{-1} for harpacticoid copepods; and from 0.07 to 0.20 d^{-1} for polychaetes. These values are only approximate. The number of samples taken was small, and patchiness in distribution was pronounced. True generation times may be slower than indicated by these values, because large numbers of eggs or larvae may already have been present. Thus these growth rates may reflect only the growth to adult stages, and not include full life cycles. These growth rates are consistent with rates reported by others. Growth rates of 0.06 to 0.14 d^{-1} have been measured for nematodes in culture (Alongi and Tietjen, 1980). Values ranging from 0.005 to 0.24 have been reported for harpacticoid copepods (Hicks & Coull, 1983).

Both the biomass and production of meiofauna were low compared to bacteria in the pond sediments (Table 9). In the ponds 11 and 29, which were stocked with prawns, meiofaunal biomass was 0.4 to 2% of bacterial biomass, and bacterial production was

higher than the estimated grazing rate by meiofauna (Table 11). Where the meiofaunal density was not limited by predation, i.e. in the pens (pond 32), the estimated requirement of carbon for production, respiration and ingestion is similar to the measured value for bacterial production (Moriarty, 1986, see Table 11). Thus bacterial population density would be expected to decline under such grazing pressure, and this did happen (Table 9). In the stocked ponds, where meiofauna density was limited by the prawns, bacterial density and production was higher than in the pens.

Table 11

Approximate carbon budget for meiofauna production in some ponds. Details of the factors used are shown for Pen 2. Only the results for estimated amount of carbon consumed by meiofauna are given for ponds 11 and 29.

	Nematodes	Copepods	Polychaetes	Others
Specific growth rate (d^{-1})	0.14	0.13	0.14	0.17
Biomass[a] ($\mu gC\ animal^{-1}$)	0.75	0.45	1.83	2.3
Respiration (30M°C) ($\mu gC\ animal^{-1} d^{-1}$)	0.04[b]	0.03[c]		
Pen 2				
Production ($mg\ C\ m^{-2} d^{-1}$)	3.0	3.2	10.8	16.5
Respiration ($mg\ C\ m^{-2} d^{-1}$)	1.1	3		
Assimilation efficiency	15%[b]	50%[d]		
Growth efficiency[e]			20%	20%
Consumption ($mg\ C\ m^{-2} d^{-1}$)	28	13	54	82
Pond 11 Consumption ($mg\ C\ m^{-2} d^{-1}$)	64	3	0	9
Pond 29 Consumption ($mg\ C\ m^{-2} d^{-1}$)	14	0.2	0	0

a Biomass: average values from unpublished work.

b From Duncan et al (1974), assuming $Q_{10} = 2$.

c From Coull & Vernberg (1970), value calculated from data for small copepods, assuming $\mu gC = \mu l\ O_2 \times 0.45$.

d A round figure from literature (Dall & Moriarty, 1983).

e Values are assumed .

Acknowledgements

We thank Mr Ti Teow Loon, Head of the Research Station, for his interest in the work. We are grateful to Mr Ismail bin Abu Hassan and Miss Chu Si Chiam for their assistance and to Mr Paul I. Boon, Griffith University, Queensland, for the nitrate and nitrite analyses. The involvement of D.J.W. Moriarty was funded by the United Nations Food and Agriculture Organisation through a contract with CSIRO.

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ICLARM
INTERNATIONAL CENTER FOR LIVING AQUATIC RESOURCES MANAGEMENT
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Printed in Manila, Philippines

Published by the International Center for Living Aquatic Resources Management, MC P.O. Box 1501, Makati, Metro Manila, Philippines with financial assistance from the Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ), GmbH, Federal Republic of Germany.

Moriarty, D.J.W. and R.S.V. Pullin, Editors. 1987. Detritus and microbial ecology in aquaculture. ICLARM Conference Proceedings 14, 420 p. International Center for Living Aquatic Resources Management, Manila, Philippines.

ISSN 0115-4389
ISBN 971-1022-29-X

Cover: Decomposing plant detritus, with associated colonies of filamentous bacteria and a fungal hypha (red). Photomicrograph by D.J.W. Moriarty.

ICLARM Contribution No. 387.

Microbial Ecology in Aquaculture

CHAIRMAN'S OVERVIEW

D.J.W. Moriarty

Extensive or semi-intensive aquaculture systems depend on microbial food webs of which there are two principal types: those dependent on microalgae (i.e., directly utilizing primary production) and those dependent on detritus (dead organic matter). The detritus is converted by bacteria and other microorganisms into nutritionally useful organic matter. In this conference we will be discussing chiefly the second food web, and the biogeochemical processes affected by it. This topic cannot be completely separated from that dealing with the generation of primary production and its utilization by animals. Many animals feed on both algae and detritus with its associated microorganisms. Furthermore, inorganic nutrients released from decomposing detritus stimulate primary production and so we will also discuss aspects of primary production in ponds.

One question that we need to consider is whether aquaculture systems that depend solely on detritus added to ponds from an external source (allochthonous systems) are more productive than those dependent on *in situ* primary production that is eaten either directly or via a detrital food chain (autochthonous systems). To answer this question, we will need to consider the biochemical composition of detritus; whether some or any of the detritus can be digested by animals or by bacteria; whether inorganic nutrients are generated from the detritus or need to be added to enhance decomposition; the types, biomass and productivity of microbes and the efficiencies of energy transfer through the food web; and the effect of detritus decomposition on water quality, especially oxygen concentration.

Considerable advances have been made in microbial ecology recently, particularly in the study of microbial activities in biogeochemical processes. The activities of bacteria in aquatic carbon and nitrogen cycles can now be quantified and this has a direct application to aquaculture. Until about 10 years ago, the role of bacteria in the ocean had been neglected by many marine biologists because bacteria were not considered to have an important function in food chains. New techniques for determining biomass and production have shown that bacteria are more numerous and grow much faster than was thought to be the case and thus do play a very important part in aquatic food chains and nutrient cycles. Thus, methodology is

important in the analysis of the role of microbes and is the subject of my review in this session.

The methods that are used must be checked critically to ensure that results obtained are indeed accurate. Some of the methods used or developed recently are discussed in this session. These are the best that are available at present for determining biomass and growth rates of microorganisms, but they are not perfect and all results need to be interpreted with caution. Improvements and new procedures are being published frequently in the aquatic and microbial ecology literature. Thus, workers new to this field will need adequate access to this literature. Some of the new techniques (e.g., lipid analysis) require expensive instruments and the assistance of trained chemists. Such methods will not be widely used, but if they were applied in aquaculture, problems could be tackled that would otherwise be difficult to study. For example, studies on the composition of detritus are needed to determine what proportions of organic matter in detritus of a given origin (e.g., phytoplankton or straw) is detritus and what proportion is the biomass of microorganisms. Furthermore, how these proportions change as detritus ages and successions of microbes occur, leading to different community structures, could also be studied. The nature and composition of detritus can be very varied and is often unknown and difficult to analyze. In particular, methods are needed to determine the amounts of nutritionally valuable compounds, e.g., protein, that are present as detritus rather than in microorganisms attached to the detritus. Accurate analyses of microbial biomass are necessary for such studies.

The production of bacteria can now be estimated with reasonable accuracy in aquatic systems, including sediment and detritus. This is particularly relevant to the theme of this conference because bacteria have a central role in the detrital carbon cycle, converting detritus in aquatic systems into useable energy, protein and vitamins for animals. A problem to be resolved in studying bacterial functions in the carbon cycle is the growth efficiency of bacteria. Growth efficiencies can be very variable, and are difficult to determine in natural systems such as the open ocean. Aquaculture ponds, where inputs and outputs are controlled and measurable, would be useful semi-natural model systems to complement laboratory microcosm studies on this problem. If the growth efficiency of bacteria can be measured or predicted for given conditions (e.g., detritus composition), then accurate estimates can be made of bacterial respiration in the presence of algae. Better estimates of net primary production, which are critical to productivity studies in aquaculture, can then be made.

Processes such as the turnover of carbon and nitrogen can be studied on a broad scale with data on the biomass and growth rates of the bacterial community as a whole. Dr. Anderson will be discussing utilization of detritus from this viewpoint. If more detailed analyses of the role of bacteria in different processes are to be made, then information on the principal types or species of bacteria and their ecophysiology is needed. Dr. Fry will discuss the role of particular bacteria in the main elemental cycles. From knowledge of environmental factors affecting bacterial species distribution and activity, it should be feasible to determine the best conditions for establish-

ing detrital food chains in aquaculture ponds. In the terrestrial sphere, fungi are the principal decomposers of detritus and it has been shown that a succession of species is involved as the decomposition proceeds. Bacteria are more important as decomposers than fungi in aquatic systems and as Dr. Fry points out, successional changes also occur, but these have not been studied at the species level. Seasonal changes in activity, and presumably species composition, of bacteria do occur. Bacterial activity varies during diel periods in some environments. Whether these changes in activity reflect changes in species composition also is not known, but it is possible if protozoan grazing activity is intense and bacterial doubling times are fast.

Anaerobic processes, discussed by Dr. Blackburn, occur in pond sediments and are significant in the decomposition of detritus. The initial decomposition of particulate material in sediment is carried out by fermentative bacteria, but little detailed information is available on this part of the process or the bacteria involved. As it is likely that much added detritus will settle to the pond bottom and be decomposed in an anaerobic environment, more research in this area would be valuable.

The second stage of anaerobic decomposition wherein small molecules are fermented, and various electron acceptors are used as electron sinks, is well studied. The flux of nutrients, oxygen and toxic products of anaerobic processes between sediment and water needs to be studied. As Dr. Blackburn points out, this is not a simple procedure, but requires detailed studies on the rates of the many interacting processes.

The activity of bacteria on particulate detritus in the water column is the subject of Dr. Kirchman's review. In contrast to sediments, most if not all, processes are aerobic. The activity of bacteria on particles is not easy to study and distinguish from that of bacteria free in the water column. In fact, some unattached bacteria may be loosely associated with particles, but separated by the filtration techniques that are used to study them. Dr. Kirchman argues that in the sea free bacteria have a more important role in the carbon cycle than attached bacteria. In ponds with much detritus that is kept in suspension by mixing or aerating equipment, bacterial activity on particles can be very intense. Dr. Schroeder, in a later session, will discuss the importance of bacteria in pond processes.

Decomposer pathways are important in aquatic food webs, as Dr. Anderson points out. Decomposition rates and production of organic matter that is useful or necessary for aquaculture depend on the nature of the detritus, microbes and environment. Through a detailed understanding of the various factors, we can formulate hypotheses concerning pond dynamics and suggest practical approaches for maximizing pond production.

Methodology for Determining Biomass and Productivity of Microorganisms in Detrital Food Webs

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Moriarty, D.J.W. 1987. Methodology for determining biomass and productivity of microorganisms in detrital food webs, p. 4-31. *In* D.J.W. Moriarty and R.S.V. Pullin (eds.) Detritus and microbial ecology in aquaculture. ICLARM Conference Proceedings 14, 420 p. International Center for Living Aquatic Resources Management, Manila, Philippines.

Abstract

Bacteria and algae in water, detritus and sediments can be counted most easily with an epifluorescent microscope after they are stained with acridine orange. Cell volume can be determined by microscopy, but may be subject to considerable error. Biomass can then be calculated using conversion factors for carbon content. Alternatively, the biomass can be determined by chemical methods, such as muramic acid and lipid analyses. Bacterial productivity can be determined from the rate at which tritiated thymidine is incorporated into DNA. Productivity values for bacteria are essential in studies on their role in food chains, because they can double their biomass in a few hours; values for biomass alone are not sufficient. More research is needed to determine the efficiencies with which various bacteria convert organic matter into biomass; values of 25% to 50% are commonly reported. If growth efficiencies are known, the rate at which oxygen is removed from the water by bacteria can be estimated. Bacterial respiration can be substantial in detrital systems. The transfer efficiencies of organic matter via bacteria to higher trophic levels are discussed.

Introduction

Bacteria decompose organic compounds that cannot be digested by animals and thereby increase the nutritive value of the organic matter. This has led many people to suggest that bacteria must play an important role in aquatic detrital food webs (for reviews see Mann 1972; Fenchel and Jørgensen 1977), but quantitative studies have been hampered until recently by lack of adequate methods to measure the biomass, growth rates and production of bacteria; such methods are now available (van Es and Meyer-Reil 1983). This paper reviews the appropriate methods for

analysis of detrital food chain dynamics in aquaculture ponds. The methods discussed are mainly applicable to heterotrophic bacteria, which may be considered as a single trophic group utilizing the primary production that has entered the detritus pool. In fact, because there are many different functional groups of bacteria, there are many trophic pathways within the bacterial component of the food web. For simplicity, all heterotrophic bacteria shall be considered as one group linking detritus with higher trophic levels.

Fungi are also decomposers of plant material, and certainly play a part in aquatic systems, especially where C:N ratios are high and complex structural compounds in higher plant leaves are degraded (Barlocher and Kendrick 1974; Suberkropp and Klug 1976). However, the methods for studying their biomass and growth in natural systems are less well developed than are those for bacteria. Fortunately, bacterial decomposition seems in general to be more important than fungal decomposition in aquatic systems (in contrast to terrestrial systems), and thus the poorer methodology is not a real drawback to studying detrital food chains.

Methods for determining primary production will be discussed briefly. Although this conference is concerned primarily with food chains based on detritus, such food webs cannot be separated entirely from those dependent on algal production in aquaculture ponds. Decomposition of detritus having a moderate to low C:N ratio will lead to mineralization of nitrogen and phosphorus and thus promote algal production in ponds. In fact, concomitant algal production may be necessary in extensive or semi-intensive aquaculture systems that make use of detrital food chains. Algae may not only improve productivity and ecological efficiency via herbivores in ponds, but also raise oxygen concentrations and perhaps supply essential dietary components that are not available from bacteria or detritus (e.g., linoleic acid).

The productivity of the cultured animals in ponds largely depends on the efficiency with which detritus and primary production are converted into biomass by the various organisms in the food chain or food web. For detrital systems that depend on bacterial decomposition, the efficiency with which heterotrophic bacteria convert their organic nutrient supply into bacterial biomass (i.e., their growth efficiency) is an important factor that may control productivity. Growth efficiencies of bacteria seem to vary widely in aquatic systems, but are difficult to measure. Growth efficiencies may be studied more easily in aquaculture ponds, where input and outputs of organic matter can be measured. This will be discussed here as it is relevant to measurements of production in ponds and to estimates of bacterial respiration, which can be so intense in ponds with large amounts of detritus that the resulting low oxygen concentrations may limit productivity.

Biomass of Microorganisms and Composition of Detritus

Bacteria

Two different strategies are available for determining bacterial biomass: 1. bacteria can be counted and their average volume estimated by microscopy; 2. a biochemical constituent that is correlated with biomass may be analyzed.

Direct counting of bacteria stained with a fluorescent dye is a quick and accurate method for enumerating bacteria in the water column or sediments (Francisco et al. 1973; Zimmerman and Meyer-Reil 1974; Hobbie et al. 1977). The dyed bacteria are clearly visible against the black background of the filter surface (Plate 1). The bacteria are also easily distinguished when they are enveloped in detritus particles (Plate 2). In my experience, acridine orange gives particularly good contrast between bacteria and detritus. Contrast is best and the image fades only slightly if the microscope is equipped for epifluorescence with a narrow-band blue excitation filter assembly (e.g., as for fluorescein isothiocyanate; Moriarty 1980). If much detritus is present, fading may be rapid and contrast poor. The concentration of acridine orange should be increased if this happens (2-5 μg/ml is usually adequate; the stained filter should not be washed). Other dyes that are supposedly more specific for DNA have been recommended for use with detritus (Colman 1980; Porter and Feig 1980; Paul 1982). All of these dyes do fluoresce with some plant compounds and I have found that bacteria in detritus are more difficult to count with them than with acridine orange.

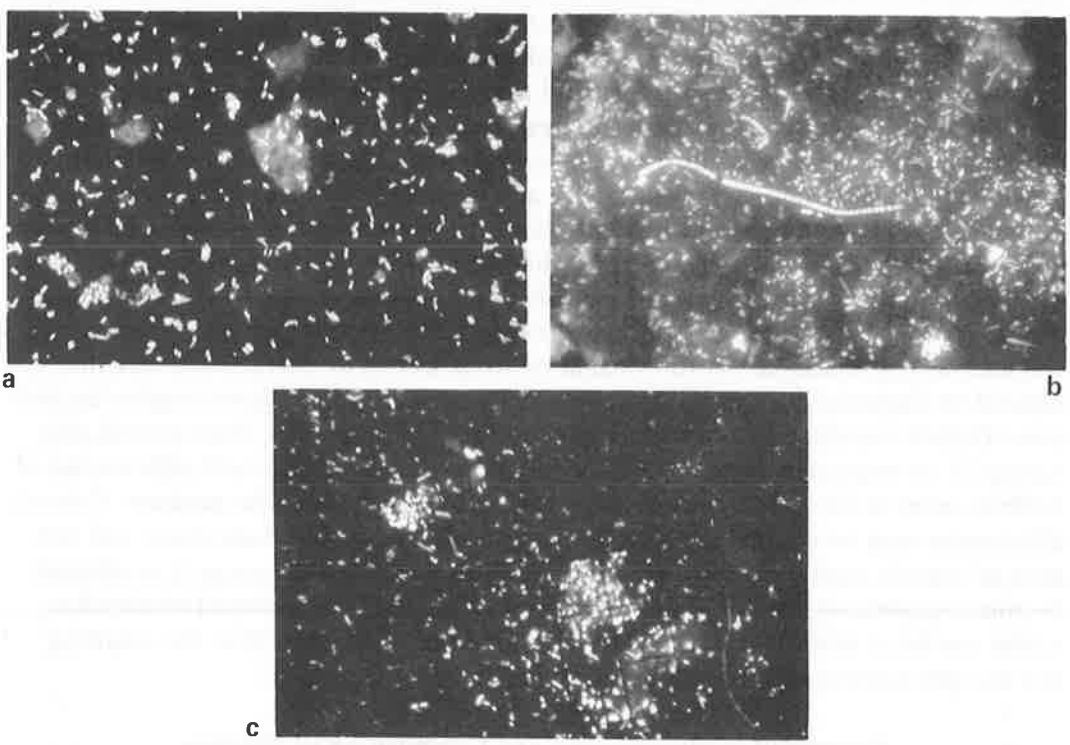

Plate 1. Bacteria from the water column stained with acridine orange and viewed with epifluorescence microscopy. a: Bacteria from water containing algal detritus. b: Bacteria from water with large amounts of algal and seagrass detritus; note the large size and different morphologies of the cells. c: Bacteria from an aquaculture pond, with particles containing attached bacteria. Scale: 1 cm = 8.6 μm.

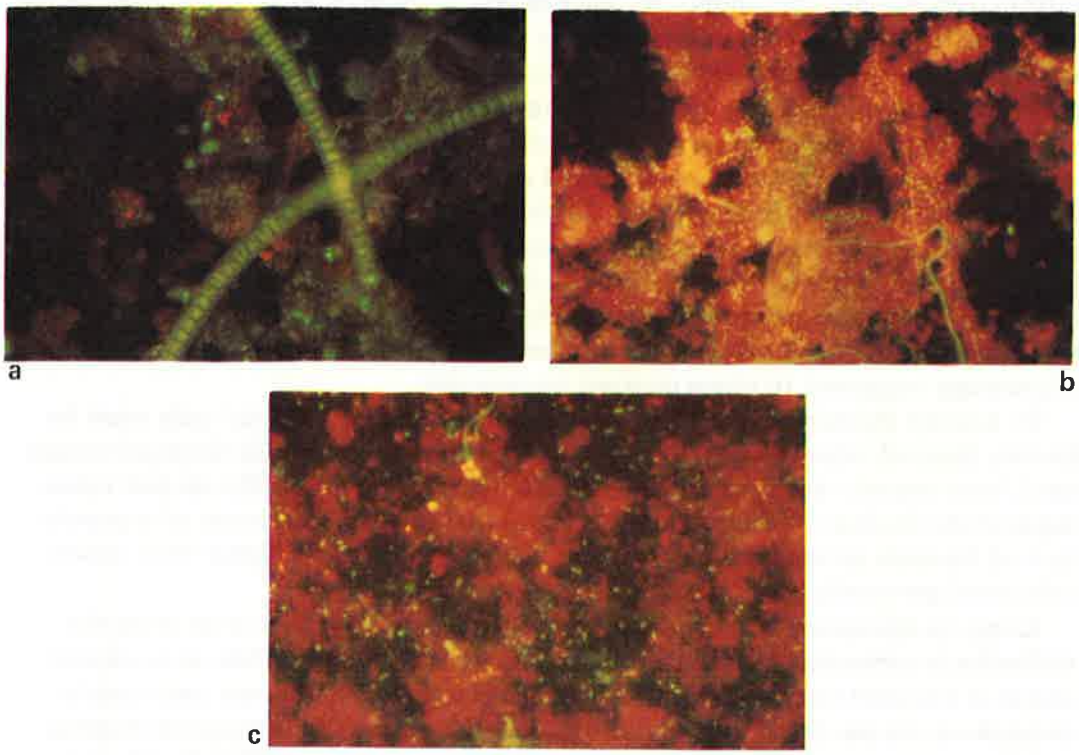

Plate 2. Microbes associated with detritus in sediments, stained with acridine orange. a: Heterotrophic bacteria (green) enveloped in slime (red) and two filaments of a cyanobacterium. Scale: 1 cm = 17.1 μm. b: Bacteria showing many different morphological forms, together with a diatom, enveloped in slime. Scale: 1 cm = 8.6 μm. c: Bacteria (green) with detritus (red) in the stomach of a tilapia from an aquaculture pond.

There are problems in counting bacteria that are patchily distributed in detritus. Particles with attached bacteria are common in aquaculture ponds and if the particles are small, bacteria can be counted (an example is shown in Plate 1c), but if the particles are large or dense counting is difficult.

Once the number of bacteria is known, their average volume must be calculated to determine the biomass. Cell volumes can be calculated from measurements of cell dimensions made under the microscope, or on negatives of photographs projected onto calibrated graph paper or by image analysis with a video monitor and computer. If an image analysis system is available, volumes can be very quickly and easily determined (Fry and Davies 1985). The other methods are, unfortunately, very tedious. Note that very small errors in measuring cell dimensions cause large errors in calculated volumes.

Although electron microscopy gives more precise values for dimensions, Fuhrman (1981) recommends epifluorescence microscopy as being more accurate, because the shrinkage of bacterial cells during fixation is less variable. Some shrinkage may occur, however, if bacteria are fixed with formaldehyde for epifluorescence microscopy and thus volumes may be underestimated. Volumes may also be underestimated with epifluorescence microscopy because acridine orange stains nucleic acids with much greater intensity than other cell constituents. Thus, the cell wall may not be easily distinguished from detritus. In the sea, many bacteria are small, which makes it difficult to measure their size accurately. In aquaculture ponds, however, they are generally larger (Plate 1). Other factors that may cause bacterial cell volumes to be underestimated have been discussed by Bratbak (1985). Bakken (1985) found that the volumes of soil bacteria were underestimated by 25% with epifluorescence microscopy compared to phase contrast microscopy.

To convert volumes to biomass, the carbon content of bacterial cells must be known. Revised values for carbon content, which are about double those previously used, have recently been published (Bratbak and Dundas 1984). The revised values support the finding of Robinson et al. (1982) that the carbon content of a population of bacteria growing on detritus was actually three times higher than values calculated previously.

Errors in estimating biomass from microscopical observations arise from the difficulty in measuring cell size and carbon content accurately. Thus accurate estimates of bacterial biomass are difficult to achieve, which affects not only conclusions about the nutritive value of detritus, but also values for bacterial production and thus ecosystem dynamics. For trophic studies, a further problem is estimating the importance of capsular and slime material excreted by bacteria (Paerl 1974; 1978). The products of bacterial cells are not included in biomass estimates, but may be significant as food for animals (Paerl 1974; Moriarty and Hayward 1982). Many bacteria in sediments or detritus produce large amounts of slime (Plate 3).

Chemical methods for determining bacterial biomass have been sought, because microscopical methods are tedious and subject to many errors. Of the various biochemical constituents of bacterial cells that have been proposed or used in the estimation of biomass, muramic acid is the most useful. An approximate value for the proportions of Gram-negative and Gram-positive bacteria is needed for calculating biomass from muramic acid concentration (Moriarty 1980; Moriarty and Hayward 1982). Good correlations were observed between numbers of bacteria, counted with acridine orange, and muramic acid content of surface marine sediments (Moriarty 1980). A close correlation between muramic acid concentration and direct counts was found for bacteria in the water column of an aquaculture pond (Moriarty 1986b). Muramic acid is not useful if cyanobacteria (blue-green algae) are abundant because they also contain muramic acid.

Earlier colorimetric or biochemical methods for determining muramic acid have been replaced by simple procedures using high performance liquid chromatography (HPLC) (Mimura and Delmas 1983; Moriarty 1983). Although the equipment needed for HPLC is expensive, it is now so widely used in biochemical and analytical

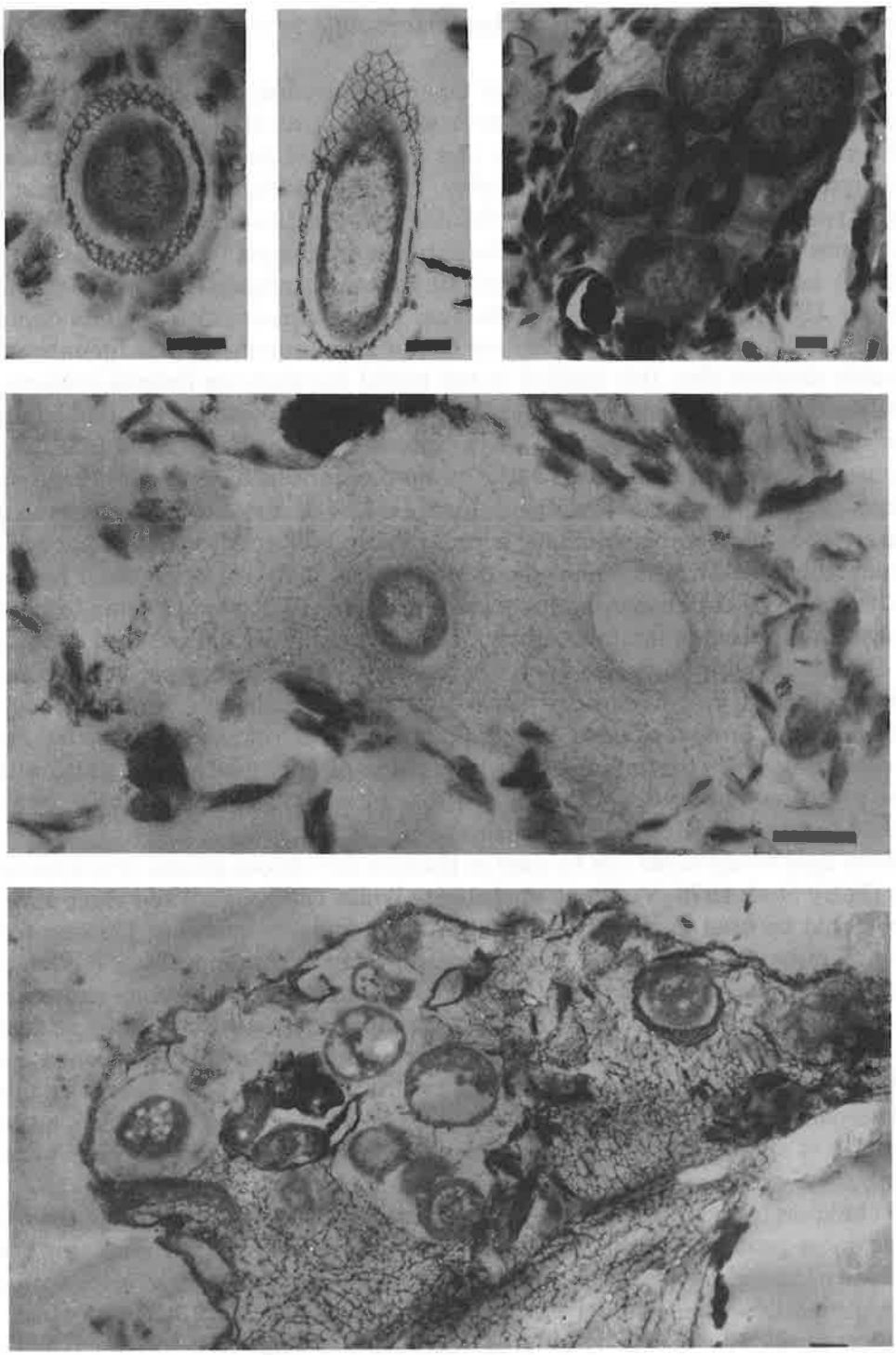

Plate 3. Electron micrographs showing single bacteria and colonies of bacteria surrounded by slime layers (glycocalyx) and mineral particles in sediment. Scale bars are 0.2 μm.

chemistry laboratories, that it is probably accessible to many researchers in the aquaculture field.

Lipopolysaccharides, components of Gram-negative bacterial cell walls, have been used to estimate biomass of bacteria in seawater (Watson et al. 1977). There are problems in calibrating the technique and these would be compounded where lipopolysaccharides are bound to detritus. Lipopolysaccharides do not persist for long in sediments once bacteria die (Saddler and Wardlaw 1980). The analytical procedures and interpretation of results in terms of biomass for bacteria bound in sediment and detritus are more complicated than for muramic acid (Saddler and Wardlaw 1980; Parker et al. 1982). Because only Gram-negative bacteria contain lipopolysaccharides, and Gram-positive bacteria are common in sediments and probably detritus also, this method is not useful for studying detrital systems.

Adenosine triphosphate (ATP) has been widely used to estimate microbial biomass since 1966 when the method was proposed by Holm-Hansen and Booth. But because all organisms contain ATP, the method cannot be used to distinguish the various groups of microbes in detritus. Furthermore, it may not be useful even as a measure of total microbial biomass, because firstly ATP concentrations per cell vary considerably with environmental conditions and secondly large organisms have so much more ATP than bacteria, that most measurable ATP would be due to them. Aquaculture systems contain a complex array of organisms and we need to study the different types of organisms and the processes in which they participate. Thus ATP is not useful as a measure of "microbial" biomass, when "microbial" could mean bacteria, Protozoa, algae, fungi, meiofauna, macrofauna, zooplankton, etc. We need to be able to determine the composition of microbial communities with more precision and accuracy than is possible with ATP. Thus it is not a useful method for studying detrital food chains.

Many lipid compounds can be used as markers for various groups of microorganisms (Perry et al. 1979; Volkman et al. 1980; White 1983; Gillan and Hogg 1984). They could be used in aquaculture studies to provide estimates of biomass for bacteria, microalgae and other microbes from fatty acid composition (Gillan and Hogg 1984). The analytical procedures are complex, time-consuming and require expensive equipment. If such facilities are available, however, some of these analyses may prove to be very useful, particularly if simpler procedures are developed. For example, the growth rates of bacteria and microalgae in sediments have been measured separately with a simplified measure of phospholipid and sulfolipid synthesis (Moriarty et al. 1985b). Similarly, the proportion of anaerobic fermenting bacteria on seagrass detritus in sediments has been measured (Moriarty et al. 1985a).

Techniques for counting numbers of bacteria that depend on culturing them (agar plates, most probable number dilution series) cannot be used for obtaining the numbers of the whole bacterial community. One of many sources of error with such techniques arises when the detritus contains an aggregation of bacteria, which produces results that are likely to be several orders of magnitude too low (van Es and Meyer-Reil 1983). Culture techniques may be useful if information is needed about a particular population of bacteria.

The direct microscopical and biochemical techniques discussed above do not distinguish dormant or dead bacteria from living or metabolically active bacteria. For trophic studies this is not important, but for studies on pond ecosystem dynamics it may be necessary to know how many bacteria are respiring or growing. There is no simple or accurate technique for doing this, even for free-living bacteria in the water column, which are the easiest to study. Respiring bacteria can be detected microscopically with a tetrazolium dye that is reduced to an insoluble formazan and deposited in the cell (Zimmerman et al. 1978). This technique does not work well with sediments, where nonbiological reduction occurs, nor does it work well with bacteria in detrital aggregates if the bacteria cannot be dispersed and separated from the aggregates (pers. obs.).

Many workers have combined autoradiography with microscopy, particularly fluorescence microscopy, to study bacteria that actively take up radioactive organic compounds (van Es and Meyer-Reil 1983). Because not all active bacteria may take up a particular organic compound at equal rates, there may be problems in interpreting the results. The techniques work reasonably well for water column bacteria although there are problems (e.g., see Fuhrman and Azam 1982). In sediments or detritus, however, the bacteria have to be well dispersed and separated from aggregates. Microautoradiography with tritiated thymidine and transmission electron microscopy could be an informative method for determining the proportion of growing bacteria in detritus.

Actively-growing bacteria in water were detected by incubating water samples with yeast extract and naladixic acid which inhibits cell division (Kogure et al. 1979). The elongated forms of bacteria were easily recognizable under the microscope.

Fungi

The fungal biomass in detritus cannot be accurately determined with currently available techniques. Direct microscopy techniques, which are probably the simplest, are discussed by Newell and Hicks (1982). Two biochemical compounds have been used: ergosterol (Seitz et al. 1979; Lee et al. 1980) and glucosamine (Ride and Drysdale 1972). As glucosamine is present in many organisms, including bacteria, it is not useful unless fungi are present in high proportions. Glucosamine can be determined by HPLC, together with muramic acid (Moriarty 1983).

Protozoa

No useful chemical methods for determining protozoan biomass in a mixed assemblage of microorganisms are available. Fluorescent dyes have been used to count flagellates in the water column (Fenchel 1982; Sherr and Sherr 1983), but fragile organisms may break apart during fixation. In sediments and detritus, it is best to extract and count flagellates and ciliates alive as many of them lyse easily during fixation. Also, I have observed that ciliates lyse when exposed to blue light

after being stained with acridine orange. Techniques for extracting Protozoa are similar to those used for meiofauna (Uhlig 1964; Schwinghamer 1981). Protozoan biomass will be underestimated if methods for examining fixed specimens, such as those used by rumen microbiologists, are used. Amoebae have not been studied extensively in natural aquatic systems, so it is difficult to recommend a method for determining their numbers or biomass, though the method of Singh (1946) might be applicable. Sieburth (1979) has discussed the distribution and types of Protozoa in marine systems and the methods of studying them.

Algae

Microscopy techniques for counting algae and determining biomass give the most accurate results. The techniques that preceded the introduction of epifluorescent microscopy are described in an IBP Handbook (Vollenweider 1969). Epifluorescent microscopy is a better technique for examining algae if they are embedded in detritus or sediment (references in section 1 above). Acridine orange is probably the most useful stain, because cell walls, as well as nuclear material, lightly fluoresce with it and cell sizes can be determined. Algae are easily recognized by the autofluorescence of their photosynthetic pigments. The color is generally a deep red where chlorophyll *a* predominates, but it may vary where other pigments predominate. For example, some cyanobacteria (blue-green algae) and coralline red algae fluoresce orange.

Filamentous algae can be counted using the procedures of Olson (1950) as described by Brock (1978). Brock recommended the dye primuline yellow, but this does not work well in detritus because the detritus fluoresces with the same color as the algae.

As with bacteria, measurements of biomass from numbers and size of cells are time consuming and liable to subjective errors in volume determinations, so chemical methods have been sought. Pigment analysis, and in particular the determination of chlorophyll *a* is the preferred technique for algae. Details of methods for extracting pigment and determining chlorophyll *a* by spectrophotometry are given by Vollenweider (1969) and Parsons et al. (1984).

If large amounts of chlorophyll degradation products (e.g., phaeophytins and chlorophyllides) are present, as may be the case in detritus, spectrophotometric techniques do not give accurate results. It is better to separate the pigments by chromatography. A number of techniques have been described for doing this with HPLC (e.g., Mantoura and Llewellyn 1983; Wright and Shearer 1984). An advantage of this procedure is that some indication of algal community structure may be obtained from the pigment analysis. The factors for converting pigment data to biomass are variable, and may give inaccurate values for biomass (Banse 1977). For benthic diatoms, conversion factors ranging from 10 to 154 have been reported (de Jonge 1980). An overall mean value that could be used is 50 (from data of de Jonge 1980).

Fatty acid composition may also be used to obtain estimates of algal biomass (Perry et al. 1979; Volkman et al. 1980; Gillan and Hogg 1984).

Microbial Composition of Detrital Systems

For each type of detrital system or even each pond or set of ponds the following questions are important: (i) what is the proportion of biomass to detritus (nonliving organic matter)? (ii) what organisms are present and how do their biomasses change with time? (iii) what is the composition of the detritus and how does it affect the community structure and succession of microorganisms associated with it? (iv) what is the nutritive value of the detritus in terms of protein, digestible carbohydrate, essential amino acids, fatty acids, vitamins, etc. These topics are discussed by Bowen (this vol.), but attention should be drawn to some points of methodology here.

The methods described above cannot give a complete description of the composition of detritus, but only an estimate of the biomass of some of the principal organisms. The size of organisms ranges so widely in detritus, from very small bacteria (0.2 μm diameter) to meiofauna, that general techniques such as ATP content are not likely to yield useful information on composition. Yet biochemical studies of biomass and community composition may be necessary to complement more direct microscopical studies. Many organisms (e.g., some Protozoa and microalgae) are very fragile and cannot be easily separated from detritus or fixed for microscopical study. Thus direct microscopical methods may underestimate the biomass of some groups. The newer techniques involving lipid composition (fatty acids, phospholipids, sterols, etc.), although requiring complex equipment and facilities, will be useful tools in microbial ecology once the biochemical composition of the various microbes is better known. When this work is undertaken, our knowledge of the detrital food chains will be enhanced. Microbial ecologists will be able to use aquaculture ponds as semi-natural model systems, where successional studies on microbial community structure may be carried out with detritus of known composition.

The question of whether detritus *per se* or the microorganisms associated with it are utilized as food by deposit or detrital feeders has been discussed in the literature; the nature of detritus is so variable that no generalization can be made. For example, neither detritus derived from vascular plants with a high C:N ratio, nor much of the organic matter present in indigestible carbohydrates will be useful directly, whereas detritus from algae may be much more digestible without microbial decomposition (Findlay and Tenore 1982; Tenore 1983).

Productivity and Growth Rates of Microorganisms

Bacteria

Growth Rates and Production

Techniques for measuring the activity and growth of heterotrophic bacteria have been reviewed by van Es and Meyer-Reil (1983), but only recently has it become

possible to measure the growth rates of heterotrophic bacteria in aquatic environments satisfactorily. Methods requiring counts of bacteria at intervals of time do not work in natural systems, due to the effects of predation as well as the difficulties associated with counting bacteria. Such methods may be used in the laboratory or in microcosms of natural systems where predators have been excluded in order to check or calibrate the technique described below (Fuhrman and Azam 1982; Kirchman et al. 1982).

A widely accepted method for measuring bacterial growth rates and production in natural environments is based on the measurement of rates with which tritiated thymidine is incorporated into DNA (Fuhrman and Azam 1980, 1982; Moriarty and Pollard 1981, 1982). It is reviewed in detail elsewhere (Moriarty 1986a). Such measurements are particularly important in studies on detrital aquaculture systems, because bacteria provide the main mechanism for transforming detritus with a low food quality into useful food material. As the method is new, and is promising for aquaculture, some details will be given here.

In principle, the thymidine method for measuring bacterial growth is simple, as there is a direct correlation between rates of DNA synthesis and cell division. Bacteria contain one chromosome, so when a cell grows and divides, every new chromosome that is synthesized represents a new bacterial cell. The rate of synthesis is calculated from the rate of incorporation of thymidine into DNA. Thymidine is one of the four nucleosides of which DNA is composed. By labelling thymidine with a radioactive isotope (tritium), this process can be simply and conveniently measured. Thymidine has the advantage that it is used in cells almost entirely for DNA synthesis; excess thymidine is not incorporated into any other macromolecules.

Methods based on adenine and adenosine triphosphate (ATP) have been developed by Karl and his colleagues for determining microbial biomass and growth rates, e.g., Karl (1982). All methods for such studies have deficiencies. The deficiencies of the adenine methods, however, are so severe, that I do not consider these methods to be useful for aquaculture studies and thus have not discussed them in detail. For a critical appraisal of these methods, see Fuhrman and Azam (1980), Fuhrman et al. (1986a, 1986b), Moriarty (1986a).

The chief disadvantages of the methods are:

1. Adenine is not taken up by all organisms, but its use in methods for measuring growth rates depends on measurements of ATP concentrations. Large errors result because ATP is in all organisms.
2. Adenine is taken up by some algae as well as bacteria and is incorporated into nucleic acids, but at probably quite different rates. The complexities of adenine metabolism make it very difficult, if not impossible, to determine meaningful growth rates in natural ecosystems from rates of labelled adenine incorporation (Moriarty 1986a).

In practice, there are some problems in the use of tritiated thymidine that need to be considered if accurate and ecologically meaningful results are to be obtained. In

detritus and sediment, thymidine should be added at very much higher concentrations than would be added to the water column, otherwise insufficient thymidine will penetrate to the growing bacteria. Problems may arise from the effects of isotope dilution if the concentration of thymidine supplied is too low because it adsorbs to detritus (Pollard and Moriarty 1984). Thymidine may be degraded within the cell and the tritium distributed into other macromolecules if bacteria are incubated for too long with the labelled thymidine. Short-term assays (generally 10 to 30 min.) are necessary to avoid the effects of predation and containment of natural samples in bottles.

Conversion factors are needed to calculate rates of cell division from rates of thymidine incorporation, and of production from rates of cell division. Rates of cell division can be calculated with reasonable accuracy (Moriarty 1986a). Estimates of production, however, may be inaccurate if the size of the growing bacterial cells is not known. The errors that apply to biomass determinations also affect productivity when cell sizes and carbon content are not known. More research is needed in this area. Aquaculture ponds are suitable experimental systems for this type of work, because it is easier here than in uncontrolled natural environments to check estimates of production from data on known rates of input and from successional studies.

The growth of most types of aerobic and anaerobic heterotrophic bacteria is specifically measured by the thymidine method. Cyanobacteria, eukaryotic algae and fungi lack thymidine kinase, the enzyme that is needed to incorporate thymidine into the biosynthetic pathway leading to DNA. Some bacteria, particularly those with strict nutritional requirements such as chemoautotrophs, are unable to use thymidine, which may be due to their lack of uptake mechanisms, rather than of thymidine kinase. Thus the method may underestimate bacterial production, but apparently not severely. Protozoa, which have thymidine kinase, lack uptake mechanisms equivalent to those of bacteria, and thus their DNA is not labelled in short-term experiments with nanomolar concentrations of thymidine. The specificity of the thymidine method for bacterial growth rates is discussed in more detail elsewhere (Moriarty 1986a).

Other proposed methods of estimating bacterial production in aquatic systems include those that are based on measurements of the dark fixation of $^{14}CO_2$, the frequency of dividing cells and the uptake of small compounds such as amino acids or sulfate. These are more difficult than the thymidine method to calibrate or interpret, and are not suitable for use with detrital systems. For methods that measure the synthesis of cellular constituents other than DNA, the bacteria must be in a state of balanced growth or conversion factors will be invalid. Balanced growth occurs when all components of the cell (e.g., DNA, RNA, protein, lipid) increase at the same rate, which is unlikely to be the case in natural systems.

The fixation of $^{14}CO_2$ in the dark was proposed by Romanenko (1964) as a method for measuring heterotrophic bacterial growth. Its use has been reviewed by van Es and Meyer-Reil (1983). Results obtained with the method were generally up to an order of magnitude too high. It is unlikely that accurate values can be obtained in natural systems because the method requires long incubation times, all

organisms fix small amounts of CO_2 in the dark and the conversion factors are variable.

The frequency of dividing cells was suggested as a measure of bacterial growth rates in the water column (Hagström et al. 1979). Seasonal changes in the frequency of dividing cells were covariant with the uptake by bacteria of phytoplankton exudates. The method is, however, difficult to calibrate (Hagström 1984), requiring culture studies that match laboratory values with field estimates (Riemann et al. 1984). Problems with interpretation of the method have been discussed by Newell and Christian (1981). The method would not work with detritus or sediments, where filamentous bacteria abound and cannot be distinguished from dividing cells. Fallon et al. (1983) found that the frequency of dividing cells method gave values for bacterial production in sediments that were over an order or magnitude too high.

Protein Synthesis

The microorganisms associated with detritus are usually the main source of protein for detritivores, as detritus itself generally has a low protein content if it is aged or is derived from vascular plants (Fenchel and Jørgensen 1977). In studies of detrital decomposition, the production of microbial biomass, measured using techniques described above, can be used to estimate protein production. If growth is not balanced, the rates of protein synthesis will not be equivalent to the rates of cell division. Under starvation conditions, bacteria may still synthesize DNA and divide, but they become smaller and their protein content declines (Kjelleberg et al. 1982). Alternatively, where nutrients are plentiful, the bacteria increase in size and synthesize protein faster than DNA.

For studies of trophic dynamics in detrital systems, direct measurement of the rates with which bacteria synthesize protein would be preferable. Protein synthesis is often determined in the laboratory from rates of ^{3}H-leucine or ^{14}C-leucine incorporation. This technique is also useful as an index of rates of protein synthesis of bacteria in natural aquatic systems (Kirchman et al. 1985). There are problems in determining the specific radioactivity of ^{14}C-leucine within the bacteria, and thus obtaining an accurate rate of synthesis; however, further work should resolve these problems.

Kirchman and Hodson (1984) have discussed the relationships between protein synthesis and the uptake and utilization of dissolved organic carbon compounds. The turnover of organic carbon in aquatic systems is not necessarily directly correlated with rates of bacterial growth. For a fuller understanding of the carbon cycle and trophic dynamics in ponds, processes such as protein and lipid synthesis, bacterial respiration and bacterial growth rates need to be studied.

Algae

The two principal and well-known methods for measuring primary production depend on oxygen production or $^{14}CO_2$ fixation (Vollenweider 1969; Parsons et al.

1984). For quantitative studies on detrital food chains, net primary production must be measured. True net primary production is much more difficult to measure than gross production, because the respiration of algae and other organisms in the community is difficult to measure during photosynthesis. There has been considerable discussion in the literature over the last few years on measurements of net primary production and the accuracy of $^{14}CO_2$ method (Peterson 1980; Davies and Williams 1984). Davies and Williams (1984) concluded that the $^{14}CO_2$ method can give values for primary production that agree with gross production measured by the oxygen method, provided that certain conditions are met. Much of the respiration in planktonic communities is probably due to bacteria (e.g., see Williams 1981), so unless bacterial respiration is measured or estimated separately from algal respiration, calculations of net primary production will not be accurate. In aerobic environments, estimates of bacterial respiration can be obtained from growth rates (thymidine technique), but a value for growth efficiency has to be assumed or chosen from published values. Aquaculture pond systems would be useful model environments for studying this problem.

Growth or Ecological Efficiencies and Food Chains

In the fields of aquaculture and microbial ecology in general we need to know how much detrital organic matter is converted into bacterial biomass and how much is respired or mineralized. In other words, what is the growth efficiency of the bacteria? And furthermore, what is the overall ecological efficiency of the pond, that is, how much of the organic matter added to the pond is converted into biomass in the end product?

Biomass Production

In a grazing food chain where a herbivore feeds on plants, the plant biomass is usually very high in proportion to the biomass of the herbivore. Biomass values alone are sufficient to determine whether the animals are food limited and to compare one food chain with another. With food chains that are based on bacteria, biomass values are not sufficient. Bacteria can double very rapidly and so production values are essential for a full understanding of the importance of bacteria in food chains. Consider a case where bacteria have a doubling time of 12 hours and are grazed at a rate that keeps the biomass constant. A measurement of biomass would underestimate by twofold the amount of food available each day to animals. The rate of bacterial biomass production must also be measured.

When bacterial production is measured with the thymidine method, the conversion efficiency must be known to assess the relative amounts of detritus consumed and mineralized. Conversion or growth efficiencies of bacteria have been extensively studied in culture, where an average value of 60% was commonly found (Payne 1970). Conversion efficiencies vary with such factors as temperature, pH and nutrient

consumption (Payne and Wiebe 1978). Quite high values for carbon assimilation have been found (e.g., 85% for *Enterobacter aerogenes*), usually when nutrient complexity is high (Payne and Wiebe 1978). Where simple or structural carbohydrates are supplied with inorganic nitrogen, efficiencies are generally low. Because so many different interacting factors affect growth yields, culture studies cannot be extrapolated to the natural environment.

Payne and Wiebe (1978) pointed out that growth efficiencies have to be determined *in situ*. One technique they discussed measures growth and respiration with ^{14}C-labelled substrates having a low molecular weight. They make an important point; in any work with radioisotopes one must be aware that isotope dilution can be variable and changeable in short-term experiments. For respiration studies, it is necessary to check that the specific radioactivity of the respired $^{14}CO_2$ is the same as that of the substrate. Although the problems of isotope dilution are recognized by biochemists, it seems that aquatic microbial ecologists have overlooked them (King and Berman 1984).

Microcosm studies where mass balances can be determined may be a better approach for studying growth efficiency. Koop et al. (1982) calculated that about 30% of carbon from a decomposing kelp *(Ecklonia)* was converted into bacterial carbon over a 9-day period. Their values were based on bacterial biomass conversion factors that have since been reported as being too low (Bratbak and Dundas 1984). If the newer conversion factor is accepted, the growth efficiency of bacteria on the kelp would be around 50 to 60%. In that case, twice as much food (bacterial biomass) would be available to higher trophic levels.

Robinson et al. (1982) measured bacterial conversion efficiencies during degradation of another kelp *(Laminaria)*. They found that bacterial biomass measured directly was about 3 times higher than that calculated from measurements of cell size. Conversion efficiencies were about 45% for the first 2 to 3 days of degradation, but declined to about 20% after 36 days, as the percentage of refractory detritus increased with the depletion of the readily digestible material.

Lower values for conversion efficiencies of bacteria that were degrading phytoplankton detritus have been reported (Bauerfeind 1985). Respiration was measured directly (i.e., O_2 uptake which avoids isotope dilution problems with $^{14}CO_2$) and was found to account for 73 to 83% of carbon uptake. Bauerfeind's (1985) values were calculated on the basis that bacterial carbon was 10% of the cell mass, but as mentioned above, Bratbak and Dundas (1984) have reported that carbon content is higher (around 20%). Using this higher value, the respiration was 57 to 72% or, in other words, conversion efficiencies were around 35%. From these studies, it seems that bacteria may be marginally more important as mineralizers of organic matter than as converters into useful biomass, but there is much variation, depending on the nature of the organic substrates and the availability of nitrogen and phosphorus. Williams (1984) has discussed this problem with reference to marine water columns and points out that a single conversion efficiency for all conditions is too simplistic.

Conversion efficiencies need to be measured for each environment. Thus, it is not possible to give a general answer to the question posed above, namely, what propor-

tion of detrital organic matter is mineralized by bacteria, and what proportion is made available to the rest of the food web? Research programs in aquaculture will need to include experimental studies to determine conversion efficiencies in each system, and to determine whether these efficiencies can be increased by manipulating nutrient concentrations.

The need for accurate conversion efficiencies in understanding pond ecosystem dynamics is illustrated by some studies on ponds used for the culture of penaeid shrimp, where an excess supply of pelleted food became a high quality detritus for a bacterial-based food chain (Moriarty 1986b; Moriarty et al. 1987). The bacterial production in the water column was very high compared to natural marine systems, and production in the sediment was also high, but not as great as in the water (Table 1). The production values were clearly correlated with food input.

To determine how closely correlated they were, conversion efficiencies are needed to calculate the total amount of organic matter utilized by the bacteria. Production values for the control pond were subtracted, and then total organic matter utilized was calculated, assuming conversion efficiencies of 25 and 50% (Table 2). It can be seen that, with an efficiency of 50%, rates of food input and utilization agree closely in ponds and 11 and 23, whereas with 25% there is a large discrepancy. In pond 29, a conversion efficiency of 42% is necessary to balance the two. A conversion efficiency of 40 to 50% is quite possible in these ponds, because the detritus, being pelleted food, was nutritionally of high quality.

Table 1. Bacterial production in shrimp aquaculture ponds supplied with pelletized food (from Moriarty 1986b). Units: food and production: g C m^{-2} day^{-1}; doubling time: days.

Pond	Food input	Water column		Sediment (0-10 mm)	
		Production	Doubling time	Production	Doubling time
Control	0	0.43	0.25	0.26	4
11	1	1.07	0.17	0.24	9
29	3.2	1.32	0.17	0.41	4
23	4.0	2.10	0.34	0.50	8

Table 2. Comparison of pelletized food input and organic matter utilization by bacteria in shrimp aquaculture ponds. Bacterial production was measured, and values for utilization are calculated, assuming either 25 or 50% bacterial growth efficiency. Values are g C m^{-2} day^{-1}. Values for a control pond have been subtracted: from Moriarty (1986b).

Pond	Input	Water column		Sediment	
		25%	50%	25%	50%
11	1.0	2.6	1.3	0	0
29	3.2	3.6	1.8	1.0	0.5
23	4.0	6.6	3.3	1.4	0.7

Oxygen Uptake

Besides the direct relevance to biomass production, studies of conversion efficiencies are important in other ways. Aerobic bacterial growth is, of course, accompanied by oxygen utilization; the more efficient the conversion, the less oxygen is used. Bacteria may account for the bulk of oxygen uptake in ponds where detrital food chains operate. Their respiration, simply by lowering oxygen concentration in the water, may be the most important factor that limits the production of fish or prawns on detrital food chains.

An example of such effects is seen in the data from the shrimp aquaculture ponds discussed above. In all ponds that were studied, respiration was greater than gross production; gross production ranged from 0.8 to 1.6 g C m^{-2} day^{-1} and respiration from 2.3 to 3.6 g C m^{-2} day^{-1} (Moriarty et al. 1987). Bacterial respiration, estimated from production measurements, accounted for a considerable proportion of the total respiration (Table 3). In pond 29, for example, bacterial respiration was about 70% of the total. With the bacterial respiration known, a maximum value for net primary production can be calculated; in this case, 0.5 g C m^{-2} day^{-1} (Table 3). The large respiratory activity in this pond was reflected in the low oxygen concentration in the water, particularly the bottom waters (Table 4). Such low values stress animals and limit their production. The amount of detritus that can be added each day to ponds would be limited by the degree of aeration.

Table 3. Primary production and respiration in shrimp aquaculture ponds. Values for bacterial respiration are calculated assuming a 42% conversion efficiency. Values are g C m^{-2} day^{-1} (from Moriarty et al. 1987).

Pond	Gross production	Respiration		Net primary production
		Total	Bacterial	
29	1.2	2.5	1.8	0.5

Table 4. Oxygen production, respiration and concentration in a shrimp aquaculture pond (from Moriarty et al. 1987).

Depth cm	Gross production mg l^{-1} h^{-1}	Respiration mg l^{-1} h^{-1}	Concentration mg l^{-1}
10	0.49	0.34	4.61
60	0.43	0.39	3.38
80	0.08	0.20	0.92

Higher Trophic Levels

Now that the biomass and production of bacteria can be measured with some assurance of accuracy, it is possible to study the efficiency with which that production is transferred to higher trophic levels. The control of bacterial growth by grazers can also be studied.

The ecological efficiency of a pond depends on the feeding strategy of the cultured species and the number of levels in the food chain to those species. Organisms that feed on bacteria and microalgae, such as tilapia, should be produced more efficiently than carnivores (e.g., penaeid shrimp). Even so, there are likely to be variations in the efficiency of tilapia production because a complex food web operates at the microbial level. Protozoa, microzooplankton and meiofauna compete with larger "detrital" feeders for bacteria and microalgae and can have a considerable effect on the ecological efficiency. The rates of grazing on bacteria by Protozoa and meiofauna in sediments are unknown, but need to be determined, because a large proportion of bacterial production may be removed by them. An extension of such studies would be to determine whether bacterial production and detritus decomposition are stimulated by grazing on the bacteria.

To illustrate the effects of multiple trophic levels and controls on pond production by productivity of autotrophs and bacteria, consider a hypothetical example of a food web in a pond (Fig. 1). A pond used for the culture of penaeid shrimp in

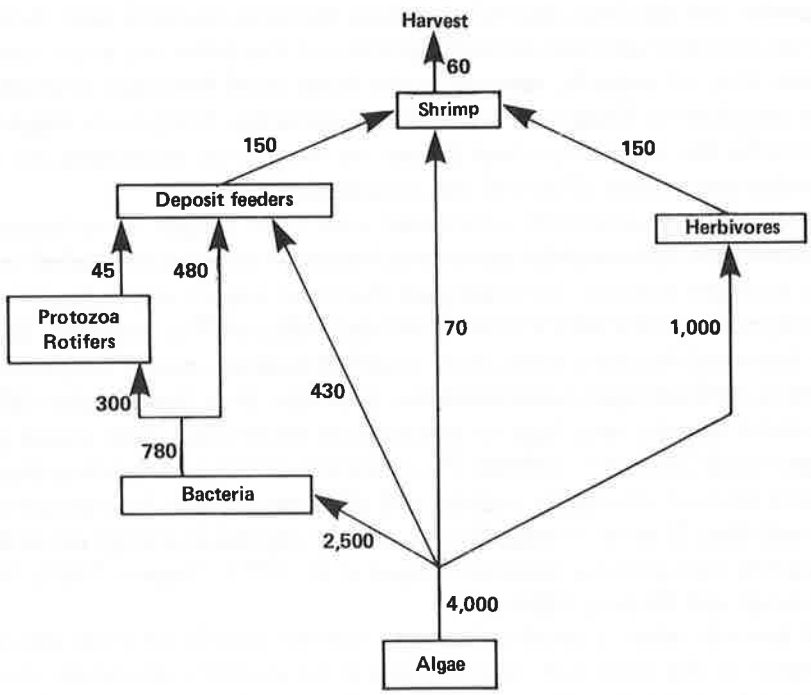

Fig. 1. Hypothetical food web for a penaeid shrimp pond based on known production of fish and shrimp of 60 mg C m^{-2} day^{-1}. Values are mg C m^{-2} day^{-1}.

Thailand has no inputs except for 30% water exchange each day from a productive estuary. It produces about 1,800 kg wet weight of shrimp and 1,000 kg of fish per month. Extensive mats of benthic algae grow in the pond and are one source of production, either directly, or as detritus when they decay. The monthly production of fish and shrimp is approximately equivalent to 60 mg C m^{-2} day^{-1}. The hypothetical food web and values constructed for this pond indicate that primary production would need to be about 4 g C m^{-2} day^{-1}, which is high, but not unrealistic for the tropical environment concerned. Some organic matter would have come from the estuary also. It has been assumed that a little over half the primary production enters the detrital food web directly, and that growth efficiencies are 30% for bacteria, 20% for Protozoa and rotifers and 15% for other organisms. The shrimp are assumed to feed mainly on deposit feeders and herbivores and also to some extent on algae. Only a small fraction of the original primary production entering the detrital food web would be needed to increase prawn production significantly based on deposit feeders. In other words, detrital food chains are much less efficient than those with herbivores for feeding carnivores.

The population density of bacteria in water columns is relatively constant, and yet the bacteria are known to grow rapidly. Zooflagellates are the main grazers on bacteria in the water column, and their density and grazing rates are sufficient to control the bacterial density (Fenchel 1982; Andersen and Fenchel 1985). Larger Protozoa, e.g., ciliates and metazoans, control the flagellate populations.

In sediments and detritus, the relationships between bacteria and their growth substrates on the one hand and bacterial grazers on the other are more complex. A much greater array of animals, ranging in size from small Protozoa to large fish, feed on bacteria attached to detrital or sedimentary particles. It has been suggested that Protozoa may be the most important grazers on bacteria in sediments, but more detailed studies are needed (Fenchel and Jørgensen 1977).

Bacterial biomass is positively correlated with food supply in sediment. Dale (1974) showed that, in intertidal sediments, bacterial biomass depended on organic carbon and nitrogen content. He suggested that food supply might limit bacterial numbers, a hypothesis for which there is strong evidence. For example, in seagrass sediments, bacterial densities were about fourfold greater around seagrasses, where organic carbon and nitrogen concentrations were also four times higher (Moriarty 1980). Bacterial density was high in aquaculture pond sediments where pelleted food was provided (Moriarty 1986b). The bacterial biomass in detritus depends not simply on the amount of organic carbon and nitrogen present, but on the composition of the detritus. It is the availability of readily digestible energy substrates and nitrogen sources that governs biomass (Lopez et al. 1977; Hanson 1982; Tenore et al. 1982; Alongi and Hanson 1985).

Bacterial growth rates or production may not be correlated with amounts of detritus present in the same way that biomass is. In an artificial system, no obvious correlations of bacterial growth rates with detritus supply were seen (Alongi and Hanson 1985). Diel changes in bacterial growth rates in seagrass sediments have been observed, which may be related to exudation of readily available organic compounds

by the seagrass (Moriarty and Pollard 1982). Thus growth rates of some bacteria in sediments may be influenced by food supply. Many bacteria may be dormant until the correct substrate or environmental conditions are provided. Others may grow slowly on degrading particulate matter and thus would not easily be detected by the thymidine method. With the thymidine method it is possible to carry out more incisive experiments on the relationships of bacteria to detritus supply and composition than was previously possible.

The biomass and growth rates of bacteria in detritus are influenced by animals grazing on them. Rates of decomposition of detritus are often increased by the effects of grazing animals (Fenchel 1970; Welsh 1975; Harrison 1977). Bacterial activity, and presumably growth rates, are increased by grazing, although only general studies have been carried out (see Fenchel and Jørgensen 1977). If bacterial growth rates are substantially increased by grazing activities of animals, values solely for biomass will not be useful in determining the role of bacteria in food chains. Production must also be measured.

In some aquaculture ponds in Malaysia, the effect of added chicken manure on microbial populations was examined (Moriarty 1986b). After one week, biomass increased and doubling times slowed. After two and three weeks, bacterial biomass fell markedly, while the meiofauna population density increased (Table 5). The bacterial doubling times also fell markedly, which supports the suggestion that bacterial growth is stimulated by the effects of grazing animals. The mechanisms by which this occurs may be various.

Table 5. Effect of chicken manure on bacteria and meiofauna in pond sediments.

Treatment	Bacteria		Meiofauna
	Biomass g C m^{-2}	Doubling time (days)	No. 10 cm^{-2}
Control	3.4	5	140
Manure, 1 week	4.3	8	300
Manure, 2 weeks	1.9	7	500
Manure, 3 weeks	0.8	3	1,500

It is now possible to investigate some of these mechanisms with the tritiated thymidine method to measure changes in instantaneous growth rates of bacteria in short time periods. A study of this type has been conducted by Alongi and Hanson (1985). They found that the polychaete *Capitella capitata* stimulated bacterial growth to some extent when detritus was in low supply, but had no effect when detritus was present in large quantities and bacteria were growing very rapidly. Alongi (1985) suggested that faster bacterial growth rates in the presence of *Capitella capitata* may be due to the effect of protozoan grazing on bacteria attached to the tube walls of the polychaete.

It has long been accepted that bacteria have a role in sediments as food for deposit feeders, but the significance of that role has been debated. With the improvement in methods for determining biomass and growth rates of microbes, and the increased values for carbon content of bacteria, it is likely that, at least in sediments and detritus, bacteria will be confirmed as having a very significant role. It has been shown, for example, that 40% of daily bacterial production was eaten by holothurians on a coral reef flat (Moriarty et al. 1985c). One of the important areas for further research is in determining growth efficiencies for the various groups of microbes, and in particular whether food chains can be manipulated to improve overall ecological efficiency.

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Discussion

MORIARTY: Regarding the efficiency of conversion by bacteria, one of the big problems in microbial ecology is what is the conversion efficiency of bacteria in a natural system? We can measure bacterial production but we don't know how much organic matter the bacteria need to take in to achieve that production. We can make hypotheses, for example, their conversion efficiency may be around 30 to 50%.

SCHROEDER: I doubt that they are that efficient. A 50% conversion efficiency is almost perfect. They are probably closer to 20%. I am assuming here that the bacteria in a pond are achieving the same efficiency as we might observe in the laboratory in a test tube or fermentation vessel.

MORIARTY: Bacterial conversion efficiencies of up to 80% have been recorded. It all depends very much on the energy value and C:N ratio of what the bacteria take in and their respiration.

SRINIVASAN: It depends on the substrate. In fermentation, if we feed 100 g of glucose to the bacteria, 50 g should be fixed as biomass and 50 g will go off as CO_2. For other substrates like amino acids and proteins, the maximum conversion efficiency to biomass is about 40 to 45%.

SCHROEDER: But it is unlikely that the conditions we are discussing are optimal conditions.

MORIARTY: The only point I am trying to make is that we do not have yet a single reliable measure of conversion efficiency. However, now that we can measure bacterial production we at least have some idea of what is going on. If we can now proceed to measure food inputs and particularly if we can measure oxygen changes as well, then we can make some better estimates of conversion efficiency. Conversion efficiency is likely to be higher in the water column than the sediment.

SCHROEDER: Why?

MORIARTY: Because in the water column aerobic processes predominate. These are more efficient than the anaerobic processes which predominate on the sediment.

SCHROEDER: But anaerobic processes are probably more efficient in producing total biomass, that is slime plus bacterial cells. Aerobic processes are only more efficient than anaerobic processes for cell production.

MORIARTY: Slime production is controlled by many factors, especially C:N ratios. Slime is also produced during aerobic processes.

PRUDER: This is a very important point which we should discuss further.

ANDERSON: If anyone is planning to use redox dyes to look for anaerobic microsites then do ask a good chemist to assist. One of the problems with these dyes is the phenomenon of poisoning. A dye itself has an oxygen demand. The best way to proceed is to reduce the dye and then let the reaction go back the other way. If you put in a dye which has to be reduced to get a color change, it can take up oxygen from the microsite and still not change color. There are some ghastly artefacts in the literature resulting from misuses of redox dyes.

COLMAN: When using the labelled-thymidine method for estimating bacterial production, could the addition of the thymidine actually increase the rate of production, because the bacteria would not have to synthesize it for themselves?

MORIARTY: No. It has no such effect. This is a fundamental aspect of the method. If it did, when we studied bacterial production over time using isotope dilution experiments, we would see curves that were not linear. The control of DNA synthesis is very much more than just the control of thymidine synthesis. Four bases go into DNA and its synthesis is controlled by the whole status of the cell with respect to energy supply and nutrient conditions. If the cell is not getting enough thymidine from an external source, it switches on some enzymes and makes some more. If it is getting too much thymidine it switches off these enzymes. The same applies to adenine, cytosine, etc. All these are rigidly controlled—in fact the concentrations of their precursors are controlled, so that more of any one has no effect on the overall rate of synthesis.

BOWEN: You mentioned that staining with acridine orange was a good method for counting, but that there were some problems with bacteria attached to particles. I have used a sonic probe to strip most of the bacteria off the particles. This is much more efficient than a blender. It also reduces the particle size and disaggregates the particles. After this, it is possible, by focusing up and down on a particle, to count the bacteria on the front and back surfaces. I am confident that by counting in this way we are not missing many bacteria. Have you or others present tried this?

MORIARTY: I have used both methods. I have used a blender (Ultra-Turrax) which is a very high speed blender. It is not like a Waring blender. It fragments particles like sand grains. I have also used a sonic probe. The problem with this is that as you give it more and more energy for a longer and longer time, you get less and less particles and everything becomes spread more evenly. If you do total counts during this process, your numbers go down and down, so it is obviously disrupting cells. In fact biochemists use sonic probes to break up bacterial cells to get the enzymes out.

BOWEN: But you can adjust the energy to an appropriate level.

MORIARTY: Yes, I agree; using a low energy for 30 seconds is very efficient, and better than a blender for sand grains. Even this does not give complete particle breakup and even distribution. I now use an ultrasonic probe! The main exception is for coral reef sediments. Coral reef sediments are difficult because, once you have removed the calcium carbonate, what is left is bound up with a lot of slime. In any situation where you have a lot of slime, neither blenders nor sonic probes can easily disperse the particles and bacteria. In these situations, if you need to do a lot of estimations, then muramic acid estimations are probably better. There is no really good technique and the techniques that we have, depend to a large extent on the artistry of researchers and fine-tuning in their respective laboratories.

BOWEN: You also mentioned the tedium of making measurements directly on images seen through the microscope and you referred to an image analyzer.

MORIARTY: Yes, these are being used in a number of laboratories. Perhaps John Fry would like to comment? I haven't got one in my laboratory.

FRY: We use an image analyzer for measuring the size of bacteria (Fry and Davies*) but we have not yet been successful in adopting this technique for making total counts. We use a Cambridge Instruments Quantimet 800, which unfortunately is no longer made. I have also used a Cambridge Instruments Quantimet Q10 which is much cheaper and is still on the market. This works equally well. We do conventional acridine orange staining and then photograph the preparation and measure the sizes of the organisms directly from the photograph. This is a reasonably quick method.

BOWEN: I have used a camera lucida to trace the images of detritus particles, bacteria and algae using different magnifications. These drawings can then be analyzed with a digitizer. A tablet costs only about two to three thousand dollars whereas image analyzers cost more than ten thousand.

We simply use the pen on the digitizer to mark the long and short axes and indicate which of several geometric shapes best approximate to the drawings. Using this approach you can quantify the volumes of a whole range of particles with reasonable precision. To guarantee accuracy, we take pure samples of bacteria or algae or sand grains and determine a conversion from measured volume to directly determined weight. With the aid of a microcomputer, this method is very quick and not at all tedious. For me this method provided something of a breakthrough. At reasonable speed and cost we can now make direct measurement not only of the biomass of various microorganisms (autotrophs and heterotrophs) but we can also quantify the amount of detritus. Formerly we had to rely on using estimates of differences for such data and 'difference' methods are always hazardous.

Finally I have a question about muramic acid determinations. What is the half-life of muramic acid in a dead organism in the natural environment?

MORIARTY: From work that I have done it is less than 24 hours in mangrove sediments. David White has done similar work in estuarine systems. I think his values were around 12 hours. It doesn't last long because it is associated with glucosamine and amino acids. Muramic acid is just one component of peptidoglycan—the macromolecule wrapped around the bacterial cell. It is very nutritious. Muramic acid from dead bacteria will be utilized rapidly in the presence of other live, actively growing bacteria. However, all these comments apply to surface sediments. In deeper sediments, it can persist for much longer. So muramic acid determination is a useful technique if you need to look at turnover in surface sediments. It is not a useful technique for deeper sediments.

MORIARTY: Should we include slime in biomass or should we call it detritus? Slime is important when we consider productivity, particularly when we compare work by different people. My view is that the term biomass should be restricted to actual living cellular material, which is shown clearly by epifluorescence microscopy. This technique does not show all slime. Slime can be seen by acridine orange staining but is very difficult to quantify. Slime is also extremely variable both in chemical composition and in its structure around the cells. You can find well-structured slime layers around some microorganisms, whereas in others, its structure can be very loose and it tends to dissociate from the microorganisms and become part of the general organic matter in the water body. Therefore, the term biomass should be restricted to living cells and biomass production to their protein and other internal biosynthetic activities. Slime production should only be considered in the definition of total organic matter production.

SCHROEDER: I agree. Slime may have an important function in making food available to the target animals. The slime can act as an ion exchange column and the large amounts of dissolved organic matter produced by phytoplankton may be absorbed on to slime. Therefore, the slime may be nutritious even though its own fiber molecules are unavailable to a target organism. It is these ionic exchange properties which allow the slime to provide organic molecules for the slime-producing bacteria. We can therefore regard slime as really having the property of changing dissolved organic matter into fixed organic matter.

GRAY: This reminds me of the long standing debate about mucus production in animals. Mucus is generally regarded as an excretory product. It is not included in standing crop estimations, so this agrees with Dr. Moriarty's definition. However there are many organisms - meiofauna, for example—which produce mucus to trap bacteria and then reingest the mucus. This has been called the 'gardening' phenomenon.

PRUDER: Regarding our definition of primary production, which refers to fixed particulate carbon, how can we frame some definitions to account for the large quantities of dissolved organic carbon which are released?

BOWEN: Ecologists generally treat dissolved organic carbon as a part of detritus, even though it is not particulate matter.

MORIARTY: Well, the definitions I have suggested here were meant to refer to bacterial production. However, as has just been pointed out, there are similar difficulties with defining terms for

the phytoplankton. The main point is to be clear about what we mean by production. If we wish to work out total net production with ^{14}C methods then, of course, we should include all the bacteria and slime, the phytoplankton and dissolved organic matter, leaching away from all organisms, that is labelled. But this would not be a production of biomass. These definitions are merely for our convenience in trying to understand these systems.

WOHLFARTH: I think we have said enough about slime. We can surely all agree that it is not alive.

SCHROEDER: Yes, biomass is cellular production—matter that is within a cell.

MORIARTY: A good definition.

ANDERSON: However many of these slimes which are mucopolysaccharide matrices do contain free enzymes. The work of Lock et al.** on epibenthic communities has shown that these are not simply microorganisms in an inert matrix. There is a holistic function served by the presence of free enzymes in that matrix. Therefore, while I agree with your definition of biomass we should not forget the importance of slime.

BOWEN: The same applies to the water column. There are many free enzymes in the water. They are concentrated on surfaces, for example, plant surfaces and roots. They have an important function in production of detritus by acting upon dissolved organic matter in the water column.

ANDERSON: Do they also act on colloidal material? The importance of colloidal material is not well understood. It is outside the conventional definitions of dissolved and particulate organic matter. Perhaps these free enzymes are important in colloidal degradation? They tend to floc on surfaces.

BOWEN: Right, the colloids tend to aggregate and precipitate. Biologists have not considered colloids to any significant extent.

SRINIVASAN: Are these free enzymes of microbial origin?

ANDERSON: I imagine so, almost exclusively. Where free enzymes are produced by bacteria in the aqueous medium, how can they control these enzymes?

KIRCHMAN: There is no good evidence for the production of free enzymes away from the cell wall.

MORIARTY: These enzymes are found with the Gram-negative bacteria which have two membranes around the cell with a space between them. The inner cytoplasmic membrane bounds the main cell, but there is a much looser outer membrane. Many of the enzymes associated with digestion of polymers are found in the space between the membranes. Thus the enzymes are in contact with the bacterial cell and the substrate and there are gradients of substrate and products. The Gram-positive bacteria lack this arrangement (Costerton and Cheng***) but again the enzymes do seem to be associated with the cell wall. One reason put forward for the production of slime by a lot of bacteria on particles is that it provides a microenvironment around the bacterial cells in which the enzymes responsible for hydrolysis can operate without being dispersed to the aqueous medium. The slime polymers provide a site for the enzymes and also help to hold their products. However, some of the enzymes are lost to the surrounding medium. There can be complex situations in which bacteria live 'on the backs' of other bacteria and make use of their enzymes. This is very difficult to study.

*Fry, J.C. and A.R. Davies. 1985. An assessment of methods for measuring volumes of planktonic bacteria, with particular reference to television image analysis. J. Appl. Bacteriol. 58: 105-112.

**Lock, M.A., R.R. Wallace, J.W. Costerton, O.R.M. Ventull and S.E. Charlton. 1984. River epilithon: towards a structural-functional model. Oikos 42: 10-22.

***Costerton, J.W. and K.J. Cheng. 1975. The role of the bacterial cell envelope in antibiotic resistance. J. Antimicrob. Chemotherapy 1: 363-377.

Role and Impact of Anaerobic Microbial Processes in Aquatic Systems

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Blackburn, T.H. 1987. Role and impact of anaerobic microbial processes in aquatic systems, p. 32-53. *In* D.J.W. Moriarty and R.S.V. Pullin (eds.) Detritus and microbial ecology in aquaculture. ICLARM Conference Proceedings 14, 420 p. International Center for Living Aquatic Resources Management, Manila, Philippines.

Abstract

The penetration of oxygen into sediments is very limited, and as a result sediments are dominated by anaerobic processes, particularly when the organic loading is heavy. Increasing sediment depth is matched by processes that occur at low redox potential. A nitrate zone is followed by sulfate and methane zones, in both of which substrates are supplied to the sulfate-reducing and to the methane-producting bacteria by fermentative bacteria. It is these fermentative bacteria that are the active hydrolyzers of organic detritus. Newly isolated strains of fermentative, sulfate-reducing and methane-producing bacteria are discussed. Knowledge of the range of substrates utilized by the fermenters and the sulfate-reducers has increased greatly in recent years. The methane-producing bacteria appear to be limited in their choice of substrates. Measurement of the rates of carbon mineralization in the nitrate, sulfate, and methane zones is discussed, followed by a short discussion on N-mineralization. The effect benthic animals have is to increase the rate of nutrient flux between sediment and overlying water.

Introduction

In the context of aquaculture it may be assumed that anaerobic processes are restricted to the sediment underlying the water column, and that because this water is aerated, there is some penetration of oxygen into the sediment. The depth of oxygen penetration will depend on the organic input to the sediment, and on the degree of water aeration and mixing, but the depth of penetration will normally be a few millimeters. This is in large measure due to the low solubility of oxygen in water. With moderate mixing, oxygen penetration was between 2 and 3 mm; vigorous mixing increased the penetration down to 3 to 4 mm (Fig. 1). This is typical of a

MANUAL OF TECHNIQUES FOR ESTIMATING BACTERIAL GROWTH RATES, PRODUCTIVITY AND NUMBERS IN AQUACULTURE PONDS

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CMFRI SPECIAL PUBLICATION
Number 42

ISSUED ON THE OCCASION OF THE WORKSHOP ON
BACTERIAL GROWTH RATES AND PRODUCTIVITY
ORGANISED BY
THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
Indian Council of Agricultural Research
HELD AT COCHIN FROM 2ND TO 15TH AUGUST 1986

PREFACE

Heterotrophic microorganisms, especially bacteria, play an important part in decomposition processes, nutrient cycling and food chains in aquatic systems. A number of different methods have been proposed for measuring microbial growth rates, but many are not specific for bacteria or do not include the whole population. It is evident that the ideal method should involve minimal handling of the bacterial population and be applied quickly so as not to alter natural or *in situ* growth rates or to be influenced by bacterial grazers. The use of radioactive nucleicacid precursors especially thymidine, to measure the rate of DNA synthesis has many of the prerequisites of the ideal method. As with all other techniques, there are disadvantages as well as advantages in using measurements of nucleicacid synthesis.

Dr. D. J. W. Moriarty, Principal Research Scientist, CSIRO Marine Laboratories, Division of Fisheries Research, Cleveland, Australia, an authority on bacterial growth rates and productivity in aquatic systems, visited the Centre of Advanced Studies in Mariculture at CMFRI, Cochin for a brief period in August 1986 as an expert consultant to afford advice and suggestions on the subject to upgrade research, especially on bacterial growth rates and productivity in aquaculture ponds and formulation of suitable course on the subject. During this period, a six day workshop on **'Bacterial Growth Rates and Productivity'** was organised and conducted by Dr. Moriarty, the course programme of which covered indepth the bacterial growth rates and productivity. Selected aspects pertaining to sampling techniques, laboratory processing of water and sediment samples, techniques in fluorescent microscopy were examined in the course of the workshop. Emphasis was laid on planning experiments for study of pond dynamics, isotope dilution and time courses for rates of labelling of macromolecules and liquid scintillation counting technique in samples taken from aquaculture ponds.

This manual was prepared in connection with the workshop conducted by Dr. Moriarty. It considers the measurement of growth rates calculated from the rate of tritrated thymidine incorporation into DNA. In India no work has been carried out on such aspects as measuring bacterial growth rates and

productivity by radio-tracer method in aquaculture systems. The techniques employed require a minimum of prior professional training. Methods requiring the use of very sophisticated equipment have also been avoided. As such, it is intended that the manual will be useful to students, scientists, environmentalists as well as aquaculture entrepreneurs.

I express my sincere thanks to Dr. D. J. W. Moriarty for preparing this manual. I also thank Dr. V. Chandrika, Scientist and counterpart to Dr. Moriarty for the assistance given in the preparation of the manual and for the keen interest shown in the conduct of the workshop.

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CONTENTS

1

INTRODUCTION

Microbial food webs are an integral part of all aquaculture ponds and have a direct impact on productivity, even where intensive, artificial feeding is practised. There are three major reasons for this. Firstly, in most or perhaps all ponds, respiration is predominantly due to bacteria. Hence the oxygen content of the water is governed by bacterial activity. Secondly, in all extensive, semi-intensive and some intensive aquaculture systems, bacteria also contribute significantly to the food web. They may be eaten directly by the target species (e.g. *Tilapia* or mullet) or by small animals on which the target species feed (e.g. larvae and juveniles of penaeid prawns). Thirdly, through the activity of the heterotrophic decomposers, nitrogen and phosphorus are recycled to stimulate primary production.

If we can quantify the productivity of bacteria, we can then make informed judgements about the above three functional roles of bacteria, and thus improve pond management to optimise productivity.

It is now possible to determine reasonably accurate growth rates for the heterotrophic bacteria, and thus estimate their productivity. The thymidine method for doing this is described in this manual. A brief discussion on microbial ecology in general is included, to show how such studies can be integrated with a broader study of aquaculture pond productivity.

Modelling Trophic Pathways

An ultimate goal for studies on microbial productivity (which includes microalgal productivity) is to be able to predict the size of the harvest from rates of production. A model is useful in helping design experiments to achieve this goal; a simple example is shown in Fig. 1.

The example is for a marine or brackishwater pond with primary production being the only source of organic matter. It will be necessary to measure inputs and losses to the pond during water exchange each day. It is also necessary to determine how much primary production is eaten by herbivorous animals and thus how much enters the detritus pool. This will not be easy to do, so an alternative procedure is to determine bacterial productivity, and estimate the amount of organic matter needed to sustain it. The rates of other processes such as ammonia release, sulphate reduction, oxygen uptake or carbon dioxide output will provide alternative estimates to check rates of transfer between compartments. From the quantitative model, it will be possible to examine the relative importance of different pathways, and to estimate the rates for pathways where no data are available.

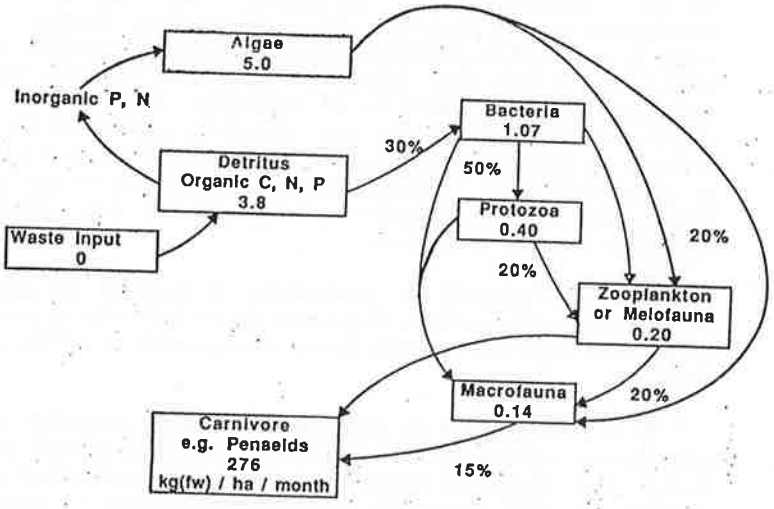

Fig. 1. A simplified conceptual model of detrital and microbial components of the food web in an aquaculture pond.

Bacteria are shown as only 1 compartment (with a central role to play) in Fig. 1, but this is a simplified concept. There are many different functional groups of bacteria, all interacting together. For a better understanding of their role in a pond, the activities and productivities of the major groups need to be studied. These groups are shown in Fig. 2 for a marine pond. Even this is a simplification of the complexities of the bacterial community, but it will be adequate at this stage.

Much organic matter is decomposed by aerobic heterotrophic bacteria in the water column and sediment surface. In the anoxic zone of the sediment, fermentation is the major process by which organic matter is broken down. Productivities of both these major groups of bacteria are determined with the thymidine method. The short chain fatty acids that are the final products of fermentation are respired to CO_2 by the sulphate-reducing bacteria.

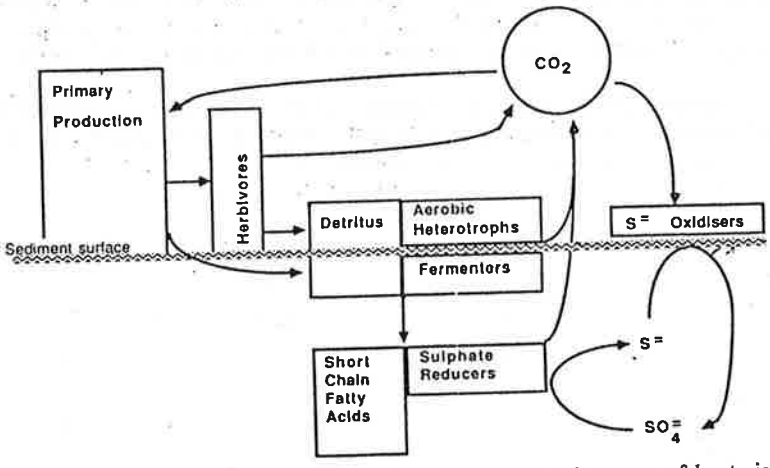

Fig. 2. Principal pathways for carbon flow and associated groups of bacteria in a marine aquaculture pond.

Most of the organic matter entering the sediment will finally be respired by these bacteria. Hydrogen sulphide is the main product of this process, and is important in the transfer of energy from lower depths of sediment to the sediment surface. Chemoautotrophic bacteria oxidise the sulphide, fixing CO_2 in the process. Thus a potentially rich source of organic matter is available for animals at the sediment surface. Chemoautotrophic production of bacterial biomass could be possibly 10% to 20% of the original organic carbon entering the sediment and is, therefore, a significant process (Howarth, 1984).

Growth Efficiencies

An area of microbial ecology where more research is needed is the growth efficiency of the various groups in the bacterial community. The growth efficiency is the amount of carbon converted into bacterial biomass divided by the total amount of carbon utilized by the bacteria; it is expressed as a percentage. Energy units

may also be used. Growth efficiencies may vary considerably with the species composition of the bacterial community, the composition of the organic matter and other factors such as oxygen, ammonia and phosphate concentrations.

Although the growth efficiency of any one species of bacterium may be low, the overall efficiency of a mixed community could be high. In sediments many bacteria may secrete large amounts of organic carbon in the form of low molecular weight compounds or as extracellular polymers. Their growth efficiency would, therefore, be low, but, because other species utilise that organic matter, the overall efficiency would be higher than that for individual species.

Quantitative studies are needed on the overall growth efficiency of bacterial communities in the water column and sediments, as well as in oxic and anoxic environments.

2

MEASUREMENT OF GROWTH RATES OF HETEROTROPHIC BACTERIA IN AQUATIC SYSTEMS

Introduction

At present, the most useful method for estimating growth rates of heterotrophic bacteria is the measurement of rates of DNA synthesis with tritiated thymidine. Most, but not all, heterotrophic bacteria are able to take up thymidine, so the method will underestimate growth rates. For very detailed or critical studies it would be necessary to obtain an estimate of the proportion of bacteria that do take up thymidine in the particular environment being studied.

Thymidine has been selected as a precursor, because it is used almost entirely for DNA synthesis only; it is not incorporated into other macromolecules. Microalgae and fungi cannot incorporate thymidine into DNA, because they lack a necessary enzyme; namely thymidine kinase (Fig. 3). Protozoa cannot take up thymidine at rapid rates at nanomolar concentrations and incorporate it into DNA. Thus labelling of DNA by tritiated thymidine is specific to bacteria.

Once replication of DNA has been initiated, the synthesis usually proceeds at a fixed rate until it is complete. Thus it is possible to carry out short term (usually 10 to 15 minutes) experiments with sediment or water without affecting rates of DNA synthesis. But changing the environment of bacteria will bring about changes in the rates of initiation of DNA synthesis and this will be evidenced by changed rates of thymidine incorporation after a period of time. It is necessary, therefore, to carry out experiments as soon as samples are collected, *i.e.* at the side of the pond.

Biochemistry of Thymidine Incorporation into DNA

Thymidine is readily incorporated into DNA via a salvage pathway, but in some bacteria the incorporation stops after a short time due to breakdown of thymidine (O' Donovan and Neuhard, 1970). *De novo* synthesis proceeds via dUMP directly to dTMP (Fig. 3). Catabolism of thymidine starts with conversion to thymine and ribose-1-phosphate by the action of an inducible phosphorylase. The best radioactive label is (methyl-^{3}H) because

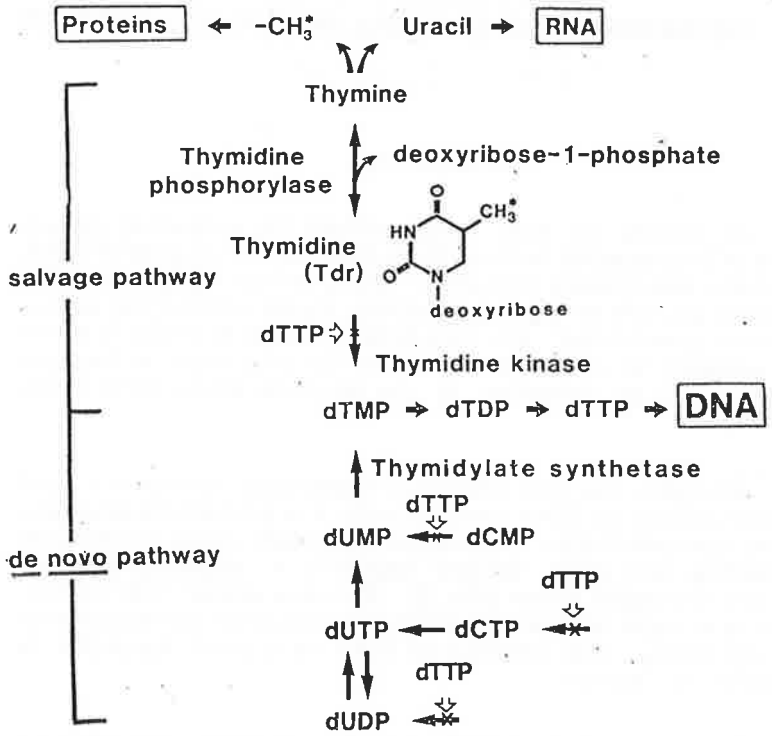

Fig. 3. Some pathways of thymidine metabolism. The asterisk shows the position of tritium labelling in thymidine. Sites of feedback inhibition by dTTP are indicated (adapted from Kornberg, 1980).

subsequent conversion to uracil removes the label. The tritiated methyl group can be transferred to a wide variety of compounds, but DNA is not labelled, as demonstrated in microorganisms that lack thymidine kinase (Fink and Fink, 1962). (2-^{14}C) Thymidine, on the other hand, does label DNA after catabolism, because the label is retained in the resulting uracil (Grivell and Jackson, 1968).

The absence of tritium incorporation into DNA in some eukaryotic microorganisms led Grivell and Jackson (1968) to show that these organisms lacked thymidine kinase. As Kornberg (1980) pointed out, thymidine meets reasonably well the criteria for pulse labelling. These are that the precursor should be rapidly and efficiently taken up by bacteria, be stable during uptake, be converted rapidly into the nucleotides and specifically label DNA with little dilution by intracellular pools. He also outlined pitfalls in its use, of which some are particularly relevant to environmental studies and are discussed in detail below.

Thymidine is converted to dTMP by thymidine kinase (Fig. 3). This enzyme must be present for labelling of DNA to occur to a significant extent. Thymidine kinase was thought to occur in most organisms (Kornberg, 1980), but some groups of microorganisms are now known not to contain it. These include fungi *(Neurospora crassa, Aspergillus nidulans* and *Saccharomyces cerevisiae)* and *Euglena gracilis* (Grivell and Jackson, 1968) and a number of cyanobacteria (blue green algae) (Glaser *et al.*, 1973). It is also absent from the nuclei of various eukaryotic algae, but may be present in chloroplasts although the amount of label incorporated from tritiated thymidine into chloroplast DNA was slight and required hours or days of incubation to be shown by autoradiography (Stocking and Gifford, 1959; Sagan, 1965; Steffensen and Sheridan, 1965; Swinton and Hanawalt, 1972).

We have been unable to obtain significant incorporation of (methyl-^{3}H) Tdr into DNA of four species of marine microalgae *(Thalassiosira, Isochrysis, Platymonas* and *Synechococcus)* which suggests that they lack thymidine kinase (Pollard and Moriarty, 1984). As there are no reports of the presence of thymidine kinase in the nuclei of small eukaryotic algae, fungi or cyanobacteria, it seems reasonable to generalize and conclude that this salvage pathway is lacking in all members of these groups of microorganisms. The lack of thymidine kinase in blue-green algae and many eukaryotic microorganisms, is a considerable advantage for studies on heterotrophic bacterial production in the marine environment. Protozoa probably do contain the enzyme (Plant and Sagan, 1958; Stone and Prescott, 1964), but as explained below their contribution to labelled DNA in short term experiments is probably small. Thus the use of thymidine provides specific information about the growth of heterotrophic bacteria that has not been available previously.

Most bacteria that lack thymidine kinase are mutants specially selected for biochemical studies. Two wild type strains of *Pseudomonas* have been reported not to incorporate thymidine into DNA (Ramsay, 1974). The technique used to demonstrate this

was autoradiography, which is insensitive compared to liquid scintillation counting of purified DNA. Ramsay's results could mean that these bacteria lacked thymidine kinase, or that they had a deficient membrane transport mechanism. A few species of *Pseudomonas* have been found not to utilize thymidine, probably due to a deficient cell membrane transport system (Pollard and Moriarty, 1984). Fuhrman and Azam (1980) have found good agreement between bacterial growth rates in seawater measured by the incorporation of thymidine and by counting the increase in cell number. The results of an autoradiographic study on bacteria in seawater support the view that most aerobic marine heterotrophic bacteria can utilize thymidine (Fuhrman and Azam, 1982). Anaerobic bacteria with strict and limited nutrient requirements may not be able to utilize thymidine, particularly if they can transport only a limited range of metabolites. *Desulfovibrio*, for example, does not appear to be able to utilize exogenous thymidine (G.W. Skring, per comm.)

The possibility that some bacteria in seawater may be unable to incorporate thymidine into DNA means that estimates of bacterial productivity may be too low, but this disadvantage is considerably outweighed by the advantages of using thymidine to measure DNA synthesis over other techniques for estimating growth rates of bacteria in natural populations.

Kinetics

Bacteria, with their active transport systems, take up organic molecules much more rapidly than do algae or protozoa and can utilize nanomolar concentrations of organic molecules in their environment more effectively than algae or protozoa (Wright and Hobbie, 1966; Fuhrman and Azam, 1980). Thus in a short time period (e.g. 10 min. at 25°C, 20 min. at 15°C) tritiated thymidine should be taken up preferentially by bacteria in a mixed community.

Uptake of labelled thymidine by organisms should not be confused with incorporation into DNA, although in bacteria the latter may be the main fate of labelled thymidine assimilated intact (Hollibaugh *et al.*, 1980; Fuhrman and Azam, 1980). As mentioned above, thymidine is readily incorporated into DNA in bacteria, but thymidine phosphorylase soon converts thymidine to thymine and deoxyribose-1-phosphate (Fig. 3). Labelled thymidine concentration within cells may be rapidly depleted, so it is important to measure the rate of label incorporation into DNA and not simply label uptake. In our work with sediments we have found that label incorporation into DNA proceeds linearly for 5–8 min. at high temperatures (27°–31°C) and 20–30 min. at lower temperatures (15°–18°C) in sediments, and over 1 hr in seawater (Fig. 4).

We had interpreted the change from the linear rate to be due to adsorption of thymidine by clay in sediments, but although this undoubtedly is a factor, degradation by thymidine phosphorylase rates may also have occurred. Experimental studies to determine growth rates must be carried out in the initial linear period of incorporation of label into DNA. Uptake of thymidine by cells and incorporation into TCA–insoluble fractions are different processes, probably with different kinetics which may be uninterpretable in a mixed population.

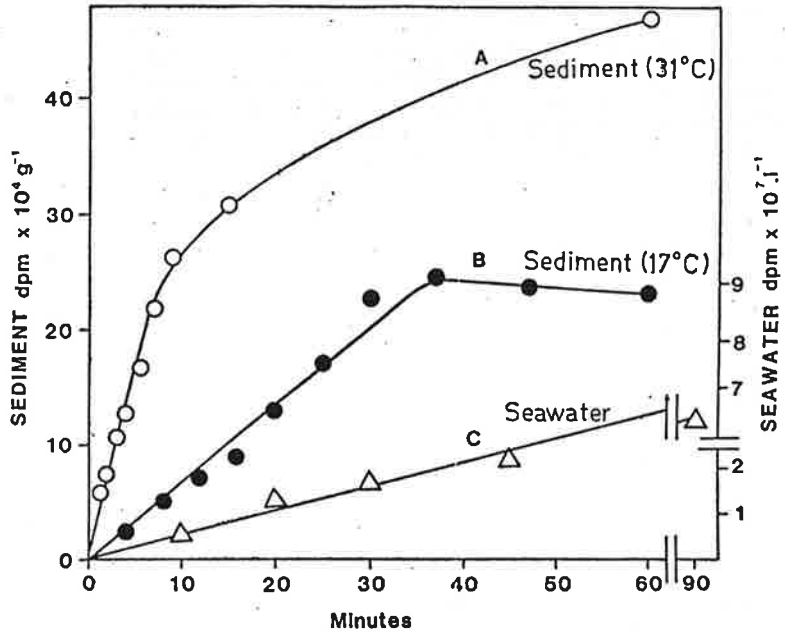

Fig. 4. Time course of incorporation of tritiated thymidine into DNA (from Moriarty and Pollard, 1981, 1982).

The kinetic studies (Fig. 4) show that thymidine is very rapidly taken up and is incorporated into DNA in less than 1 minute. We presumed that this was indicative of bacterial activity (Moriarty and Pollard, 1981), because protozoa, the other main group of microorganisms with thymidine kinase, are generally particulate feeders and probably would not have membrane transport mechanisms that are as efficient as those of bacteria.

A time course should be the first experiment carried out, as it is a good check on technique. If values are widely scattered in the first 15 minutes or so, problems in sampling, pipetting or washing may be the cause. Variable values can result from the final step with heating in 3% TCA if much water is lost by evaporation, or particles of filter or sediment are transferred to the scintillation vial. There are other reasons for carrying out a kinetic study besides the practical ones listed above. It is necessary to select an incubation period long enough to give an incorporation of tritium into DNA that is well above background adsorption; preferably at least 10 fold higher. Errors due to variable backgrounds are thus minimised.

The incubation period should be short enough to ensure that DNA is the main macromolecule that is labelled. Degradation of tritiated thymidine in the cell will eventually lead to labelling of all cellular components, but this occurs more slowly than DNA synthesis in growing bacteria. Where bacteria are not growing, degradation could be very marked. Thus a time course experiment to determine the rate of labelling of DNA itself is needed. For tropical water bodies, 15 minutes is generally a suitable time for incubation of water samples and 10 minutes for sediment.

3

ISOTOPE DILUTION

The specific radioactivity of exogenous thymidine is diluted during incorporation into DNA, primarily by *de novo* synthesis of dTMP (Fig. 3). A technique for measuring the dilution of label from an exogenous precursor during synthesis of a macromolecule is to add different quantities of unlabelled precursor as well and to measure the effect on the amount of label actually incorporated into the macromolecule (Forsdyke, 1968). This technique worked well with bacterial populations in sediments (Moriarty and Pollard, 1981). A plot of the reciprocal of isotope incorporated into DNA against total amount of thymidine added is extrapolated to give the amount of dilution of isotope in DNA itself (Fig. 5). The negative intercept on the abscissa is used to determine the specific activity of tritiated thymidine actually incorporated into DNA.

This technique measures the dilution of labelled thymidine in dTTP (the final precursor for DNA synthesis) by all other precursors of dTTP, including *de novo* synthesis. Provided DNA is purified before counting radioactivity that is incorporated, it doesn't matter if only a small proportion of the thymidine that is taken up by cells is used for DNA synthesis, because only the dilution of isotope in thymidine pools that are actually being used for DNA synthesis in growing cells is measured. Thus this method is not subject to errors inherent in trying to extract and quantify nucleotides from cells.

These problems with measuring the amount of isotope dilution by *de novo* synthesis of dTMP can be avoided by using a high enough concentration of thymidine to supply all the thymine required for DNA synthesis, in which case *de novo* synthesis is switched off by feedback inhibition (Moriarty, 1986).

Isotope dilution should be minimised or eliminated. This can usually be done by having a final thymidine concentration of

20 nM in water or by using 2 nmol/ml of sediment (dry weight 0.5 g). For aquaculture ponds, 0.5 nmol thymidine in 0.1 ml of a diluted slurry is satisfactory. The slurry is made by taking the upper 0.5 cm from a 25 mm diameter core and diluting it with 10 ml of filtered pond water.

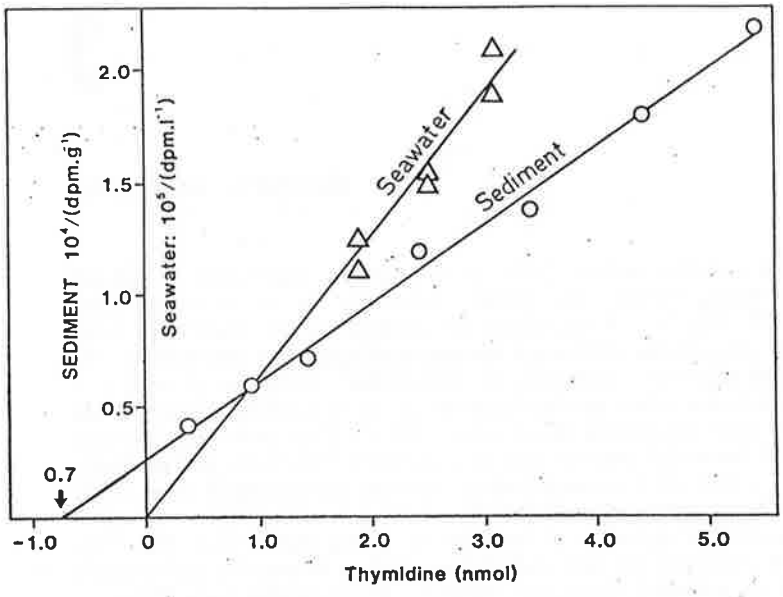

Fig. 5. Isotope dilution plots for incorporation of tritiated thymidine into DNA (from Moriarty and Pollard, 1981).

If the rate-limiting step for incorporation of tritiated thymidine into DNA is the activity of DNA polymerase, then isotope dilution can be prevented. If, however, the rate limiting step is prior to TMP synthesis, e.g. uptake into the cell, isotope dilution will occur, but will not be detectable. At present, it seems that there are not many heterotrophic bacteria in which uptake is limiting. It would be worthwhile carrying out experiments similar to those of Riemann *et al.* (1986) to check the conversion factor and thus indirectly to establish whether isotope dilution is likely to be a problem.

4

DETAILED PROCEDURES

a. Storage of tritiated thynmidine

For 3 to 4 months: store as aqueous solution (sterile) with 2% ethanol. (Decomposition rate : 4% per month). For longer times: store as 70% ethanol solution. In this case, remove ethanol before use by evaporation and reconstitute thymidine in distilled water (sterile). Ethanol helps prevent the decomposition of thymidine that is caused by free radicals generated during radioactive decay.

b. Calculating amount of tritiated thymidine

For water : use 20 nM = 0.1 nmol in 5 ml pond water

For sediment : use 0.5 nmol in 0.1 ml of diluted slurry

Thymidine will be supplied at given specific activity and concentration:

e.g. 18.4 Ci/mmol; 1 mCi/ml

thus 18.4 μCi = 1 nmol; 1 μCi/μl

therefore 0.1 nmol = 1.84 μl

Shortly before experiments are undertaken, withdraw total amount needed and dilute it 20 times with filtered distilled water.

c. Labelling

Draw up a working sheet, showing tube numbers, volumes of solutions to use, incubation time periods, etc. Plan atleast 2 or 3 blanks for each type of sample. Collect extra samples for direct counting with acridine orange (AODC no).

Example

Tube No.	Sample	Time	Comments	AODC No.
1	Surface water	Blank	e. g. time of day temperature	1
2	"	Blank		
3	"	15 min.		
4	"	15 min.		
5	"	15 min.		
6	Bottom water	Blank		
7	"	"		
8	"	15 min.		
9	"	15 min.		
10	"	15 min.		

d. Field kit

All experiments must be carried out at the pond site. Prepare a kit containing all necessary sampling equipment for water and sediment; pipettes, water bath, test-tube racks, ice container, solutions and dispensers for stopping bacterial activity or preservation.

A boat may be necessary for collecting sediment and water. Syringes and a syringe filter assembly are useful for obtaining filtered pond water on the site.

e. Procedure

Carry out experiments and store samples on ice or in a refrigerator until filtering is carried out. Detailed procedures are as follows:

Part I : Detailed procedures for standard measurement of rates of DNA synthesis in water column with TCA

1. Collect water samples; dispense 5 ml into bottles. Bottles should be teflon, polycarbonate or polypropylene.
2. Add 5 μCi (5-methyl-3H) thymidine (about 25 Ci/mmol) at known time intervals (see note i below). Store tritiated thymidine in 2% (v/w) ethanol; the ethanol does not need to be removed prior to assay.
3. Incubate at *in situ* temperature for 5–30 min. (see note ii below).

4a. Stop by adding 0.2 ml 37% formaldehyde containing 0.1 g thymidine per 100 ml and buffered about pH 8 (sodium tetraborate). Store cold until water can be filtered.

4b. Filter through polycarbonate (or cellulose nitrate) membrane filters (0.3–0.45 μm pore size)

5. As soon as filtration is complete, add 5 ml of 80% ethanol and draw it through. Repeat once, then wash with 1–2 ml of ice-cold 3% trichloroacetic acid (TCA) to the filter. Repeat cold TCA wash 4 times.

6. Remove filter, place in centrifuge tube and store if necessary. If backgrounds are high, try cutting out ring around edge that was under filter holder.

7. Add 2 ml 3% TCA, cap the tubes, heat at 90°C for 30 min. and then cool and centrifuge if particulate matter is noticeable.

8. Take 0.5 ml, place it in a mini-vial and add 4 ml of water-miscible scintillation fluid and count.

Notes: i. This amount of isotope should be adequate to overcome isotope dilution. For aquaculture ponds, 20 nM thymidine is usually adequate. If detritus is present, upto 50 nM may be needed. A preliminary isotope dilution experiment should be carried out for any new environment that is investigated.

ii. The incubation time should be determined by a preliminary time-course experiment. It should not exceed the time period during which the rate of DNA synthesis is linear. For tropical waters, 10 min is usually enough; colder temperate waters may require 20 – 30 min.

iii. Blanks should be prepared by adding formalin before tritiated thymidine.

Part II : Detailed procedures for measurement of isotope dilution during DNA synthesis in sediments

1. Shortly before use, set up isotope dilution series in polypropylene centrifuge tubes by adding the following amounts of a 100 μM

thymidine solution: 0, 5, 10, 15, 20 µl. Add 25 µCi of (5 methyl-3H) thymidine (approximately 25 Ci mmol^{-1}). Keep cold, but not frozen (see note i below).

2. Collect sediment, mix and dispense even amounts (about 10–50 mg dry weight) into tubes at known time intervals. Mix, incubate in water bath at *in situ* temperature for 5–15 min. (The time is temperature dependent; see note ii below).

3. Stop reaction with 10 ml 80% ethanol (ethanol 80 ml, water 20 ml, Na Cl 2 g, thymidine 0.01 g). After storage, remove the ethanol by centrifuging.

4. Add 2–5 ml 80% ethanol, mix and filter (see Part 1, 4b); wash out tube with more ethanol.

5. Wash filter with 2 ml 3% ice-cold TCA; repeat 4 times.

6. Transfer filter to centrifuge tube; add 2 ml 3% TCA; cap tubes; heat at 90°C for 30 min. Cool and centrifuge.

7. Transfer 0.5 ml to mini-scintillation vials (or 1 to 2 ml to large vials). Add water-miscible scintillant fluid and count radioactivity. Be careful not to transfer any sediment.

8. Blanks are prepared by mixing thymidine and ethanol before adding sediment.

Part III : Detailed procedures for standard measurement of DNA synthesis in sediment

1. Use stock (5-methyl-3H) thymidine with a specific activity of about 25 µCi mmol^{-1}, stored in 2% (v/v) ethanol. Add 25 µCi of tritiated thymidine diluted to about 100 µl. (see note i).

2. Collect sediment in small cores (e.g. 2–3 cm plastic tubing). Cores should not be stored; the assay should be carried out as soon as possible after collection.

3a. Make slurry of the top 0–5 mm with 10 ml of pond sediment (10–50 mg). Take 100 µl (0.1 ml) for each assay, including 2 blanks (add ethanol first).

3b. Repeat for deeper sediment depths if desired.

3c. For sandy sediments, do not make a dilute slurry, it is too hard to dispense. Use 1 ml syringes as corers and take 2–3 mm depth intervals.

(Alternatively, collect cores in small syringes and expel individually into centrifuge tubes set up as in No. 1 and proceed).

4. Incubate at *in situ* temperature for predetermined time (see note ii below). Stop reaction by adding 10 ml ethanol : water : Na Cl: thymidine (80 ml: 20 ml: 2 g: 0.1 g).

5. Proceed as in 3, Part II above.

6. Blanks should be prepared by mixing ethanol with the thymidine before adding sediment.

Notes : i. This is a suggested range of thymidine concentration. To prevent isotope dilution, about 1 nmol thymidine is needed per 0.2 g dry weight of sediment. I have found this to be satisfactory for a variety of sediments.

ii. For sediments with a surface temperature of about 27–30°C, 5 min. may be enough. For temperatures around 15–25°C, 15–20 min. should be adequate. Longer times may be needed for colder temperatures. A time course should be carried out first.

iii. The recovery of DNA may be measured by adding a known amount of bacteria labelled with ^{3}H–Tdr in culture during log phase. Centrifuge culture (6000 x g, 20 min.); resuspend in seawater and formaldehyde (1 % v/v and sodium tetraborate pH 8-9 (25 mg/100 ml). Centrifuge again. Resuspend in same solution, use for recovery experiments – check total dpm by filtering and washing with cold TCA as for seawater (p. 1).

iv. For short term incubations, there is no significant difference in results between anaerobic sediments that are mixed briefly as described here and anaerobic sediments that are retained in cores and injected.

v. Check for label in protein in some samples by adding perchloric acid (12 m) to final concentration of 0.5 M; heat at 100°C for 30 min; centrifuge. Count supernatant (DNA only; protein precipitates).

ADDENDUM

With some sandy sediments, recovery of DNA is poor with the method in Part II, procedure steps 4 and 5 on page 16.

If the recovery of DNA from cultured cells (note iii, page 17) is low, use one of the following procedures (Pollard, 1987).

Part IV: Alternative procedures for extracting DNA from sediment

A is the best procedure, but is time-consuming. B is simpler, but may give high background counts for some sediments.

1 and 2. As in Part II or Part III

A 3. Stop reaction with 10 ml 80% ethanol mixture (as in Part II). After storage, remove the ethanol by centrifuging. Then add 2 ml 0.6M NaOH and 10 mM thymidine. Heat at 100–105°C for 1 hr in an autoclave or 100°C for 2 hr in an oven.

A 4. Centrifuge (3,000–5,000 x g); take half the supernatant (1 ml if no evaporation losses in the oven).

A 5. Transfer supernatant to dialysis tubing (molecular weight cut-off : 6,000–8000). A series of samples can be kept in series in one long piece of tubing (Pollard, 1987).

A 6. Dialyze against running water overnight. Transfer to centrifuge tubes, centrifuge if any precipitate is present. Make up to known volume.

A 7. Transfer 0.5 ml to mini-scintillation vials (or 1 to 2 ml to large vials). Add water-miscible scintillant fluid and count radioactivity.

Note: For short term incubations of sediment where bacteria are growing rapidly, no protein is labelled. In oligotrophic sediments, where bacteria are starved, protein may be labelled. The procedure below, steps 4, 5, 6, should be followed to check if protein, which does not hydrolyze readily in 5% TCA, is present. If it is present, steps B 4, 5, 6 must be used for all samples.

B 1 and 2. As above.

B 3. Stop reaction with the 80% ethanol mixture. Centrifuge. Repeat twice to wash sediment. Then add unlabelled DNA (100 µl, 5mg ml^{-1}, 2 ml 0.6M NaOH. Heat and centrifuge as in 3 above.

B 4. Take half supernatant, chill on ice to 0°C. Add concentrated HCl (approx. 3 drops) until pH of about 1 is reached. Stand on ice for about 30 minutes while DNA precipitates.

B 5. Centrifuge at 6000 xg or filter (Whatman GF/F). Wash 4 times with ice-cold 80% ethanol.

B 6. Transfer pellet or filter to small tube, add 1 ml 5% TCA, heat at 100°C for 30 min. Cool.

7. As in 7, above.

5

DIRECT COUNTING OF BACTERIA BY FLUORESCENCE MICROSCOPY

Sample preparation

1. Collect water (5 ml) or sediment (0.5 ml).
2. Fix with formaldehyde (0.2% final concentration: add 300 µl formalin to 5 ml water).
3. Store in the refrigerator.

4a. Blend or homogenise water for about 0.5–1 min.

4b. Add 5 ml water to sediment, blend for 5 min. (or use ultrasonic probe for 0.5 min.)

Counting

1. Prepare acridine orange (AO) solution: 10 mg/10 ml filtered water, add formaldehyde to final concentration of 2%. (The AO must be high quality, specially prepared for fluorescence microscopy).
2. Prepare irgalan black solution: about 100 mg in 100–200 ml 2% acetic acid, (filtered water).
3. Filter wash water (use 0.2–0.3 µm filters for all filtration).
4. Stain filters (Polycarborate) in irgalan black.
5. Wash filter, place in filter funnel.

6. Add 1 ml filtered water and 0.5–3 ml pond water or 0.05–0.1 ml sediment. (The amount will need to be selected by trial – about 20 to 40 bacteria per grid field).

7. Add acridine orange: 10–20 µl. Stain for 1–10 min.

8. Turn on vacuum pump; draw water through and leave pump on while removing filter.

9. Place filter on a small drop of water on microscope slide. Add a drop of non-flourescent oil, coverslip then more oil.

10. Count about 10 grid squares (about 30 bacteria each, *i. e.* total of $10 \times 30 = 300$ bacteria) per filter; select grids randomly.

11. For one pond in each major experiment and each season, measure sizes of bacteria from random samples (about 200).

12. The intensity and colour of the fluorescence is dependant on the biochemical composition of the bacteria and the incubation time with the stain.

13. Calculation: calculate average ($\bar{X}$) standard error for No./grid.

$$\text{No./ml} = \frac{\bar{X} \times \text{area of filter}}{\text{area of grid}} \times \frac{1}{\text{vol. filtered (ml)}}$$

$$\frac{\text{area of filter}}{\text{area of grid}} = \text{constant factor}$$

6

CALCULATION OF RESULTS

i. Convert cpm to dpm.

Automatic scintillation counters can usually be programmed to convert counts per minute to disintegrations per minute. A set of quench standards is necessary.

ii. Subtract values for blanks.

iii. Calculate number of cells dividing (N) using formula given below:

I. *To calculate rate of bacterial division from rate of DNA synthesis or rate of tritiated thymidine incorporation.*

1. Proportion of thymine in 4 bases (A, G, C, T) average: $p = 0.25$.

2. Amount of DNA per cell (range 1.7 to 5 fg; average: $w = 2 - 2.5$ fg (10^{-15}g).

3. Amount of Tdr incorporated (mol Tdr x 318) (318 = molecular weight)

$$N = \frac{\text{mol Tdr} \times 318 \times 1/p}{w}$$

N = number of bacteria dividing

4. Thus N = mol Tdr incorporated x 6×10^{17}.

6×10^{17} is the theoretical factor; many measured values are higher, so we use 1×10^{18} which best fits experimental data. It is possible that mol Tdr incorporated is underestimated, due to isotope dilution and poor uptake of Tdr by some bacteria.

II. *To calculate mol T incorporated into DNA*

1. Isotope Dilution

Need to know the specific activity (S.A.) ^{3}H Tdr of the radioactive isotope (tritiated thymidine) after it is converted into DNA. Thus we need to determine the S.A. of TTP.

If we give the bacteria enough thymidine to stop them synthesising their own TTP, then the S.A. of TTP will be the same as that of the thymidine we add. That is, there will be no isotope dilution.

2. Calculate amount of radioactivity (distintegrations per minute = dpm) from counts pm (cpm).

3. mol T incorporated (T) $= \frac{\text{dpm}}{\text{S.A.}} = \frac{\text{dpm x mol}}{\text{ci x } 2.22 \text{ x } 10^{12}}$

picomol $= 10^{-12}$ mol

Usual units: p mol Tdr incorporated

$$= \text{dpm x } \frac{\text{mmol x } 10^{-3}}{\text{Ci x } 2.22} \text{ or x } \frac{1}{1000}$$

S.A. is usually quoted as Ci/mmol.
1 Ci $= 2.22 \text{ x } 10^{12}$ disintegrations per minute (dpm).

III. *Complete calculation*

No. of bacteria dividing per hour

$$= \text{Nh}^{-1} = \frac{\text{dpm}}{\text{S.A.}} \text{ x } 1 \text{ x } \frac{1.62}{\text{t}} \text{ x } 10^4$$

where t = incubation time in minutes

Derivation:

N $= 6 \text{ x } 10^{17}$ x mol Tdr incorporated into DNA

$$= 6 \text{ x } 10^{17} \text{ x dpm x } 1 \text{ x } \frac{10^{-3}}{\text{Ci x } 2.22 \text{ x } 10^{12}} \text{ x } \frac{60}{\text{t}}$$

where t = time (min.); $1 \text{ x } \frac{10^{-13}}{\text{Ci x } 2.22 \text{ x } 10^{12}} = \frac{1}{\text{S.A.}}$

N.B. A liquid scintillation counter with a set of quench standards for tritium is needed to calculate dpm from cpm.

IV. *Specific growth rate* (μ) is obtained by dividing the values for number of cells produced in a given time (N) by the total number present (N_t).

$$\mu = \frac{N}{N_t}$$

Generation time, or doubling time, (g) is the reciprocal of μ times the natural log of 2:

$$g = \frac{\ln 2}{\mu}$$

V. *To obtain productivity in terms of carbon* : the average cell volume (V) should be calculated from size measurements under the microscope. Assume specific gravity is 1.1 and carbon content is 22% of wet weight (Bratbak and Dundas, 1984).

Thus carbon content (C) = V x 1.1 x 0.22

e g. if V = 0.5 μm^3

$C = 0.12 \times 10^{-12}$ g

$= 1.2 \times 10^{-13}$ gC per cell

N x 1.2×10^{-13} = gC produced per hour (unit time).

Equipment List

Isotopes:	(methyl ^{3}H) thymidine (e.g. Amersham Cat. No. TRK 418 or CEA (France) Cat. No. TMM 199C).
Chemicals:	Ethanol, NaCl, thymidine, Trichloroacetic acid, Liquid scintillation counting fluid for aqueous samples and formaldehyde.
Other Items:	2–3 ml disposable plastic (polypropylene) syringes (for coring sediment), 10 ml disposable plastic tubes (e.g. blood sample vials from hospitals), General lab glassware, plasticware, pH meter, oven (100°C), autoclave or large pressure cooker, refrigerator, crushed ice, centrifuge (3000 – 5000 g), centrifuge tubes to fit, 10 ml and 20 ml capacity scintillation counting vials (5 ml capacity preferably), vacuum pump, filtration apparatus 47 mm, 25 mm diameter (separate for ^{3}H, ^{14}C), filters 47 mm, 25 mm (membrane, 0.45 μm pore size), racks to hold centrifuge tubes, etc. polypropylene bottles or tubes, 20 to 50 ml capacity and liquid scintillation counter.
Enumeration of Bacteria:	
	10–20 ml plastic vials or tubes, ultrasonic disruptor, acridine orange (BDH - highest quality available) irgalan black (black cloth, dye) (Ciba - Geigy), acetic acid, formalin, measuring cylinders, pipettes - 1 and 5 ml, automatic pipettes, filter apparatus (25 mm) single unit, Buchner flask, vacuum pump, filters: polycarbonate (Nuclepore) 0.2 μm poresize x 25 mm diameter, microscope slides, coverslips, forceps, non-fluorescent immersion oil, epifluorescence microscope (FITC filter set) (narrow band blue excitation, green emission) grid for eyepiece, eyepiece micrometer and calibration stage micrometer.

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