The physiological response of hermatypic corals to nutrient enrichment

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DECLARATION

I declare that the research discussed in this thesis has been carried out by myself unless otherwise cited or acknowledged. It has not, in whole or in part, been submitted for any other degree.

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ABSTRACT

Nutrient enrichment of tropical waters constitutes an increasing threat to the health and biodiversity of coral reefs. In order to manage these ecosystems effectively, the onset of nutrient pollution has to be closely monitored. This thesis examined the possibility of using some physiological responses of hermatypic corals as an early-warning bio-assay, to detect nutrient enrichment before reef deterioration has taken place.

To this aim, the physiology of the common branching coral *Porites porites* and the massive coral *Montastrea annularis* was studied both in the laboratory and on the reef under different nutrient conditions. By measuring the organic and inorganic productivity of corals and by constructing carbon budgets, it was hoped to relate differences in the fixation, allocation and utilisation of carbon to differences in nutrient regimes.

Nubbins of *Porites porites* and explants of *Montastrea annularis* were chosen as the experimental units. Nubbins were obtained by cutting coral tips (approx. 20 mm.), grounding their cut surface flat, and gluing them onto a perspex tile with cyanoacrylate glue. To obtain explants, a coral head was cored under a drill press fitted with a hole saw. Cores were then cut to fit, and sealed into polyethylene cups with underwater epoxy putty.

A new culturing system was developed to grow corals successfully in the laboratory under completely controlled and repeatable conditions. This system (the 'photostat') consisted of glass aquaria (30x21x18 cm) placed in a constant temperature water-bath under metal halide lamps. The aquaria were fitted with specially designed air lines and coral trays to maintain a strong water motion around the corals, independent of the rate of water-flow. A peristaltic pump ensured a daily water turn-over.

A new improved carbon budget methodology was developed by comparing the well established methods of Davies (1984) and Muscatine *et al.* (1984) on *Porites porites*. These methodologies differed in the measurements of zooxanthellae respiration rate (R_Z) and zooxanthellae growth rate (μ). $R_{Z,\,DAVIES}$ was found to be twice as small as $R_{Z,\,MUSCATINE}$ ($R_{Z,\,DAVIES}$ = 18.1 $\mu gC\ cm^{-2}d^{-1}$ vs. $R_{Z,\,MUSCATINE}$ = 33.1 $\mu gC\ cm^{-2}d^{-1}$), but this accounted for a difference of only 3% when R_Z was expressed as a percentage of the total daily carbon input. By comparison, a 25-fold difference between methods occurred in the component of carbon required for the daily growth of the zooxanthellae. Davies' method measured the net rate of zooxanthellae growth (μ_{NET}) from the increase in surface area, assuming a constant zooxanthellae population density. In this case μ_{NET} was only 1.65 $\mu gC\ cm^{-2}d^{-1}$. Muscatine's method measured the gross rate of zooxanthellae growth (μ_{GROSS}) from the mitotic index of freshly isolated zooxanthellae, assuming a duration of cell

division (t_d) of 11h. This accounted for a daily expenditure of 41.1 μgC $cm^{-2}d^{-1}$. The assumption of t_d might make this method prone to error. However, assuming that the measurement of μ_{GROSS} is correct, two new budget components had to be introduced to account for the large difference between μ_{GROSS} and μ_{NET}. These were expulsion and digestion, which had not been previously recognised. The latter had important consequences on the shape of the carbon budget because any carbon fixed in zooxanthellae that are digested constitutes an intrinsic part of total carbon translocated to the host. Therefore, Davies' budget, using μ_{NET}, overestimated translocation by the amount of carbon lost in expulsion, and Muscatine's budget, using μ_{GROSS} underestimated translocation by the amount contained in digested zooxanthellae. The new methodology incorporated these components. The carbon fixed by gross photosynthesis was still assumed to be the only source of carbon to the system. Carbon expenditure by the zooxanthellae was then divided into respiration, net growth and expulsion. The remaining carbon made up the component of total translocation, integrating the processes of translocation of fixed carbon from zooxanthellae to host, and digestion of zooxanthellae. Carbon was used by the host for respiration and growth, and any surplus was assumed to be lost from the symbiosis.

The effects of elevated nitrate on the budget components were tested for both *Porites porites* and *Montastrea annularis* in a month-long laboratory experiment. Corals were grown in the photostat under oligotrophic seawater and under three concentrations of nitrate (1, 5 and 20 μM). The response was the same in both coral species, and similar to previous reports on the effects of elevated levels of ammonia. Under higher nitrate concentration (5 and 20 μM), corals had a higher rate of photosynthesis per surface area, and a higher zooxanthellae population density. The freshly isolated zooxanthellae had a higher nitrogen, protein and chlorophyll content per cell when corals were grown in enriched seawater than in oligotrophic seawater. This is further evidence that zooxanthellae *in hospite* in oligotrophic seawater are nitrogen limited. The amount of carbon fixed in photosynthesis and available for translocation to the host was found to increase with nitrate enrichment. Hence, the overall organic productivity of corals appeared to be enhanced by nitrate. In contrast, the growth rate of corals measured by buoyant weighing was significantly reduced by nitrate enrichment. The average growth rate of *Porites porites* decreased from 1.24 mg $cm^{-2}d^{-1}$ (control) to 0.68 mg $cm^{-2}d^{-1}$ (20 μM NO_3), and that of *Montastrea annularis* decreased from 1.14 mg $cm^{-2}d^{-1}$ (control) to 0.51 mg $cm^{-2}d^{-1}$ (20 μM NO_3). It was suggested that, under elevated nitrate, the increased carbon requirements of the higher zooxanthellae population density promoted carbon competition between zooxanthellae and calicoblastic cells. Since zooxanthellae are in the gastrodermal cells closer to the pool of dissolved inorganic carbon in seawater, they had a competitive advantage over the calicoblastic cells, and calcification was reduced. This was defined as the 'endogenous carbon limitation of calcification'.

A similar experimental design was used to test the effects of phosphate enrichment on corals. Phosphate was added to oligotrophic water to give four treatments of 0, 0.2, 1 and 5 μM PO_4. Overall, no significant change in the organic productivity of corals was measured. Phosphate enrichment resulted in a significant reduction of the daily calcification rate of *Porites porites*, but not of *Montastrea annularis*.

In order to test if changes in water quality on the reef affected coral physiology, nubbins of *Porites porites* and explants of *Montastrea annularis* were grown for a month at three sites along a eutrophication gradient on the west coast of Barbados. The most oligotrophic site was the offshore one (OS) with low nutrient concentration and high light. The intermediate site (BRI) was characterised by higher nutrient concentration and high light. The most polluted site (SG) had both high nutrients and low light penetration. At the end of the exposure period corals of both species could be discriminated between sites on the basis of their physiological characteristics alone. Corals at OS showed some evidence of nitrogen limitation with a significantly lower zooxanthellae population density, lower nitrogen and chlorophyll content per zooxanthella, and lower photosynthetic efficiency than at the other sites. At BRI, corals attained significantly higher rates of gross photosynthesis and calcification, and their zooxanthellae contained significantly higher amounts of photosynthetic pigments. Corals at SG were characterised by a high zooxanthellae population density, high nitrogen and photosynthetic pigment content per cell, and relatively low primary productivity and calcification. Thus corals at each site were found to respond to both nutrient enrichment and irradiance levels in a combined manner.

The use of discriminate function analysis was pivotal in identifying those physiological variables that are most sensitive to nutrient enrichment ('primary' characters), and those that are highly dependent on irradiance and only secondarily on nutrient levels ('secondary' characters). Photosynthetic pigments' concentration constituted 'primary' characters. These were found to increase with nutrient concentration (from OS to BRI), and remain high as environmental degradation brought about a decrease in irradiance (from BRI to SG). The rates of gross photosynthesis, respiration and calcification corresponded to 'secondary' characters. These were related to environmental degradation by a single-humped curve, increasing with nutrient enrichment and decreasing again as the reduction in light developed. Thus corals in the most oligotrophic site (OS) and the highly degraded site (SG) could not be separated on the basis of 'secondary' characters alone. Therefore in contrast to expectations, this study found that a reduction in the growth rate or in the organic productivity of corals *per se* cannot be taken to imply the presence of stress factors.

The carbon budgets and the simple ratio of $dayP_{gross}/24hR_c$, were found to be entirely dependent on the rate of photosynthesis when corals from different nutrient environments were compared. This was the case because the budget expenditure components that were found to differ

significantly between treatments (for example, zooxanthellae population density), were very small when compared to photosynthesis. Therefore, in relation to nutrient enrichment, carbon budgets and the ratio dayP_{gross}/24hR_c were included with photosynthesis among the 'secondary' characters.

Among the physiological parameters measured in this study, the 'primary' characters and in particular the photosynthetic pigment content per surface area, were identified as the parameters with the highest potential for the development of a bio-assay to detect the onset of nutrient enrichment on coral reefs.

Chapter 1

GENERAL INTRODUCTION

1.1 Coral reefs

The average nutrient concentration of tropical oceans is characteristically low, particularly when compared to temperate waters or areas of upwelling (D'Elia & Wiebe, 1990; Mann & Lazier, 1991). Low nutrients limit the primary productivity of the water column despite the fact that tropical oceans benefit throughout the year from a high daily solar radiation, a euphotic zone greater than 100m, and warm seawater temperatures (Lewis, 1977; Fogg & Thake, 1987). It is in these oligotrophic waters that coral reefs are found to flourish (Achituv & Dubinsky, 1990). In obvious antithesis with the paucity of life of the surrounding water, coral reefs are highly productive ecosystems with the most stunningly diverse marine communities on earth.

Coral reefs occupy approximately 15% of the shallow continental shelf, and dominate the coastal marine environment of about 110 countries, most of which have been classed as low and low-middle income countries according to their GDP per capita (IUCN/UNEP/WWF, 1991; Davies & Brown, 1992).

The economic importance of coral reefs relies on their high productivity and on their biological richness or 'biodiversity' meaning the variety of life encompassing the genome, species and ecosystem levels. Coral reefs provide fish, molluscs, crustaceans and echinoderms for local consumption and for domestic and export markets. The importance of fisheries lies both in the magnitude of the catch and its market value, and in the opportunity it offers for employment in coastal areas with low income and few job alternatives (Russ & Alcala, 1989). Many organisms with little value for consumption are harvested for the aquarium and curio trade (Wood, 1985). Seaweeds are farmed in areas adjacent to coral reefs and collected for local consumption and for the agar and alginate industries (Batista de Vega, 1996). Commercial biotechnology has focused on the high reef biodiversity in its search for new genes and molecules from living organisms to develop into new food, pharmaceutical and industrial products. Coral reefs are mined to provide construction material in areas where no other terrestrial alternative is available. However, the benefits offered by coral reefs are not limited to what can be physically extracted from them. Coral reefs play a key role in the ever-flourishing tourist industry which has become the major revenue earner for many countries and promotes local and international investment in all related infrastructures (hotels, restaurants, diving facilities, airports, harbours etc.). The amount of revenue earned by tourism can be phenomenally high, as for example in the Great Barrier Reef

Marine Park, where an estimated 2,000,000 visitors spend in excess of A$ 1,000 million per year (Kelleher & Craik, 1993).

Lastly, coral reefs are essential in protecting beaches and coastal environments as initially recognised by Darwin (1842). Low-lying coastal areas are particularly affected by changes in near-shore hydrodynamics when the natural protection offered by coral reefs is damaged (Wells & Edwards, 1989; Bateson, 1995). Increased erosion, which is the most obvious direct result, coupled with the predicted rise in sea level brought about by global warming may be particularly disastrous for atoll nations such as the Maldives, Marshall Islands, Tonga, Tuvalu and Kiribati (Wells & Edwards, 1989; Hanley, 1994).

The scale with which human activities are dependent on reefs emphasises the need for the maintenance of healthy coral reef communities. Strategies for coastal management are being developed and implemented to enable the long-term utilization of natural resources in a sustainable manner to support human societies at an adequate quality of life (Craik *et al.*, 1990; Davies & Brown, 1992; Chakalall, 1994; Olsen, 1994).

1.2 Reef degradation

A coral reef is unique in being a biogenic structure produced by those very same organisms that occupy it, the hermatypic corals. In fact hermatypic corals are simultaneously the major living components of the coral reef ecosystem and the major contributors to the formation of the reef structure. This dual role of hermatypic corals has been amply acknowledged by the attention given to these organisms by both ecologists and geologists.

Reef accretion occurs only when processes of carbonate production (through skeletogenesis, mainly by hermatypic corals but also by crustose coralline algae and hydrozoan *Millepora* colonies) exceed those of carbonate erosion (mechanical erosion by waves and biological erosion by both borers and grazers). Any factor, be it natural or man-induced, that has a negative impact on hermatypic corals but does not inhibit the concurrent rate of carbonate erosion will disturb the dynamic balance between these antagonistic processes and have a detrimental effect on the whole coral reef ecosystem and in turn on all human activities benefiting from it.

Coral reefs are regularly subjected to many natural disturbances: hurricanes, cyclones, typhoons (Harmelin-Vivien & Laboute, 1986; Mah & Stearn, 1986; Aronson *et al.*, 1993; Lugo-Fernandez *et al.*, 1994), earthquakes (Cortes *et al.*, 1992), volcanic eruptions (Grigg & Maragos, 1974), ENSO events (Glynn *et al.*, 1988), freshwater floods (Byron, 1993) and prolonged extreme low tides (Loya, 1976; Fadlallah *et al.*, 1995) have all been observed to have devastating effects. Further reasons for concern about the status of reefs are provided by biological phenomena such

as the infestations by the coral predator *Acanthaster planci* in the Indo-West Pacific region (Endean & Cameron, 1990) or the spreading of the white band disease of *Acropora palmata* (Bythell & Sheppard, 1993) in the Caribbean region. The pathogen-induced die-off of the common sea-urchin *Diadema antillarum* across the whole Caribbean (Lessios *et al.*, 1984) has also been reported to have devastating effects to the reef community because of the sea-urchin function as a key herbivore.

In addition, there is the anthropogenic factor: the intense human development of coastal areas and coastal construction afflicts coral reefs with increased sediment loads (dredging, filling, deforestation and land erosion) and with chronic sources of organic (sewage and fertilisers), chemical (herbicides, pesticides, industrial effluents, desalination plants) and thermal pollution. Furthermore, over-exploitation of fish and invertebrate populations, coral mining, vessel groundings, coral collection, anchor damage and reef abuse by snorkellers/divers are some of the most common human activities that have a direct harmful impact on coral reefs. With the exception of a few illegal activities (dynamite and cyanide fishing), the solution to reef degradation does not lie in banning the human use of marine resources but rather in limiting it to a sustainable level within the framework of an integrated coastal management plan.

Since natural and man-made causes of degradation might be acting simultaneously, it is essential for reef managers to be able to discriminate between them. As suggested in the reviews by Johannes (1975), Pearson (1981), Brown & Howard (1985) and Kinsey (1985; 1988) the impact of any environmental stress is highly dependent on its duration, thus it is necessary to differentiate also between 'acute' and 'chronic' stresses. For example, a hurricane has a devastating but instantaneous impact and in the absence of other influences coral communities can be quickly re-established through the recovery of damaged colonies and the settlement of new recruits. Brown *et al.* (1990) reported on the full recovery of an intertidal reef flat that had shown pronounced degradation from high sedimentation loads over 11 months of dredging operations. In fact such acute disturbances could play an essential dynamic role by freeing space for settlement, (a limiting resource for recruits on well-established reefs), and thus maintaining a highly diverse coral community by disrupting the progress towards ecological equilibrium (Connell, 1978; Rogers, 1993). On the other hand, chronic disturbances (such as a prolonged exposure to increased nutrient levels, over-fishing, high turbidity and sedimentation) can shape coral diversity and distribution. For example chronic sedimentation has been correlated with reef degradation, coral mortality, loss of species diversity, reduction of coral calcification and reproductive output. The different tolerance capabilities of coral species to sediment deposition and burial play a key role in their zonation and distribution (Stoddart, 1969; Stafford-Smith, 1990). One could interpret the high siltation input of the Orinoco and Amazon river deltas as an extreme case of chronic

sedimentation resulting in the absence of coral reefs below latitude 5°N in the Western Atlantic (Achituv & Dubinsky, 1990).

1.2.1 Nutrient enrichment on coral reefs

The contribution of each natural/anthropogenic stress factor to reef degradation is location specific but some problems have a more globally important urgency. In this respect, nutrient enrichment and consequent eutrophication require special attention. In the late 1980s eutrophication was found to correlate with the degradation of coral reefs in more than 40 countries (UNEP/IUCN, 1988-1989) and the incidence of such reports continues to increase (Ginsburg, 1993).

The major nutrients involved in eutrophication are nitrogen and phosphorus mainly from domestic sewage and agricultural run-off. Sewage dumped on land through dry toilets, suck-well systems, shallow injection wells, and septic tanks reaches coastal waters through seepage, a particularly effective process on porous coral rock islands (Lewis, 1985). In some continental areas river discharges enhance nutrient transport so that effective management strategies have to include the condition of the entire catchment area (Goreau *et al.*, 1996). The effect of sewage discharges on coastal ecosystems is crucially dependent on the level of treatment and on the position of the outfall (Parnell, 1992; Grigg, 1994). Kanehoe Bay (Hawaii) is the best case study of the effects of sewage diversion (Marszalek, 1987) and deserves a detailed description.

Kanehoe Bay is a relatively sheltered bay known as "coral gardens" in the 1930s before undergoing major destruction from urbanisation, channelling and high sewage inputs (from two sewage outfalls). The coral community was replaced by one dominated by the green bubble alga *Dictyosphaeria cavernosa* and filter/deposit feeders. After 30 years of discharge, the outfalls were diverted offshore. Nutrient levels, turbidity, phytoplankton and filter feeders abundance decreased rapidly (Smith *et al.*, 1981) and by 1983 the benthic community responded with an increase in coral cover and a reduction of *D. cavernosa* to 1/4 of its previous abundance (Maragos *et al.*, 1985). No further coral improvement was observed in a repeated survey in 1990 (Hunter & Evans, 1993).

It is a well established fact that elevated nutrient levels enhance primary productivity of plankton and benthic algae, resulting in light attenuation and blooms. Algal blooms represent the biggest threat to coral reefs from nutrients, albeit indirectly. High plankton loads reduce light for photosynthesis and calcification of hermatypic corals and induce a shift in benthic community towards one dominated by particle-feeders, zooanthids, soft corals and sponges (Banner, 1974). Boring sponges, enhancers of the rate of dissolution of reefs, are most common on rubble (Holmes, 1996) and coalesced coral colonies (Cuet & Naim, 1992) in areas affected by eutrophication. Benthic algal blooms affect the invertebrate community at all stages of their life

history. Larvae of corals, sea-urchins and other invertebrates require suitable substrate to settle: encrusting coralline algae are known to facilitate metamorphoses (Morse *et al.*, 1988) but these are outcompeted by fleshy algae when nutrient limitation is lifted. In a study along a eutrophication gradient on the West Coast of Barbados, Hunte & Wittenberg (1992) found that the rate of coral settlement on artificial plates was inversely related to eutrophication while juvenile mortality was positively correlated with it (Wittenberg & Hunte, 1992). Hughes (1993) identified high algal cover in Jamaica as the cause of long-term coral recruitment failure, and Rogers *et al.* (1984) observed that high rates of recruitment were associated with low algal biomass. Also healthy adult coral colonies can suffer overgrowth by macro algae, mainly by broad-leaved ones such as *Ulva lactuca* (Woodley, pers.comm.) or *Dictyosphaeria cavernosa* (Banner, 1974) and Johannes *et al.* (1983) suggested competition with macroalgae as key factor in the latitudinal limits of coral reefs. All studies in which nutrient levels and benthic communities have been monitored have noted that increased nutrients are positively correlated with macro-algae abundance and inversely correlated to coral cover and coral diversity (Tomascik & Sander, 1987a; Cuet *et al.*, 1988; Lapointe *et al.*, 1992; Littler *et al.*, 1992; Naim, 1993; Tomascik *et al.*, 1993).

Conditions on the reef however are complex and the observed correlation between high algal cover/low coral cover and high nutrients does not directly imply that nutrients *per se* are the main cause of reef degradation. To substantiate the general theory of the effect of an acute stress on chronically stressed reefs, Kinsey (1988) used the case of eutrophication as a chronic stress. Well-developed reefs are expected to be quite resistant to chronic nutrient pollution but only if no other factors are afflicting them. This is because chronic eutrophication drastically impairs the recovery capability of a reef so that in its presence, the impact of any short-term acute stress (hurricane, low tides, *Acanthaster* etc.) will not be followed by a successful recovery and the benthic community will tend towards those species that are most favoured by high nutrient levels. Unfortunately the chances of a eutrophic reef to never encounter any acute stress are dangerously close to zero. Commonly, nutrient enrichment does not occur as a single perturbation on reefs: turbidity and increased sedimentation are common co-factors amongst many others. In particular algal growth is favoured when grazers are removed either by over-fishing or by pathogens. The latter case has been particularly well documented in the Caribbean (Hughes, 1993; Steneck, 1993) where the high populations of the top grazer sea-urchin *Diadema antillarum* suffered mass mortality between 1983-1984 (Lessios *et al.*, 1984; Lessios, 1988). The combination of high nutrient inputs and loss of grazers has a synergistic effect with devastating consequences (Allard, 1994).

1.3 Damage assessment on coral reefs

In view of the economic importance of coral reefs and the potential impact on all human activities that benefit from them (especially in areas of the world afflicted by extreme poverty and the lack of other resources), reef degradation is a very urgent issue. Nonetheless, there are considerable differences of opinion about the significance of observed changes on reefs. Too often, a genuine lack of knowledge about the complex reef ecosystem exacerbates these opinions into opposing theses which advocate either that no action should be taken unless there is irrefutable proof that the observed change is anthropogenically induced, or that any change observed has been induced by Man. Neither of these unbalanced alternatives can be of any use to the management of coral reefs.

Thus the development of methodologies for the assessment of the degree of reef degradation and for the identification of its causes is crucial.

1.3.1 Reef monitoring

Most studies involved with measuring reef degradation consist of quantitative/qualitative analyses of the composition and structure of reef communities. Many sampling techniques have been developed from the basic quadrat and plotless methods (Stoddart, 1969; UNESCO, 1984). These can be carried out directly by divers in the field or by the use of video and photography (Porter & Meier, 1992; Carleton & Done, 1995). The information collected for corals or for the whole benthic community is most commonly expressed as percentage cover or as indices of species diversity. At times these measurements are not correlated and, although species diversity is most often measured, there is some confusion about its degree of sensitivity to pollution (Brown & Howard, 1985; Brown, 1988). Such studies carried out in the past have become the baseline for comparative monitoring (Bell & Tomascik, 1993; Ogden & Ogden, 1993; Vicente, 1993) while permanent sites have been set-up for future long-term monitoring world-wide (CARICOMP, 1996). These temporal comparisons have yielded a good estimation of change but in most cases they lack information on environmental factors and therefore cannot be used to interpret the causes of change. On the other hand spatial comparisons have been carried out to correlate community structure with environmental factors. The first and most thorough study of this sort was carried by Tomascik & Sander (1985) who measured several key environmental parameters at 7 sites along the West coast of Barbados. They found a gradient of eutrophication and correlated it to benthic community structure, coral growth and reproduction (Tomascik & Sander, 1985, 1987a,b).

Whatever the method, interpretation of these studies is complex: while temporal studies are long-term, spatial comparisons have the problem that different sites might not have been subjected to

the same history of damage (Grigg & Maragos, 1974; Pearson, 1981). Most importantly, once the change has been observed, damage has already occurred.

1.3.2 Bioassays

What is urgently required is a sub-lethal measure of damage: a parameter that can be easily monitored and which occurs in direct response to a known stimulus, thus acting as an early detection mechanism of pollution (Brown, 1988). This search has brought about a shift in research interests from the analysis of damage at the community level, to that at the organism level.

A variety of bioassay techniques have been suggested to monitor the "health" of a coral reef community focusing on the physiology of the reef-building hermatypic corals: growth rates, carbon budgets, reproductive output, zooxanthellae loss, zooxanthellae division rates, production of stress proteins and tissue regenerating capacities (Brown, 1988; Davies & Brown, 1992). In all cases more research is required to test their potential value in response to specific stresses.

This thesis focuses on nutrient enrichment and aims to improve on the current knowledge of the physiology of corals subjected to high nutrient regimes. In particular, coral responses will be investigated by monitoring changes in skeletal growth rate and primary productivity and by constructing carbon budgets to evaluate the potential of these techniques as bioassay tools for the early detection of nutrient pollution on reefs.

1.4 Hermatypic coral physiology

The 'physiological status' of a coral is the product of a continuous flux of organic and inorganic substances between four compartments: seawater, zooxanthellae, host and skeleton. Complexity arises from the interaction between compartments and external stimuli through a series of feedback mechanisms. The essential features of coral physiology that relate to the flux of nutrients are depicted in Fig.1.1, and the numbers within the diagram are referred to in the following description of coral physiology.

1.4.1 Symbiosis

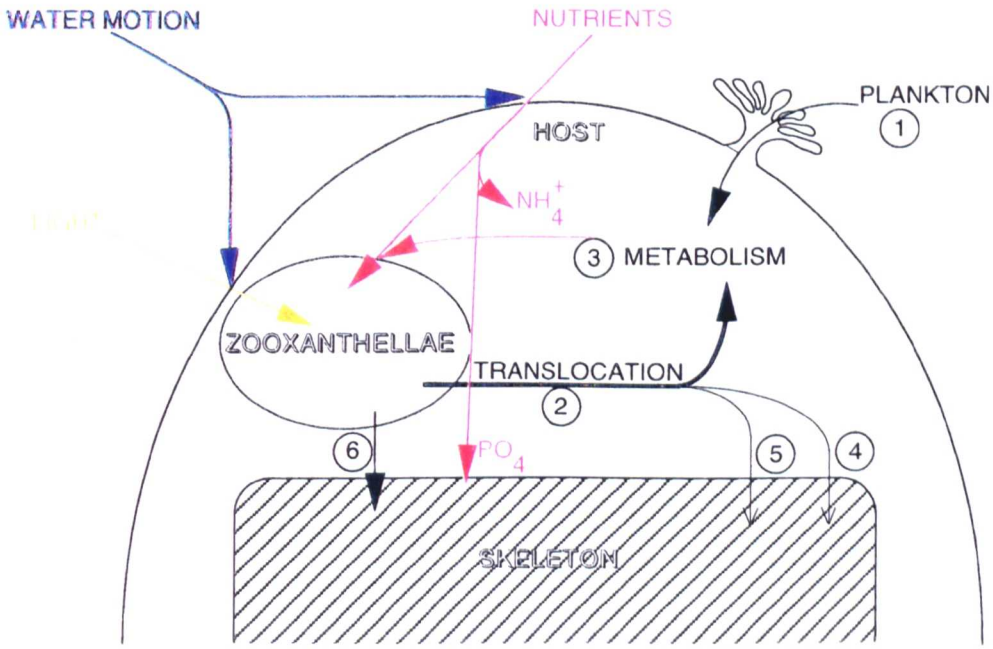
Cnidarians are very effective heterotrophic feeders **(1)** able to ingest a selection of food particles (including zooplankton and mucus-associated bacteria) using a combination of feeding strategies such as tentacle capture, ciliary movements and mucus filament entanglement (Porter, 1974; Lewis & Price, 1975).

The endosymbiosis with the dinoflagellate alga *Symbiodinium* spp. (zooxanthella), which separates reef-building hermatypic corals from other stony corals of the order Scleractinia, provides the host with a suite of reduced carbon molecules fixed during photosynthesis and translocated **(2)**. Hermatypic corals are not the only invertebrates to benefit from symbiotic association with dinoflagellates: within the phylum Cnidaria (Coelenterata) to which corals belong, the symbiotic association with zooxanthellae is widespread also among sea-anemones (Actinaria), jellyfish (Rhizostomae and Coronatae) and soft-corals (Alcyonacea). Symbiosis with dinoflagellates also occurs in clams Tridacnidae (phylum Mollusca) and clionids sponges (phylum Porifera) and although these associations differ at the cellular level, translocation of carbon-rich molecules from zooxanthellae to the host is the essential feature. In fact the role of the symbiosis in host nutrition is crucial to the success of these organisms and in particular to hermatypic corals in oligotrophic waters and is directly dependent on light intensity. Under optimal light conditions photosynthesis is maximal and the amount of photosynthate exceeds the respiratory requirements of both symbiotic components (McCloskey *et al.*, 1978; Muscatine *et al.*, 1984; Davies, 1991), thus ensuring host survival independently from external organic carbon sources. In conditions of reduced light intensity zooxanthellae maximise their photon-harvesting capacity by undergoing photo-adaptive processes similar to those of free-living dinoflagellates by changing the size and number of the photosynthetic units in their chloroplasts (Prézelin, 1987). Nonetheless colonies living at depth or in shaded shallow areas have drastically reduced photosynthetic production and translocation so that exogenous sources of carbon are required to maintain host respiration and growth (Davies, 1977; 1991; Muscatine *et al.*, 1984; McCloskey & Muscatine, 1984; Porter, 1985). The proportion of photosynthetically fixed carbon that can be translocated to the host is very high in symbiotic zooxanthellae because algal growth *in hospite* is slow in comparison to free-living dinoflagellates (Wilkerson *et al.*, 1988) even though primary productivity remains maximal. In fact, if zooxanthellae growth is restricted but photosynthesis is not, the fixed carbon that would otherwise have been utilised in cellular growth, accumulates and can leak from the symbiont to the host.

The host might be actively involved in enhancing the rate of translocation from the zooxanthellae via a 'Host Factor' identified as a set of free amino acids (Gates *et al.*, 1995). Translocation has been observed also in freshly isolated zooxanthellae, but it is abundant only in the presence of host homogenate (Muscatine, 1967; Trench, 1971c; Hinde, 1987, 1990; Sutton & Hoegh-Guldberg, 1990). Nevertheless the evidence in favor of a host factor remains equivocal (Davies, pers. comm.).

It has been suggested that the host is actively "farming" zooxanthellae for its own benefit derived both from translocation of photosynthate and from active digestion. The hypothesis that algae are digested *in hospite* has been discussed several times (Boschma, 1925; Yonge & Nicholls, 1931c;

Fig. 1.1 A diagrammatic representation of the physiological mechanisms involved in the organic and inorganic growth of a hermatypic coral colony in relation to environmental factors (light, nutrient concentration, water velocity). The energy requirements of corals are satisfied by the holozoic feeding of the host (1), and the photosynthetic activity of the zooxanthellae. Photosynthetically fixed carbon molecules are translocated from the zooxanthellae to the host (2) where they serve as an essential carbon source for metabolism (3). The primary production of zooxanthellae depends directly on light through photosynthesis, and on the flux of nutrients taken up from seawater and from the host cytoplasm. Photosynthesis plays a central role in the inorganic growth of the colony by directly enhancing the availability of carbonate ions for deposition(6), and by providing the host, via translocation, with essential molecules for the structural organic matrix of the skeleton (4) and for the energy demanding processes of skeletogenesis (5). Water velocity affects the thickness of the boundary layers present on the coral surface and thus affects the flux of inorganic carbon and nutrients from seawater.


WATER MOTION
NUTRIENTS
PLANKTON
1
HOST
NH_4^+
3 METABOLISM
ZOOXANTHELLAE
TRANSLOCATION
2
6
PO_4
5
4
SKELETON

Muscatine & Pool, 1979; Fitt & Trench 1983; Muscatine *et al.*, 1986), but a thorough investigation is still missing.

Porter (1976) proposed that different coral species are likely to rely on autotrophic and heterotrophic nutrition to different extents. In particular, the observation that the size of coral polyps is inversely correlated to their surface area to volume ratio prompted Porter (1976) to suggest that the skeletal morphology of corals has been developed through the evolutionary process of niche separation that maximises the use of the available resources. Thus, corals with large polyps and a low surface area to volume ratio (massive, solitary) are best suited for zooplankton capture while corals with small polyps and a high surface area to volume ratio (branching corals) rely more heavily on the photosynthetic capacities of their symbionts. It follows that the photosynthesis to respiration ratio is lower in massive than in branching corals (Porter, 1976).

Whatever their polyp size, the adequacy of zooplankton in satisfying the food requirements of corals has been the subject of contention (Lewis, 1977). Early work during the Great Barrier Reef Expedition (1928-1929) found that corals were highly specialised carnivores, with fast enzymatic digestion capacities (Yonge & Nicholls, 1931a) and suggested that zooplankton biomass might be sufficient to support the coral population (Russell, 1934). On the other hand, more recent estimates of zooplankton biomass and capture rate have failed to balance the energy requirements of corals (Johannes *et al.*, 1970; Johannes & Tepley, 1974; Porter, 1974), thus implying that all hermatypic corals are opportunistic heterotrophs receiving essential trace elements from zooplankton and obligatory autotrophs satisfying the bulk of their respiratory requirements with photosynthate.

The other heterotrophic sources of organic matter, including bacteria and detritus, are even more difficult to quantify *in situ*.

Recently Fabricius *et al.*(1995, 1996) found that the azooxanthellate soft coral *Dendronephthya hemprichi* obtained most of its carbon from phytoplankton and suggested that it could represent an important previously overlooked source of energy for all reef cnidarians.

As the host receives a direct benefit from the symbionts in the form of photosynthate, zooxanthellae benefit from the inorganic metabolites in the host cytoplasm **(3)**: this nutritional 'exchange' fuelled the concept of a mutualistic relationship between the two symbiotic partners. However the actual exchange mechanisms are elusive. Zooxanthellae are located in vacuoles within host gastrodermal cells and are tightly surrounded by host membrane so that any substance flux from/to zooxanthellae has to cross host tissue. When cnidarians are cultured in oligotrophic conditions their zooxanthellae appear to show physiological characters indicative of nutrient limitation such as a high carbon content per cell (Berner & Izhaki, 1994; Muller-Parker *et al.*, 1994a; Snidvongs & Kinzie, 1994) because the bulk of carbon fixed is used for carbohydrate

synthesis rather than for protein synthesis and growth. In addition, low nitrogen content per cell (Cook *et al.*, 1988; Snidvongs & Kinzie, 1994), low cell division rates (Cook & D'Elia, 1987; Wilkerson *et al.*, 1988), low clorophyll *a* content (Cook *et al.*, 1988; Hoegh-Guldberg & Smith, 1989; Dubinsky *et al.*, 1990; Snidvongs & Kinzie, 1994) and a high enhancement of dark carbon fixation by ammonium enrichment (Cook *et al.*, 1994) are specific signs of N limitation. Active acid phosphatases is used to identify P limitation (Jackson *et al.*, 1989).

Host heterotrophic feeding has a stimulatory effect on symbionts (Cook & D'Elia, 1987; Cook *et al.*, 1988) which results in the reversal of N limitation and increased zooxanthellae population density. Thus it is suggested that the effect of host feeding on the zooxanthellae population is mediated by the enhanced ammonia levels produced by the catabolic activity of the host. When fed on *Artemia*, colonies of the branching small-polyped coral *Stylophora pistillata* appear to be able to sustain a larger zooxanthellae population density than if starved (Dubinsky *et al.*, 1990) although in a similar experiment, Muscatine *et al.* (1989) were not able to replicate the result. The most striking effect of feeding has been obtained with symbiotic anemones: biomass of zooxanthellae can double when the host is fed abundantly (Janssen & Moeller, 1981).

In view of the N limitation of zooxanthellae *in hospite*, the fact that the photosynthate translocated to the host contains not only carbohydrates but also amino acids (Muscatine & Cernichiari, 1969; Trench, 1971b; Markell & Trench, 1993) seems paradoxical, but in a steady-state model of the flux of C and N between symbiotic partners Falkowski *et al.* (1993) predicted a translocate C:N ratio of as high as 25, thus suggesting that N translocation is discriminated against.

In relation to N metabolism, the flux of molecules between host and zooxanthellae could lead to either N recycling (Lewis & Smith, 1971; Muscatine & Porter, 1977), or N conservation (Rees & Ellard, 1989). In the 'recycling' hypothesis, N produced by host metabolism is taken up by the zooxanthellae, fixed into amino acids and translocated back to the host. In the 'conservation' hypothesis the release of photosynthate by the zooxanthellae is used for respiration and gluconeogenesis while amino acids are diverted preferentially to protein synthesis so that host deamination can be minimised (Rees, 1989).

1.4.2 Skeletal growth

The skeleton of hermatypic corals is a porous ordered mesh of aragonite crystals deposited by calicoblastic endodermic cells on a structural organic matrix **(4)**. The organic matrix dependency of crystal formation allows for the genetic determination of the skeletal micro-architecture relied upon by coral taxonomists. One component of the matrix comprises individual sheaths that envelope each forming aragonite crystal: they are present only transiently at the growth surface and Johnston (1980) suggested that they are the site of crystal nucleation.

For deposition to occur, calcium and carbonate ions from seawater have to reach the site of nucleation and accumulate into a supersaturated solution. Simple diffusion of Ca^{2+} from seawater across the epidermal layers to the site of deposition could occur only if the intracellular concentration of Ca^{2+} was higher than that in the supersaturated solution. This has been ruled out because of the overwhelming evidence that low and well regulated levels of Ca^{2+} are critically important for many cell functions such as mitochondrial activity, enzymatic functions, membrane transport etc. (Goreau & Bowen, 1955; Simkiss, 1976; Kempe & Kazmierczak, 1994). Furthermore, the kinetics of calcium transport do not fit with the simple diffusion hypothesis (Chalker, 1975; Chalker & Taylor, 1975).

Thus calcium transport during calcification must involve some energy requiring process. Among the mechanisms for active transport **(5)** proposed by Simkiss (1976), the hypothesis of paracellular transport of ions within vesicles (Johnston, 1980) was the most likely but it has been recently contradicted by Tambutté *et al.* (1996). Instead, they hypothesised that Ca^{2+} crosses the upper tissue layer by diffusion via an intercellular pathway, thereafter being actively taken up by the calicoblastic epithelium and deposited in association with the organic matrix.

The carbonate moiety of skeletal aragonite is derived from the same source as for photosynthesis: both total seawater dissolved inorganic carbon (DIC) and respired CO_2 (Pearse, 1970). In seawater dissolved CO_2 is hydrated to form H_2CO_3 (acidic), in continuous equilibrium with bicarbonate and carbonate (alkaline) as shown below:

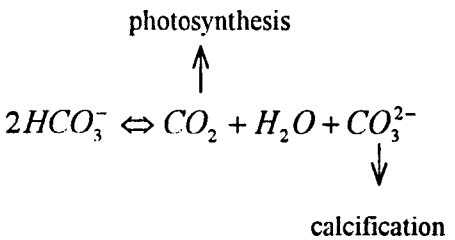
$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \Leftrightarrow CO_3^{2-} + 2H^+$$

In addition, the proportion of each form varies with pH. The addition of CO_2 lowers pH and the amount of CO_3^{2-} available is correspondingly decreased, while the removal of CO_2 increases pH , and the amount of CO_3^{2-} available will be increased thus favouring precipitation.

The predominant form of DIC at seawater pH of 8.2 is bicarbonate (1800 μM) which requires either facilitated diffusion or active transport to cross cell membranes. On the other hand, dissolved free CO_2 can diffuse into cells but at pH 8.2 it is present in low concentration (10 μM). The extracellular conversion of HCO_3^- into CO_2 which occurs according to its equilibrium constant, pH and temperature was found to be insufficient to sustain optimal photosynthetic rates (Benazet-Tambutté *et al.*, in press) in *Anemonia viridis* and by extrapolation should not be sufficient in hermatypic corals where carbon is simultaneously required for both optimal photosynthesis and calcification. The enzyme carbonic anhydrase (CA) which catalyses the conversion of HCO_3^- into CO_2 to enhance diffusion has been found in host gastrodermal cells in close proximity to sites of photosynthesis (Weis *et al.*, 1989; Weis, 1993; Benazet-Tambutté *et*

al., in press) suggesting that CA is directly involved in photosynthesis and only secondarily in calcification (Weis *et al.*, 1989).

A direct link between calcification and photosynthesis **(6)** was first proposed by Goreau & Goreau (1959): their model defined the following reaction



where the removal of CO_2 by photosynthesis shifts the equilibrium of the reaction towards CO_3^{2-} and thus enhances precipitation of $CaCO_3$. However, this reaction can take place only if high pH and high bicarbonate concentration are maintained at the site of skeletogenesis (Chapman, 1974), otherwise, the removal of CO_2 would reduce the concentration of bicarbonate ions available for skeletogenesis.

Studies on the porous branching coral *Acropora cervicornis* suggested that the two processes involved in calcification follow a diel pattern (Gladfelter, 1983). At night, the organic matrix deposits the fusiform crystal framework resulting in apical linear extension. This is followed during the day by the nucleation of new crystals unto the framework and by their calcium carbonate accretion by epitaxial growth. This final process, resulting in an increment in skeletal density, is typical of inorganic precipitation and thus it benefits most by the change in pH brought about during photosynthesis. In *Acropora cervicornis* this secondary mineralisation process never ceases (although its rate is reduced) because the high porosity of the skeleton ensures a continuous movement of ions around the inner portions of the skeleton (Gladfelter, 1982).

1.4.3 External stimuli

Light

As previously mentioned, light intensity determines the rate of photosynthesis, and this in turn has an effect on many facets of coral physiology. A high daily irradiance sustains a high rate of daily carbon fixation and translocation **(2)** that can exceed the zooxanthellae and host requirements for both respiration and growth (Davies, 1977; Muscatine *et al.*, 1984; McCloskey & Muscatine, 1984). Comparative studies along depth gradients found that the rates of photosynthesis, respiration and skeletal growth decreased with depth and suggested that, in shallow water, corals can maintain a higher rate of metabolism **(3)** and growth because of the optimal rate of

translocation (Davies, 1980; Muscatine *et al.*, 1984). It could follow that the rate of respiration in corals is directly related to translocation but this assumption requires experimental testing.
By enhancing photosynthesis, light enhances both the organic and inorganic processes of calcification. The former consists of the translocation of photosynthetically fixed organic molecules used by the host in the formation of the organic matrix **(4)** and in the provision of ATP for energy requiring ion transport **(5)**. From areas of high zooxanthellae density (high photosynthesis), photosynthate moves readily through the coral colony towards the faster growing tissue of active calcification sites. For example in the branching coral *A.cervicornis*, the apical region is the centre of skeletal growth and is void of zooxanthellae but its calcification rate can be accelerated in the light by the photosynthetic activity of algae lower down the branch (Pearse & Muscatine, 1971; Chalker, 1975). Attempts to enhance calcification by incubation of branch tips in high exogenous concentrations of organic molecules have failed (Chalker, 1975).
The light enhancement of the inorganic process of calcification consists in the removal of CO_2 by photosynthesis thus producing a pH shift towards a more alkaline environment with a higher availability of carbonate ions for deposition, as theorised by Goreau (1961) **(6)**.

Water velocity

This factor is important in relation to its effect on the thickness of the boundary layers on the surface of corals. Corals, like any other sessile organism, require the transport of nutrients, O_2 and CO_2 towards their surfaces and that of waste substances away from them. In the absence of water motion, molecular diffusion is the only process available for either supply or removal of gases and nutrient. Coral epithelium is ciliated but the movement of water over the surface thus produced is not sufficient to optimise mass flow. As water velocity is increased, the length of the diffusive path diminishes. Shashar *et al.*, (1993) and Kuhl *et al.* (1995) have measured the thickness of diffusive boundary layers surrounding corals and their reduction as water velocity was increased.
Water velocity has been positively correlated to photosynthesis and respiration (Patterson *et al.*, 1991; Newton & Atkinson, 1991; Rex *et al.*, 1995), to calcification (Dennison & Barnes, 1988) and to nutrient uptake (Atkinson & Bilger, 1992; Atkinson *et al.*, 1994). This is evidence that the rate of all these processes can be limited by the physical process of diffusion.

Nutrient enrichment

While aposymbiotic cnidarians have been found to excrete waste products (e.g. NH_4^+, phosphate), symbiotic associations in the light take up phosphate (Yonge & Nicholls, 1931b,c; D'Elia, 1977; Muscatine, 1987; Wilkerson & Kremer, 1992) and ammonium (Kawaguti, 1953;

D'Elia & Webb, 1977; Muscatine & D'Elia, 1978; Muscatine *et al.*, 1979; Burris, 1983) and in some cases also nitrate (Franzisket, 1974; Webb & Wiebe, 1978; Wilkerson & Trench, 1986; Bythell, 1990).

The precise mechanism of nutrient uptake by symbiotic zooxanthellae has not been fully elucidated. D'Elia (1977) proposed the 'depletion- diffusion' model whereby zooxanthellae deplete the nutrient pool in the host cytoplasm allowing further diffusion from seawater to take place. However, unlike ammonium, phosphate and nitrate are ionized in the physiological pH range and thus their transport across membranes must be carrier-mediated (Miller & Yellowlees, 1989). Because of the intracellular position of zooxanthellae, the host must be involved in nutrient transport from seawater and this provides the basis for the hypothesis that algal growth is regulated by the host through the regulation of nutrient supply at the symbiosome membrane (Miller & Yellowlees, 1989; Rands *et al.*, 1993).

The physiological response of corals to nitrogen enrichment has been investigated in growth experiments under high NH_4^+ levels (Muscatine *et al.*, 1989; Hoegh-Guldberg & Smith, 1989; Dubinsky *et al.*, 1990; Muller-Parker *et al.*, 1994; Snidvongs & Kinzie, 1994). High seawater NH_4^+ levels tend to free zooxanthellae from nitrogen limitation and promote their growth rate to a higher zooxanthellae population density. Falkowski *et al.* (1993) hypothesised that with more nitrogen available for zooxanthellae growth, quantitatively less photosynthate will be available for translocation to the host. This in turn will force the host to depend more on heterotrophic feeding to satisfy its metabolic requirements. This failing, the growth rate of the host might be reduced while that of the symbionts enhanced. In the worst case scenario this could lead to the algae outgrowing their host: this process has never been observed in corals but has been reported for symbiotic green hydra (Neckelmann & Muscatine, 1983).

As yet, no physiological study has focused on the effects of nitrogen in the form of nitrate rather than ammonium, in spite of the fact that nitrate is the form in which N accumulates in coastal environment subjected to sewage pollution.

On the other hand, zooxanthellae biomass or photosynthetic production do not appear to respond to phosphate enrichment (Muscatine *et al.*, 1989; Stambler *et al.*, 1991).

Evidence is accumulating regarding a detrimental effect of nutrient enrichment on skeletogenesis. Tomascik & Sander (1985) found that the linear extension rate, measured by X-radiography on colonies of *Montastrea annularis*, was negatively correlated to water quality along the eutrophication gradient on the west coast of Barbados. Growth experiments have shown significant reductions in the rate of calcification of corals incubated under high NH_4^+ or PO_4 levels in comparison to control colonies in oligotrophic conditions (Rasmussen, 1988; Stambler *et al.*, 1991; Stimson, 1992; Ferrier-Pagès *et al.*, 1996). It has been suggested that the effect of nitrogen on calcification is indirect and results from the reduction in the amount of

photosynthetically fixed carbon translocated to the host (Stambler *et al.*, 1991). Conversely, phosphate is known to act as a direct inhibitor of crystal formation (Reddy, 1977). Simkiss (1964) suggested that zooxanthellae uptake of phosphate ensures that high levels of this ion do not reach the calicoblastic region of the coral thus proposing this as a mechanism explaining the increased calcification observed in symbiotic corals in the light.

1.5 The assessment of the 'health' status of hermatypic corals in relation to nutrient enrichment

Growth is typically considered an index of viability of an organism. In the case of hermatypic corals 'growth' can be subdivided into three components:

1- the growth of the zooxanthellae, or 'primary production'

2- the growth of the host biomass, or 'secondary production'

3- the growth of the skeleton, or 'inorganic production'.

The zooxanthellae component presents the added complexity that growth (increase in biomass) and productivity (carbon fixation) do not match because a large proportion of the carbon fixed in photosynthesis is translocated to the host rather then used in cellular division.

To determine the physiological condition of corals in an integrated manner, two approaches have been adopted, one focusing on the organic production (1+2), and the other entirely on the inorganic production of a coral colony.

Studies on organic production have been developed from the commonly used photosynthesis to respiration ratio (P/R) into complete energy or carbon budget methodologies able to quantify the carbon requirements of each metabolic component and express them as a percentage of the gross primary productivity. These budgets have been measured in corals growing at different depths and light intensities (Davies, 1977; Porter *et al.*, 1984; McCLoskey & Muscatine, 1984; Muscatine *et al.*, 1984; Davies, 1991) and sediment regimes (Edmunds & Davies, 1989; Riegl & Branch, 1995). The use of a measure of organic growth as a bioassay in relation to nutrient enrichment is attractive and requires careful evaluation. The evidence for nitrogen enhancement of zooxanthellae growth and photosynthesis, and the hypothesis of Falkowski *et al.* (1993) that the increase in zooxanthellae population density results in a reduction of translocation to the host suggest that a budget methodology could be best suited in identifying nitrogen pollution.

Studies on inorganic production are based on the direct measurement of skeletal growth, for which a variety of methods are available. Since skeletal growth is related to surface area, it can also be used to estimate indirectly the growth rate of organic tissue. Linear extension of corals has been estimated by X-radiography (Hudson, 1981; Dodge & Brass, 1984; Houston, 1985;

Tomascik & Sander, 1985), alizarin staining (Lamberts, 1974; Stambler *et al.*, 1991) and time-lapse photography (Barnes & Crossland, 1980). Mass accretion has been determined by X-radiography (Dodge & Brass, 1984) and more commonly by the bouyant weighing method which does not damage the coral and therefore allows for repeated measures on the same colony (Bak, 1973; Jokiel *et al.*, 1978; Edmunds & Davies, 1986; Davies, 1989).
A better understanding of the dynamics of skeletal growth in response to nutrient enrichment are necessary before this measure can be used to assess water quality conditions on the reef.

1.6 Thesis outline

The purpose of this project is to identify a bioassay for the early identification of nutrient enrichment on coral reefs. The measurement of skeletal growth by buoyant weighing and the construction of carbon budgets appear to be the most promising and attractive techniques because they are based on the ecologically critical processes of coral productivity.
While buoyant weighing is a well established technique, carbon budgets require further methodological developments before they can be used to assess the effects of nutrient enrichment (Ch2). In particular, there are two approaches available in the literature to the calculation of carbon budgets and both have been successfully applied to evaluate the importance of autotrophism in hermatypic corals growing at different sites. It was therefore thought appropriate to compare these two methodologies and find the most sensitive one (Ch.3).
In order to identify nutrient effects in the complex condition of the open sea it is necessary to first study corals in controlled experimental conditions in the laboratory where nutrient concentration can be varied while all other parameters (light, temperature, water velocity) are kept constant. A setup was developed to grow corals satisfactorily in the laboratory: it was tested against corals growing on the reef to ensure that laboratory result could be extrapolated to the field in future and results are presented in Ch. 4.
In comparison with the knowledge acquired about the effects of nutrient enrichment on coral reef communities, very little is known about their direct effects on hermatypic corals. The effects of nitrogen have been tested on coral physiology but only in the form of ammonia: however, on the reef, NH_4^+ is quickly taken up by phytoplankton and algae and nitrogen concentration build up as nitrate. Therefore Ch.5 and Ch.6 have been devoted to the investigation of the effects of different concentrations of nitrate and phosphate (respectively) on coral physiology.
Finally, three sites were chosen on the West coast of Barbados corresponding to low, medium and high nutrient enrichment and corals were grown in these conditions for a month before

measurements of skeletogenesis, photosynthesis and biomass growth were taken (Ch. 7). The information acquired in the laboratory was used to interpret the results from the field.
The final chapter assesses the use of buoyant weighing and coral carbon budgets as bioassay for nutrient enrichment and proposes a new technique.

Chapter 2

METHODS

2.1 Introduction

As mentioned in the General Introduction (Ch. 1), the effects of nutrients will be assessed by examining their effects on each component of the carbon budgets of the experimental corals. The determination of the carbon budgets is dependent upon measuring the carbon fixation in photosynthesis and the subsequent partitioning of this between the requirements of the algae and of the host for respiration and growth. Two somewhat different methodologies to construct carbon budgets have been developed by Davies (1984 *et seq.*) and Muscatine *et al.* (1984 *et seq.*). Their comparison in the succeeding chapter will generate an improved methodology. A concise version of it will be used in all nutrient enrichment experiments.

In this chapter, all methods used in developing the improved carbon budget are collectively described. In some cases, standard methodologies were adopted; in others existing methods were evaluated and/or subjected to further development.

The methods may be broadly arranged under three headings as follows:

Experimental corals: nubbins and explants

Processes:
- growth rate
- photosynthetic carbon fixation
- respiratory carbon expenditure

Biomass:
- colony surface area
- tissue dry weight
- isolation of zooxanthellae
- carbon content of tissue, zooxanthellae and mucus
- protein content
- zooxanthellae population density
- mitotic index and growth rate of zooxanthellae
- zooxanthellae volume
- chlorophyll content

2.2 Experimental corals

Physiological experiments on hermatypic corals, both *in situ* and in the laboratory, have been carried out on a variety of coral sizes: single excised polyps as in the microcolonies of *Galaxea fascicularis* (Al-Moghrabi, 1992), nubbins i.e. tips of branching corals broken off and glued onto perspex tiles (Davies, 1984) and explants i.e. cores of massive corals fitted into plastic holders (Davies, 1990), single cut branches (Fitzgerald & Szmant, 1988; Chalker & Taylor, 1975), small detached colonies (Porter *et al.*, 1984; Hoegh-Guldberg *et al.*, 1987 ; Riegl & Branch, 1995;) and large adult colonies *in situ* (Atkinson & Grigg, 1984).

In the development of a methodology for ecotoxicological studies it is of paramount importance to choose the coral unit that:

1. best reflects the physiological behaviour of the intact colony
2. facilitates its handling without any damage to the tissue
3. can be easily replicated
4. maximizes experimental sample size while minimizing the amount of coral removed from the reef

Nubbins and explants were thought to possess the above characteristics. They are removed from an adult colony and continue to grow (in the case of *Porites porites* nubbins) at a rate comparable to that of intact colonies (Davies, 1989).

Preparation followed the methodology in Davies (1984, 1990) and was further improved in the case of explants.

2.2.1 Species choice

The entire practical work was carried out in Barbados (West Indies) at the Bellairs Research Institute of McGill University, on the leeward side of the island. The Institute is adjacent to a well-developed and thoroughly studied fringing reef (Stearn *et al.*, 1977; Lewis, 1981; Mah & Stearn, 1990; Tomascik & Sander, 1987a; Bateson, 1995) that lies within the protected area of the National Marine Park. Coral collection took place at reefs North of the Marine Park in close proximity to the Institute and corals were transported back submerged in large seawater insulated containers via sea or land.

In studying the effects of nutrients on coral physiology, I chose to work with two coral species. Porter (1976) proposed that the degree of autotrophy is inversely related to polyp size and to the surface area to volume ratio. Thus I wished to compare a branching small-polyped coral with a massive large-polyped one to test if measurements of carbon budgets corroborate Porter's theory.

If this is the case, such a comparative approach could be useful to observe if the effect of nutrient enrichment relates to the degree of autotrophy of a species.

On the fringing reefs of Barbados there are three common branching species: *Porites porites*, *Acropora cervicornis* and *Madracis mirabilis*. The former was chosen for this study because:

-it is the most abundant species (Lewis, 1960)

-it has been used extensively in experimental work (Edmunds & Davies, 1986;1987;1989; Davies, 1990)

-the columnar shape of its branch tip facilitates measurement of the surface area of the nubbin

-it has a relatively tougher skeleton, and is very resistant to handling

-survival rate after nubbin preparation (see below) was always higher than 95%.

The only difficulty in working with *P. porites* is the periodical formation of mucous tunics (Coffroth, 1985; 1990; 1991) which envelop the coral and reduce growth rate to a minimum (Davies, 1989).

On the contrary, fragility is the main problem with *M. mirabilis* and *A. cervicornis*. The former have thin, often branched tips and the latter have a very fine tissue layer and extremely fragile sclerosepta. In both cases damage during transport, preparation and maintenance was highly increased. For this reason, it was decided to use nubbins of *P. porites*, despite the disadvantages associated with periodic mucous tunic formation.

Among the massive corals there are only two common species: *Montastrea annularis* and *Siderastrea siderea*. The former was chosen for the following advantages:

-its growth rate has been extensively studied in relation to reef degradation

-it has a relatively porous skeleton that facilitates coring.

-colonies of the morphotype 2 (Knowlton *et al.*, 1991) have a large flattened uniform surface that increased the ratio between tissue used in explants and wasted.

-survival rate after explant preparation (see below) was always higher than 95%.

The main problem with *S.siderea* was the high density of the skeleton. Severe damage to the tissue occurred during coring because the heat produced while drilling could not be dissipated.

2.2.2 Nubbins for branching corals

Small coral branches (3 cm long) were removed from the parent colony with metal cutters while underwater. Care was taken in choosing unbranching tips of similar width. During collection and transport, they were held in place by elastic bands attached to a wooden board to minimise any damage to the tip. In the laboratory each branch was carefully cut to a length of approx. 20 mm and the cut surface ground flat using a rotary grindstone. This was carried out in air, holding the piece of coral as lightly as possible. The nubbins were then placed back in running seawater to

remove excess mucus and skeletal dust. Finally their exposed surface was blotted dry on tissue paper and glued on a pre-weighed 3x3cm perspex tile using a drop of cyanoacrylate glue (superglue® gel). Careful handling throughout the procedure ensured minimal tissue damage. It was observed that polyps were expanded after less than one hour from the end of preparation. The tiles were fitted into a plastic tray which was then firmly bolted onto a concrete building block placed on the reef (Davies, 1995) and surrounded by a chicken wire cage (of approx. 2 cm mesh size) to protect the corals from grazing fish. Nubbins were left on the reef at a 'nursery' site on the North Bellairs reef at a water depth of 2.5 ± 0.5 m for at least one week before starting any experiment. By this time any damaged tissue had regenerated and sealed the nubbin onto the tile. The perspex tiles were subject to settlement by filamentous and calcareous algae, thus trays were brought back to the laboratory at regular intervals to enable these to be scraped off.

2.2.3 Explants for massive corals

A coral head (approx. 15-25 cm in diameter) was removed from the reef with hammer and chisel and carried back to the laboratory workshop where it was placed under a drill press fitted with a hole saw: different sized wooden frames were used to immobilize the head during drilling. The coral tissue was continually wetted with seawater from a squeeze bottle to minimise damage to the tissues from heating during the drilling process. Each core was drilled to a depth of 20-25mm, broken off at the base using a small chisel inserted laterally and washed in seawater to get rid of any excess mucous and coral dust.

Only the upper surface of the colony was used since the lateral parts presented a much higher skeletal density, a different pigmentation, a larger polyp size and a much reduced survival rate after coring: round colonies were not collected since too much side tissue would have been wasted.

The explant was sawn to the desired length with a small hacksaw while holding it with a clamp. Initially cores were drilled with a 23mm corer and sawn to 20mm to make a relatively tight fit in 20x23mm PVC tubes which were glued onto 30x30mm perspex tiles, with the polyp surface flush with the top of the tube (Davies, 1990). However, it was found that occasionally sediment accumulation and bacterial growth occurred in the space between the skeleton and the tube. This was accompanied by gas release, which made buoyant weighing (see below) prone to error.

A successful improvement was developed. In place of the PVC tubes, 24x22mm low density polyethylene cups were used. To reduce the problem of bacterial growth beneath the skeleton, the cores were sealed into the cups with underwater epoxy putty. The two parts of the putty (PC11, Protective Coating Co., Allentown, Pa, USA) were mixed, inserted into the cups, whilst excluding any air bubbles and the cores were then inserted therein. This stage was carried out under water

to ensure that no air bubbles were introduced into the putty. Prior to insertion, the cores were ground to the desired length rather than being sawn, smoothing the edges to facilitate the incorporation into the cup. Trays were built to hold the cups containing the explants during the recovery period on the reef. They consisted of a perspex sheet to which was glued a set of cups 4mm larger than the explants each containing a ring of Velcro® strip (the loop component). The cups containing the explants could easily be inserted into these larger cups and pushed out again subsequently by pressing from below. After preparation, trays with explants were brought to the same 'nursery' site as described for nubbins and maintained in those same conditions for at least two weeks. The cups of the explants were subjected to fouling but the insertion of these into the larger cups significantly reduced the problem.

2.3 Processes

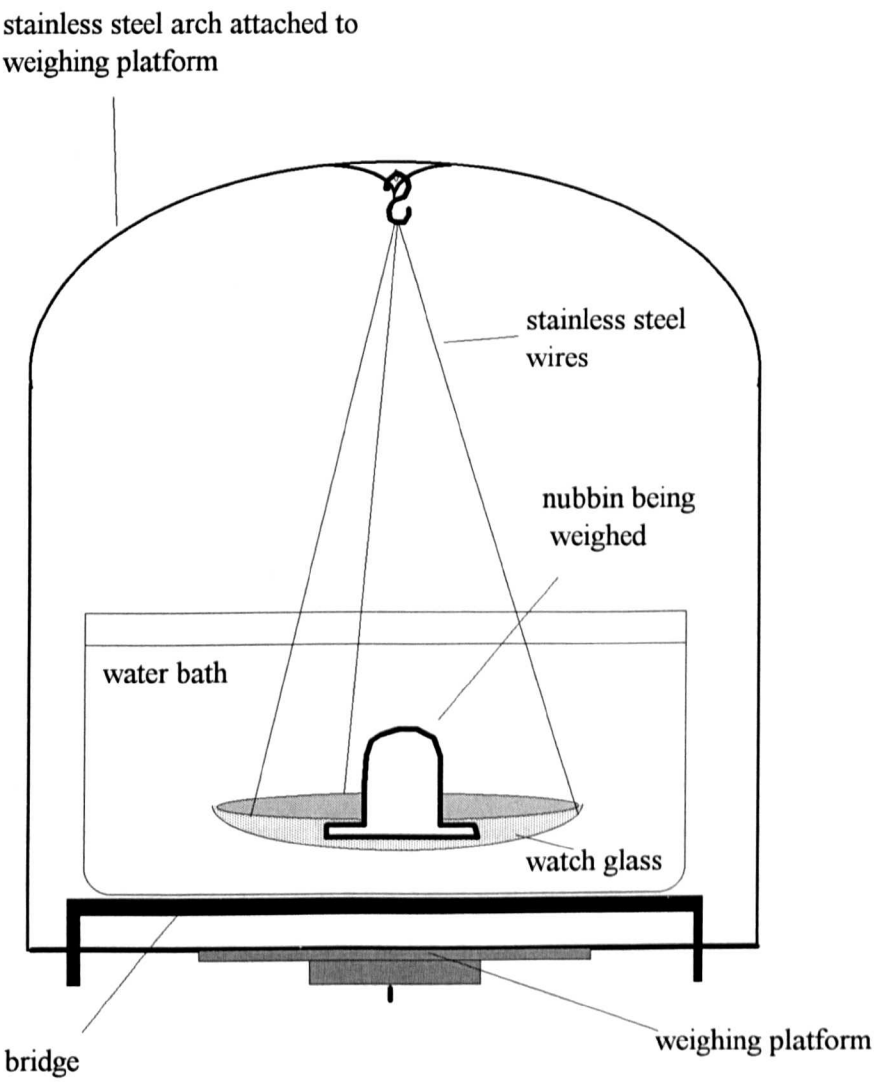
2.3.1 Skeletal growth: the buoyant weighing technique

Skeletal growth was measured using the buoyant weighing technique which allows the weight of an object to be determined accurately underwater. It follows from Archimede's Principle that the weight of an object in air is equal to the object's weight in a liquid medium plus the weight of the liquid displaced by the object. In order to transform the weight in water into an actual weight in air it is necessary to know the density of the object and the density of the water in which it is weighed.

The buoyant weighing was first used to measure skeletal growth in corals by Franzisket (1964) and by Bak (1973). A detailed procedure was given by Jokiel *et al.* (1978) who underlined its major advantage: corals are not damaged in any way by buoyant weighing so that repeated measures over time are possible and using a high precision balance, intervals between weighing can be as short as 12 hours (Jokiel *et al.*, 1978). Davies (1989) developed the procedure for use with *P.porites* nubbins determining the errors associated with some of the original assumptions of Jokiel *et al.*(1978) and showing that the growth rate of nubbins was almost identical to that of branch tips of an intact colony. More recently this technique has been widely appreciated and applied in coral growth studies (Davies, 1990; Edmunds & Davies, 1986, 1989; Ferrier-Pagès *et al.*, 1996; Takabayashi & Hoegh-Guldberg, 1995).

The buoyant weighing procedure was modified from that of Davies (1989) which required the use of a specially constructed chamber to enable weighing to be conducted beneath the balance. Instead a standard density determination kit was fitted to a Ohaus electronic balance (reading to

Fig. 2.1 Diagram of the standard density determination kit used in buoyant weighing.



0.1 mg). It consisted of a modified weighing platform (Fig. 2.1) with a high metal arch from which a hook is hung: a 5 cm watchglass is suspended by three 0.05mm diam stainless steel wires from the hook into a 15x11x6 cm perspex water bath held above the weighing platform by a bridge.

All weight measurements are dependent on accurate knowledge of the density of the seawater in the water bath. This can vary during the weighing period with temperature, salinity and mucus released from the corals. To minimize this error, corals were weighed in a room with stable temperature close to that of the sea, and the water in the weighing bath was regularly changed. During a weighing session, the density of seawater was determined at regular intervals (5 min) by weighing a solid glass stopper of known air weight and density since:

$$Density_{water} = Density_{object} * \left(1 - \frac{buoyant\ weight_{object}}{airweight_{object}}\right)$$

The density of the glass stopper was determined using the following equation:

$$Density_{object} = \frac{Density_{water}}{\left(1 - \frac{buoyant\ weight_{object}}{airweight_{object}}\right)}$$

by weighing the stopper first in air and then in distilled water. The density of distilled water was obtained from Table F5-6 and F11 of the CRC Handbook of Chemistry and Physics (1984).

In order to transform the buoyant weight of a nubbin (tile + aragonite) or of an explant (cup + epoxy + aragonite) into air weight of the aragonite skeleton, the density and weight of all components as well as the density of aragonite have to be known since:

$$Airweight_{aragonite} = \frac{\left\{buoyant\ weight_{nubbin} - \left[airweight_{tile} * \left(1 - \frac{Density_{water}}{Density_{tile}}\right)\right]\right\}}{\left(1 - \frac{Density_{water}}{Density_{aragonite}}\right)}$$

The density of the tile (cup or epoxy) was derived simply as for the glass stopper. To determine the density of the aragonite all coral tissue had to be removed from samples of the skeleton. Coral tips and cores were sacrificed, and soaked in a 20% solution of commercial bleach in seawater for 24 h. Any residual organic matter was washed off by waterpiking underwater so that no air bubbles remained trapped in the skeleton and all bleach was washed out of the skeleton. They were then weighed in seawater of known density, washed in distilled water and dried to constant weight at 70 °C.

I have assumed that the coral is made only of aragonite, hence ignoring the contribution of the tissue to the weight of the coral. Davies (1989) estimated that this assumption introduces an

absolute error of approx. 3% but since growth rates were being determined the error would be constant and would contribute very little to the overall error of the method.

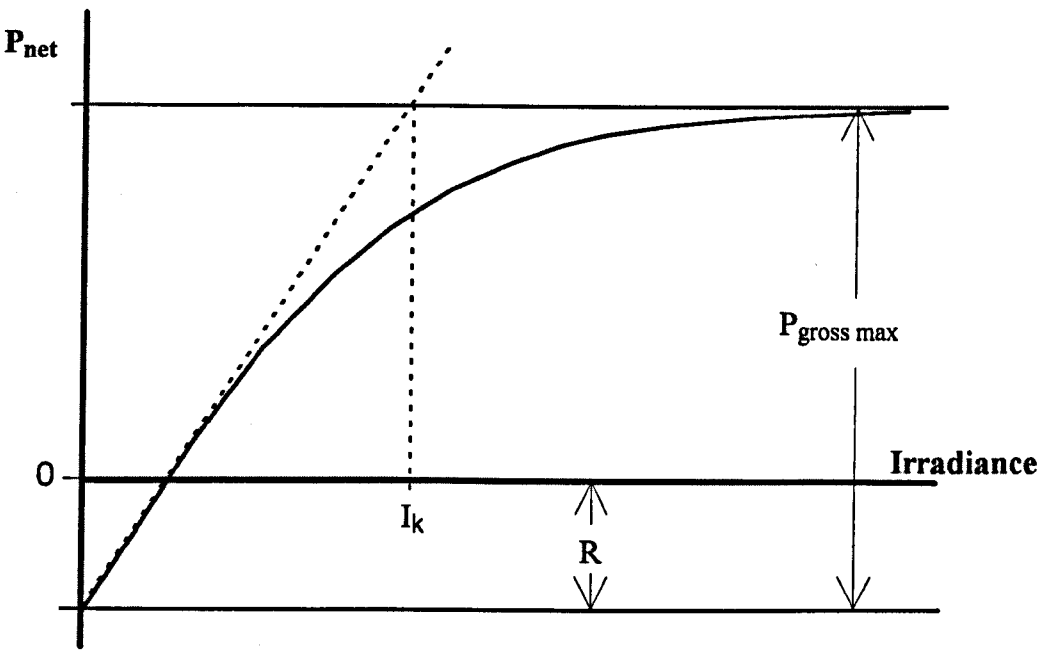
2.3.2 Photosynthesis

Photosynthesis is measured to determine the amount of dissolved inorganic carbon that is fixed into organic carbon by the zooxanthellae. It may be measured indirectly as oxygen flux in closed or semi-closed respirometer chambers placed directly on the reef under natural sunlight (Porter *et al.* 1984) or in the laboratory under artificial illumination (Davies, 1984, Edmunds & Davies, 1986). Either way, it can be measured only as net photosynthesis since respiration of both algae and host occurs simultaneously: gross photosynthesis can only be inferred by measuring respiration of the intact association during darkness and adding it to the net oxygen fluxes. The accuracy of gross photosynthetic data has therefore to rely on the assumption that respiration in darkness is the same as during photosynthesis. This assumption is subject to error and will be evaluated (see 2.3.3).

Photosynthetic rates were measured in a twin chambered closed respirometer constructed of transparent acrylic. The 50 ml chambers were maintained at 27 ± 0.05°C by a surrounding constant temperature waterjacket and were fitted with microcathode oxygen electrodes, connected to oxygen meters (Strathkelvin Instruments, Glasgow), whose output was displayed on a two channel chart recorder. The respirometer was located within an aluminium foil-lined hood under an overhead bank of high energy fluorescent light tubes (Philips high frequency regulation luminaires). Corals were incubated in filtered seawater that had been fully aerated at the start of each run: lids were closed and oxygen measurements made in darkness until the dissolved oxygen was reduced to 75% of saturation. Salinity of seawater was measured with a hand refractometer (Atago, S/MILL). Tables of oxygen solubility in seawater (Green & Carritt, 1968) were used to determine the amount of oxygen at saturation in the chambers. The lights were then switched on and their intensity increased sequentially from 25 to 50, 100 and 200 μmol photons m^{-2} s^{-1} bringing oxygen saturation back to 100%; a second respiration run was performed before increasing the lights further to 350 and 600 μmole photons m^{-2} sec^{-1}. The power to the lights was varied using a Philips LPS100 control potentiometer. Respiration rate was calculated as the average from the two runs in darkness and added to the net photosynthetic rates in order to calculate values for gross photosynthesis.

It was found that the increase in light intensity affected water temperature within the chambers and that in turn affected the electrode output: a temperature variation of as little as 0.1 °C shifted the electrode reading of about 0.5%. To minimize this error, chambers were run as controls

Fig. 2.2 Generalised hyperbolic tangent function fitted to a P/I curve



$$P_{net} = P_{gross\ max} * \text{Tanh}\,(I/I_k) + R$$

where: P_{net}: net oxygen evolution at an irradiance I

$P_{gross\ max}$: maximum gross photosynthesis

R: oxygen consumption

I_k: the irradiance at which the initial slope of the curve intercepts the horizontal asymptote

without nubbins and any change in output was used to correct the rates of photosynthesis measured at each light intensity.

Net photosynthesis vs. irradiance data were fitted by an iterative algorithm to Chalker's (1981) model based upon the hyperbolic tangent function. The obtained P/I relationship has a characteristic shape (Fig. 2.2): initially the rate of net photosynthesis (P_{net}) is directly proportional to light intensity (I), thereafter the curve rapidly approaches the horizontal asymptote referred to as maximum photosynthetic rate ($P_{net\ max}$). The slope (alpha) of the tangent to the initial part of the curve is a measure of photosynthetic efficiency. The irradiance at which photosynthesis saturates is defined by I_k, the irradiance level at which the initial linear part of the curve would intercept the horizontal asymptote.

In order to calculate the daily photosynthetic rate it is necessary to know the light intensity experienced and the P/I curve of each coral. Experimental corals grown in the laboratory (ch4-6) were subjected to constant irradiance ($I_{EXPERIMENTAL}$) over the hours of daylight and daily net photosynthesis was easily computed from the P/I curve as $P\ I_{EXPERIMENTAL}$ ($\mu lO_2 cm^{-2} h^{-1}$) * daylight length (h). On the other hand, corals grown on the reef experienced changing light intensity related to time of day (from dawn to dusk), cloud cover and turbidity. It was not possible to monitor light intensity on the reef for the duration of growth observations. However a measure of maximum underwater light intensity was obtained on a day of full sunshine, calm sea and high water quality for the 2.5 m deep 'nursery' site where nubbins used in the development of the carbon budget methodology were grown. An underwater cosine quantum sensor (Skye Instruments Ltd.) was attached to a cement block next to the coral trays. The sensor was connected to a light meter attached to a battery-operated data logger (Data Hog, Skye Instruments Ltd.) kept dry on a boat anchored nearby. The average light intensity over 15 min. periods was logged automatically from sunrise to dusk, when recording was terminated and the equipment recovered from the reef. The integrated daily net photosynthetic rate was calculated with a computer program by combining the data in the P/I curve with the light intensity measurements at each 15 min. interval. The integrated gross daily photosynthetic rate was calculated by summing the integrated daily net photosynthetic oxygen production to the respiration rate over the daylight hours.

2.3.3 Respiration

Intact symbiosis

Respiration rate of nubbins and explants was measured in short-term dark incubations during the day as outlined in the section on photosynthesis above (2.3.2).

With the common assumption that respiration is constant throughout the day and night, the measured respiration rate was then extrapolated to daily respiratory carbon requirement (McCloskey *et al.*, 1978). However this basic assumption has been challenged in symbiotic associations: Porter *et al.* (1984) noticed that *in situ* respiration of *Stylophora pistillata* was higher after sunset than just before sunrise; Edmunds & Davies (1988) showed a respiration rate increase of 39% above pre-illumination levels when nubbins of *Porites porites* were artificially illuminated for a 3 h period and Muller-Parker (1984) found a 50% increase after light exposure in the sea anemone *Aiptasia pulchella*; most recently Harland & Davies (1995) investigated the effects of exposure to light on the respiration and photosynthesis of the sea anemone *Anemonia viridis* and compared them to the effects of hyperoxia: they suggest that nighttime respiration is limited by oxygen diffusion from the water while during the day photosynthesis oxygenates the tissues offsetting oxygen limitation hence resulting in an increased respiration rate which in turn provides a CO_2-rich intracellular environment that enhances photosynthetic rate. In this study, respiration rates obtained during the night and during the day were compared to allow for a better estimation of the actual carbon requirements over a 24h day.

Respiration rate for six nubbins was measured in the respiration chambers in darkness at 03:00 and the following day at 15:00. Between measurements nubbins were kept in the laboratory where artificial lights were switched on from 05:30 to 18:30 at a constant intensity of 300 µmol photons m^{-2} s^{-1}. Corals at night were therefore measured after more than 8 h of dark respiration while during the following day they had been exposed to light and had photosynthetised for at least 9 h.

Respiration during the night was 6.45 ± 1.604 µl O_2 cm^{-2} h^{-1} and during the day it increased by 42% to 9.16 ± 1.736 µl O_2 cm^{-2} h^{-1}. It was also noted that corals at night tended to partially retract their polyps so that the actual surface area was reduced in respect to the same nubbins during the day. The 40% increase fits well with the data of Edmunds & Davies (1986) and it was therefore taken into consideration when estimating total 24h respiration expenditure in the carbon budget calculations.

Isolated zooxanthellae

To measure the respiration rate of freshly isolated zooxanthellae (section 3.2.5), a suspension (2 ml) was pipetted into a 3 ml water-jacketed glass cell fitted with the same oxygen electrode as in 2.3.2. Respiration was measured in darkness and standardized to cell number after estimating zooxanthellae density in the suspension, by sample counts on a Neubauer haemocytometer.

2.4 Biomass

2.4.1 Surface area

Due to the very complex forms of many corals, especially the branching ones, surface area is a difficult parameter to measure. The most commonly used method is the aluminum foil technique introduced by Marsh (1970) which involves wrapping a coral with foil and weighing the foil. It is very successful on massive or encrusting corals and was used to measure the surface area of the *M.annularis* explants. With *P.porites* nubbins, as with all branching corals, this technique is very time consuming, thus another one was sought. Stimson & Kinzie (1991) dipped cleaned skeletons into hot paraffin wax and the weight of the wax adhering to the coral was compared to that of skeleton blocks of known surface area. Similarly, McCloskey and Muscatine (1984) dipped bare skeletons in hot distilled water, then in a solution of methylene blue dye and measured spectrophotometrically the dye eluted when dipped again in hot water: dye concentration was calibrated with similarly treated pieces of coral of known surface area. Rinkevich & Loya (1983) working on branch tips of *Stylophora pistillata* calculated surface area by taking measurements with calipers and then using the geometric formula for cylinders and hemispheres.
Since the least damaging method was preferred, the procedure by Rinkevich & Loya (1983) was adapted to nubbins by considering them a half sphere on a cylinder and calculating their surface area as: $S = \left[(ht - r)*2\Pi r\right] + \left(4\Pi r^2 / 2\right)$

where ht = height of nubbin and r = (length+width)/4. All measurements were taken with calipers to 0.1mm accuracy.
A comparison between the caliper and the wrapping methods was carried out and no significant difference found in the surface area measurements: the caliper was less time consuming and not damaging to the coral and was therefore adopted.

2.4.2 Tissue dry weight

The most common procedures used in estimating total tissue dry weight in corals involve either fixation and decalcification (Edmunds & Davies, 1986) or waterpiking in distilled water (Johannes & Wiebe, 1970) and drying.
To identify the technique that would best suite the requirements of a carbon budget study, the following investigation was carried out comparing the total dry weight per surface area and the percentage ash free weight obtained from different methods.

Nubbins of *P.porites* were retrieved from the 'nursery' site on the North Bellairs Reef. After measuring their surface area, they were assigned to one of five treatments (n = 5 per treatment).
A- fixation in carbon free Zenker's fixative (50g mercuric chloride, 25g potassium dichromate, 10g sodium sulphate in 1l distilled water) for 4 weeks, followed by decalcification in 10% HNO_3.
B- Fixation in formalin for 24h followed by decalcification in 10% HNO_3.
Tissue fixed by these methods was examined under a dissecting microscope after decalcification for presence of endolithic algae which were then removed with forceps.
C- Decalcification in 10% HCl without fixation: the slurry obtained was centrifuged before drying.
D- Waterpiking in distilled water (Johannes & Wiebe, 1970). Coral tissue was removed from the skeleton using strong intermittent jets delivered by a Water Pik® (dental cleaning device). A nubbin was held by its tile inside a thin plastic bag to prevent loss by spattering. The blastate was homogenized in a glass mortar and pestle and subsamples were either (D) collected in a pre-weighed aluminum pan or (D_{bk}) filtered onto glass fiber filters (GF/C) before drying. The skeletons were then treated as in B to estimate how much tissue remained after waterpiking.
All samples were dried at 60 °C to constant weight and then ignited at 450 °C for 5h to obtain the ash free weight of the tissue (AFDW). Data are summarised in Table 2.1. Results from a one-way ANOVA indicate significant differences in dry weight estimation between treatments ($F=12.7$; $P<0.0001$). To evaluate specific differences the Tukey-HSD multiple comparison test was used: as expected, the dry weight of homogenate collected on GF/C was significantly lower than all others. In terms of percentage AFDW, excluding the Zenker's fixed tissue (no data available), there was a significant difference ($F=23.5$; $P<0.0001$): the Tukey-HSD multiple comparison test identified the %AFDW collected on filter as significantly higher from all others as well as the dry tissue after formalin fixation being higher than that after waterpiking.
The average weight and %AFDW of the tissue remaining after waterpiking were measured as 6.7 mg cm^{-2} and 60% respectively.
Only 20% of coral homogenate from waterpiking is collected on GF/C filters and of that a very high proportion (90%) is organic matter. Even though there was no significant difference between techniques for tissue extraction in terms of dry weight, fixation in formalin and waterpiking in distilled water had a higher outcome than either fixation in Zenker or direct decalcification in HCl. It is also worth pointing out that both direct decalcification and fixation in Zenker's had a much higher standard deviation suggesting a reduced accuracy and the potential requirement for larger sample sizes.
The most striking result is that the sum of the dry weight from waterpiking (D=15.8 mg cm^{-2}) plus that of the tissue remaining inside the skeleton after waterpiking (D_{extra}=6.7 mg cm^{-2}) gives a much higher total dry weight estimation than any other method: this suggests that all methods

used in the literature may give results that are underestimates. However if waterpiking is an underestimation because 30% of the tissue is left within the skeleton, the fixation methods must be undergoing a loss during decalcification of as much as 30%. Davies (1980) estimated that in the sea anemone *Actinia equina* a 24h fixation in formalin followed by 24h immersion in 10% HCl reduced the dry weight by approximately 11% due to a loss of small molecular weight organics. The higher loss observed in this experiment could be due to the poorer fixation of tissue deep in the skeleton compared to that of an anemone.

In conclusion, this comparison has shown that when only the dry weight of tissue is required, tissue extraction by either waterpiking or by formalin fixation and decalcification (and a combination of both) are preferable to any other method.

However, in the interest of a carbon budget methodology development, fixation in carbon free Zenker's solution was chosen instead. In fact both waterpiking and fixation in formalin might introduce an additional source of carbon into the tissue: in the former the risk comes from the skeletal inorganic carbon and in the latter from contamination with carbon in formalin. Even though there was no significant difference between fixation in Zenker's and direct decalcification the former was preferred because it did allow for removal of endolithic algae before drying. The carbon budget methodology will take into consideration the 30% underestimation of dry weight discussed above.

Table 2.1 Dry weight (mg cm^{-2}) and percentage ash free dry weight as means ± one standard deviation as a comparison between tissue extraction techniques (n = 5).

Treatment	dry weight (mg cm^{-2})	%AFDW
A- Zenker's	12.1 ± 4.325	-
B- Formalin	14.1 ± 1.462	69.7 ± 3.90
C- HCl	10.5 ± 4.695	58.2 ± 11.41
D- Waterpik	15.8 ± 2.562	53.9 ± 6.21
D_{bis}- Waterpik +GF/C	2.8 ± 0.661	90.5 ± 6.63

2.4.3 Zooxanthellae isolation

Once coral tissue has been homogenised, the isolation of zooxanthellae from coelenterate host material is carried out routinely by centrifugation (Muscatine, 1967; Gattuso *et al.*, 1993; Streamer *et al.*, 1986; Cook *et al.*, 1988). Zooxanthellae are heavy and settle quickly whilst host mucus, nematocysts and other tissue debris tend to remain in suspension. However even when centrifugation is repeated several times, some host material remains to contaminate the zooxanthellae suspension. When zooxanthellae isolation is required for analytical measures of zooxanthellae protein, carbon or nitrogen content, host contamination may introduce a large error. The problem exists for the analysis of all symbiotic coelenterates. Thus other techniques have been put forward to improve isolation and minimize host contamination. Franker (1970) purified zooxanthellae from the anemone *Anthopleura elegantissima* by centrifuging in density gradients of sucrose to study DNA properties. Tytler & Davies (1983) used density gradients of silica sol Percoll produced by ultracentrifugation to isolate zooxanthellae from *Anemonia sulcata*. The zooxanthellae remained viable and were able to photosynthesise at rates comparable to those measured before centrifugation. McAuley (1986) centrifuged and washed symbiotic *Chlorella* with sodium dodecyl sulfate (SDS) to remove hosts' impurities and measured the success of its technique by measuring the apparent protein content of cells. Those washed with SDS had a lower protein content than those washed in the standard medium and the difference was attributed to the loss of contaminating host protein.

In this study, zooxanthellae were isolated to measure respiration rate (2.3.3), carbon, nitrogen (2.4.6) and chlorophyll content (2.4.9). Therefore a viable and uncontaminated suspension of cells was required. Since ultracentrifugation facilities were not available and the additions of sugar or SDS were avoided to minimise potential carbon contamination or carbon loss respectively, a novel technique was sought. Differential filtration appeared most promising since it does not require centrifugation or chemical addition. The effectiveness of the filtration technique was tested against the standard method of centrifugation with both *P. porites* and *M. annularis* tissue.

After waterpiking a nubbin or explant, the tissue slurry was homogenized and then divided into two portions. Each was assigned to either the filtration or the centrifugation procedure to obtain a suspension of zooxanthellae. The duration of the two procedures was approximately the same.

Centrifugation

Coral homogenate was centrifuged at 500 x g for five minutes: after discarding the supernatant, the zooxanthellae pellet was washed 3 times in 40ml of 35‰NaCl w/v before centrifuging for 5 min at 500 x g. The final pellet was resuspended in about 20 ml of 35‰NaCl w/v.

Filtration

The homogenate was first passed through a 12μ Nucleopore membrane filter, which retained nematocysts and cell debris but allowed zooxanthellae to pass through. The filtrate was then added to a 5μ Nucleopore filter which retained the zooxanthellae. The zooxanthellae were resuspended from the filter using a jet of NaCl solution (about 30 ml) from a syringe and rehomogenised. This procedure was satisfactory for *P. porites*, but not for the mucus-rich homogenate of *M. annularis* which quickly clogged up the 12μ Nucleopore filter. Thus for this species, an initial centrifugation (10 min. at 500 x g) and resuspension (in 50 ml) step was introduced before starting with the filtration procedure.

The resulting sample of isolated zooxanthellae from each method was compared by visual observations under a light microscope and by measuring the apparent protein content per cell (McAuley, 1986). The visual measure of contamination (standardized to number of algal cells) was estimated by counting the numbers of algal cells, nematocysts and other debris in a Neubauer haemocytometer cell. Protein content was analysed on two 1 ml aliquots of zooxanthellae suspension and the higher the protein content per cell from the same coral the more contaminated with host material the sample was considered to be.

From visual observations, filtration produced a cleaner sample in both species (Table 2.2) with less contaminating tissue debris or nematocysts than centrifugation. This difference was observed also in the protein content per cell of zooxanthellae isolated from *P. porites* where filtration reduced protein content by approx. 35%. Zooxanthellae isolated from *M. annularis* had the same protein content irrespective of isolation technique.

Table 2.2 Comparison of the degree of host contamination of zooxanthellae isolated from *P.porites* and *M. annularis* using either the standard centrifugation or the novel differential filtration method. Mean ± SD, (n = 4).

Species	**Method**	nematocysts (numbers cell^{-1})	debris (numbers cell^{-1})	protein content (pg cell^{-1})
P.porites	**Centrifugation**	0.015	1.224 ± 0.727	360 ± 43.6
	Filtration	0.005	0.481 ± 0.385	247 ± 43.1
M.annularis	**Centrifugation**	0.003	0.778 ± 0.321	175 ± 44.7
	Filtration	0	0.426 ± 0.169	183 ± 28.3

The method of zooxanthellae isolation by differential filtration succeeds in producing a sample of zooxanthellae that is less contaminated by host debris than after standard centrifugation. Although the advantage of this novel methodology appears to be somewhat reduced by the mucus-rich tissue of *Montastrea annularis*, it was an improvement. It is especially advantageous when working in areas remote from laboratory facilities or when no chemical addition is acceptable as in the case of carbon budget determination, and was therefore adopted throughout the study.

2.4.4 Zooxanthellae population density

Comparative studies on the physiology of corals and especially carbon budget estimations have focused much attention on the population of zooxanthellae (*Symbiodinium microadriaticum*) and on their density in relation to host tissue.

The precision of zooxanthellae counts depends largely on the method with which coral tissue is extracted from the skeleton. Most commonly, tissue is waterpiked (see 2.4.2) and homogenized before zooxanthellae are counted in a haemocytometer. Since waterpiking does not extract the deeper parts of the tissue in corals such as *Porites porites* other methods are presented for comparison.

The following techniques were tested on a set of nubbins, randomly allocated:

A- fixation in carbon free Zenker's solution for 4 weeks, followed by decalcification in 10% HNO_3.

B- Fixation in formalin for 24h followed by decalcification in 10% HNO_3.

C- Fixation in both Lugols' solution and formalin for 24h followed by decalcification in 10% HNO_3. Formalin-fixed zooxanthellae lose their pigmentation and become more difficult to recognize among a tissue slurry, the addition of Lugols' (which stains the algae) prior to formalin fixation was investigated to minimize this problem.

In both A, B and C the fixed tissue was homogenized in 20ml of distilled water before counting.

D- Waterpiking (as in 2.4.2)

E- Anaesthetizing the nubbin in a solution of $MgCl_2$ before waterpiking. A *P. porites* nubbin was placed in a beaker with seawater: when all polyps were extended, a 1M solution of $MgCl_2$ was slowly pipetted into the beaker taking care not to disturb the coral. Within 10 min the coral was anaesthetized and could be brought into air and waterpiked with the polyp tissue still unretracted. While counting, it was observed that living brown coloured zooxanthellae in waterpiked samples are easy to identify so that a x10 magnification is sufficient, and counts can be carried out relatively quickly. The translucent zooxanthellae in fixed samples on the other hand are more

difficult to discriminate so x40 magnification and, in the case of Zenkers' fixed zooxanthellae, phase contrast was also used.

Four nubbins were used in each treatment (Table 2.3). Fixation in formalin produced the highest zooxanthellae counts, especially when in combination with Lugol's. Waterpiking had the lowest counts and the anaesthetising treatment was an improvement. Fixation in Zenkers' was intermediate.

Table 2.3 Zooxanthellae population density per surface area of *P porites* nubbins from the 'nursery'site on the North Bellairs Reef. Means ± SD (n = 4)

Treatment	($*10^6$ cell cm^{-2})
A- Zenker's	3.59 ± 0.951
B- Formalin	4.06 ± 0.935
C- Lugol's + Formalin	4.86 ± 1.265
D- Waterpik	2.91 ± 0.353
E- Anaesthetic + Waterpik	3.55 ± 0.464

When tested with a one-way ANOVA, no significant difference was found between treatments probably due to the small sample size (n = 4 per treatment) and relatively large standard deviation. Thus, to increase sample size, I chose to concentrate on the difference between formalin fixation (treatments B + C: $4.46*10^{-6} \pm 1.116*10^{-6}$ cell cm^{-2}) and waterpiking (D + E: $3.23*10^{-6} \pm 0.547*10^{-6}$ cell cm^{-2}). A Student's *t* test was performed on the pooled treatments: formalin fixation resulted in a 30% higher zooxanthellae count than waterpiking and the difference was found to be statistically significant ($t = 2.80$; $P<0.05$).

In conclusion, methods utilizing coral tissue fixation and decalcification are different from those involving waterpiking: the former have a significantly higher cell count per surface area (30%) and a higher standard deviation which possibly reflects the increased identification difficulties during counting. Waterpiking underestimates total cell counts by the amount that remains within the skeleton. Assuming that counts from formalin-fixed tissue are the best estimate, then waterpiking underestimates total zooxanthellae population density by 30%. This will be taken into consideration in the carbon budget formulation (Ch.3).

2.4.5 Protein content

Protein content of homogenised coral blastate (2.4.2) and of isolated zooxanthellae (2.4.3) was determined using the Markwell *et al.* (1978) modification of the Lowry (1951) method. A solution of 35 ‰ w/v solution of NaCl rather than filtered seawater was used for waterpiking and for zooxanthellae resuspension because this protein assay contains sodium dodecyl sulfate (SDS) which in the presence of Ca^{2+} ions (as in seawater) would precipitate out of solution. The homogenised coral blastate required dilution 1:1 with NaCl solution before determining protein content. Two 1 ml aliquots were measured for both coral and zooxanthellae protein content for each coral.

2.4.6 Carbon and nitrogen content

Coral tissue

Corals were fixed in Zenker's solution (see 2.4.2) decalcified in 10% HCl v/v and dried to constant weight. Total (= host + zooxanthellae) carbon and nitrogen content were measured in a C:H:N elemental analyzer.

Zooxanthellae

Zooxanthellae were isolated by filtration (2.4.3): the suspension was filtered onto pre-combusted GF/C filters that had been previously washed in distilled water and combusted at 500 °C for 7 hours to get rid of any organic residue. The carbon and nitrogen content of the suspension were measured with a C:H:N elemental analyzer and standardized to cell number after estimating cell density on a haemocytometer.

Mucus

Cnidarians excrete carbon (particulate and dissolved) mainly as mucus and mucus-lipids (Crossland *et al.*, 1980) and a further loss is produced by the expulsion of zooxanthellae. When carbon loss has been calculated by subtraction in order to balance carbon budgets, it has been estimated that in optimal light conditions it can account for as much as 50% of the carbon fixed in photosynthesis (Davies, 1984; Muscatine *et al.*, 1984). Mucus production is involved in some heterotrophic feeding activity (Lewis & Price, 1975) and in surface clearing and the rate of production is enhanced by high sediment loads (Stafford-Smith, 1990; Rogers, 1983). Excreted mucus might be an important source of organic carbon for the reef community (Benson & Muscatine, 1974) and in particular for the coral-associated bacterial fauna (Ducklow, 1979;

Wahbeh & Mahasneh, 1988). In some studies investigating the quantity and quality of coral secretions, mucus has been collected from corals in air (Wahbeh & Mahasneh, 1988; Ducklow & Mitchell, 1979) and under stressful artificial conditions (Meikle *et al.*,1988). Since the composition and concentration depends upon the degree of stress (Benson & Muscatine, 1974) these methodologies have to be avoided when attempting to measure 'natural' levels of carbon loss. Crossland *et al.* (1980) studied mucus secretion in intact corals by continuous incubations with inorganic ^{14}C and suggested a rate of mucus output for mucus-polysaccharides of 49 ± 7 μgC.mg protein^{-1}.d^{-1} and for mucus-lipids of 324 ± 220 μgC.mg protein^{-1}.d^{-1}. A direct measure of mucus production including both particulate and dissolved organic carbon by *Acropora variabilis* and *Stylophora pistillata in situ* (Crossland, 1987) gave estimations of 71.6 and 72 μgC mg protein^{-1} d^{-1} respectively.

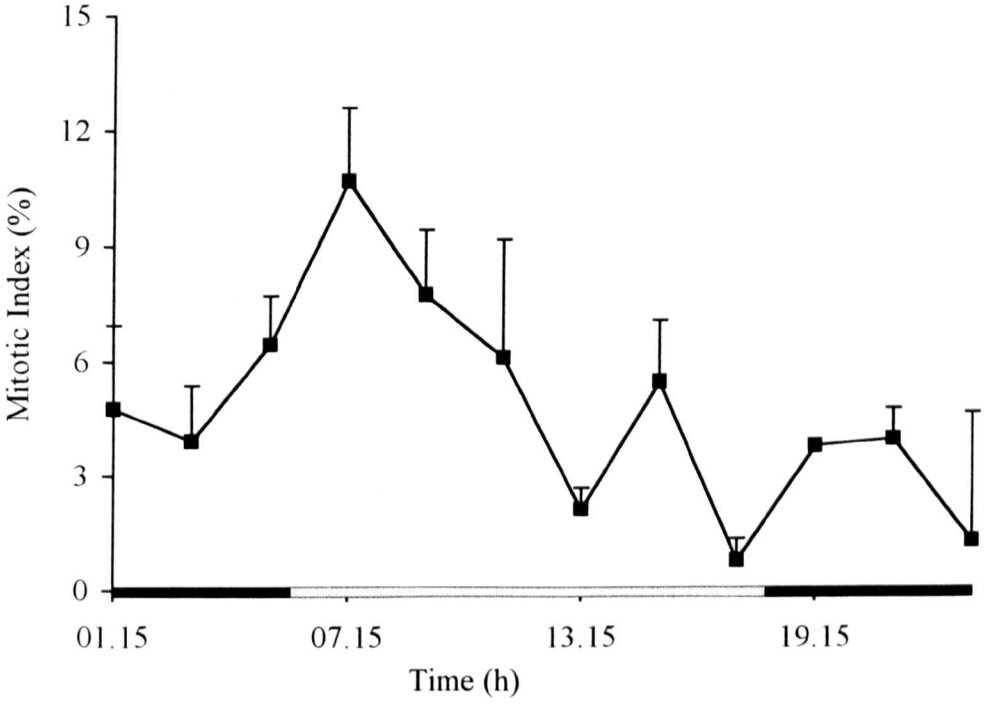
In this study, the estimation of total carbon loss as a component of the carbon budget was obtained in 24 h incubations as follows. Nubbins of *P. porites* (n = 8) from the 'nursery' site were brought into the laboratory and their tiles cleaned of any algal fouling. They were placed in 100 ml Pyrex glass beakers containing 50 ml of GF/C filtered seawater. In each beaker, a stream of fine bubbles was expelled from a needle attached to an air line to provide water motion and oxygenation without inducing polyp retraction. Beakers were placed in a water bath at 27 °C under two THORN luminaires with 400 Watt metal halide bulbs providing light intensity of 250 μmol photons m^{-2}sec^{-1} for 12 h per day. Corals were incubated in these conditions for 24 h and then removed from the beakers. Before replacing them on the reef, their surface area was determined (2.4.1). Beakers containing only seawater provided experimental controls (n = 2). At the end of the experiment, incubation water was collected into 100 ml glass tubes. Sufficient concentrated HCl was added to make a 10% solution in each tube. This succeeded in oxidizing the mucus during the following 8 h incubation period at 95 °C. Tubes were covered with inverted crystallizing dishes to minimise evaporation. All glass material (beakers, boiling tubes, crystallizing dishes) had been coated with dimethyldichlorosilane solution to avoid mucus attachment. Tubes were left to cool at room temperature and samples were frozen (-20 °C). Carbon analysis was carried out on a Shimadzu TO500 total organic carbon analyser (TOCA). On average a nubbin released 720 ± 391.5 μgC d^{-1}. For comparison with published data, this amount was standardized to surface area and to the average coral protein content measured on a different set of nubbins growing at the same site (3.2.5). Thus carbon lost in mucus and mucus-lipid was 125 ± 68.1 μgC cm^{-2} d^{-1} and 57.1 μgC mg protein^{-1} d^{-1}, a value within the range of those published.

2.4.7 Mitotic Index

The growth rate of the endosymbiotic zooxanthellae *in hospite* has been widely investigated because of its crucial role in the biology of the symbiosis. As in all unicellular organisms, the most important component of zooxanthellae growth is the increase in the number of cells as opposed to the increase in size of each cell (Prescott, 1976). Measurement of cell division has centred on the estimation of the mitotic index, i.e. the proportion of cells undergoing mitosis (MI). MI can be converted into cell growth rate (see 3.1.2) if the duration of mitosis (t_d) is known. Estimation of t_d requires synchronously dividing cells (McDuff & Chisholm, 1982). A characteristic phased division was observed in the zooxanthellae of jellyfish *Mastigias* sp. (Wilkerson *et al.*, 1983), while among corals, division of zooxanthellae appears to be asynchronous (Wilkerson *et al.*, 1983; Muscatine *et al.*, 1984; Wilkerson *et al.*, 1988) with few exceptions (Hoegh-Guldberg & Smith, 1989; Hoegh-Guldberg, 1994). Overall a relationship between host nutritional status (or nutrient availability in seawater) and cell division has been observed (Cook & D'Elia, 1987; Cook & Fitt, 1990; Fitt & Cook, 1990; McAuley & Cook, 1994; Muscatine & Marian, 1982). The highest MI *in hospite* was measured for zooxanthellae in fed temperate anemones (Cook *et al.*, 1987) while the lowest was measured from shaded *Stylophora pistillata* from the Red Sea (Muscatine *et al.*, 1984). Most strikingly cell division *in hospite* is always lower than when cultured *in vitro* implying the presence of some host tissue characteristics involved in the regulation of zooxanthellae growth rates (Muscatine *et al.*, 1984). In all the above studies, mitotic index was based on the proportion of cells appearing as doublets with a distinct cell plate (cytokinesis). Brown & Zamani (1992) introduced the standard cytochemical technique of staining nuclear material with Feulgen solution and successfully applied it to zooxanthellae in anemone tentacles. This method provides a more appropriate mitotic index because it is based directly on karyokinesis (i.e. the presence of 2 mitotic figures per cell) which occurs prior to cell plate formation. The comparison between cell plate and mitotic figures methods (Brown & Zamani, 1992) showed the latter to be an improvement. It produced a much higher (3 to 5 times higher) and more precise mitotic index estimation. Furthermore mitotic index is usually measured in order to estimate cell growth rate from the equation of McDuff and Chisholm (1982) which was derived from work on phytoplankton based on karyokinesis rather than just cytokinesis. Therefore to determine the mitotic index of zooxanthellae from *P. porites* in the carbon budget evaluation study, the technique of Brown & Zamani (1992) was applied for the first time to hermatypic corals.

A tray of nubbins was removed from the 'nursery' site and kept in a laboratory aquarium with running seawater. Sampling started at 15:15 on 26th November and continued at two hour intervals for 24 h with the exception of the sample at 03:15 which was missed and therefore

Fig. 2.3 Mitotic index (%) based on karyokinesis as a function of time of day for zooxanthellae symbiotic with *P. porites* in nubbins grown at the 'nursery' site on the North Bellairs Reef. Mean + SD; n=3. The dark area on the x-axis corresponds to the hours of darkness



repeated the following night, together with one other sample on either side. Polyps were collected by anaesthetizing the coral with MgCl solution (see 2.4.6) and cutting single polyps off the nubbin with a pair of very fine scissors: two subsamples of 4 polyps were obtained from two nubbins at each time interval. Polyps were treated similarly to the tentacles of anemone by Brown & Zamani (1992). They were fixed in 3:1 alcohol/glacial acetic acid for 2 h and transferred to 70% alcohol for storage at 4°C in Eppendorf tubes. The staining procedure required the hydrolysis in 1N HCl for 18min (a shorter or longer hydrolysis was found to be unsuccessful) in a water bath set at 60°C, followed by immersion in Feulgen stain for 3 h. A drop of 45 % (v/v) acetic acid was placed on a slide, a treated polyp was placed in it and then macerated with the coverslip. The cells with paired mitotic figures were counted out of a minimum of 500 cells per sample using a phase-contrast Zeiss microscope at x400 magnification. This value was expressed as a percentage of total number of cells observed.

As shown in Fig. 2.3, cell division varied during the 24 h period between 1% and 11% with an average mitotic index of 4.76%. A tendency for higher division rates just after sunrise (similar to cultured zooxanthellae of McAuley & Smith, 1995) is present. More investigations with this staining method are required to understand if the lack of phase found in coral studies is a real phenomenon or due to a dampening of a synchronized division due to the increased inaccuracy of cell plate identification (Brown & Zamani, 1992).

Eight species of Caribbean corals sampled at 1.5 m by Wilkerson *et al.* (1988) gave a very similar average mitotic index of 4.3% even though they used counts based only on cytokinesis. Regrettably no direct measurement of doublet cells was taken concomitantly to Feulgen staining on *P. porites* in this study, and would be inappropriate to make any deduction on the success of Feulgen staining in corals by comparing different studies.

2.4.8 Zooxanthellae volume

Corals were waterpiked and samples of the resulting homogenate were transferred to a haemocytometer cell: two measurements of cell diameter normal to one another were taken on 50 cells per nubbin or explant using a calibrated ocular micrometer and a binocular microscope (x1250).

2.4.9 Chlorophyll content

A known volume of zooxanthellae suspension was filtered on to precombusted 27mm GF/C filters. These were wrapped in aluminum foil and kept at 4°C until analysis. After extraction in 100% acetone, chlorophyll*a* and chlorophyllc_2 pigments were determined spetrophotometrically

using the equations by Jeffrey & Humphrey (1975) for dinoflagellates. The density of cells in the suspension was estimated in order to obtain the pigment content per cell.

Chapter 3

AN IMPROVED CARBON BUDGET FOR HERMATYPIC CORALS

3.1 Introduction

In several cnidaria-zooxanthellae associations, the transfer of photosynthetic substances from algal symbiont to host has been demonstrated in experiments with a ^{14}C-labelled precursor as a source of inorganic carbon for photosynthesis. *In vivo* incubations have proved that this photosynthetically fixed carbon becomes an integral component of host biomass (Muscatine & Hand, 1958; von Holt & von Holt, 1968; Lewis & Smith, 1971; Trench, 1971a; Sutton & Hoegh-Guldberg, 1990) and *in vitro* experiments with freshly isolated zooxanthellae have showed that appreciable translocation occurs only in the presence of host homogenate (Muscatine, 1967; Muscatine & Cernichiari, 1969; Trench, 1971c). In qualitative terms there is evidence for translocation of carbon in the form of glucose, glycerol, alanine, fatty acids and triglygerides: these compounds are either utilized by the host as a rapidly metabolized pool or fixed into immobile structural components such as tissue proteins and lipids (Muscatine & Cernichiari, 1969; Trench, 1971b; Benson & Muscatine, 1974; Schmitz & Kremer, 1977; Crossland *et al.*,1980; Patton *et al.*, 1983).

In addition to utilizing photosynthate, the host can absorb glucose and amino-acids from seawater (Stephens, 1962), feed on bacteria (Sorokin, 1973), capture and ingest zooplankton (Yonge & Nicholls, 1931a; Porter, 1974). Porter (1976) suggested that Caribbean corals display a range of growth forms correlated with their nutritional strategy, so that coral species with small polyps have a high surface to volume ratio and maximize autotrophy, and those with large polyps and a small surface to volume ratio tend towards heterotrophy.

Interest has therefore focused in determining to what extent the carbon fixed in symbiont photosynthesis meets the host requirements for maintenance and growth. This quantitative approach has followed two main lines of research: the CZAR model by Muscatine and colleagues (Muscatine, 1980; Porter, 1980; Muscatine *et al.*, 1981; Muscatine *et al.*, 1983) and the energy/carbon budget model by Davies and colleagues (Davies, 1984; Edmunds & Davies, 1986; Al-Sofyani, 1991; Harland, 1992). Both models have been successfully applied to studying how environmental factors such as light intensity (McCloskey & Muscatine, 1984; Muscatine *et al.*, 1984; Porter *et al.*, 1984; Al-Sofyani, 1991; Davies, 1991), seasonality (Al-Sofyani, 1991) or water quality (Edmunds & Davies, 1989) can affect the carbon transfer in a symbiotic relationship.

The protocols employed in these two models have both advantages and disadvantages. It was therefore appropriate to reassess them in order to combine the best of both into a new improved procedure for carbon budget determinations.

3.1.1 The Energy/Carbon Budget model

This model is a direct development of the widely used energy budget design (Brafield & Llewellyn, 1982) which equates the amount of energy or total carbon entering a system to the amount spent in metabolic processes and that secreted.
In the case of hermatypic corals, the following components can be identified to construct a carbon budget:

	Energy Input		*Energy expenditure*
Zooxanthellae:	Photosynthesis	=	Respiration
			Growth
			Translocation
Animal host:	Translocation	=	Respiration
	heterotrophic feeding		Growth
	DOM absorption		Reproduction
			Losses: mucus and mucus-lipid secretion

All the above components were measured by Edmunds & Davies (1986) with the exception of the absorption of dissolved organic matter (DOM), and expressed in energy units (Joules).
The rate of photosynthesis and respiration of the whole association were derived from short-term incubations in respiration chambers in the laboratory, under artificial illumination. The obtained oxygen flux data were used to calculate daily photosynthetic and respiratory rates using light intensity recordings from the reef. They were converted into energy units by assuming that glucose was the substrate for photosynthesis (2817 kJ obtained from each mole of glucose) and lipid was the substrate for respiration (19.63 J ml O_2^{-1}). Zooxanthellae respiration (R_Z) was measured as oxygen flux directly on freshly isolated cells in darkness and the animal respiration (R_A) obtained by subtraction of R_Z from total coral respiration (R_C). Zooxanthellae growth was measured from skeletal growth by assuming a constant proportion of zooxanthellae to surface area and a constant relationship between skeletal weight and surface area: skeletal growth was

measured at regular intervals by buoyant weighing and surface area was estimated with the aluminum foil method of Marsh (1970). Energy content of zooxanthellae, tissue samples and planulae were determined by wet oxidation. Once respiration and growth of zooxanthellae had been accounted for, all the remaining energy produced by photosynthesis was expressed as translocation. Microscopic inspection of decalcified and dissected polyps was used to evaluate the degree of heterotrophy and of reproductive expenditure by counting any zooplankton and planulae present in each polyp. Animal growth was measured from skeletal growth by assuming a constant relationship between skeletal weight and tissue weight. Finally, the energy released by nubbins over 24 h, was measured by filtering incubation water through pre-combusted GF/C filters and assessing their energy content by wet oxidation. In a subsequent study on *Anemonia viridis* by Harland (1992) all budget components were expressed in carbon units rather than energy units. Oxygen flux measurements were converted into carbon units with the following formula:

$$\text{mg C} = 1.428 * \text{ml } O_2 * 12/32 * [/ \text{ PQ or } * \text{ RQ}]$$

where PQ, the photosynthetic quotient, is the ratio of oxygen produced to carbon dioxide consumed during photosynthesis, and RQ, the respiratory quotient, is the ratio of oxygen consumed to carbon dioxide produced in respiration. In that study both PQ and RQ were assumed to be based on glucose with a ratio of 1 in both cases and biomass carbon and mucus released were measured with a C:H:N analyzer.

3.1.2 The CZAR model

The main objective in the development of this model was not the evaluation of the budget components, but the determination of how much of the host carbon requirement for respiration is supplied by algal translocation on a daily basis. Initially, photosynthesis to whole colony respiration ratios (24h P:R) greater than 1 were interpreted to endow self-sufficiency with respect to carbon (Davies, 1977). However, Muscatine *et al.* (1981) dismissed this as an unsatisfactory oversimplification; in fact, a symbiotic association with a 24h P:R > 1.0 could still be dependent on a heterotrophic carbon source if photosynthetically fixed carbon is primarily used for zooxanthellae growth and respiration. Therefore estimating the contribution of symbiont photosynthesis to host respiration requires both the amount of fixed carbon translocated and the fraction of total respiration due to the animal to be carefully evaluated.
The following equation was constructed to measure rigorously the fractional contribution of translocated zooxanthellae carbon to animal daily respiratory requirements or CZAR (Muscatine *et al.*, 1981), in the absence of any heterotrophic input:

$$CZAR = \frac{P_Z * T}{R_A}$$

where P_Z : net photosynthesis = gross carbon fixed by zooxanthellae - zooxanthellae respiration,

T: % of net photosynthetically fixed carbon translocated to the animal

R_A : carbon respired by the animal

Photosynthesis and respiration of the coral were measured *in situ*, over an entire day, with an underwater self-contained respirometer (Porter, 1980). The changes in oxygen concentration in the chamber were converted into oxygen fluxes and then converted into carbon equivalents using the photosynthetic quotient (PQ) and respiratory quotient (RQ).

To evaluate the amount of carbon respired by the zooxanthellae (R_Z) separately from that respired by the animal (R_A), it was assumed that respiration of both components is directly proportional to their relative biomass. Coral biomass was measured in terms of total protein by the method of Lowry on tissue homogenate (Lowry *et al.*, 1951). Zooxanthellae protein was determined indirectly from measurements of mean cell radius following these steps (Muscatine *et al.*, 1984):

1. mean cell volume (V): $V = 4/3\pi r^3$,
2. carbon per cell (C), from Strathmann equation derived for phytoplankton:

   $\log C = (\log V) * 0.866 - 0.46$
3. nitrogen per cell (N), assuming a C:N of 6.1 (from data in D'Elia *et al.*, 1983):

   $N = C/(C:N)$
4. protein per cell was estimated as N*6.25

and animal protein corresponds to the difference between total and algal protein.

The assumption that the ratio of alga-coral respiration is proportional to that of alga-coral biomass was tested by McCloskey & Muscatine (1984). They compared R_Z values obtained in the manner outlined above with R_Z measured directly as oxygen flux on freshly isolated zooxanthellae as in Davies' model and found a 8-fold difference (see section 3.3).

Translocation was initially assayed in short-term ^{14}C studies (Muscatine & Cernichiari, 1969; Muscatine *et al.*, 1981) by incubating either intact coral colonies (Muscatine & Cernichiari, 1969) or freshly isolated zooxanthellae (Muscatine *et al.*, 1981) in the light with $NaH^{14}CO_3$ and measuring the rates of release of fixed organic ^{14}C. Disadvantages with this methodology include: ^{14}C recycling between photosynthesis and respiration, lack of equilibrium between the initial pulse of ^{14}C and the soluble pools of ^{12}C compunds in the zooxanthellae and error of

extrapolating short-term results to daily totals. Therefore this methodology was abandoned in favor of a growth rate method (Muscatine *et al.*, 1984). The growth rate method is based on the fact that, once respiration of zooxanthellae has been accounted for, all daily fixed carbon either goes into new cells or has to be translocated to the host. Therefore, the total daily translocation expressed as a percentage of net carbon fixed is: $T = \frac{\mu_C - \mu}{\mu_C} \times 100$

where μ_C is the net carbon increment added per day to the zooxanthellae population by photosynthesis or carbon specific growth rate and μ is the daily growth rate of zooxanthellae.

μ_C is estimated from : $\mu_C = \frac{1}{C'} \times P_z$

where C' is the standing stock zooxanthellae cell carbon

and μ is estimated from: $\mu = \frac{1}{t_d} \ln(1+f)$ in McDuff & Chisholm (1982)

where t_d , the duration of cell division in days, has the value of 0.46 (i.e. 11h in a 24h day) calculated as described by Wilkerson *et al.* (1983) for *Mastigias* spp., since no direct measurement on corals is available. And finally f, the average mitotic index is calculated as the number of cells undergoing cytokinesis (doublet cells) per 100 cells.

When CZAR is larger than 100, it means that more carbon is translocated from zooxanthellae to the host than is used in animal respiration and some can be used for host tissue growth and/or released as mucus: when these components are also taken into consideration a complete carbon budget can be calculated. Muscatine *et al.* (1984) estimated the amount of carbon secreted from the association into seawater by ^{14}C tracer studies. In that paper the amount of carbon assimilated in host growth was not directly measured but predicted by subtraction when all the other components of the carbon budget were accounted for. However, following the Davies methodology (1984), Muscatine *et al.* (1985) estimated animal tissue growth by assuming a direct measurable relationship between skeletal surface area and tissue biomass.

Thus, the CZAR model developed into a complete budget: the only input into the system consists of the C fixed in photosynthesis which has to equate with the C used by zooxanthellae (zooxanthellae respiration + zooxanthellae growth) and by host (animal respiration + animal assimilation + secretion).

3.1.3 Comparison

The Muscatine papers present an overwhelming profusion of formulas, assumptions and convoluted redefinitions of many budget parameters so that at first sight, one is made to believe

that the approach is utterly different from that of Davies. However, this is not the case and the only identifiable differences are the following:

1. measurement of photosynthesis and respiration: short-term incubation under artificial lighting (Davies) vs. 24-h incubations with *in situ* respirometers (Muscatine).
2. zooxanthellae respiration: dark incubation of freshly isolated zooxanthellae (Davies) vs. calculation from biomass ratio of zooxanthellae to host (Muscatine).
3. zooxanthellae growth rate: estimation from skeletal increment (Davies) vs. measurement of cytokinesis with assumption of 0.46 days long cell division (Muscatine).
4. mucus/lipid secretion: direct energy/carbon content measurement (Davies) vs. extrapolation from ^{14}C tracer studies (Muscatine).

In comparing the two protocols, I was not able to compare the two methods for the measurement of photosynthesis (1) and for the estimation of mucus secretion (4) since the necessary equipment was not available. Carbon budgets were therefore determined using laboratory- based measurements of photosynthesis and colony respiration, and mucus secretion was determined directly by C analyses of incubation water. Estimations of zooxanthellae respiration (2) and zooxanthellae growth (3) were determined for both methods and the two resulting carbon budgets were compared.

No attempt was made to estimate carbon used in reproduction.

3.2 Methods & Results

The carbon budget was determined for nubbins of *Porites porites* grown at 2.5 m depth on the North Bellairs Reef, between November and January 1993.

3.2.1 Photosynthesis, respiration and light saturation curve

Photosynthetic and respiratory rates were measured as in 2.3.2. on 10 nubbins. The mean light saturation curve obtained was plotted in Fig. 3.1 as gross photosynthesis vs. irradiance. Mean dark respiration rate for the intact association was 11.59 ± 2.019 µl $O_2 cm^{-2} h^{-1}$ as measured during daylight hours ($R_C^{daylight}$).

The maximum net photosynthetic rate ($P_{net\ max}$) was 45.73 ± 14.736 µl $O_2 cm^{-2} h^{-1}$ while the maximum gross photosynthetic rate ($P_{gross\ max}$) was estimated to be 57.32 ± 16.55µl $O_2 cm^{-2} h^{-1}$ since $R_C^{daylight} + P_{net} = P_{gross}$. Alpha was 0.282 ± 0.0656 µl $O_2 cm^{-2} h^{-1}$ µmol photons$^{-1} m^2$ sec^1 and I_K was 199.8 ± 41.81 µmol photons m^{-2} sec^{-1}.

Fig. 3.1 Mean gross photosynthesis vs. irradiance curve for *Porites porites* nubbins grown at the 'nursery' site on the North Bellairs Reef at 2.5 m depth. Plotted values are means ± SE, n = 10. Line was derived from the best-fit hyperbolic tangent function.

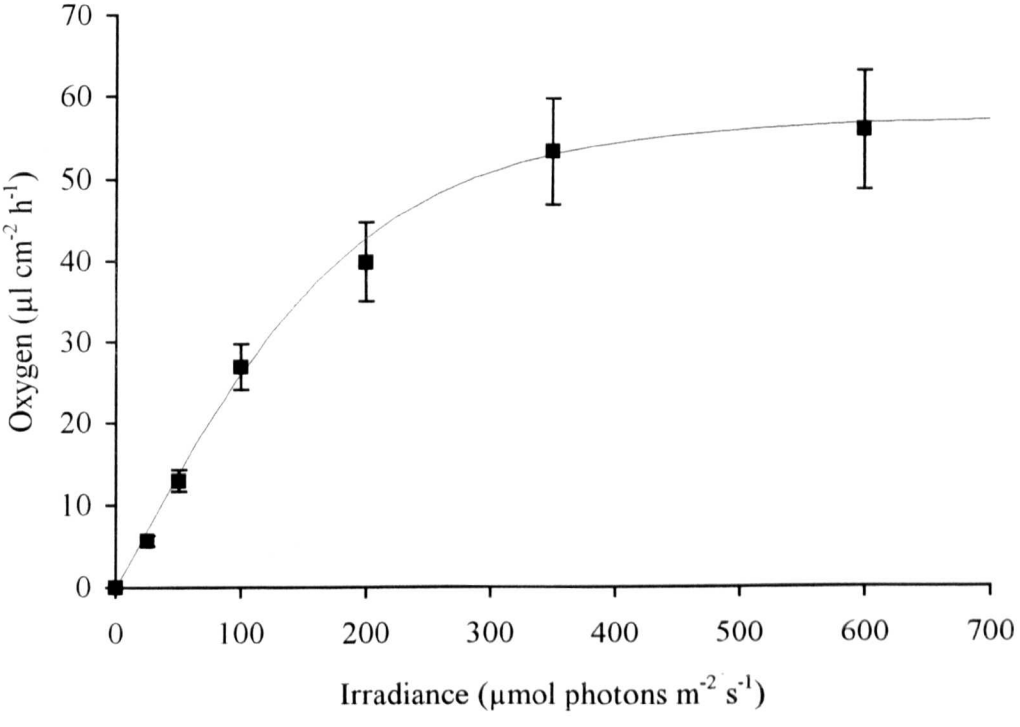
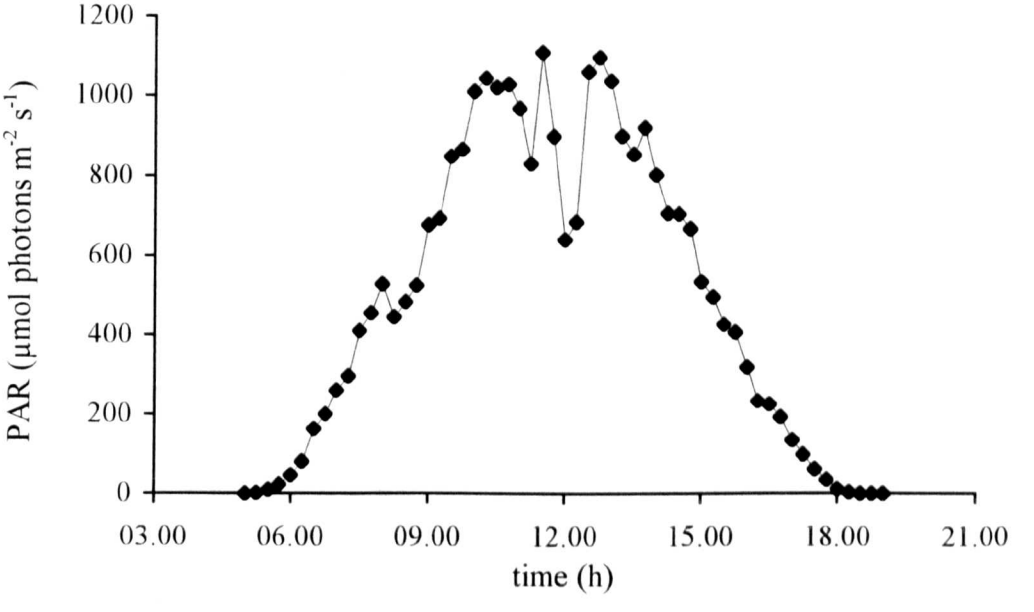


Fig. 3.2 Daily photosynthetically active radiation (PAR) at the 'nursery' site on the North Bellairs Reef at 2.5 m. depth on the 8th June 1994.



3.2.2 Daily integrated photosynthetic rate

Light intensity at 2.5 m depth on the reef was measured with an underwater light meter (2.3.2) on the 8th June 1994: the sun rose at 5:15 am and set at 6:40 p.m. with a daylength of 13.25 hours, reaching a maximum intensity of about 1100 μmol photons m^{-2} sec^{-1} in the middle of the day as shown in Fig. 3.2. The total daily irradiance was 25 mol photons m^{-2} d^{-1}. From the average coral photosynthesis vs. irradiance curve and the daily light intensity curve, a computer program calculated the integrated daily net productivity: for these *P. porites* nubbins, it was estimated to be 0.458 ml O_2 cm^{-2} d^{-1} over 13.25 h. To obtain the daily integrated gross productivity, respiration rate over 13.25 h was added to the net rate giving a value of 0.611 ml O_2 cm^{-2} d^{-1} : this corresponded to the total photosynthetic input into the budget.

3.2.3 Skeletal growth and surface area

The average skeletal density, determined as in 2.2b.1, was 2.775 g cm^{-3}(n = 10). A set of 10 nubbins were brought into the laboratory for weighing (2.3.1) at weekly intervals in the first month and then again after 4 and 6 months: their buoyant weight was transformed into actual dry weight using the above determined skeletal density. Initially these nubbins weighed 1.3 grams but grew to double their skeletal weight in about 5 months. The rate of growth was constant over 6 months, as shown in Fig. 3.3 where weight was plotted against time and a regression model was fitted to the data obtaining the following equation (with $R^2 = 0.99$):

weight (grams) = 0.0096 x time (days) + 1.3066

Surface area was calculated from measurements of nubbin diameter and height using calipers (2.2c.1). On the 25th of November 1993, both the surface area and the weight of 33 nubbins were measured in order to obtain a conversion factor to estimate surface area from weight. Fitting a linear regression through the data as in Fig. 3.4 gave the following equation (with $R^2 = 0.95$):

surface area (cm^2) = 2.697 x weight (grams) +2.015

When standardised to surface area, the average skeletal growth was 1.51 mg $cm^{-2}d^{-1}$ and the average surface area increment was 0.41 mm^2 $cm^{-2}d^{-1}$.

3.2.4 Zooxanthellae population density

Nubbins were waterpiked in 35‰ NaCl w/v. The tissue blastate was then homogenized in a glass pestle and mortar, its volume measured and two 1ml subsamples fixed in Lugol's solution.

Fig.3.3 Skeletal weight of *Porites porites* nubbins at the 'nursery' site on the North Bellairs Reef at 2.5 m depth over the observation period. Values are means ± SD (n = 10). Line is fitted using a linear regression model.

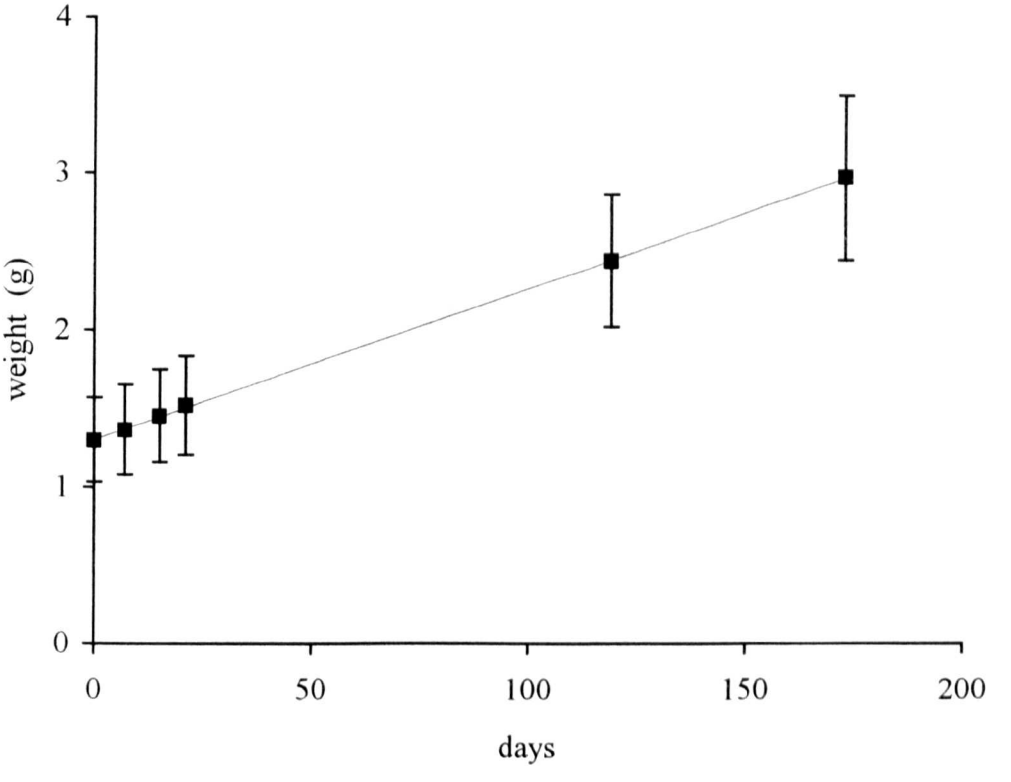
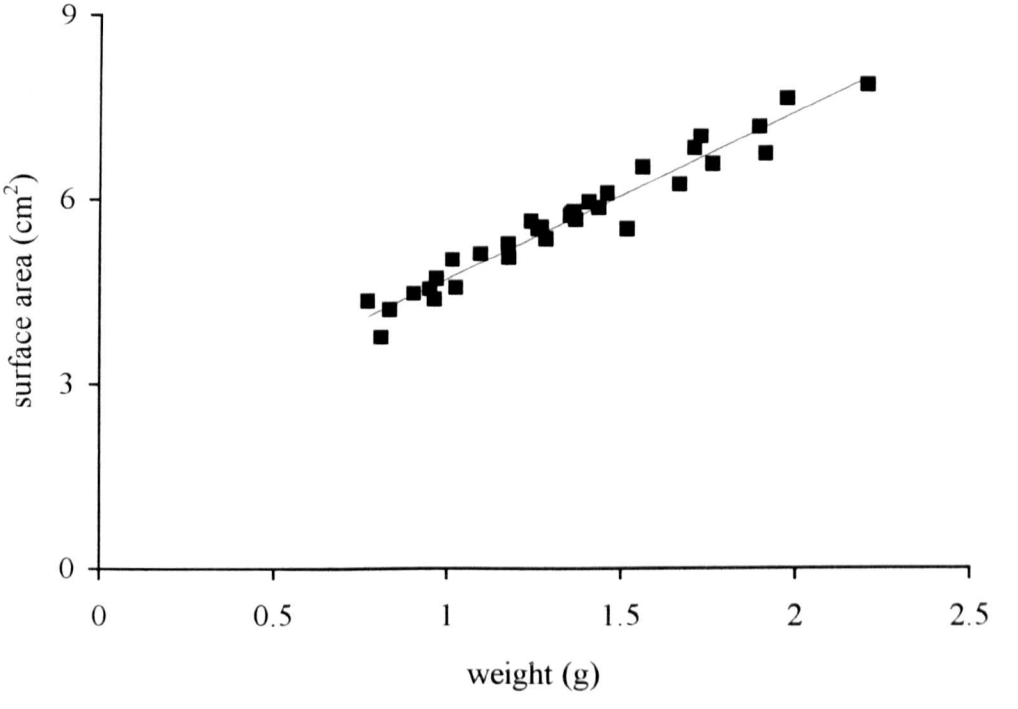


Fig.3.4 Relationship between surface area and skeletal weight for nubbins of *Porites porites* . A linear regression model was fitted to the data (n = 33).



Zooxanthellae cell number was determined with a haemocytometer on fixed samples and standardized to surface area. The average value obtained from ten nubbins was 2.96 ± 0.634*10^6 cell cm^{-2}.

3.2.5 Zooxanthellae (R_Z) and animal respiration (R_A)

Davies' Method

Ten suspensions of freshly isolated zooxanthellae were incubated in darkness to measure respiration rate as oxygen flux (2.3.3). Mean R_Z was 0.87 ± 0.127 µl O_2 *10^6 $zoox^{-1}$ h^{-1} or 2.57 µl O_2 cm^{-2} h^{-1}. By subtracting this amount from total coral respiration measured during light hours, animal respiration was estimated to use 9.01µl O_2 cm^{-2} h^{-1}.

Muscatine's Method

Total coral protein was measured (2.4.5) on the same coral tissue homogenate used for zooxanthellae density determination. The average protein content was 2.19 ± 0.276 mg protein cm^{-2}. The same zooxanthellae suspensions used in the respirometer, were placed in a haemocytometer cell to measure zooxanthellae size (2.4.8). The average diameter of a zooxanthella was 10.44 ± 1.107 µm which resulted in an average volume of 596 μm^3 and a protein content (see 3.1.2) of 89.9 pg protein $cell^{-1}$. Using the density of zooxanthellae determined by waterpicking of 2.96*10^{-6} cell cm^{-2}, the protein due to zooxanthellae per unit surface area was 266 µg protein cm^{-2}. When this value is compared to the total coral protein content of 2.19 ± 0.276 mg cm^{-2}, zooxanthellae account for 12% of total protein. Therefore, the animal has to account for 88%. Since protein content is assumed to be directly related to respiration, R_Z and R_A can be calculated by proportionality from $R_C^{daylight}$ as 1.39 and 10.2 µl $O_2cm^{-2}h^{-1}$ respectively.

3.2.6 Zooxanthellae growth rate (μ_Z)

Davies' Method

Following the assumption that zooxanthellae density remains constant over time, zooxanthellae growth was estimated from surface area increase to produce 12070 new cells cm^{-2} d^{-1}. The carbon content of zooxanthellae measured on freshly isolated cells (2.4.6) was 136.7 pg $cell^{-1}$. A daily production of 12070 cells therefore required a total of 1.65 µg C cm^{-2} d^{-1}. Since growth rate is the proportion of new cells to cell density, μ_Z = 12070/2.96*10^6 = 0.0041 d^{-1}. Another useful measurement to put growth rate into perspective is the population doubling time or G = ln2/μ = 170 days.

Muscatine's Method

In this study, mitotic index was measured not from the number of cell doublets (i.e. cells undergoing cytokinesis) but from staining DNA material and counting all cells undergoing karyokinesis for reasons given in 2.4.7. On average over a 24 h period *P. porites* polyps contained zooxanthellae with a mitotic index of 4.76%. Replacing this value in the equation for growth rate (3.1.2), $\mu = (1/0.46)*\ln(1+0.0476) = 0.1015\ d^{-1}$ which, with a density of $2.96 * 10^{6}$ cell cm^{-2} accounts for an increment of 300440 new cells $cm^{-2}d^{-1}$. The average carbon content per zooxanthella (derived from zooxanthella diameter, see 3.1.2) was 87.7 pg $cell^{-1}$. The growth rate of the symbiont population therefore required a total of 26.3 μg C $cm^{-2}d^{-1}$.

3.2.7 Animal growth rate

Assuming that tissue dry weight per unit surface area remained constant over time, the carbon used in animal biomass daily increment was estimated from the increase in surface area. In order to measure both dry weight and carbon content from the same set of nubbins, corals were fixed in carbon-free Zenker's solution and decalcified in 10% HCl (see 2.2c.2 for evaluation). On average *P. porites* tissue weighed 12.1 ± 4.325 mg cm^{-2} and carbon comprised 28.7% of total dry weight. Since surface area increased each day by 0.41 $mm^2cm^{-2}\ d^{-1}$, the carbon fixed into new coral tissue biomass was 14.16 μg C $cm^{-2}d^{-1}$: this value comprises of both animal and zooxanthellae carbon content and therefore to obtain the amount of carbon required in animal growth, the carbon required in zooxanthellae growth (from Davies' Method for net zooxanthellae growth: 1.65 μg C $cm^{-2}d^{-1}$) has to be subtracted from the total leaving 12.51μg C $cm^{-2}d^{-1}$.

3.2.8 Mucus secretion

Mucus and mucus-lipid secreted by nubbins in aerated 100ml beakers was measured over 24 hours (2.4.6). On average a nubbin released 720 ± 391.5 μg C d^{-1}, which standardized to surface area equals 125 ± 68.1 μg C $cm^{-2}d^{-1}$.

3.3 Carbon budgets

From the values calculated in the previous section, it is possible to compute the carbon budget components for both models. The result, expressed in μg C $cm^{-2}d^{-1}$, is shown diagramatically in

the following table. All oxygen flux data were transformed into carbon units giving arbitrary values of 1 to both PQ and RQ using the formula in 3.1.1.

Translocation (T) = gross photosynthesis - zooxanthellae respiration - zooxanthellae growth.

	Input	Expenditure	Davies	Muscatine
Zooxanthellae	Photosynthesis		327.3	327.3
		Respiration	**33.1**	**18.1**
		Growth	**1.65**	**26.34**
Host	Translocation		292	283
		Respiration	115.9	130.86
		Growth	12.5	12.5
		Mucus/lipid	125	125

The two different methodologies resulted in different estimations of the amount of carbon used both in zooxanthellae respiration and zooxanthellae growth.

Respiration rate obtained from freshly isolated zooxanthellae ($R_{Z,\ DAVIES}$) was 33.1 μgC $cm^{-2} d^{-1}$, almost twice as high as the respiration estimate from biomass proportionality ($R_{Z,\ MUSCATINE}$) which estimated a carbon requirement of 18.1 μg C $cm^{-2} d^{-1}$. $R_{Z,\ MUSCATINE}$ could be recalculated using as the basis for protein estimation the direct measurement of carbon and nitrogen content per zooxanthella rather than that predicted from cell volume. Thus, when measured directly, each cell contained 136.7 pg C $cell^{-1}$ and 18.7 pg N $cell^{-1}$: protein content (= N * 6.25) was 116.9 pg $cell^{-1}$ which would result in a R_Z of 23.5 μg C $cm^{-2} d^{-1}$. Edmunds & Davies (1984) measured respiration by isolated zooxanthellae to be 23.6 μgC $cm^{-2} d^{-1}$ in *Porites porites* and by assuming the protein content per cell to be the same as in this study, the respiration obtained from biomass can be calculated to require 13 μgC $cm^{-2} d^{-1}$, i.e. $R_{Z,\ DAVIES}$ was again about twice $R_{Z,\ MUSCATINE}$. McCloskey & Muscatine (1984) compared the two methods similarly and found *in vitro* respiration of zooxanthellae of *Stylophora pistillata* (81.3 μgC $cm^{-2} d^{-1}$) to be 8 times as high as that predicted from biomass ratios (10 μgC $cm^{-2} d^{-1}$): however no replicate measurements *in vitro* were available in their study. They argued that the isolation procedure could induce increased respiration. In fact it appears that freshly isolated zooxanthellae *in vitro* do not behave in the same manner as *in situ* and may have an increased growth rate (Suharsono & Brown, 1992). On the other hand, $R_{Z,\ MUSCATINE}$ is based on the untested assumption that the respiration rate of an algal cell per protein is the same as the respiration rate of an animal standardized to its protein content.

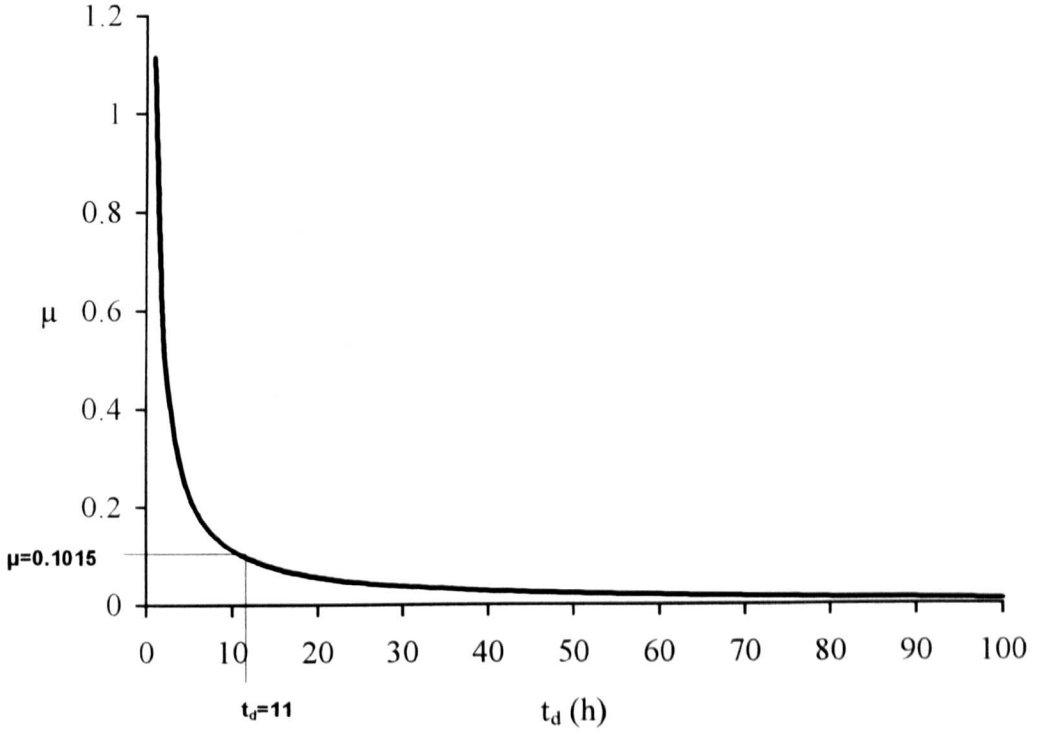
Obviously, it is impossible to determine what is the correct respiration rate for zooxanthellae by simply comparing the two methods. In view of the large difference between methodology and assumptions it is actually remarkable that in this study on *Porites porites* $R_{Z,\ DAVIES}$ is only less than twice $R_{Z,\ MUSCATINE}$ and within the context of the carbon budget this difference accounts for only 3% of the total carbon input per day. In the calculation of carbon budgets in subsequent chapters therefore, $R_{Z,\ MUSCATINE}$ was chosen above $R_{Z,\ DAVIES}$ because the latter measurement *in vitro* was found too time consuming.

The difference in zooxanthellae growth rate is much more marked: Davies' model required only 1.65 μg C $cm^{-2} d^{-1}$ in comparison to 26.34 μg C $cm^{-2} d^{-1}$ required in Muscatine's. This difference is produced both by the method used in estimating the number of new cells and the use of different carbon contents per cell: the Davies' method accounted for 12070 new cells $cm^{-2} d^{-1}$ containing 136.7 pg C $cell^{-1}$ (C:H:N measurement on freshly isolated cells), while Muscatine's method estimated an increment of 300440 cells $cm^{-2} d^{-1}$ with only 87.7 pg C $cell^{-1}$ (from the Strathmann's equation). In order to focus attention on the difference in the actual estimation of growth rate, the directly measured carbon content of zooxanthellae was used also for Muscatine's method, producing an even larger carbon requirement for zooxanthellae growth of 41.06 μg C $cm^{-2} d^{-1}$. The two methods have a 25-fold difference.

The Davies method considers zooxanthellae density per unit surface area to be constant over time and assumes that new zooxanthellae are produced only to fill new animal cells measured by an increase in surface area. This method obviously measures only net growth of zooxanthellae (μ_{NET}) and therefore it ignores zooxanthellae lost (via death and expulsion). If zooxanthellae density is a constant, new cells must be produced at the same rate with which they are lost and therefore zooxanthellae growth has to occur even if surface area does not increase.

The Muscatine method is based on the *in situ* growth rate of zooxanthellae estimated from diel mitotic index data and therefore corresponds to the gross growth rate (μ_{GROSS}). The duration of cell division (t_d) is a critical parameter of the growth equation (McDuff & Chisholm, 1982) and is likely to change between species (Chisholm, 1981). Unfortunately it can only be calculated in populations with phased division and since zooxanthellae from corals had shown exclusively asynchronous division, a value for t_d = 11 h measured in the tropical pelagic jellyfish *Mastigias* sp. with its synchronously dividing zooxanthellae was used. Obviously, the assumption that t_d in hermatypic corals could be constant and equal to that for *Mastigias* sp. was as unjustifiable (McDuff & Chisholm, 1982) as it was necessary. A small change in t_d can have a large impact in the estimation of cellular growth rate as shown in Fig.3.5 where μ was estimated for different values of t_d, while maintaining the measured mitotic index as constant. Interestingly the duration of 11 h for cell division is on the edge of the most responsive part of the curve where a small

Fig. 3.5 Growth rate of zooxanthellae (μ) calculated as a function of the cellular division rate (t_d). See section 3.1.2 for details.

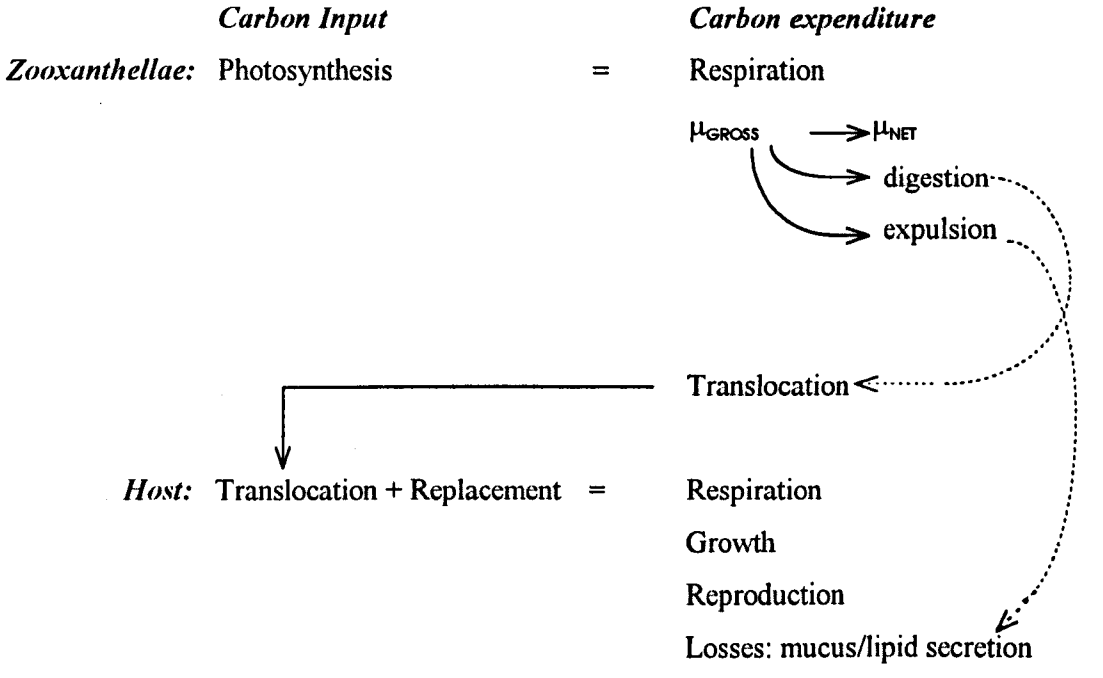


change in the independent variable will have a large effect in growth estimation. Nonetheless in order to obtain the same value of μ as in Davies' method, t_d would have to be increased from 11 h to 11 days. Only recently, Hoegh-Guldberg & Smith (1989) and Hoegh-Guldberg (1994) found some phased division in zooxanthellae populations from *Seriatopora hystrix* and *Pocillopora damicornis* and calculated values for t_d of 13.4 and 8.4 h respectively, within the range of *Mastigias* sp.

Thus, accepting Muscatine's assumption about the duration of t_d, the difference between the two methods has to be interpreted in terms of zooxanthellae "loss".

Since Muscatine's method measured the actual intrinsic cellular growth (μ_{GROSS}), while Davies' measured only net growth (μ_{NET}), the difference between the two has to correspond to the amount of zooxanthellae which are either expelled from the association or digested by the host. Hence, $\mu_{GROSS} - \mu_{NET}$ = cells expelled and digested. Therefore in this study, (μ_{GROSS} = 300440, μ_{NET} = 12070) 288370 cells cm^{-2} are either expelled or digested every day. Daily expulsion rate was estimated by Hoegh-Guldberg *et al.* (1987) and by Stimson & Kinzie (1991) to be about 0.1 % and 1% of cell standing stock respectively. By using the latter estimation as a maximum expulsion rate under normal conditions, about 29600 cells cm^{-2} would be expelled each day by *P. porites* nubbins in this study, leaving 258770 to be digested by the host. To maintain a constant density, expelled and digested cells need to simultaneously replaced. Since zooxanthellae density is $2.96 * 10^6$ cm^{-2}, replacement rate (= cells replaced / cells standing stock) would be 0.097 d^{-1}. Muscatine *et al.* (1985) measured the growth rate of animal tissue from surface area increment (corresponding to Davies' method for μ_{NET} in this study) and growth rate of zooxanthellae from mitotic index (μ_{GROSS}): they estimated a 3- to 9-fold difference between the two. Since zooxanthellae were growing faster than the host, but cell density was constant, they implied that the host was in control of zooxanthellae population possibly by digestion. All cells that are replaced but not expelled must be digested by the host. Even though the subject has not been fully clarified, the ability of hosts to digest zooxanthellae (host "farming" zooxanthellae) has been suggested (Boschma, 1925; Yonge & Nicholls, 1931a) as a possible mechanism of host control of the algae. For example, Steele and Goreau (1977) presented evidence for zooxanthellae degeneration induced by protein extracts of ruff, oral disc and tentacles of the symbiotic anemone *Phyllactis flosculifera*. However, rather than implying an active host control, the difference between μ_{GROSS} and μ_{NET} can be explained in terms of zooxanthellae mortality rate *in situ*. Fitt and Trench (1983) working with scyphistomae of *Cassiopeia xamachana* found that healthy zooxanthellae actively prohibit phago-lysosome fusion by the host to the vacuole in which they reside while senescent symbionts (or those treated with the photosynthetic inhibitor DCMU) become subject to enzymatic attack.

These last considerations affect the form of the carbon budget because on the one hand the amount of carbon in senescent cells digested by the host would add to that translocated and on the other hand, all carbon in expelled zooxanthellae would be measured with the carbon of the mucus/lipid secretion from the host (hence the dotted lines in the following diagram).
Thus, the budget can be re-written as:



And recalculated in carbon units (μg C cm^{-2} d^{-1}) as follows:

	Input	Expenditure	
Zooxanthellae	Photosynthesis		327.3
		Respiration	23.5
		μ_{NET}	1.7
		Digestion (D)	35.4
		Expulsion (EX)	4.1
		Translocation (T)	262.5
Host	TT = T + D		298.1
		Respiration	125.5
		Growth	12.5
		Mucus/Lipid	120.9

The respiration rate for zooxanthellae used above is that of $R_{Z,\ MUSCATINE}$ calculated from measured concentrations of C and N per cell.

Translocation (T) has been calculated by subtraction of all carbon used by the zooxanthellae components of the budget from total gross carbon fixed: it accounts for 262.5 μgC $cm^{-2}d^{-1}$. However for reasons given above, this is not the only carbon that moves from zooxanthellae to host: all digested cells (D) enter the animal compartment too, while expelled cells (EX) would end up in the measure of carbon loss by secretion. Input into the host consists of both T and D with a total translocation (TT) of 298.1 μgC $cm^{-2}d^{-1}$.

To further improve the accuracy of the budget the following corrections need consideration:

1. Respiration at night is only 70% of respiration measured in daylight hours (2.3.3). The daily carbon requirement for coral respiration shown above was calculated from daylight respiration only: corrected for 10.75 h of respiration in darkness, R_C would become 128.93 μgC $cm^{-2}d^{-1}$.
2. The comparison between techniques to measure zooxanthellae density (2.4.4) showed that waterpicking has the potential to underestimate density by 30% : if this is taken into account *P. porites* could have as many as 3.85 $*10^{-6}$ zooxanthellae cm^{-2}.
3. The comparison between techniques to measure tissue dry weight (2.4.2) showed that Zenker's fixation also has the potential to underestimate density by 30%: taking this into account *P. porites* could have up to 15.7 mg tissue dry weight cm^{-2}.

The final budget recalculation in μg C cm^{-2} d^{-1} results in:

	Input	**Expenditure**	
Zooxanthellae	Photosynthesis		327.3
		Respiration (R_Z)	26.5
		μ_{NET}	2.1
		Digestion (D)	48.1
		Expulsion (EX)	5.3
		Translocation (T)	245.3
Host	TT = T+D		293.4
		Respiration (R_A)	102.4
		Growth	16.3
		Mucus/Lipid	119.7

In order to balance the budget, all net output components (R_Z + μ_{NET} + EX + R_A + host growth + mucus) were subtracted from the photosynthetic input. Out of a daily carbon input of 327.3 μg C cm^{-2} d^{-1}, the measured output accounted for a total of 272.3 μg C cm^{-2} d^{-1} (83%). Therefore 55 μg C cm^{-2} d^{-1} were unaccounted for. This corresponds to only 17% of total input: this is a small amount considering how each component was measured. In particular, the daily photosynthetic input was estimated for a day with optimal light conditions and it is therefore an overestimation in comparison to the growth rate measurement which was derived from reef conditions over a month long period.

3.4 Conclusion

A new improved carbon budget was constructed after description and comparison of Davies' and Muscatine's widely used methodologies.

In particular, the two methods were found to differ in the measurement of 1. oxygen fluxes, 2. zooxanthellae respiration (R_Z), 3. zooxanthellae growth (μ_Z) and 4. carbon lost in mucus/lipid secretion. Due to equipment constraints only 2 and 3 were compared.

Nonetheless it would be interesting to study 1. in more detail: to date, there is no report comparing respirometer measurements under artificial conditions with those obtained with underwater respirometers placed on a reef. Furthermore, while evidence is building up regarding the effect of water motion on many physiological responses including O_2 and CO_2 fluxes, there is still no information relating underwater semi-closed respirometer measurements on the reef to actual reef conditions. If the carbon budget is used as a bioassay in comparative ecotoxicological studies than the laboratory methodology employed in this study is satisfactory.

2. It was measured that $R_{Z,\,MUSCATINE}$ was only twice as large as $R_{Z,\,DAVIES}$ but since this component is very small in comparison to the rest of the budget, the difference in R_Z measurement was found to account for only 3% of the total daily carbon input. The comparison cannot identify the correct method but considering the small difference, $R_{Z,\,MUSCATINE}$ was chosen simply because it can be obtained quickly and easily, an important prerequisite if the new carbon budget is used as a bioassay.

3. The comparison between zooxanthellae growth estimation showed that Davies' method measures μ_{NET} while Muscatine's measures μ_{GROSS} : the difference between the two was 25-fold and was interpreted as growth of cells required to balance both expulsion and digestion, i.e. $\mu_{GROSS} - \mu_{NET} = \mu_{EXPULSION} + \mu_{DIGESTION}$. Expelled zooxanthellae were not counted directly in this experiment but estimated from published data, however their carbon content was measured as

part of mucus/lipid secretion. The component of zooxanthellae digestion is new to the budget. Authors have suggested that the measurement of zooxanthellae growth derived from MI (μ_{GROSS}) is always higher than host growth rate and the difference cannot be accounted for by the rate of expulsion which is relatively low in normal conditions. Therefore either μ_{GROSS} is wrongly computed or/and zooxanthellae digestion must occur. Since μ_{GROSS} depends upon the duration of cell division, the problem of how to determine t_d accurately requires further investigation. A component of zooxanthellae digestion is especially important because it consists of carbon used in algal growth and then absorbed by the host and therefore it constitutes an intrinsic part of the total translocated carbon. In this study in optimal light conditions, $\mu_{DIGESTION}$ was as high as 20% of actual translocation and was sufficient to provide enough carbon to satisfy 50% of animal respiration. The significance of this carbon source might be much higher at depth where translocation is reduced but the mitotic index is constant or even increased (Wilkerson *et al.*, 1988). More research is urgently needed to evaluate the mechanisms of zooxanthellae digestion and its role in the metabolism and growth of the host.

3.5 Carbon budget methodology for use as a bio-assay

In order to use a carbon budget as a bio-assay, a more succinct form was required. Ideally a bio-assay should be easy, cheap and fast and should minimise the amount of coral necessary for measurements.

Thus, the following budget was constructed:

	Input	**Expenditure**
Zooxanthellae	Photosynthesis (Pgross)	
		Respiration (R_Z)
		μ_{NET}
		Expulsion (EX)
		Translocation (TT)
Host	TT	
		Respiration (R_A)
		Growth
		Mucus/lipid

This budget differs from the complete one of p.61 because:

1. Mitotic index is not measured and therefore the estimation of μ_{GROSS} is missing. As previously explained, this is not important in the overall budget because $\mu_{DIGESTION}$ is an intrinsic part of TT which is calculated by subtraction.
2. Mucus/lipid secretion is estimated only by subtraction (mucus/lipid = TT- R_C - growth) as the photosynthetically fixed carbon lost by the association.

3.5.1 Summary of methodology for carbon budgets used as a bio-assay

Photosynthesis- Oxygen flux measurements obtained in a laboratory respirometer chamber under increasing light intensity were used to construct a P/I curve. Oxygen flux was converted into carbon units with the assumption of a PQ and RQ of 1.0. Daily rate of carbon fixation was derived from the integration of P/I and the light recorded at the site of growth of the corals under optimal conditions.

Respiration of the coral- As photosynthesis. The daily R_C was calculated given a 40% difference between night and day respiration rates. R_Z and R_A were computed from the R_C by direct proportionality to the protein content of zooxanthellae and host components (Muscatine's method). Protein content of zooxanthellae was measured directly (see 2.4.5) on a suspension of isolated zooxanthellae, rather than deriving it from carbon content.

μ_{NET}- The zooxanthellae population density was measured by waterpiking (given a 30% underestimation by this method) and standardised to surface area. It was assumed to be constant over the period of study. Thus μ_{NET} was calculated from the increase in surface area of the coral. Daily change in surface area was derived from the change in skeletal weight (measured by buoyant weighing) after the relationship between weight and surface area had been determined. Carbon content per algal cell was measured on a C:H:N analyser after isolation by differential filtration.

Expulsion- Assumed to be always 1% of algal standing stock

Translocation- Calculated by subtraction

Growth- The surface area of the coral was measured before fixation in Zenker's solution and decalcification (it was assumed that 30% of tissue was lost in this procedure). The tissue was then dried to constant weight and its carbon content measured with a C:H:N analyser. From this estimate of carbon content per unit surface area, the carbon content due to zooxanthellae was subtracted to leave the measure of host carbon content. The daily increment in surface area derived from weight change was used to determine the daily amount of carbon used in host growth

Losses- Calculated by subtraction

Carbon budgets were calculated in this manner for *P. porites* nubbins grown in the photostat and on the reef in the nutrient enrichment experiments (Ch.5, 6 and 7). For comparison, carbon budgets for *M. annularis* were also calculated but this required the acceptance of the following untested assumptions:

1. the difference in the rate of respiration between day and night is the same as in *P. porites*.
2. waterpicking and fixation in Zenker's result in a 30% underestimation of the zooxanthellae population density and tissue dry weight respectively as in *P. porites* .
3. the net measurement of growth for both zooxanthellae and host carbon is directly proportional to the increase in surface area which is determined from the daily increase in weight once the conversion factor 'surface area:weight' is known. With different sized nubbins of *P.porites*, the conversion factor used was the slope of the regression line fitted to the surface area and weight measurements of each coral. In *M. annularis*, however, the weight of each explant depends on the depth of the core and has no relationship to surface area. I therefore assume that each core is a part of a coral colony with a perfectly hemispherical shape and a surface area of 10 cm^2 and that growth occurrs at the same rate in all directions. The bulk density of this 'standard' colony is 0.542 gcm^{-3}: this was calculated from the yearly linear extension rate of a coral at the BRI site (0.77 cm y^{-1}, measured by Tomascik & Sander, 1985) and the yearly weight increase extrapolated from my data of explants grown at BRI (0.418 mg $cm^{-2}y^{-1}$). Since a hemisphere is assumed to be the shape of this coral, the relationship between surface area and weight can be calculated from geometrical equations, and thus each increase in weight corresponds to a known surface area increase. It has to be stressed that surface area and weight in a hemisphere are not linearly related. Therefore, the same change in weight per day in a 10 cm^2 colony results in a much bigger increment in surface area than in a 100 cm^2 colony. Budgets for *M. annularis* are size specific. The budgets presented in Ch.8 to test the potential of this technique as a bio-assay have been calculated for a 10cm^2 colony for both coral species.

Chapter 4

LABORATORY CULTURE OF NUBBINS AND EXPLANTS

4.1 Introduction

A major obstacle to the study of coral ecophysiology is the difficulty of keeping corals alive and healthy in laboratory aquaria for long-term studies. Since the initial experimental work by Yonge and Nicholls during the Great Barrier Reef Expedition (1928-29), the difficulties of culturing corals have been overcome with seawater flow-through systems. The continuous renewal of water around the corals ensures that the levels of the essential elements such as oxygen, carbon, calcium, and inorganic nutrients as well as pH and temperature in the aquaria remain relatively constant. Although strong aeration can be employed to produce the necessary water motion around the corals, the directional flow produced by a high rate of flushing in the experimental tanks is a useful asset. Flow-through systems are supplied with fresh seawater pumped directly from an adjacent reef and thus some major constraints are introduced: the necessity for close proximity to the sea, the likelihood of the water being nutrient enriched from the adjacent coastal environment (although algal scrubbers can be employed to decrease nutrient concentration as in Stambler *et al.*, 1994a), and the high costs of pumping.

In ecotoxicological or pathobiological studies requiring the addition of substances to the water, the rapid throughput of water would necessitate continuous addition, which could be expensive and (since the water is usually returned directly to the sea) environmentally unacceptable.

It is crucial to overcome these problems and an essential part of the current study was the development of a laboratory culture system which would enable corals, for the first time, to be grown in the laboratory with minimal water replacement under totally artificial and repeatable conditions. Some useful information on long-term maintenace of corals in closed circuit aquaria was gathered from husbandry methods developed for aquarists (Yates & Carlson, 1992; Frakes, 1994; Jaubert, 1989).

The following qualities were required by the new system:

- artificial lighting providing illumination of an intensity similar to that on a shallow reef
- strong and turbulent water motion
- constant temperature, salinity, pH, dissolved inorganic carbon and dissolved oxygen
- minimal water replacement

It was also important to construct a system that would suite the growth requirements of both *Porites porites* and *Montastrea annularis*.

The success of the system can be initially estimated by monitoring the corals visually for any sign of stress (prolonged polyp retraction, tunic formation, zooxanthellae expulsion, mesentrial filaments extrusion, tissue necrosis). However this is only a measure of survival. A study of the corals' physiology was necessary to give the confidence in the system needed for the establishment of nutrient enrichment experiments. Since light plays such a key role in coral physiology, the effect of two levels of irradiance was also worth investigating. Thus, the success of the culture system was tested by comparing the physiological qualities (photosynthesis, respiration, skeletal growth rate) of corals growing in the laboratory under two light regimes, with those on the reef at the shallow 2.5 m deep 'nursery' site.

4.2 Materials and Methods

Initially a shallow (<10cm) 1 litre aquarium was equipped with magnetic stir bars and air stones, and placed in a constant temperature water bath under an overhead bank of fluorescent light tubes. This setup was successful with *P. porites* nubbins but failed to culture explants of *Montastrea annularis*. Explants suffered immediately from protozoan infections and within three days, 'bleaching' and tissue necrosis were obvious. Changes in the chamber design focused on increasing the flow of seawater around the corals. After unsuccessful attempts at using water pumps rather than stir bars in the same small aquaria, a new system was constructed and tested as described below.

4.2.1 Design of the culture system

As shown in Fig. 4.1 and 4.2a, a glass aquarium (30x21x18 cm) was fitted with an overflowing gap to maintain a water depth of 11 cm and a total chamber volume of 7 litres. Water was pumped into the chamber from a seawater reservoir with a peristaltic pump, at a rate of 300ml per hour, producing total water replacement once in 24h.

The most important feature of the new chamber was the strong, turbulent and spasmodic water motion. This was created by two glass tubes connected to an air pump and fixed beneath a central glass tray so that their fan-shaped mouths were facing each other (see Fig. 4.2b). Air bubbles were trapped by the tray and increased in size until they were large enough to escape through the gap between the central and the lateral trays. The size of these gaps as well as the height of the trays were critical in determining the velocity of escape of the air bubbles and thus the water motion in the chambers. A gap size of 4mm and trays positioned at 10mm above the aquarium floor were chosen. Corals were held in place on the glass trays by purpose-built perspex holders.

Fig. 4.1 The photostat chamber, with explants of *Montastrea annularis*.

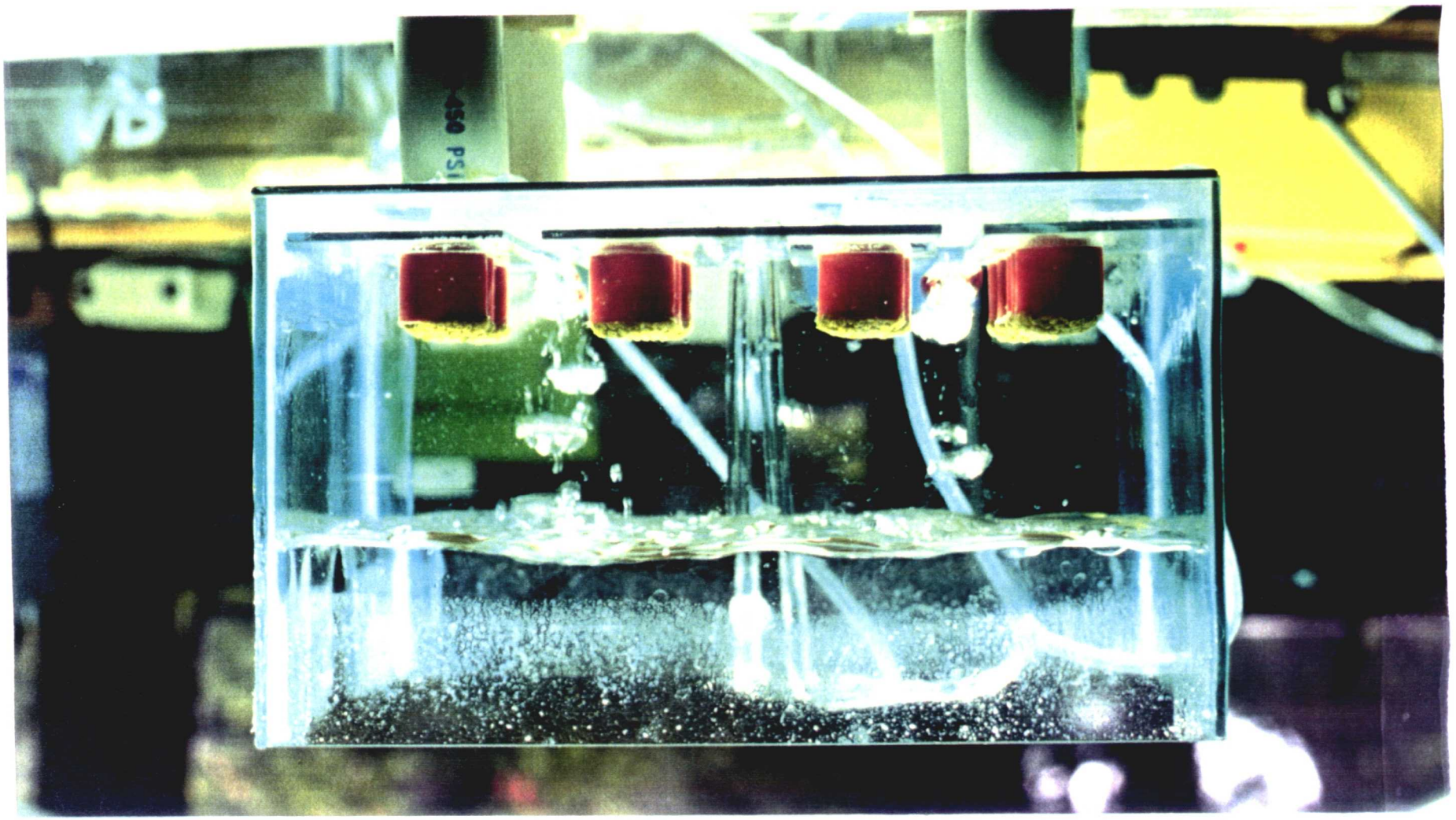


Fig. 4.2 Diagrams of the photostat chamber in side view (a) and top view (b)

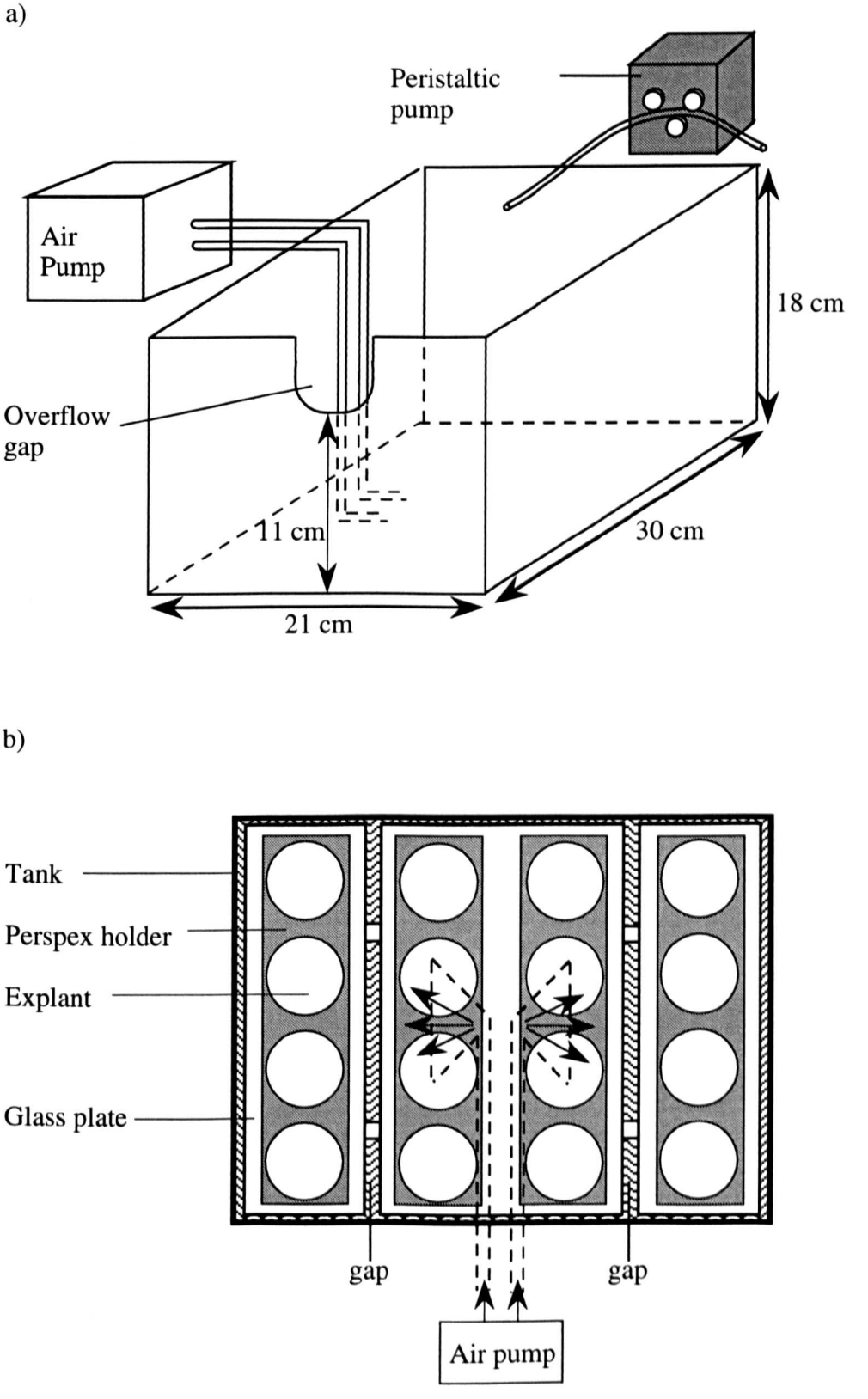
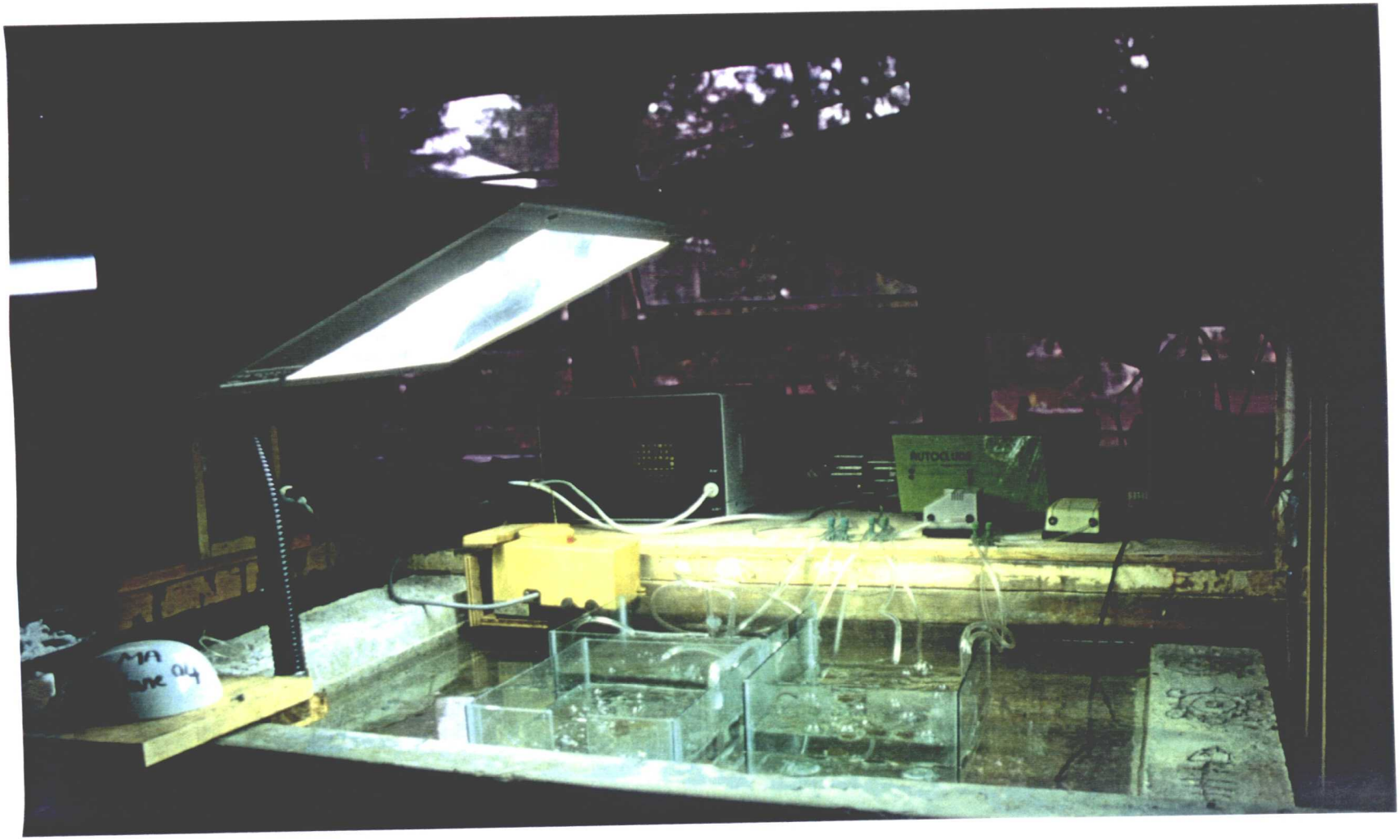


Fig. 4.3 The complete photostat system


AUTOCLAVE

A maximum of 16 explants or 20 nubbins, arranged in 4 rows, fitted each chamber: the central tray (15x21cm) held two rows, while the side trays (7x21cm) held one row each.
The turbulence produced at the surface was sufficient to maximise gas exchange between water and air and pH was approximately constant at 8.2 (±0.1).
Chambers were supported in a water bath (Fig. 4.3) with a cooling coil and a constant temperature heating unit set at 27°C. The maximum temperature fluctuation observed between day and night was ±0.5°C. A maximum of 4 chambers could be held in the water bath. Light was provided by two THORN luminaires with 400 Watt metal halide bulbs placed approx 60 cm above the water surface to provide corals with an irradiance of approx 400 µmol photons $m^{-2}s^{-1}$. Light intensity in the chamber was measured with a 'cosine' sensor and a light meter (Skye Instruments Ltd.). The luminaires were connected to a timer programmed to switch on between 5:30 and 18:30 hrs, thus providing constant illumination 13 h per day.
The overall setup is referred to as a coral "photostat", a term derived from continuous phytoplankton cultures grown under constant light.

4.2.2 Experimental comparison between corals held on the reef and in the photostat at two different light regimes

In this experiment the following treatments were compared:
1. growth in the photostat under 400 µmol photons $m^{-2}s^{-1}$ for 13 h per day ("High" light group). This was the maximum light intensity that could be provided by the system with the lamps held at a sufficient height above seawater to minimise their effect on the temperature in the water bath. The total daily irradiance was 18.7 mol photons $m^{-2}d^{-1}$.
2. growth in the photostat under 200 µmol photons $m^{-2}s^{-1}$ for 13 h per day ("Low" light group). Reduced light intensity was provided by shading this photostat chamber with neutral density shade cloth. This light regime provided a total daily irradiance of 9.36 mol photons $m^{-2}d^{-1}$ which can be compared with that received by corals in shallow water on over-cast days (Davies, 1991).
3. growth on the reef at 2.5 m depth ("Reef" group). These corals experienced the natural variations in light intensity produced by the daily cycle of the sun, cloud cover and turbidity. On a optimal clear day the total daily irradiance at the 'nursery' site was measured as 25 mol photons $m^{-2}d^{-1}$ (section 3.2.2), with maximum irradiance reaching levels above 1000 µmol photons $m^{-2}s^{-1}$.
Nubbins of *Porites porites* and explants of *Montastrea annularis* were retrieved from the reef adjacent to the Bellairs Research Institute, brought into the laboratory and cleaned: 10 nubbins and 6 explants were randomly assigned to each of the three experimental groups.

Corals were buoyant weighed (2.3.1) in the laboratory every four days: "Reef" group corals were retrieved, weighed and replaced back on the reef within one hour.

P.porites can form mucus tunics (Coffroth, 1991) that tend to block calcification (Davies, 1989) and therefore nubbins were scored daily for tunic formation and daily growth rate was corrected accordingly.

The water motion in the chamber and on the reef was measured by the rate of dissolution of plaster of Paris clods following the method by Doty (1971). A 10:9 (wt:v) mixture of calcium sulphate and water cast in oblong round-bottomed polyethylene ice-cube trays produced 18g clods, which were then cemented with epoxy to perspex tiles. From the change in dry weight over a 24h incubation, the average daily dissolution of clods in the chambers (n=6) was estimated as 4.6 ± 0.45 g d^{-1}. A control rate (diffusion) of dissolution was obtained for clods in still water over 5 days (n=5). The dissolution rate in the chambers was 8.3 times higher than in control. For comparison with the conditions on the reef during the comparative experiment, clods (n = 12) were placed for 24 h at the 'nursery' site over a two week period. On the reef clods lost 12.4 ± 0.845 g d-1 or 22 times more than control clods. Thus in the chamber water motion accounted for 37% of that on the reef.

Nubbins of *Porites porites* and explants of *Montastrea annularis* were kept under these experimental conditions for 17 and 25 days respectively. At the end of the experiment their photosynthetic parameters were assayed as described in section 2.3.2 (n = 4 in each species and treatment).

All data were normalised to surface area. In *Porites porites*, surface area was calculated from measurements made with calipers at the end of the experiment (as in 2.4.1), while the explants of *Montastrea annularis* were assumed to have a surface area of 4 cm^2 derived from the planar area of the core because the actual measurements were lost.

All data were tested for normality and homogeneity of variance and in all cases these assumptions were not violated. A one-way analysis of variance was employed to test for differences between treatments. When the hypothesis of equal means was rejected, a Tukey HSD test was employed.

4.3 Results

4.3.1 Skeletal growth

Histograms (Fig. 4.4) present calcification rate expressed as daily growth per surface area of coral in the three experimental groups and the relevant data are in Table 4.1a,b.

Fig. 4.4 Daily calcification rate for *Porites porites* (n=30) and *Montastrea annularis* (n = 15) during 17 and 25 days of growth, respectively, under three culturing regimes: in photostat chambers at 400 µmol photons $m^{-2}s^{-1}$ (High) and 200 µmol photons $m^{-2}s^{-1}$ (Low) and on the 'nursery' on the North Bellairs Reef at 2.5 m depth (Reef). Bars are means + SD.

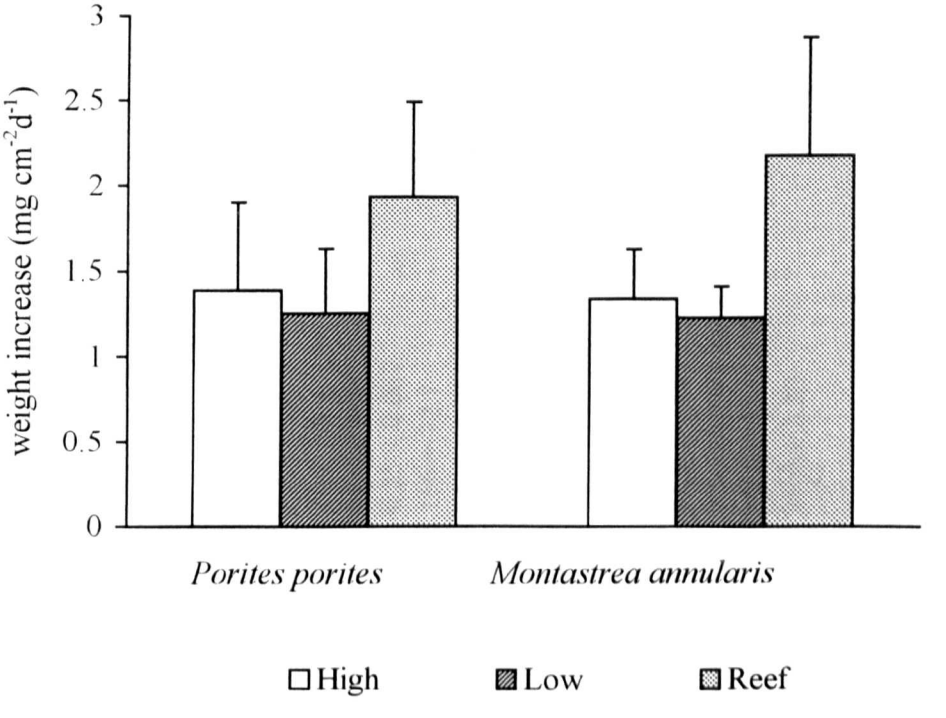
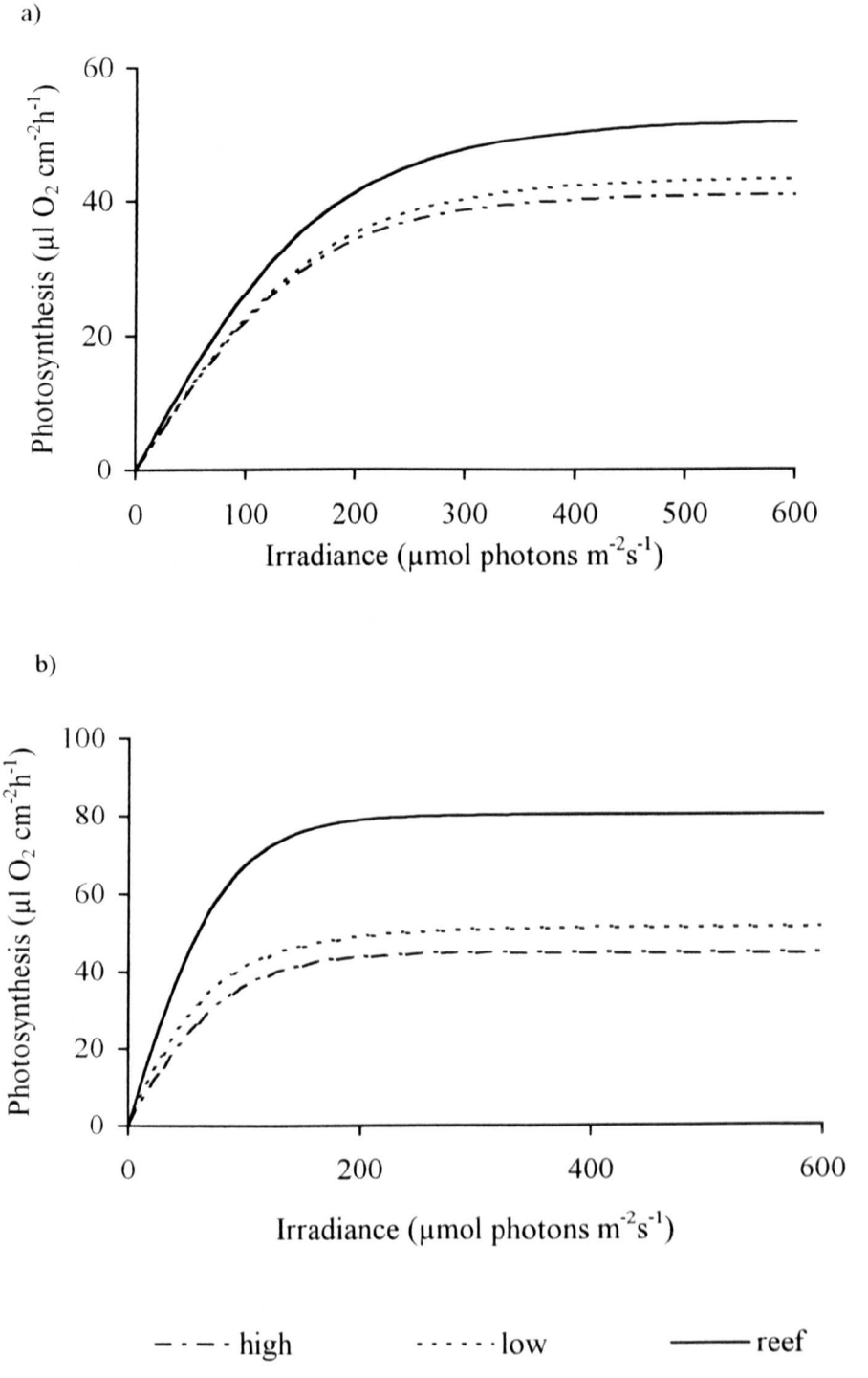


Table 4.1 Calcification rate and photosynthetic parameters of (a) *Porites porites* and (b) *Montastrea annularis* after growing for 17 and 25 days respectively under three culturing regimes: in photostat chambers at 400 μmol photons^{-1}m^{-2}s^{-1} (High) and 200 μmol photons^{-1}m^{-2}s^{-1} (Low) and on the reef in front of the Bellairs Research Institute at 2m depth (Reef). Means ± SD. Groups are significantly different ($P<0.05$) when identified by different letters. In each treatment: $^{\otimes}$ n = 10; $^{\Psi}$ n = 4; $^{\nabla}$ n = 5

	High	Low	Reef
a) *Porites porites*			
Daily calcification rate (mg cm^{-2} d^{-1})$^{\otimes}$	1.39 ± 0.354 a	1.25 ± 0.248 a	1.93 ± 0.291 b
Tunic frequency (%)$^{\otimes}$	25 ± 7.8 a	28 ± 10.7 a	9 ± 7.3 b
Maximum gross photosynthesis (μlO$_2$ cm^{-2}h^{-1})$^{\Psi}$	41.0 ± 9.63	43.5 ± 10.15	52.1 ± 15.73
alpha (μlO$_2$ cm^{-2}h^{-1} μmol photons^{-1}m^{2}sec^1) $^{\Psi}$	0.25 ± 0.067	0.26 ± 0.056	0.30 ± 0.032
I_k (μmol photons m^{-2}sec^{-1}) $^{\Psi}$	166 ± 32.7	172 ± 38.3	175 ± 49.7
Respiration (μlO$_2$ cm^{-2}h^{-1}) $^{\Psi}$	14.7 ± 1.98 b	9.0 ± 2.06 a	8.9 ± 0.92 a
b) *Montastrea annularis*			
Daily calcification rate (mg cm^{-2} d^{-1}) $^{\nabla}$	1.34 ± 0.292 a	1.23 ± 0.179 a	2.17 ± 0.701 b
Maximum gross photosynthesis (μlO$_2$ cm^{-2}h^{-1}) $^{\Psi}$	44.7 ± 5.44 a	51.1 ± 5.17 a	80.4 ± 8.52
alpha (μlO$_2$ cm^{-2}h^{-1} μmol photons^{-1}m^{2}sec^1) $^{\Psi}$	0.50 ± 0.041 a	0.64 ± 0.243 a	0.99 ± 0.083 b
I_k (μmol photons m^{-2}sec^{-1}) $^{\Psi}$	89 ± 13.0	92 ± 46.9	82 ± 15.0
Respiration (μlO$_2$ cm^{-2}h^{-1}) $^{\Psi}$	20.0 ± 2.20	20.1 ± 4.16	20.9 ± 1.64

Fig. 4.5 Plot of gross photosynthesis vs. irradiance for *Porites porites* (a) and *Montastrea annularis* (b) after 17 and 25 days of growth, respectively, under three culturing regimes: in photostat chambers at 400 µmol photons $m^{-2}s^{-1}$ (High) and 200 µmol photons $m^{-2}s^{-1}$ (Low) and in the 'nursery' on the North Bellairs Reef at 2.5 m depth (Reef). n = 4 in each treatment. Line fits were derived from the hyperbolic tangent function. Values are omitted for clarity.



Daily growth rates for *P. porites* were corrected for the cessation of calcification whilst mucous tunics were present. Nubbins in the photostat chambers spent 25% and 29% of the experimental period ("High" and "Low" respectively) covered by tunics, while those on the reef had tunics for only 9% of the time.

After 20 days in the photostat, some explants of *M. annularis* were found to lose weight between measurements. These values were not used in calculating daily calcification rate.

The null hypothesis that treatment has no effect on coral calcification rate was rejected for both species (for *P.porites* $F_{2,29}$= 14.12, P<0.001; for *M.annularis* $F_{2,15}$= 6.667, P<0.01). The corals grown on the reef had the fastest growth rate (Tukey HSD for either species: P<0.05, Reef>High=Low), 30% and 40% higher for *P. porites* and *M. annularis* respectively than when grown in the photostat. There were no significant differences between growth rates at the two different light regimes in the photostat.

4.3.2 Photosynthetic parameters and respiration

The parameters (Table 4.1a) of the light saturation curve for *Porites porites* (Fig.4.5a) were tested against the null hypothesis H_o that the means of each treatment are equal, and in all cases H_o was accepted.

The dark respiration rates of the nubbins showed a significant difference between treatments ($F_{2,11}$= 14.47, P<0.01), with those grown at the higher light intensity in the photostat having a significantly higher respiration rate (Tukey HSD, P<0.05, High>Low=Reef).

The photosynthetic parameters of explants of *Montastrea annularis* were significantly different between treatments (Table 4.1b and Fig.4.5b). In those maintained on the reef the maximum photosynthetic rate is almost twice as high as in the photostat ($F_{2,11}$= 33.67, P<0.001; Tukey HSD, P<0.05, High=Low<Reef) and alpha is increased ($F_{2,11}$= 11.19, P<0.01; Tukey HSD, P<0.05, High=Low<Reef). The irradiance I_k at which photosynthesis becomes maximal is the same between treatments. Respiration rate was not affected by treatment in this species ($F_{2,11}$= 0.12, P>0.1).

4.4 Discussion

Observations prior to this comparative evaluation indicated that the photostat could sustain coral growth in the absence of heterotrophic feeding with no sign of tissue necrosis or bleaching for at least 9 weeks (when the test was terminated). During this month-long trial there was a 100% survival rate. All corals presented a normal appearance throughout the experiment and the polyps

of *Montastrea annularis* showed maximal expansion at night similar to colonies on the reef, while those of *Porites porites* showed their normal behaviour pattern of expansion throughout the day. Tunics were observed more frequently in the photostat but were regularly shed.

4.4.1 'High' and 'Low' light in the Photostat

The zooxanthellae within corals are known to readily photoadapt (Falkowski *et al.*, 1990; Porter *et al.*, 1984) and change the size of the photosynthetic units in order to maximize the photon-harvesting process (Falkowski & Dubinsky, 1981) as has been observed in free-living dinoflagellates (Prezelin, 1987). In fact the most commonly reported result of decreased light intensity (due to depth or shade) is the relative increase in photosynthetic pigments per zooxanthella (McCloskey & Muscatine, 1984; Porter *et al.*, 1984; Zvalinskii *et al.*, 1980; Falkowski & Dubinsky, 1981; Dustan, 1982; Harland & Davies, 1994), per unit surface area (Battey & Porter, 1988), or per unit dry weight (Jaubert, 1981; Harland & Davies, 1994). Any photoadaptational change in pigment content is reflected on the P/I curve by a change in the value of alpha, i.e. the photosynthetic efficiency (Chalker *et al.*, 1983). Surprisingly in this experiment in the photostat, *P. porites* and *M. annularis* subjected for 17 and 25 days respectively to a 'high light' and a 'low light' treatment did not show any difference in their photosynthetic response, including the value of alpha. The light intensity at which corals attained maximum photosynthesis (I_k) in both groups was lower than the light intensity experienced by the 'low light' group. This means that the higher light intensity of the 'high light' group did not produce any further photoadaptational stimulus over that of the 'low light' group and therefore suggests that the intensity of 200 μmol photons $m^{-2}s^{-1}$ is sufficiently high for maximal photosynthesis. Shallow reef corals have been reported to have I_k close to 200 μmol photons $m^{-2}s^{-1}$ (Wethey & Porter, 1976; Chalker *et al.*, 1983; Chalker & Dunlap, 1983; Porter *et al.*, 1984; Davies, 1991) even though ambient light intensity can reach levels in excess of 1000 μmol photons $m^{-2}s^{-1}$ in shallow water (Dubinsky *et al.*, 1990; Al-Sofyani, 1991 and as shown in Ch.2). Calcification rate was the same in both light treatments. No explanation can be found for the higher rate of respiration attained by *P.porites* (but not *M.annularis*) from the 'high light' group.

The 24h P_{gross}/R ratio for the two photostat groups was estimated from data in Table 4.1 as 1.51 and 2.62 for the "high light" and "low light" groups respectively. Such high daily P/R ratios are characteristic of shallow reef corals where phototrophy is more than enough to satisfy the respiratory requirements of the host. This result gives confidence in the successful use of the photostat as a culture system.

4.4.2 Photostat and Reef

The environment in the photostat chambers (water velocity, light regime, plankton and nutrient concentration) is very different from that on the reef and especially so during this experimental period which was characterized by strong wind, overcast sky, very strong currents, and high sediment loading. Since coral growth is dependent on the quality of the environment (Tomascik & Sander, 1985; Dodge & Brass, 1984), the observation that skeletogenesis in both species was significantly different between the photostat and the reef is not surprising. In the photostat, skeletogenesis was lower by about 30% in *P. porites* and 40% in *M. annularis*. However, differences of this magnitude in the growth rate of corals have been commonly recorded in the field (Barnes & Crossland, 1980; Barnes & Chalker, 1990; Tomascik & Sander, 1985) and thus they do not imply *per se* that corals in the photostat are under any impending stress. In fact in the photostat, water motion was estimated to be 37% of that on the reef by monitoring the rate of dissolution of plaster blocks. This technique integrates both turbulent agitation and unidirectional flow into a single dimensionless index which is directly related to mass transfer (Jokiel & Morrissey, 1993). Since there is evidence that mass transfer and calcification are positively correlated (Dennison & Barnes, 1988), the higher water motion experienced by corals on the reef could have played an important part in enhancing their rate of calcification above that of corals in the photostat.

In *Porites porites* the rate of calcification was the only measured parameter that significantly discriminated between laboratory and reef grown corals, and the P/I curve parameters for this coral were equal in all treatments. Thus the constant irradiance provided by the metal halide lamps did not have any effect on the photosynthetic response of the symbiont. Unfortunately no light measurements were recorded directly at the 'nursery' site during this experiment. However, the sky was often over-cast and turbidity high so that on average corals were more likely to receive a similar flux of light energy as experienced in the photostats (9.36 and 18.7 mol photons $m^{-2}d^{-1}$) than the optimal daily flux for this site (in 3.2.2) estimated under optimal conditions to 25 mol photons $m^{-2}d^{-1}$.

In comparison, the P/I curve of *Montastrea annularis* (Fig. 4.5b) shows a significant difference between treatments. In particular, the $P_{max\ gross}$ and the efficiency of photosynthesis represented by alpha are 1.7 times higher on the reef than in the photostat. The enhanced photosynthetic rate of *M.annularis* on the reef could be a contributing factor to the higher rate of calcification too. Such photosynthetic variations have been observed naturally on the reef in corals adapted to different light environments (Falkowski & Dubinsky, 1981; Zvalinskii *et al.*, 1980; Porter *et al.*, 1984; Falkowski *et al.*, 1990). In laboratory experiments with flow-through systems, similar results have been obtained not only through photoadaptation, but also in corals subjected to nutrient

enrichment as will be discussed in more detail in Ch.5. Anticipating data presented in the experimental chapters, I suggest that the lower photosynthetic rate of *M. annularis* explants in the photostat results in part from a decreased zooxanthellae population density brought about by increased nutrient limitation of zooxanthellae *in hospite*. Nitrogen availability enhances algal biomass and in the photostat both dissolved nitrogen uptake and heterotrophic feeding were likely to be reduced in comparison to the condition on the reef. Since in *P. porites* photosynthesis did not change between treatments it appears that *M. annularis* is the coral species more dependent on heterotrophic feeding, in agreement with Porter's model (Porter, 1976).

4.4.3 Conclusions

The photostat design proved to be successful in culturing corals for experimental analysis. The physiological parameters measured confirmed that the change in environmental conditions from the exposed reef to the chambers is reflected in the corals' growth rate and photosynthetic activity. Provision of zooplankton as a heterotrophic source of nutrients might be useful when working with less autotrophic coral species in long-term experiments. However, there is no evidence to suggest that the degree of change observed is much more pronounced in the photostat than it would be in nature considering the high diversity of reef conditions (i.e. shallow vs. deep, exposed fore-reef vs. lagoons, oligotrophic vs. eutrophic, low vs. high latitude etc).
Since no difference was observed in the photosynthetic response of corals from the two light regimes in the photostat, the lower light intensity was adopted in subsequent experiments by raising the lamps further away from corals and thus providing a larger and more constant light field over the chambers.

Chapter 5

THE EFFECT OF NITRATE ON THE PHOTOSYNTHESIS AND CALCIFICATION OF HERMATYPIC CORALS

5.1 Introduction

Coral reefs thrive in tropical waters generally with low nutrient concentrations, low phytoplankton growth and very high light penetration. The paradox of high reef productivity and low nutrient concentration of the surrounding waters has been explained in terms of high efficiency in retaining and recycling nutrients by corals and other symbiotic benthic biota. As coastal eutrophication is spreading world-wide, there is increasing evidence that nutrient enrichment can have catastrophic effects on reefs, suppressing community calcification and stimulating algal growth (Kinsey, 1987).

While eutrophication has been shown to change the community structure, very little is known of the causal mechanisms involved or how individual organisms respond to nutrient enrichment. Experimental work on hermatypic corals has largely centred on the effects of elevated levels of ammonia: the well established fact that cnidarians do not excrete ammonia when in symbiosis (Muscatine & D'Elia, 1978; Muscatine *et al.*, 1979; Burris, 1983) has fuelled the concept of tight nutrient recycling between host and algae (Muscatine & Porter, 1977; Muscatine & D'Elia, 1978; Davies, 1984). In attempting to determine the nutrient status of zooxanthellae *in situ*, physiological studies have shown that exposure to high concentrations of NH_4^+ (approx. 20 μM) results in changes of zooxanthellar C:N ratio, and doubling of the population of zooxanthellae (Muscatine *et al.*, 1989; Muller-Parker *et al.*, 1994a,b; Snidvongs & Kinzie, 1994), suggesting that in oligotrophic water symbionts are nutrient limited. When zooxanthellae concentration is enhanced by external ammonia enrichment a reduction in the rate of photosynthesis per algal cell (Hoegh-Guldberg & Smith, 1989; Dubinsky *et al.*, 1990; Stimson & Kinzie, 1991) and even a reduction in the rate of skeletogenesis (Stambler *et al.*, 1991; Ferrier-Pagés *et al.* ,1996) have been observed. Falkowski *et al.* (1993) and Dubinsky & Jokiel (1994) speculated that eutrophication could disrupt the growth equilibrium of the two symbiotic components at the expense of the host.

However, *in situ*, corals are not normally exposed to elevated levels of ammonia. Inorganic nitrogen in its reduced form is rapidly removed and it is in the oxidised form, as nitrate, that concentrations build up. Very little information is available on the effects of elevated levels of nitrate on corals. Earlier studies on the uptake of nitrate by symbiotic anthozoans produced equivocal results. Some corals appear to remove nitrate from seawater (e.g. Franzisket, 1974;

Webb & Wiebe,1978; Bythell, 1990; Atkinson, *et al.*, 1994), while some others, and all symbiotic anemones, do not (Wilkerson & Trench, 1986). The methodology of these nitrate depletion experiments has been questioned by Miller & Yellowlees (1989) who suggested that depletion could result from bacterial assimilation. Furthermore, on theoretical grounds, they argued that uptake into the host cytoplasm would require the presence of a specific nitrate carrier mechanism in the host cell membranes purely to satisfy symbiont requirements. Nitrate assimilation by the algae would require the induction of nitrate reductase, and this normally only occurs when ammonia is limiting (Syrett, 1981). It is perhaps because of the experimental and theoretical ambiguities surrounding nitrate uptake, that there is a dearth of information on the effects of nitrate on coral physiology.

In this chapter I investigate the effects of a month exposure to elevated levels of nitrate on nubbins of *Porites porites* and explants of *Montastrea annularis.* If nitrate is taken up by the symbiosis and utilised by the symbionts as a source of nitrogen, the effects of nitrate on coral physiology should be similar to those observed under ammonium enrichment. This will be tested by measuring how nitrate affects the zooxanthellae biomass and the rate of photosynthesis *in hospite*. Thus the hypothesis that zooxanthellae *in situ* in oligotrophic seawater (i.e. control corals) are truly nitrogen limited will be examined. Measurements of respiration, host biomass and skeletal growth will be carried out in order to understand the degree to which any change in symbiont biomass associated with elevated levels of nitrate might affect the growth of the host, and hence the well-being of the coral as a whole.

5.2 Material and Methods

5.2.1 Corals, experimental chambers and treatment regimes

Nubbins of *Porites porites* and explants of *Montastrea annularis* were brought into the laboratory after a 3 week recovery period on the reef. Four groups of 8 nubbins and 6 explants were randomly selected and assigned to each experimental chamber. Four photostat chambers (see Ch. 4) were used: the control chamber received oligotrophic water (PO_4^{2-} = 0.05 µM; NO_3^{2-} = 0.2 µM) freshly collected from 3 km offshore while the three experimental chambers received seawater enriched with KNO_3 to give inflow concentrations of 1, 5 and 20 µM. A peristaltic pump maintained the continuous flow of water with a residence time in the chambers of approx. 20h. The actual nitrate concentrations in the chambers was measured at intervals of 4 days, by the cadmium reduction method of Strickland and Parsons (1972). The four photostats were kept in a constant temperature water bath at 27 ± 0.5°C. The rate of water motion produced by the

strong air bubbling was judged to be 37% of that of corals on the fringing reef, based on measurements of the comparative rates of dissolution of plaster of Paris clod cards (Doty, 1971). The photostats were illuminated by two metal halide lamps, providing 250 ± 50 μmol photons $m^{-2}s^{-1}$ for 13 h per day. To minimize confounding sources of variation, the position of the photostat chambers in relation to light source and air pumps was shifted daily. This ensured that over a 4-day cycle each chamber had been subjected to each possible irradiance and water motion condition. On every fourth day, the corals were removed for 15 min to aquaria containing the same concentrations of nitrate, to allow the photostat chambers to be cleaned and refilled. Nubbins and explants were grown simultaneously in the photostats: physiological measurements began after 30 days and took 8 days per species. Corals were grouped by species during the measurement period to concentrate further on differences between treatments. *M. annularis* was the randomly chosen species to be analysed first while *P.porites* was kept under experimental conditions for 40 days before initiating measurements. Within each species, the sequence of analysis was random across treatments with no more than two corals from the same treatment analysed consecutively.

5.2.2 Measurement of photosynthesis and respiration

Rates of respiration and photosynthesis were measured at the end of the experimental period in a closed respirometer fitted with oxygen electrodes (2.3.2); the respirometer chambers were surrounded by a waterjacket to maintain a constant temperature of 27°C ± 0. 5°C. Each chamber had a volume of 90 ml and contained water of the same nitrate concentration as the treatment water. The power to the lights was varied to provide illumination levels in the chambers of 25, 50, 80, 120, 200, 300 and 440 μmol photons $m^{-2}s^{-1}$. Dark respiration was measured over an approx. 30 min period during which the oxygen in the chambers was depleted to about 70% saturation. The lights were then switched on at the lowest level of irradiance and the illumination was increased sequentially. The O_2 level in the chambers at the end of a photosynthesis measurement run was not more than 110% saturation.

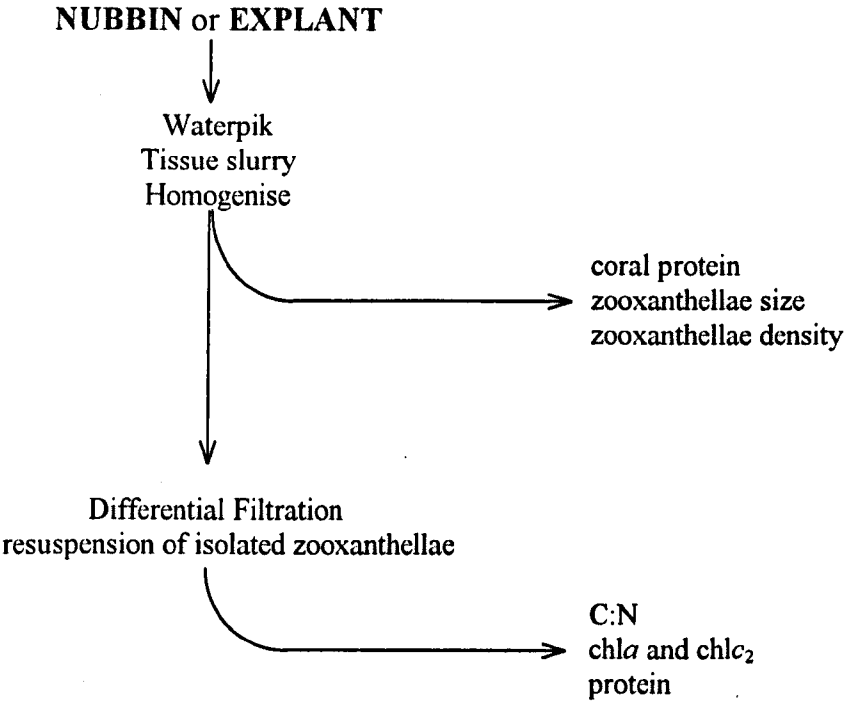
5.2.3 Measurement of growth of skeleton

The skeletal weight was measured at intervals of 6 days by buoyant weighing (2.3.1), using the method of Davies (1989). Final daily growth rates, normalised to surface area, were obtained from regression equations of weight against time over the last 3 weeks of the experiment. In *P. porites,* growth rate was corrected for any days in which mucus tunics (Coffroth, 1991) were present, since calcification almost ceases on those days (Davies,1989).

5.2.4 Biometry and biomass measurements

At the termination of the experiment, after determining the surface area of nubbins and explants (2.4.1), the procedures described in Fig. 5.1 were carried out in order to obtain all the necessary host and zooxanthellae biomass measurements following methods described in ch.2.

Fig. 5.1
Summary of analytical procedures used to obtain all the required animal and zooxanthellae biomass parameters.



5.2.5 Statistical analysis

The question posed by this study can be addressed in two steps: (1) does nitrate addition to oligotrophic seawater have any effect on coral physiology, and if it does, then (2) what is the critical threshold along the nitrate concentration gradient (treatment variable) at which the significant change in the physiological response occurs.

The effect of nitrate on each parameter measured was tested using ANOVA. The second stage of the analysis in those cases where the null hypothesis was rejected, would normally use a multiple comparisons test such as Tukey's test. However, treating data in this manner neglects the fundamental ordinal nature of the treatment variable (i.e. $0\mu M < 1\mu M < 5\mu M < 20\mu M$). It was

also judged inappropriate to fit the data to a linear regression model because in practice the four nitrate concentrations did fluctuate over time (see Table 5.1 in the Results section) and therefore cannot be precisely reduced to four points on the independent variable (i.e. 0, 1, 5 and 20 μM NO_3 are their nominal values rather than the exact concentration in each treatment).

A solution was found by coding the four treatment levels using three "dummy" variables (Zar, 1984) and analysing the resulting distribution by multiple regression (Walter *et al.*, 1987). The coding scheme is as follows:

Treatments		Dummy Variables		
levels	NO_3 (μM)	D_1	D_2	D_3
1	0	0	0	0
2	1	1	0	0
3	5	1	1	0
4	20	1	1	1

Since in this multiple regression model the response variable is dependent upon the three dummy variables, their relationship between response and treatment level is expressed by the following equation:

$$Y = a + b_1 D_1 + b_2 D_2 + b_3 D_3 \,. \qquad \text{Eq. 5.1}$$

Substituting the appropriate dummy variable value for each treatment level (e.g. for treatment 1, $D_1 = 0$, $D_2 = 0$ and $D_3 = 0$) into equation 5.1, it then follows that:

for treatment 1: $Y_1 = a + b_1*0 + b_2*0 + b_3*0 = a$;

for treatment 2: $Y_2 = a + b_1*1 + b_2*0 + b_3*0 = a + b_1$ and so on.

The difference between Y_2 and Y_1 therefore is b_1, and the difference between Y_3 and Y_2 is b_2 and so on. The multiple regression analysis then employs the Student *t* statistic to test that the regression coefficients b_1, b_2, and b_3 are different from 0 and this corresponds to testing for differences between treatments (i.e. if $b_1 \neq 0$ then treatment 1 ≠ treatment 2; if $b_2 \neq 0$ then tr. 2 ≠ tr. 3 and if $b_3 \neq 0$ then tr. 3 ≠ tr. 4)

In the initial ANOVA, if the interaction term between treatments and species was significant, the two species were treated separately. If there was no significant interaction, the two species were tested in the multiple regression above, using a fourth dummy variable to distinguish them.

All data sets were tested for normality and homogeneity of variance but no serious departure from either assumption was observed (Zar, 1984). Analysis were carried out with the statistical package SPSS 6.1 for WINDOWS.

5.3 Results

5.3.1 Experimental chambers and treatment regimes

The actual concentration of nitrate in the chambers was determined at 4-day intervals. In each of the 3 experimental chambers the actual concentration of nitrate was below that of the inflow (Table 5.1). This probably results from the uptake activity by both corals and the surface film of algae which developed between cleaning, and the long residence time (20 h).

Table 5.1
Range of nitrate concentrations of the inflow stock solutions and as measured in the photostats at 4 day intervals immediately before chambers were cleaned and refilled.

	Treatment No.			
Treatment	1	2	3	4
NO_3 inflow (μM)	0.1	1	5	20
NO_3 outflow (μM)	0.1-0.3	0.2-0.5	0.5-3	5-17

Therefore over each 4-day period between refillings, nitrate concentration fluctuated within each chamber. In subsequent sections, each treatment was identified by the notional inflow concentration of 0, 1, 5 and 20 μM NO_3, however the actual average concentration experienced by the corals was somewhat lower in each treatment.

5.3.2 Measurement of photosynthesis

The photosynthesis vs. irradiance curves for *P. porites* and *M. annularis* after 40 and 30 days exposure to the treatments is shown in Fig. 5.2a,b, and all photosynthetic parameters are presented in Table 5.2. In *P. porites* values for maximum gross photosynthesis ($P_{gross\ max}$) normalised to surface area, increased from 44.2 $\mu lO_2\ cm^{-2}\ h^{-1}$ in the control to 61.8 in the 20μM nitrate group. In *M. annularis* the values increased from 39.5 to 49.5 $\mu lO_2\ cm^{-2}\ h^{-1}$. ANOVA showed that there was no significant interaction between species and treatments, and so the two species were combined for analysis of treatment effects. There was a significant positive relationship between $P_{gross\ max}$ and treatment levels of nitrate ($F_{4,41} = 10.21$; $P < 0.0001$). While no significant change in response was observed between control and 1μM NO3 treated corals, a

Fig. 5.2 Plots of gross photosynthesis vs. irradiance for *P. porites* (a) and *M.annularis* (b) after incubation for 40 and 30 days, respectively, in photostat chambers with 4 different nitrate concentrations. Lines are derived from the hyperbolic tangent function fitted to the original data. Mean values and error bars are omitted for clarity. n = 6 in each treatment.

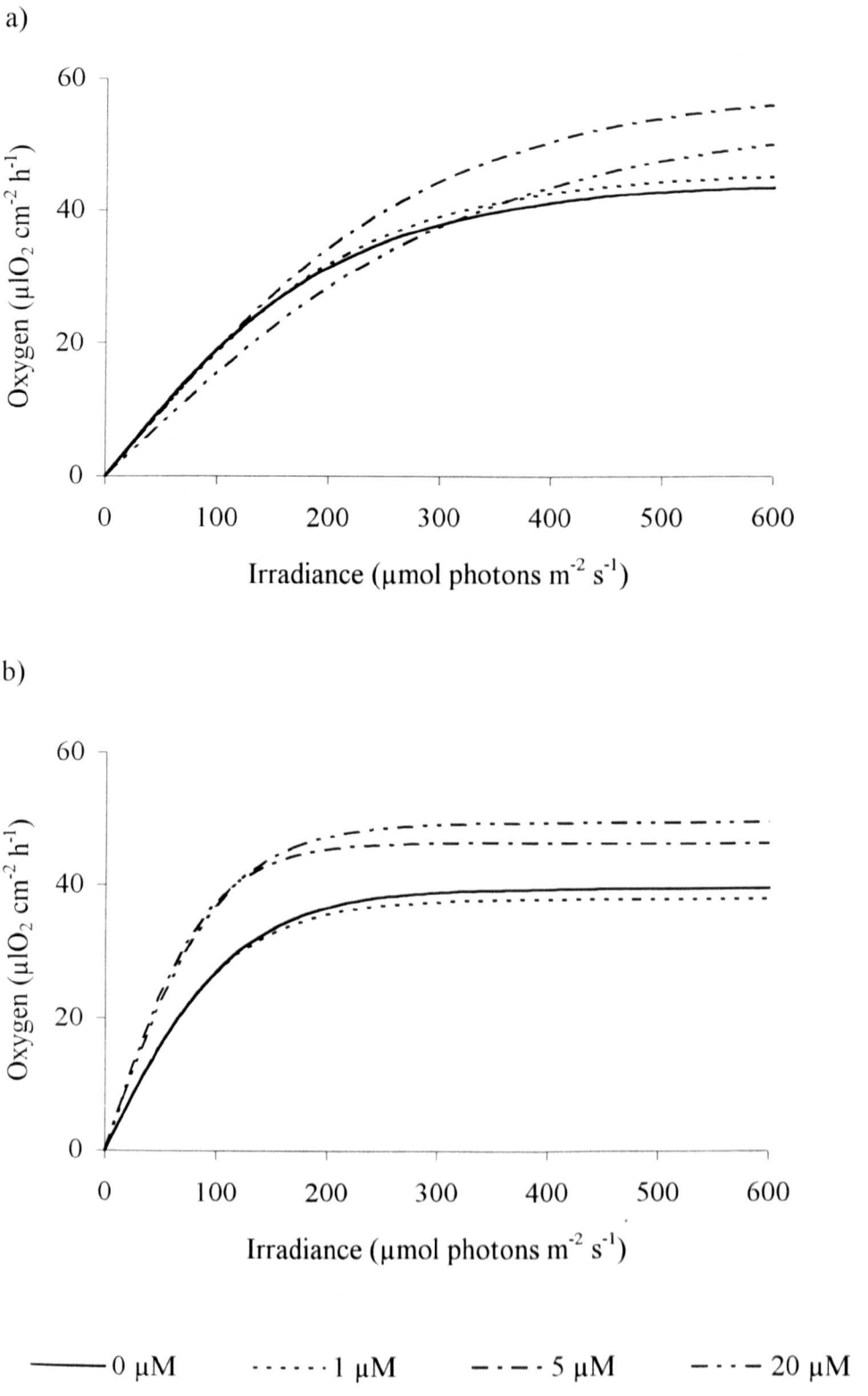


Table 5.2 *Porites porites* and *Montastrea annularis* Photosynthetic parameters and respiration after 40 and 30 d exposure, respectively, to control oligotrophic seawater and three concentrations of nitrate. Values are means ± SD, n = 6 in each treatment. Data were analysed using multiple regression model (see Materials and methods, Statistical analysis). Position of asterisks indicates where significance between nitrate treatments first appeared (* significant at $P < 0.05$; ** significant at $P < 0.01$ and *** significant at $P < 0.001$). In Analysis A, ANOVA interaction between species and treatments is significant, and each species is analysed separately. In Analysis B, ANOVA interaction is not significant, so common multiple regression obtained by combining data of the two species is used, in such case, the asterisk is shown between rows for the two species

Parameter	Analysis	Species	Treatment (µmol.l^{-1} NO_3)			
			0	1	5	20
Photosynthesis						
Max. gross photosynthesis per surface area (µl O_2 cm^{-2} h^{-1})	B	*P. porites*	44.2 ± 13.47	45.8 ± 4.62 } ***	{ 58.6 ± 5.8	61.8 ± 14.32
		M. annularis	39.5 ± 5.10	37.9 ± 5.17 }	{ 46.4 ± 6.40	49.5 ± 4.66
Max. gross photosynthesis per algal cell (µl O_2 cell x10^{-6} h^{-1})	B	*P. porites*	14.75 ± 4.789	20.33 ± 3.669	16.98 ± 3.00	15.83 ± 4.72
		M. annularis	10.19 ± 1.542	10.38 ± 1.445	10.58 ± 1.661	10.44 ± 0.753
Alpha (µlO_2 cm^{-2} h^{-1} µmol photons^{-1}m^{2}s^1)	A	*P. porites*	0.207 ± 0.0276	0.199 ± 0.0246	0.198 ± 0.0392	0.165 ± 0.0170
		M. annularis	0.338 ± 0.0511	0.346 ± 0.0309 ***	0.529 ± 0.0611	0.491 ± 0.0975
I_k (µmol photons m^{-2}s^{-1})	A	*P. porites*	215 ± 67.0	232 ± 37.4	304 ± 63.1 *	382 ± 116.2
		M. annularis	119 ± 25.4	111 ± 22.9	88 ± 10.4	104 ± 21.5
Respiration						
Respiration rate per surface area (µl O_2 cm^{-2}h^{-1})	A	*P. porites*	10.9 ± 1.26	10.2 ± 1.07	9.6 ± 2.23	8.5 ± 0.99
		M. annularis	14.8 ± 1.12	13.6 ± 0.52	14.4 ± 1.20	15.0 ± 1.40
Respiration rate per protein content (µl O_2 mg^{-1}h^{-1})	B	*P. porites*	3.78 ± 0.30	3.55 ± 0.37	3.44 ± 0.47	2.93 ± 0.52
		M. annularis	2.64 ± 0.27	2.68 ± 0.32	2.19 ± 0.49	1.73 ± 0.22

highly significant increment of $P_{gross\ max}$ occurred with 5 μM NO3 (t = 3.347, P < .001). There was no further increase in response in those maintained at 20 μM nitrate. Since increase in maximum photosynthetic rate corresponds to increase in the number of photosynthetic units (Prezelin,1987), the analysis was repeated using photosynthetic measurements normalised to number of zooxanthellae. In this case, there was no significant difference in $P_{gross\ max}$ ($F_{3,35}$ =1.675, P > 0.05), suggesting that the increase in photosynthesis had not resulted from an increase in the number of photosynthetic units or thylakoid membranes per algal cell, but from an increase in algal cells per surface area.

The second essential parameter of the P vs. I curve is alpha, the slope of the tangent to the initial part of the curve, which is a measure of photosynthetic efficiency. ANOVA showed a significant interaction between species and treatments ($F_{3,42}$ = 13.5, P < 0.001), so separate multiple regression analyses were used to assess the effect of nitrate addition in the two species. In *P. porites* values of alpha ranged from 0.207 to 0.165 $\mu lO_2\ cm^{-2}\ h^{-1}$.μmol photons$^{-1}m^2$ sec., with no significant differences between treatments. In *M. annularis*, however, nitrate had a very significant effect on alpha ($F_{3,20}$ = 14.35, < 0.001), with significant differences appearing at 5 μM NO_3 (t = 4.987, P < 0.001).

The photosynthetic saturation may be estimated from I_k which is the point of intersection of the initial slope of the curve with the saturated asymptote. There was again a significant interaction between species and treatments ($F_{3,42}$ = 7.42, P < 0.001) and so separate multiple regression analyses were used. In *P. porites*, I_k increased from 215 in the control to 382 μmol photons cm^{-2} sec^{-1} at 20 μM NO_3 showing a significant treatment effect ($F_{3,18}$ = 5.55, P < 0.01). In *M. annularis* there was no significant effect of nitrate upon I_k.

5.3.3 Respiration rate

Dark respiration rate values were normalised to surface area, and also to protein content, in view of the possibility of biomass per unit surface area changing during the course of the experiment (Table 5.2). Since no significant interaction between species and treatments was found, data for both species were analysed together. Multiple regression analysis showed no significant relationship between nitrate concentration and respiration rate when data were normalised to surface area.

However, when the biomass normalised data were tested, respiration rate was found to fall from 3.78 to 2.93 $\mu lO_2\ cm^{-2}\ h^{-1}$ in *P. porites* and from 2.64 to 1.73$\mu lO_2\ cm^{-2}\ h^{-1}$ in *M. annularis* as nitrate concentration increased ($F_{4,35}$ = 30.24, P < 0.001), with a very significant decrease occurring in the corals treated with 20 μM NO_3 (t = 9.358, P < 0.01).

5.3.4 Measurement of growth of skeleton

Fig. 5.3 a,b present the absolute weight increase as measured every 6 days. In both species, corals in the control treatment maintained the fastest growth rate: in *P. porites*, in nitrate enriched corals skeletogenesis started to decrease after 15-20 days of treatment; in *M. annularis* on the other hand it was the untreated group of explants that increase growth rate after about 15 days in the photostat.

Overall growth rate decreased significantly with an increase in nitrate levels ($F_{4,45} = 36.42$, $P < 0.001$) and there was no interaction between treatments and species, showing that nitrate addition affected growth rate of both species in the same way. In *P. porites* the growth rate declined from 1.24 mg cm^{-2} d^{-1} in the control to 0.68 in those exposed to 20 μM NO_3, whilst in *M. annularis* the decline was from 1.14 to 0.51 mg cm^{-2} d^{-1} (Table 5.3).

Table 5.3

Daily calcification rate (mg $cm^{-2}d^{-1}$) for *Porites porites* and *Montastrea annularis* after a 40 and 30 day exposure respectively to control oligotrophic seawater and three concentrations of nitrate. Means ± SD; n = 6 in each treatment. ANOVA interaction not significant so a common multiple regression is used. ** significant at $P < 0.01$ and *** significant at $P < 0.001$. See text for details of statistical analysis.

	Treatment (μM NO_3)			
	0	1	5	20
P. porites	1.24 ± 0.186	0.91 ± 0.061	0.65 ± 0.122	0.68 ± 0.107
M. annularis	1.14 ± 0.14	0.53 ± 0.085	0.43 ± 0.229	0.51 ± 0.074
	***	**		

The negative relationship obtained was not linear but was most strongly affected by the lower nitrate concentrations (Fig.5.4): a significant difference appeared at 1μM NO_3 ($t = 6.965$, $P < 0.001$) and again at 5μM NO_3 ($t = 2.648$, $P = 0.01$), where calcification was half that of control corals, but no further suppression was obtained with 20 μM NO_3 ($t = 0.023$, $P > 0.05$).

5.3.5 Biometry and biomass measurements

In all biomass measurements there was no interaction between species and treatment in the initial ANOVA so that the relationship between physiological response measured and nitrate concentration was the same for both species. It was found that all measured parameters of algal biomass were positively correlated with nitrate concentration (Tables 5.4 & 5.5).

Fig. 5.3 Absolute skeletal weight increase for *Porites porites* (a) and *Montastrea annularis* (b) during a 40 and 30 days incubation, respectively, in photostat chambers with 4 different nitrate concentrations. Error bars are omitted for clarity. For P.*porites* n = 32 and for *M.annularis* n = 18.

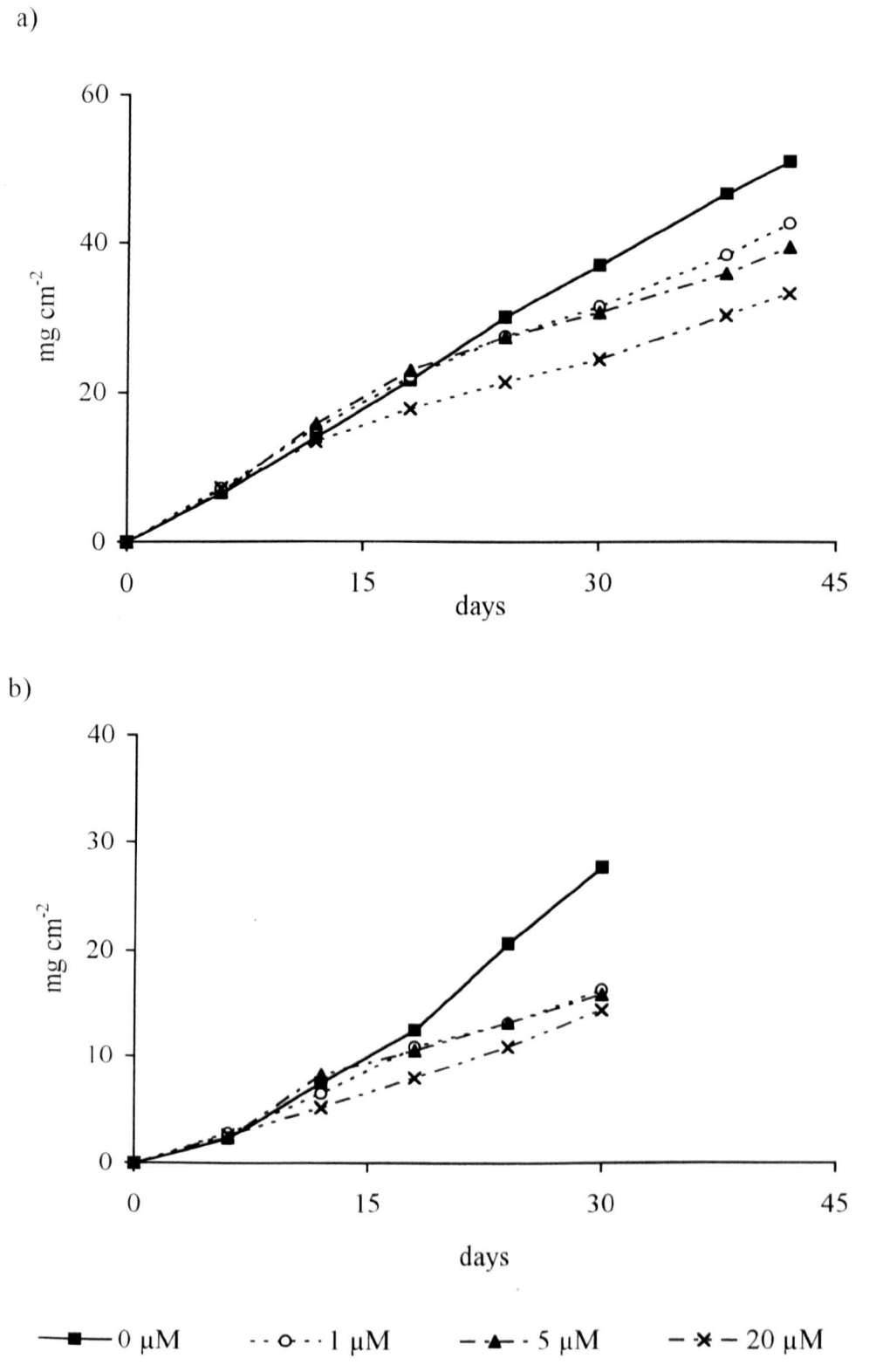


Fig. 5.4 Daily calcification rate for *Porites porites* and *Montastrea annularis* during incubation for 40 and 30 days respectively in photostat chambers with 4 different nitrate concentrations. Bars are one standard deviation. For *Porites porites* n = 32 and for *Montastrea annularis* n = 18.

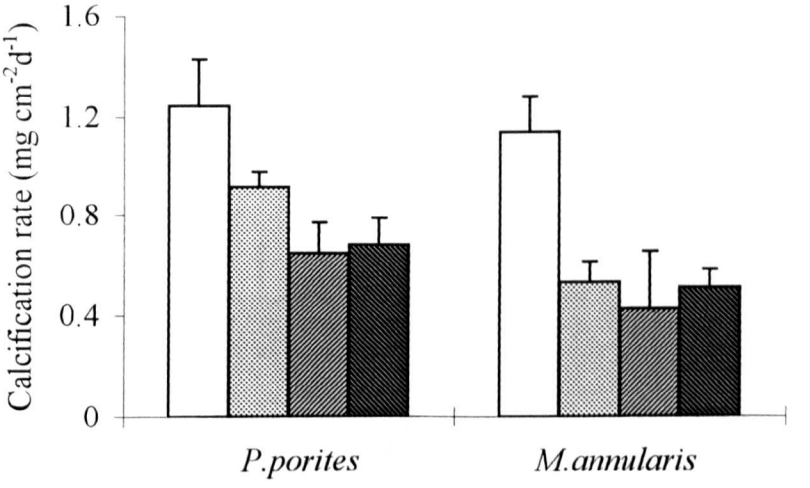


Fig. 5.5 Chlorophyll a content per zooxanthellae isolated from *Porites porites* and *Montastrea annularis* exposed for 40 and 30 days respectively to 4 different nitrate concentrations. Bars are one standard deviation;
n = 20 in each species.

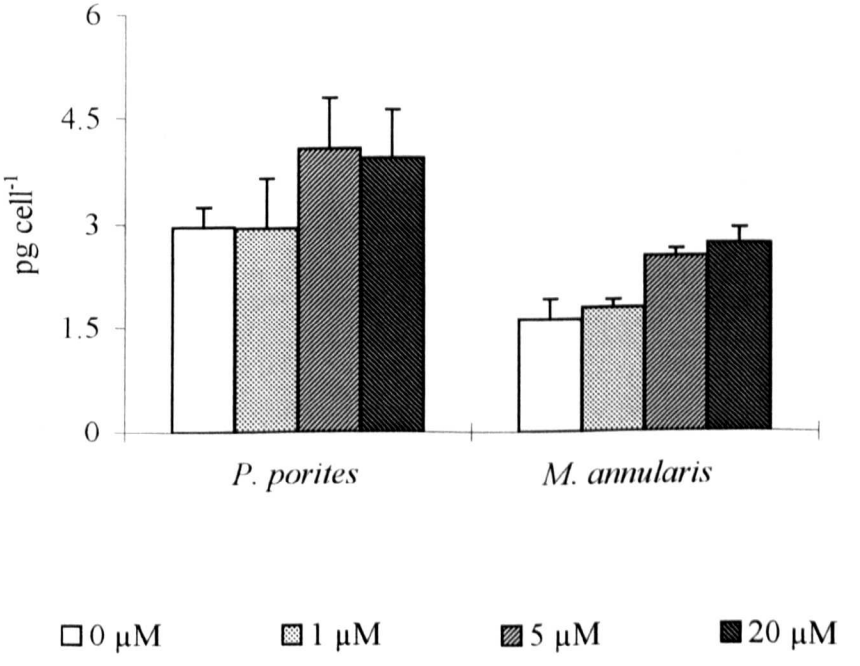


Fig. 5.6 Volume of zooxanthellae isolated from *Porites porites* and *Montastrea annularis* after 40 and 30 days exposure, respectively, to 4 different nitrate concentrations. Bars are one standard deviation; n = 20 in each species.

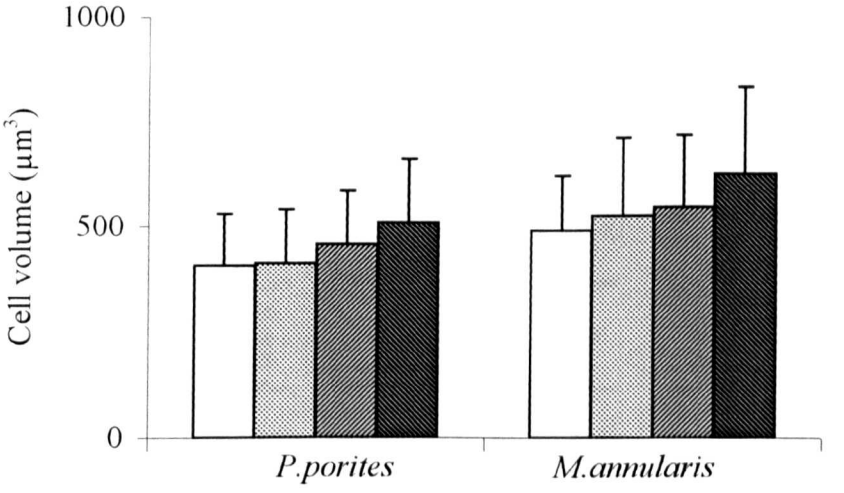


Fig. 5.7 Population density of zooxanthellae standardised to surface area in *Porites porites* and *Montastrea annularis* exposed for 40 and 30 days respectively to 4 different nitrate concentrations. Bars are one standard deviation; n = 20 in each species.

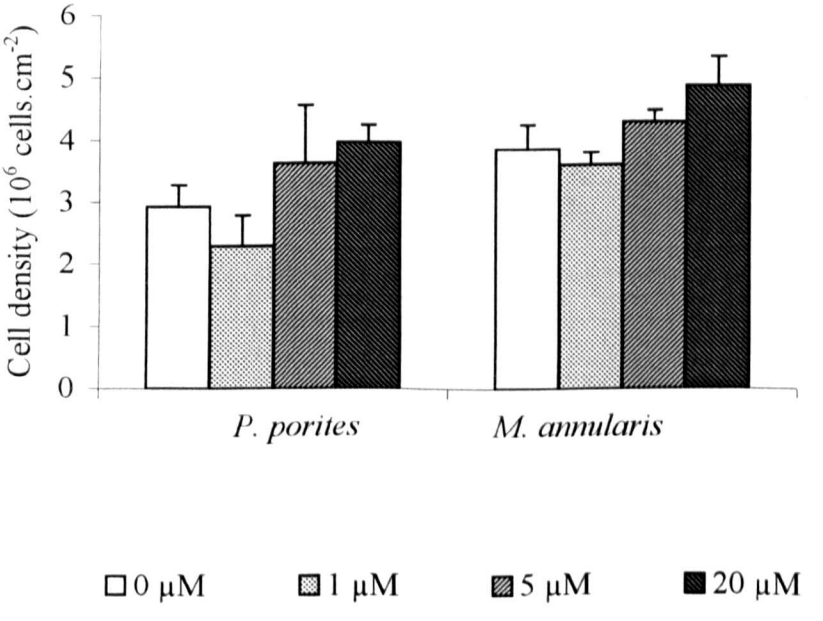


Table 5.4 *Porites porites* and *Montastrea annularis*. Biomass characteristics after 40 and 30 d exposure, respectively, to control oligotrophic seawater and three additional concentrations of nitrate. Values are means ± SD; n = 5 in each treatment. In all cases ANOVA interaction is not significant so common multiple regression obtained by combining the data of the two species is used, and asterisks are shown between rows for the two species. Further details as in legend to Table 5.2.

Parameter	Species	Treatment (μM NO_3)			
		0	1	5	20
Chlorophyll *a* (pg $cell^{-1}$)	*P. porites*	2.95 ± 0.281	2.93 ± 0.712 } *** {	4.06 ± 1.013	3.93 ± 0.671
	M. annularis	1.62 ± 0.285	1.79 ± 0.113 } *** {	2.53 ± 0.475	2.71 ± 0.220
Chlorophyll c_2 (pg $cell^{-1}$)	*P. porites*	0.82 ± 0.084	0.83 ± 0.312 } * {	1.20 ± 0.421	1.22 ± 0.280
	M. annularis	0.39 ± 0.131	0.46 ± 0.055 } * {	0.60 ± 0.168	0.68 ± 0.076
Zooxanthella protein (pg $cell^{-1}$)	*P. porites*	170.4 ± 7.309	164.8 ± 30.826	191.8 ± 27.908 } *** {	230.7 ± 18.121
	M. annularis	90.3 ± 27.96	126.4 ± 58.62	122.8 ± 22.26 } *** {	216.3 ± 71.56
Zooxanthella volume (μm^3)	*P. porites*	491.3 ± 27.84	527.3 ± 42.94 } * {	547.7 ± 28.66 } *** {	627.2 ± 35.76
	M. annularis	406.9 ± 28.87	413.1 ± 47.74 } * {	457.1 ± 39.57 } *** {	510.5 ± 21.93
Zooxanthellae density ($x10^6$ cm^{-2})	*P. porites*	2.93 ± 0.348 } * {	2.30 ± 0.487 } *** {	3.63 ± 0.934 } * {	3.96 ± 0.274
	M. annularis	3.86 ± 0.392 } * {	3.61 ± 0.198 } *** {	4.29 ± 0.194 } * {	4.86 ± 0.466
Coral protein (mg cm^{-2})	*P. porites*	4.23 ± 0.568	3.77 ± 0.550 } * {	4.58 ± 0.503 } ** {	4.96 ± 0.199
	M. annularis	3.96 ± 0.066	3.84 ± 0.434 } * {	4.25 ± 0.445 } ** {	5.19 ± 1.037
Host protein (mg cm^{-2})	*P. porites*	3.73 ± 0.504	3.39 ± 0.535	3.89 ± 0.492	4.04 ± 0.221
	M. annularis	3.62 ± 0.091	3.38 ± 0.535	3.73 ± 0.392	4.13 ± 0.818

Table 5.5 Elemental characteristics of zooxanthellae isolated from *Porites porites* and *Montastrea annularis* after a 40 and 30 day exposure respectively to oligotrophic water and three concentrations of nitrate. Values are means ± SD. n= 4 in each treatment. In all cases ANOVA interaction is not significant so common multiple regression is used. * significant at $P < 0.05$; ** = significant at $P < 0.01$ See text for details of statistical analysis.

Parameter	Species	Treatment ($\mu M\ NO_3$)			
		0	1	5	20
C content (pg C $cell^{-1}$)	*P. porites*	159.4 ± 14.56	155.5 ± 27.31	155.9 ± 28.55	157.6 ± 13.17
	M. annularis	108.9 ± 10.55	118.3 ± 35.13	134.7 ± 29.07	168.1 ± 57.36
N content (pg C $cell^{-1}$)	*P. porites*	32.3 ± 3.44	45.3 ± 8.52	45.3 ± 15.36	43.7 ± 1.55
	M. annularis	15.8 ± 2.20	25.5 ± 12.37	27.7 ± 10.44	47.0 ± 14.36
C:N ratio	*P. porites*	4.50 ± 0.870	3.44 ± 0.111	3.69 ± 1.004	3.61 ± 0.361
	M. annularis	6.97 ± 1.000	5.30 ± 2.068	5.12 ± 1.280	3.62 ± 0.714

Chlorophyll a and c_2. Photosynthetic pigment concentration increased with nitrate concentration as shown in Fig.5.5 ($F_{4,35} = 23.56, P < 0.001$ for Chlorophyll$_a$, $F_{4,35} = 16.59, P < 0.001$ for chlorophyll c_2). It did not show a difference between control and 1 μM NO_3 but was found to be significantly enhanced by the addition of 5 and 20 μM NO_3 ($t = 3.936$, $P < 0.001$ for chlorophyll *a*; $t = 2.562$, $P < 0.02$ for chlorophyllc_2).

Zooxanthellae protein content. This was also significantly affected by nitrate ($F_{4,35} = 23.56, P < 0.001$) but an addition of 20 μM NO_3 was required to observe a significant increase of 66.22 pg protein cell^{-1} above the content of zooxanthellae isolated from corals treated with 0, 1 and 5 μM NO_3 ($t = 3.754$, $P < 0.001$).

Zooxanthellae volume. The volume of zooxanthellae increased with nitrate concentration ($F_{4,35} = 38.97, P < 0.001$): the average cell size in the control chamber was 490 μm^3 when isolated from *P. porites* and 400 μm^3 when isolated from *M. annularis* (for species: $T = 9.315$, $P < 0.001$). The regression model shows a non significant increase in size of 21 μm^3 between control and 1μM NO_3 ($t = 1.369$, $P = > 0.05$), a first significant increase of 32.2 μm^3 occurs with 5μM NO_3 ($t = 2.092$, $P < 0.05$) and a final very significant increase of 66.4 μm^3 appears with 20μM NO_3 ($t = 4.312$, $P < 0.001$) suggesting a linear positive relationship between algal volume and nitrate (Fig.5.6).

Zooxanthellae population density. The density of zooxanthellae was significantly elevated by nitrate addition ($F_{4,35} = 24.57, P < 0.001$) from 2.9 (control) to 4 x 10^6 cells per cm^2 (20 μM NO_3) in *P. porites* and from 3.9 to 4.9 x 10^6 in *M. annularis* (Fig. 5.7): the corals treated with only 1μM NO_3 actually showed a small but significant decrease in density (a loss of about 0.4 million cells per cm^2 with $t = 2.108$, $P < 0.05$); with 5 μM NO_3 there was a 1 million cell increase per cm^2 ($t = 4.807$, $P < 0.001$) and with 20 μM NO_3 a further increase of 0.46 million cells per cm^2 ($t = 2.198$, $P < 0.05$).

C and N of zooxanthellae. The treatment did not have an effect on the carbon content per cell isolated from either coral species, but a significant positive relationship was established between N content and NO_3 addition ($F_{4,27} = 6.82, P < 0.001$): zooxanthellae isolated from *P. porites* had 32 pgN cell^{-1} when in oligotrophic water and 44 pgN cell^{-1} after the highest nitrate treatment, whilst those from *M.annularis* showed a cellular N increase from 16 to 47 pgN cell^{-1} . No significant interaction between species and treatment occurred so both species were combined in the multiple regression analysis: a significant effect of treatment appeared with the lowest nitrate concentration ($t = 2.13$, $P < 0.05$). Accordingly to the change in N, zooxanthellae C:N decreased with increase nitrate enrichment ($F_{4,27} = 7.73, P < 0.001$) with 1 μM NO_3 responding significantly ($t = 2.93, P < 0.01$).

Protein content of the coral. The treatment also had a significant effect in enhancing the total protein content per unit surface area of coral tissue ($F_{4,35} = 7.787$, $P < 0.001$): corals treated with 5 μM NO_3 showed a significantly higher protein content than control and 1 μM NO_3 treated corals ($t = 2.547$, $P < 0.02$) and a further enhancement was found in those treated with 20 μM NO_3 ($t = 2.748$, $P < 0.01$). Coral protein per surface area is the measure of both animal and algal protein content: since nitrate was positively correlated both with algal protein per cell and with the density of zooxanthellae, in order to study the effect of nitrate on animal protein only, the protein content due to algal biomass was subtracted from the measurement of total coral protein. Once the zooxanthellae contribution to the total protein content was removed, no relationship was found between host protein and nitrate concentration (Table 5.4).

5.4 Discussion

This is the first study that investigates the effects of nitrate enrichment on the physiology of hermatypic corals. Previous research has focused on rates of nitrate depletion from enriched seawater by hermatypic corals and clams (Franzisket, 1974; D'Elia & Webb, 1977; Webb & Wiebe, 1978; Wilkerson & Trench, 1986; Szmant *et al.*, 1990) or from unenriched seawater (Bythell, 1990). In this experiment, significant nitrate depletion occurred in the experimental chambers. Some of this could be attributed to the growth of algae and bacteria on the surfaces of the photostat, but it is obvious from the physiological results obtained that nitrogen was being taken up by the corals. In *Acropora cervicornis* and *Montastrea annularis* nitrate depletion was found to occur during the daytime and to continue for 20 h of darkness after which it ceased (Szmant *et al.*, 1990). This suggests that depletion is associated with assimilation by zooxanthellae during photosynthesis. This in turn suggests that nitrate is in fact transported across host cell membranes (c.f. Miller and Yellowlees, 1989), and that the algae are equipped with the nitrate reductase enzymes required for nitrate assimilation. Syrett (1981) showed that in phytoplankton, nitrate reductase was only activated when ammonia was limiting. In *Acropora acuminata* and *Goniastrea australensis,* Crossland and Barnes (1977) showed that nitrate reductase of the symbiotic algae was induced by light and high exogenous nitrate (500 μM NO_3) and depressed by incubation with ammonia. Muscatine *et al.* (1984) failed to demonstrate nitrate reductase activity in *Stylophora pistillata* from the Red Sea suggesting that this may have been correlated with the low nitrate concentration in seawater at the time of sampling. Wilkerson and Kremer (1992) measured $^{15}NO_3$ incorporation in the coronate scyphozoan *Linuche unguiculata* at a rate which was an order of magnitude less than ammonium uptake rates, whilst Wilkerson and Muscatine (1984) showed that the temperate anemone *Aiptasia pallida* would not deplete nitrate

from enriched seawater unless starved for over one month. A rather controversial result was presented by Cates and McLaughlin (1979): they compared the removal of phosphate, nitrite and nitrate from seawater in symbiotic and aposymbiotic *Condylactus* sp. over 18-h incubations. Whilst both phosphate and nitrite uptake occurred only in the presence of zooxanthellae, nitrate depletion in aposymbiotic anemones took place at an even higher rate than in symbiotic ones. The authors concluded that nitrate-reducing bacteria in the coelenteron or body walls had to be responsible for nitrate uptake. However, even though some bacteria can denitrify in aerobic conditions, this only occurs at oxygen levels below 50-90% of air saturation (Robertson & Kuenen, 1984) and commonly the opposite process, i.e. bacterial nitrification occurs (Kaplan, W.A., 1983; Wafer *et al.*, 1985). It is likely that some of the inconsistency (see review by Miller & Yellowlees, 1989) found in the literature regarding nitrate depletion could be explained by variation in the activation of nitrate reductase by zooxanthellae, which would in turn, depend upon the concentration of ammonia and nitrate in the surrounding seawater. The level of heterotrophic feeding and hence rate of excretory ammonia production by the host may also play a part.

In this experiment nubbins of *Porites porites* and explants of *Montastrea annularis* were cultured in oligotrophic oceanic water enriched with four different levels of nitrate. At the end of the incubation period, all physiological parameters measured showed a significant correlation with nitrate concentration in the seawater. The extremely low nutrient concentration of the control treatment was pivotal to the successful demonstration of the effects of nitrate enrichment on the physiology of corals.

5.4.1 Photosynthesis

Maximum gross photosynthesis ($P_{gross\ max}$) normalised to coral surface area increased with increase in nitrate in both species (Table 5.1, Fig 5.2a,b). This can be largely explained by the associated increase in the population density of zooxanthellae expressed on a surface area basis. Hence, when $P_{gross\ max}$ is normalised to number of algal cells, it is found that there is no significant change with nitrate concentration. In *Stylophora pistillata* and *Seriotopora hystrix* Hoegh-Guldberg and Smith (1989) showed a similar increase in $P_{gross\ max}$ following incubation in 20μM NH_4^+, which was also attributed to increase in population density of zooxanthellae; however $P_{gross\ max}$ per cell was reduced and this was attributed to light limitation induced by shading of the deeper algae by the more superficially located ones. In *S. pistillata*, Dubinsky *et al.* (1990) demonstrated an increase in $P_{gross\ max}$ normalised to surface area when the corals were treated with 100 μM ammonium, 10 μM phosphate + 100 μM ammonium or fed with *Artemia* nauplii for 18

days. The $P_{gross\ max}$ per algal cell declined and the authors suggested that this was due to competition among the enlarged population of algae for CO_2.

The photosynthetic efficiency, as measured by the value of alpha, the initial slope of the P vs. I curve, increased with increasing nitrate concentration in *Montastrea annularis* but not in *Porites porites*. The increased alpha of *Montastrea annularis* is similar to that observed in the ammonium enrichment experiments on *S. pistillata* and *S. hystrix* (Hoegh-Guldberg and Smith, 1989) where it was interpreted as a photoadaptation response by the deeper-dwelling zooxanthellae to the reduced light resulting from increase in population density. The reasons why this was not observed in *Porites porites* in the present experiment remain obscure.

5.4.2 Respiration

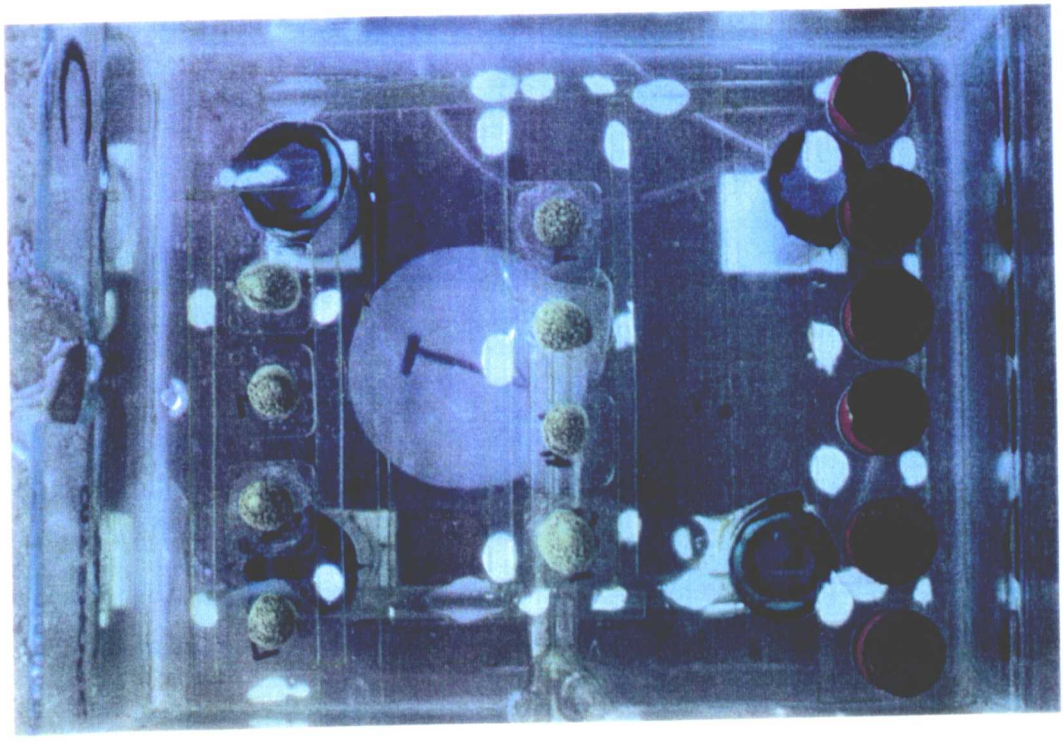
There was no change in the rate of colony dark respiration expressed on a surface area basis, with different concentrations of nitrate. Respiration tends to be closely correlated to protein content and the analysis of protein biomass per surface area showed that nitrate enrichment did not have any effect on host biomass. However if one accepts the common assumption (Muscatine *et al.*, 1981; Muscatine *et al.*, 1984) that the relationship between respiration and protein biomass is constant between host and symbiont (see Ch.3) it follows that coral respiration should be closely correlated to the total protein content of the intact symbiosis and not just to animal protein. Recalculating the respiration rates on a unit protein biomass basis showed that in both species the respiration rate fell with increase in nitrate. This could be attributed to the limitation of respiration by oxygen diffusion (Harland & Davies, 1995) which is exacerbated as the biomass per unit area increases and is likely to have a similar effect on both host and symbiont respiration.

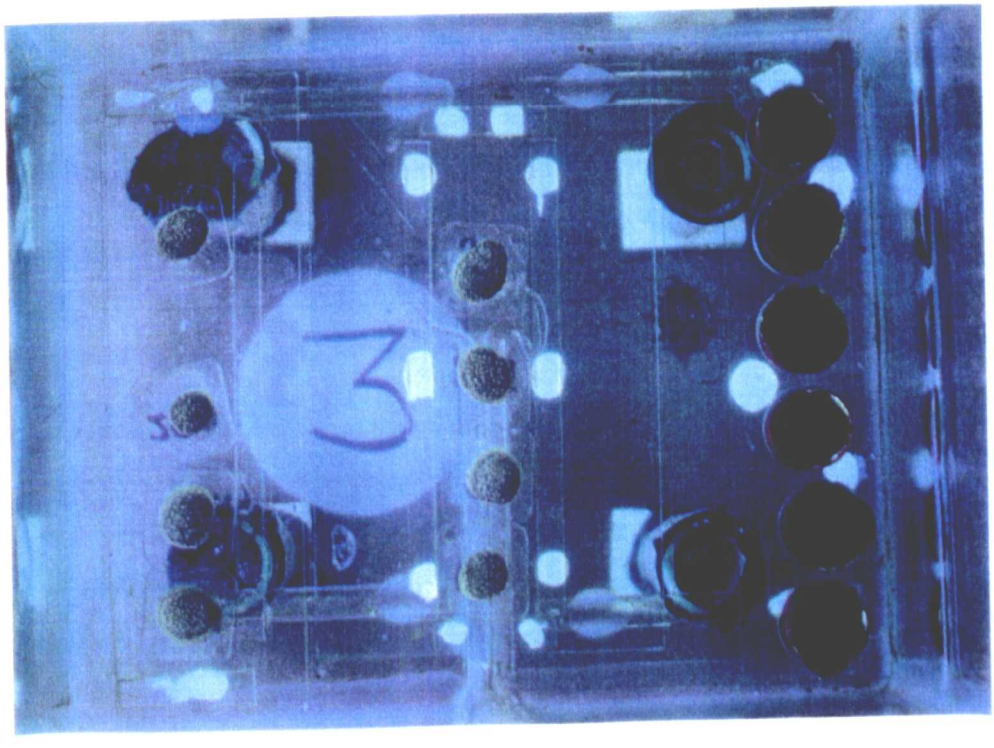
5.4.3 Biomass changes

After a 30-40 day exposure to nitrate, both species appeared to be darker in colour as shown in Fig.5.8. This is attributable to an increase in both the population density of zooxanthellae and the concentration of chlorophyll *a* and chlorophyll c_2 in each algal cell.

This is the first record of increased population density of zooxanthellae resulting from exposure to nitrate although similar increases have been observed with incubation of corals in seawater with elevated levels of NH_4^+ (e.g. Cook *et al.*, 1988; Hoegh-Guldberg & Smith, 1989; Stambler *et al.*, 1991; Stimson & Kinzie, 1991; Muller-Parker *et al.*, 1994b). Also heterotrophic feeding by the host is known to increase zooxanthellae population density as a result of increased production of excretory ammonia (Clayton & Lasker, 1984; Szmant-Froelich and Pilson, 1984; Cook *et al.*, 1988), but even fed corals further enlarge their zooxanthellae biomass when

Fig. 5.8 Aerial view of the photostat chambers for the control treatment (top) and the 5 μM NO_3 treatment (bottom) after 30 days exposure. Nubbins of *Porites porites* are on the left and central tray, and explants of *Montastrea annularis* are on the right tray. Note the difference in the pigmentation of the tissue of both coral species between treatments.

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inorganic nitrogen in seawater is increased (Muscatine *et al.*, 1989). All these cases attest that nitrogen is a limiting factor to zooxanthellae growth *in situ*, especially when corals are grown in oligotrophic waters.

The increase in the population density of zooxanthellae might induce self-shading of symbionts *in situ* (Schomwald *et al.*, 1987) and this in turn could result in a corresponding increase in cellular pigmentation since zooxanthellae are known to readily photo-adapt to different irradiance levels (Chang *et al.*, 1983; McCloskey & Muscatine, 1984; Porter *et al.*, 1984). In this case the positive response of cellular pigments to nitrate enrichment would be indirect (Dubinsky *et al.*, 1990). However, since nitrogen is an essential element in the production of photosynthetic pigments and especially chlorophylls, its increased availability can directly stimulate pigment production as established in many phytoplankton studies (Fogg & Thake, 1987). It is likely that the positive correlation established using nitrate (this study) and ammonium (Hoegh-Guldberg & Smith, 1989; Muller-Parker *et al.*, 1994b; Snidvongs & Kinzie, 1994) between the nitrogen concentration of seawater in which symbioses are incubated and the chlorophyll content of zooxanthellae is the combined result of nitrogen availability and photo-adaptation.

The exact manner in which N limitation and photo-adaptation interplay in the production of chlorophyll needs further investigation. It may be interesting to consider the ratio of chl*a* to protein per cell. This is a species specific ratio used in phytoplankton studies as a measure of the degree of pigmentation. This ratio decreases hyperbolically with decreasing irradiance (Chan, 1978) since at lower light levels, proportionally more nitrogen is invested in the production of chlorophyll rather than other structural proteins. In this experiment, both nitrogen and protein per cell increased with nitrate enrichment so that the ratio chl*a*:protein remained constant in all treatments (0.0174 and 0.0182 in *P.porites* and *M.annularis* respectively): this therefore suggests that the increase in cellular pigments is directly related to increased incorporation of nitrogen into zooxanthellar tissue rather than to a photo-adaptive response.

Phytoplankton ecologists commonly use the ratio of intracellular C:N:P as an index of nutrient limitation: values close to the "Redfield ratio" of C_{106}:N_{16}:P_1 characteristic of oceanic phytoplankton are attained when growth rates are high and nutrient limitation is not operating (Goldman *et al.*, 1979). This correponds to a C:N ratio of 6.6. On the other hand when N becomes limiting and amino acid production is inhibited, photosynthesis results in the accumulation of endogenous carbohydrate (starch reserves): C content per cell increases while N decreases (Droop, 1983; Turpin, 1991).

Zooxanthellae freshly isolated from *Stylophora pistillata* were found to have a C:N ratio of 7.7 when grown in oligotrophic seawater but this ratio was reduced to 4.4 by growing corals in 20μM NH_4^+ (Muscatine *et al.*, 1989). The increase in N content per cell (proportionally higher than the decrease in C content) was found to be closely coupled to protein synthesis underlying

the fact that N is not simply accumulated but used in structural growth. Similar results have been obtained by Snidvongs & Kinzie (1994): zooxanthellae from *Pocillopora damicornis* grown at 15 μM NH_4^+ for 8 weeks had 15% less C and 60% more N than controls so that the overall C:N ratio was only 5 in comparison to the 9.4 ratio observed in the controls. When the host is cultured in low nutrient water, zooxanthellae from the anemone *Aiptasia pallida* respond to endogenous levels of nitrogen produced by the metabolic activity of the host and starvation induces an increase in C:N ratio (Cook *et al.*, 1988). In agreement with the studies mentioned above, zooxanthellae from both *M. annularis* and *P. porites* in control oligotrophic water must be experiencing nitrogen limitation and those under high nitrate enrichment have a lower C:N ratio. This was mainly a result of increased cellular N since C did not significantly change between treatments. The observation that the carbon content remains constant and independent of nitrogen availability in this experiment might be an artefact of C translocated to the host and therefore not accumulated under nutrient limitation. The elemental ratio of C:N in zooxanthellae is not as good an index of nutrient saturation as it appears to be in phytoplankton and should be used cautiously, in making direct comparisons. This is because the large proportion of photosynthate constantly translocated to the host is especially rich in carbon, (Falkowsky *et al.*, 1993) and its translocation therefore results in an artificial decrease in the C:N ratio of the remaining cellular components (Rees, 1991). Conversely, some evidence of C accumulation in oligotrophic water was found in *P.damicornis* by both Berner & Izhaki (1994) who measured high starch reserves and by Muller-Parker *et al.* (1994a) who detected a C:N ratio of 20. However, this study was characterised by an average cellular C of 1000 pg $cell^{-1}$, that is an order of magnitude higher than all other studies on zooxanthellae. The difference in C accumulation observed could be explained if the elemental composition of zooxanthellae and translocate varies between species.

Having established that the zooxanthellae biomass is dependent on nitrogen, it is interesting to find that in both *P. porites* and *M. annularis* there was no change in the host protein. Zooxanthellae population density is usually well correlated with host protein (Cook *et al.*, 1988), but under high nitrate the increased zooxanthellae biomass was not matched by a an increase in host protein. Some evidence of host enhancement was found in *P. damicornis* exposed to 20 μM ammonium (but not when exposed to 50 μM) after 8 weeks of exposure (Muller-Parker *et al.*, 1994b). The duration of the experiment could be very important and possibly 4-5 weeks are not sufficient to measure any change.

5.4.4 Calcification

In both *P. porites* and *M. annularis*, the rate of skeletal growth fell abruptly with increasing nitrate concentrations in the photostats even when concentrations of nitrate were as low as 1μM.

Furthermore the rate in 5 and 20 μM NO_3 was approx. 50% of that of controls in oligotrophic water. Inhibition of calcification by ammonia was reported in *Pocillopora damicornis* by Stambler *et al.* (1991) and Stimson (1992) and in *S. pistillata* by Ferrier-Pages *et al.* (1996). Stambler *et al.* (1991) interpret the lower growth rate as due to either a reduced translocation of organic carbon from algae to host, or to an increased competition for inorganic carbon between algae and host for photosynthesis and skeletogenesis respectively. The amount of C translocated to the host has been evaluated for the average coral in each treatment (data are presented in Ch.8) and in both species zooxanthellae translocate more carbon to the host under high nitrate. Therefore, to explain the inhibition of calcification by nitrate, the second hypothesis of Stambler *et al.* (1991) can best account for my data, that is to say that algae and host compete for inorganic carbon. This rationale may be carried a step further.There is abundant evidence for CO_2 limitation of photosynthesis in corals, arising from studies of carbonic anhydrase activity (Weis *et al.*, 1989; Lesser, *et al.*, 1994), of ^{13}C fractionation (Muscatine *et al.*, 1989), of stimulation following enrichment of seawater with HCO_3^- (Burris *et al.*, 1983) and of the increased rate of photosynthesis associated with light-enhanced respiration (Harland & Davies, 1995). In relation to nitrogen enrichment, Dubinsky *et al.* (1990) showed that in *S.pistillata* P_{max} increased per surface area under high NH_4^+ but was reduced on a per cell basis; hence they suggested that the increased zooxanthellae biomass had become carbon limited. Unfortunately they did not measure the rate of skeletogenesis. Corals growing at high levels of nitrogen (c 5 μM DIN) in the Waikiki aquarium did not appear (there were no controls in the study) to show inhibition of calcification (Atkinson *et al.*, 1995) and the authors suggested that the abnormally high pCO_2 of the inflow water might have counterbalanced any intracellular carbon limitation. The problem is exacerbated in situations of low water movement by the increase in the thickness of the boundary layer which reduces the rate of inward diffusion of CO_2 (Dennison & Barnes, 1988; Lesser, *et al.*, 1994).

Following incubation in elevated levels of nitrate, maximum gross photosynthesis per surface area was found to increase, as a result of the increase in the population of zooxanthellae but no change in the rate per cell was observed. If photosynthesis and skeletogenesis are two processes drawing upon the same pool of dissolved inorganic carbon, it follows that if one process is enhanced the other will be proportionally limited (Fig.5.9). Since water motion is the same in all experimental chambers, boundary layer thickness and mass transfer rate cannot confound the comparison between treatments. I postulate that photosynthesis has a competitive advantage over calcification because zooxanthellae located in the gastrodermal cells are closer to the CO_2 diffusing into the tissues from seawater than the calicoblastic epithelial cells responsible for calcification thus, calcification is carbon limited at high nitrate.

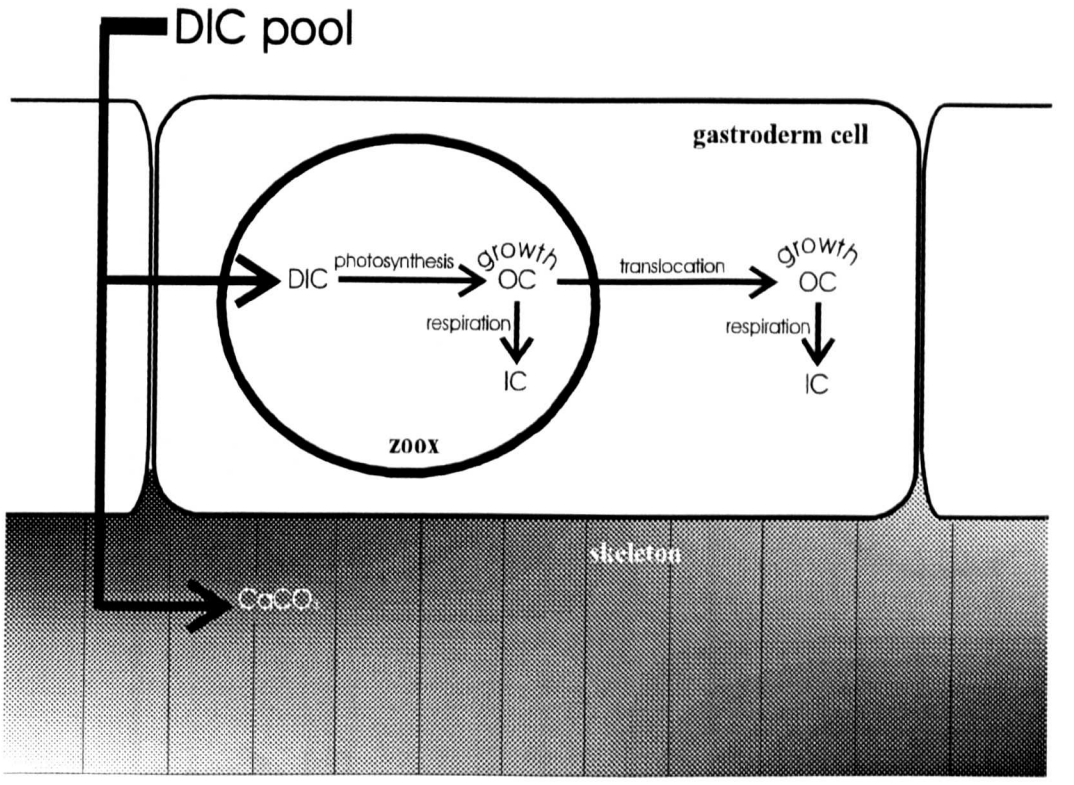


Fig. 5.9 Diagram illustrating the flux of inorganic carbon from seawater into the coral. The same pool of dissolved inorganic carbon (DIC) provides for both photosynthesis by zooxanthellae and skeletogenesis. Carbon fixed by the symbiont into organic carbon (OC) is used for algal growth and respiration and translocated to the host to satisfy its metabolic requirements. The inorganic carbon (IC) produced by respiration might be recycled and used either in photosynthesis or skeletogenesis.

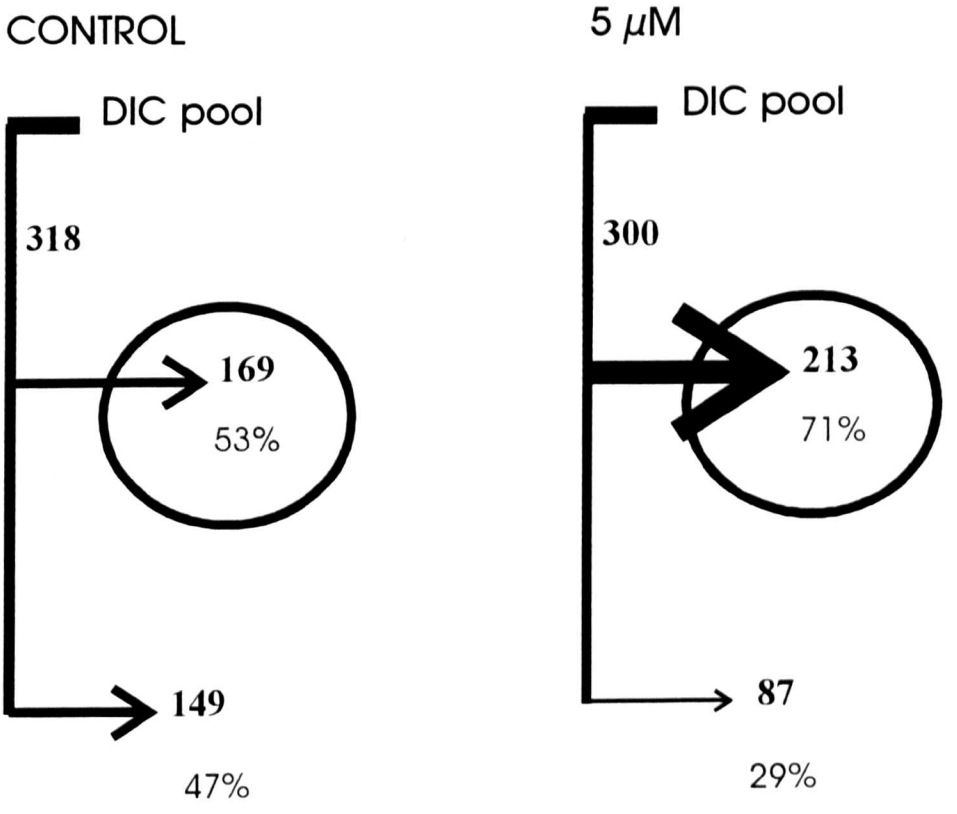


Fig. 5.10 Daily inorganic carbon flux in *Porites porites* nubbins after a 40 day incubation in photostat chambers with oligotrophic water (control) and 5 μM NO3. There is no significant difference in the size of the total net DIC flux into the corals but while in control corals this is shared equally between photosynthesis and calcification, in the nitrate treated corals photosynthesis has increased by 20% to fix as much as 70% of the available DIC into organic carbon and calcification is concomitantly reduced by 20%.

To test this hypothesis, the requirement for carbon for photosynthesis was compared with the carbon required for skeletogenesis using the data of Tables 5.2 and 5.3, (photosynthetic rates were converted from oxygen to carbon assuming a PQ and RQ of 1, calcification rates were converted using the atomic weight of C in $CaCO_3$). Net photosynthesis over daylight hours ($dayP_{net}^{c}$ = Pgross - 13h R) was used because it was assumed that respiratory carbon dioxide was recycled and that calcification (Calc) at night was minimal. The results of this inorganic carbon budget are shown in Table 5.6 and Fig.5.10 for the control and 5μM groups and the two processes do match. In *P.porites*, the inorganic carbon used in net photosynthesis increased by 44 μgC $cm^{-2}d^{-1}$ whilst that used in calcification fell by 62 μgC $cm^{-2}d^{-1}$. A similar pattern is seen in *M.annularis* although here the decline in calcification is greater than the increased use in photosynthesis.

Table 5.6 Mean values for net inorganic carbon fixed in photosynthesis and in skeletogenesis by *Porites porites* and *Montastrea annularis* after respectively 40 and 30 days exposure to oligotrophic water and 5 μM NO_3.

	0 μM NO_3	5 μM NO_3	difference
Porites porites			
Daily net photosynthesis ($dayP_{net}^{c}$) μg C cm^{-2} d^{-1}	169	213	+44
Daily calcification μg C cm^{-2} d^{-1}	149	87	-62
Montastrea annularis			
Daily net photosynthesis ($dayP_{net}^{c}$) μg C cm^{-2} d^{-1}	162	220	+58
Daily calcification μg C cm^{-2} d^{-1}	137	51	-86

Fig. 5.11 presents, for all treatments, the stacked histograms of $dayP_{net}^{c}$ and Calc which together represent the total net inorganic carbon flux into the tissue (net DIC flux). With the exception of explants of *M. annularis* in 1μM NO_3, this net DIC flux is similar for both species and constant across treatments (ANOVA: for *P.porites*: $F_{3,21}=2.12$, $P>0.05$ and for *M.annularis*: $F_{3,17}=3.93$, $P<0.05$, Tukey HSD test: 1uM<0 ,5 and 20uM). This indicates that the net DIC flux into the control corals is maximal and the increased demand by the high nitrate treated corals

Fig. 5.11 Histograms showing the daily dissolved inorganic carbon flux (DIC flux) used in net photosynthesis and calcification by *Porites porites* and *Montastrea annularis* after respectively 40 and 30 days exposure to 4 different nitrate concentrations. Bars are one standard deviation; n =5 in each treatment.

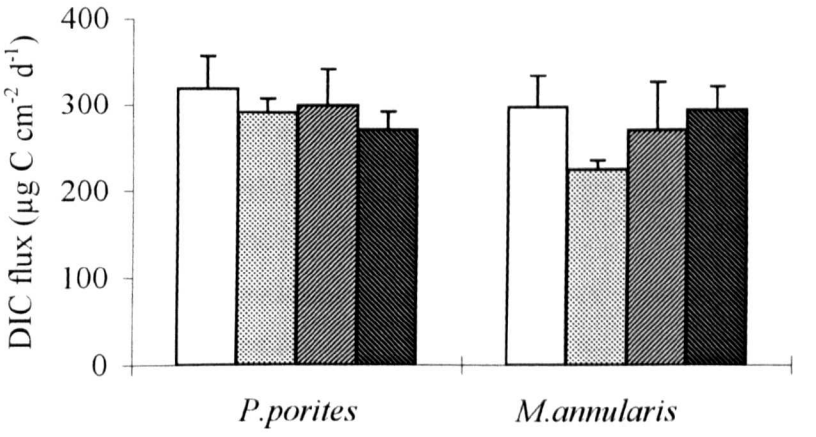
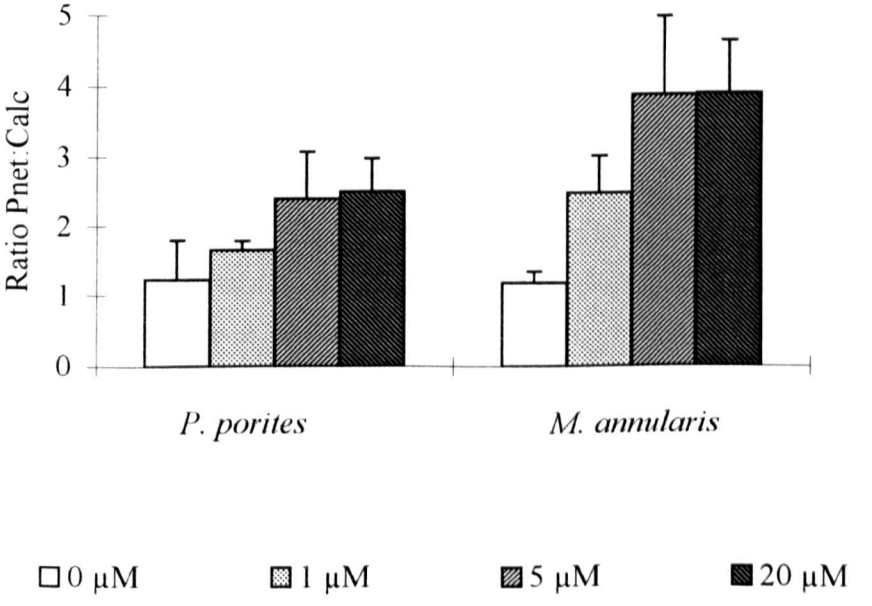


Fig. 5.12 The carbon limitation test. Histograms of the ratio of carbon fixed in net photosynthesis to that fixed in calcification by *Porites porites* and *Montastrea annularis* after respectively 40 and 30 days exposure to 4 different nitrate concentrations. Bars are one standard deviation; n = 5 in each treatment.



cannot enhance the flux any further. The limiting step is therefore the rate of mass transfer of DIC from seawater into the tissue which is known to respond to the thickness of diffusive boundary layers and therefore to water velocity. Considering that the water motion was 40% of that on a very exposed reef, corals growing in low energy areas (such as back reefs and lagoons) and showing reduced calcification rates (Lesser *et al.*, 1994; Jokiel, 1978) are also likely to be most susceptible to decreased skeletogenesis if nutrient enrichment takes place.

What changed between treatments is the proportion of carbon allocated to either photosynthesis or calcification, i.e. the ratio of dayP_{net}^{c}:calc. It increased with high nitrate as proportionally more available carbon was fixed by photosynthesis and less reached the site of calcification. Thus the ratio of dayP_{net}^{c}:calc can be used as an index for endogenous carbon limitation (see Appendix 1). In the high (5 and 20 μM) nitrate treatments this ratio is much higher than in either control or 1μM corals (Fig. 5.12). I therefore infer that a dayP_{net}^{c}:calc ratio of 2.4 in *P. porites* and 3.9 in *M. annularis* attest to severe intracellular carbon limitation for skeletogenesis.

I feel it is worth considering that this rationale of carbon limitation can be applied to corals from control, 5 and 20 μM nitrate but not to those in the 1μM group. Another explanation must be sought here to understand the decrease in calcification when no change in photosynthetic rate was observed: this requires further investigation and could result in important understanding of coral physiology.

Specific experiments set out to directly test the hypothesis of carbon limitation of skeletogenesis and its relationship with nutrient enrichment and coral growth *in situ* are urgently called for especially considering the environmental implications of the physiological mechanisms that can inhibit coral calcification.

Tomascik and Sander (1985) found that the skeletal extension rate of *Montastrea annularis* was negatively correlated with nutrient levels of a eutrophication gradient along the west coast of Barbados. In an earlier study on artificial fertilisation of a patch of reef at One Tree Island, Australia, with 2 μM phosphate and 20 μM urea + ammonia, there was an increase in production and a 50% decline in calcification (Kinsey & Davies, 1979). This reduced calcification was attributed to the effects of phosphate. However, it seems from studies on ammonia and from this experiment on nitrate with *Porites porites* and *Montastrea annularis* that nitrogen elevation could also be responsible for the decline in calcification. Increased levels of nitrate in seawater surrounding coral reefs could result in a dramatic decrease in skeletogenesis, and hence in the rate of growth of reefs as a whole.

Chapter 6

THE EFFECT OF PHOSPHATE ON THE PHOTOSYNTHESIS AND CALCIFICATION OF HERMATYPIC CORALS

6.1 Introduction

The rate of organic production in the sea is controlled by the availability of both nitrogen and phosphorus (Ryther & Dunstan, 1970). Oceanic seawater is typically characterised by a nitrogen to phosphorus atomic ratio of 15:1 (Redfield, 1958), but in the euphotic zone this ratio becomes highly variable due to differential uptake by primary producers. Nutrient enrichment of coastal tropical waters is characterised by increased concentrations of both elements. Usually terrigenous inputs tend to be relatively higher in phosphorus (domestic sewage, guano, rum refinery outfalls, fertilisers) inducing a decrease in the nitrogen to phosphorus ratio and potentially shifting nutrient regulation of productivity from P to N. Phosphate concentrations of oligotrophic waters surrounding coral reefs have been measured to be as low as 0.06 μM (Sander & Moore, 1979; Kinsey, 1987) but concentrations of about 1 μM have been reported on fringing reefs in a section of the Gulf of Aqaba (Red Sea) where pollution from both sewage and loading of phosphate dust occurs (Walker & Ormond, 1982). Along the west coast of Barbados, phosphate concentrations vary from 0.06 to 0.21 μM along a pollution gradient which has shown a strong correlation with reef degradation (Tomascik & Sander, 1987a).

There is abundant evidence that cnidaria symbiotic with unicellular dinoflagellates take up phosphate from seawater (Yonge & Nicholls, 1931b,c; D'Elia, 1977; Muller-Parker *et al.*, 1990; Wilkerson & Kremer, 1992) and as with nitrogen, assimilation is by the zooxanthellae. Release of phosphate is observed in aposymbiotic associations and after prolonged darkness both in isolated zooxanthellae and intact symbioses (D'Elia, 1977; Cates & McLaughlin, 1979; Jackson & Yellowlees, 1990). At the range of pH of seawater and cytoplasm, phosphate is always a charged ion ($H_2PO_4^-$ and HPO_4^{2-}) hence transport into the cells must be carrier-mediated: this could allow for host regulation of phosphate availability to the zooxanthellae (Miller & Yellowlees, 1989; Belda & Yellowlees, 1995).

Few studies have addressed the physiological response of symbiotic cnidarians to phosphate enrichment. Muscatine *et al.*, (1989) found that supplementing colonies of *Stylophora pistillata* with 2μM PO_4 for 14 days did not have any effect on either the zooxanthellae or the host biomass. The experiment by Stambler *et al.* (1991) on *Pocillopora damicornis* came to the same conclusion. On the other hand after 8 weeks of phosphate enrichment to 1.2 μM PO_4, Snidvongs

and Kinzie (1994) found a significant decrease in both cellular C and P in zooxanthellae isolated from colonies of *P. damicornis*. Their interpretation was that phosphate enrichment induced CO_2 limitation of zooxanthellae indirectly by inhibiting $CaCO_3$ deposition which may act as an important endogenous supply of CO_2 for photosynthesis (Ware *et al.*, 1991). Unfortunately no data on calcification were available in their study to support this hypothesis of a mechanism linking calcification to photosynthesis in relation to phosphate enrichment.

Nonetheless phosphate ions are known to inhibit the formation of calcium carbonate crystals *in vitro* (Reddy, 1977). Experimental evidence of an inhibitory effect of phosphate on calcium carbonate deposition has been reported in studies with giant clams (Belda *et al.*, 1993b), coccolithophorids (Paasche & Brubak, 1994) and coralline algae (Bjork *et al.* 1995). Also experiments with hermatypic corals carried out by Lamberts (1974), Rasmussen (1988) and Ferrier-Pagès *et al.* (1996) found evidence for an inhibitory function of phosphate on calcification. However the study by Stambler *et al.* (1991) on *Pocillopora damicornis* suggested that phosphate enrichment did not produce any change in skeletal growth rate.

The growth rate of hermatypic corals is the critical component in the maintenance and accretion of coral reefs (Barnes & Chalker, 1990; Bateson, 1995). Hence the effect of phosphate on coral calcification requires further investigation, especially in view of the fact that *in situ* phosphate concentrations have been negatively correlated with the calcification rate of reef communities (Kinsey & Davies, 1979), calcareous algae (Delgado & Lapointe, 1994; Bjork *et al.* 1995) and coral colonies (Tomascik & Sander, 1985).

In this study, nubbins of *Porites porites* and explants of *Montastrea annularis* were grown in photostat chambers in oligotrophic water and with three different concentrations of phosphate enrichment in order to investigate the effect of phosphate on both the calcification and the symbiont and host biomass. The viability of the mechanism suggested by Snidvongs & Kinzie (1994) connecting calcification to photosynthesis in relation to phosphate enrichment will be discussed.

6.2 Materials and Methods

Explants of *Montastrea annularis* (n = 24) and nubbins of *Porites porites* (n = 32) were retrieved from the reef, and any encrusting or filamentous algae cleaned off before being randomly assigned to one of four photostats. The four photostat chambers were supplied with oligotrophic seawater (control treatment) and KH_2PO_4 enriched seawater (experimental treatments) to give inflow concentrations of 0.2, 1 and 5 μM PO_4. The actual phosphate concentration in the chambers was measured five times during the experiment by the ascorbic

acid method of Strickland & Parsons (1972). The duration of the experiment was 4 weeks for both coral species. Methods for the maintenance of the photostats and measurements of photosynthesis, respiration, skeletal growth and biomass are exactly the same as in the nitrate experiment (see Ch. 5). Elemental analyses of zooxanthellae were carried out only on algae isolated from corals in control and 5μM PO_4 treated groups. No N content values are available because samples were lost during analysis.

Statistical analyses were performed by multiple regression with treatments coded by "dummy" variables as described in the previous chapter for all variables with the exception of the carbon data. In this latter case, data were available for only two treatment groups and Student's *t*-test was used. Tests of the assumptions of normality and homogeneity of variance were performed prior to analysis. In all cases, no serious violation was observed (Zar, 1984).

6.3 Results

6.3.1 Experimental chambers and treatment regimes

As with nitrate (Ch. 5), phosphate concentrations in the chambers were lower than in the inflow stock solution (Table 6.1). Algae and bacteria were undoubtedly responsible for some of the phosphate uptake. Again the nominal inflow concentration of 0, 0.2, 1 and 5 μM PO_4 will be used in all future sections to identify the treatments but it should be borne in mind that the actual concentrations experienced by the corals were somewhat lower.

Table 6.1 Range of phosphate concentrations of the inflow stock solutions and as measured in the photostats immediately before chamber cleaning and refilling.

	Treatment No.			
	1	2	3	4
PO_4 inflow (μM)	0.02	0.2	1	5
PO_4 outflow (μM)	0-0.03	0.01-0.04	0.1-0.8	3.0-4.8

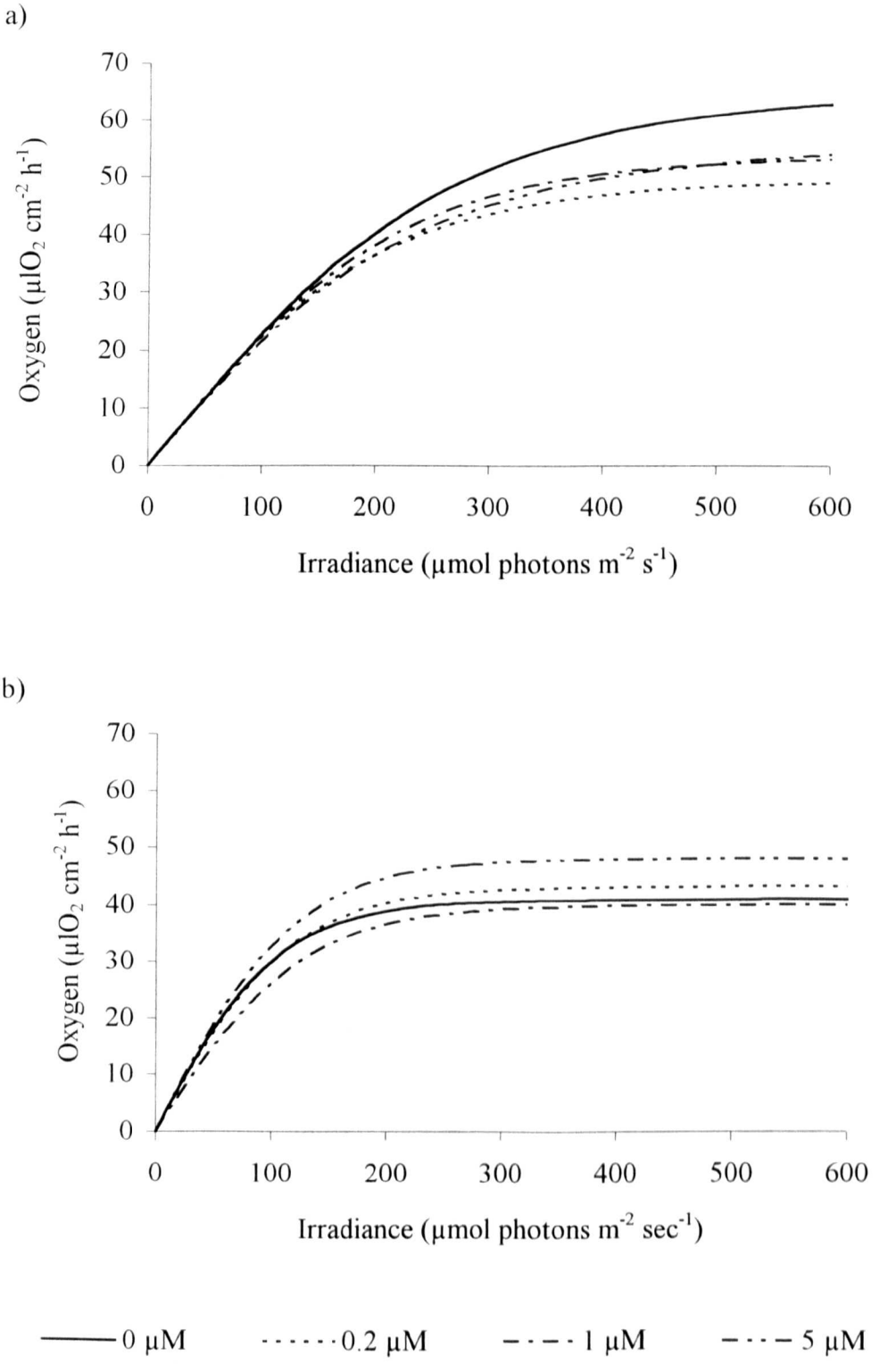
6.3.2 Measurement of photosynthesis and respiration

Gross photosynthesis vs. irradiance curves for both coral species in all four treatments are presented in Figs. 6.1 and the mean values for all photosynthetic parameters are shown in Table 6.2. All parameters were tested against the null hypothesis H_0 which stated that there is no

Table 6.2 Respiration and photosynthesis of *Porites porites* and *Montastrea annularis* after a 30 day exposure to control oligotrohic seawater and three additional concentrations of phosphate. Values are mean ± SD; n = 24 in each species.

		Treatment ($\mu M\ PO_4$)			
		0	0.2	1	5
Photosynthesis					
Max. gross photosynthesis per surface area ($\mu lO_2\ cm^{-2}\ h^{-1}$)	*P. porites*	64.6 ± 16.16	49.6 ± 15.26	53.8 ± 11.40	56.6 ± 13.78
	M. annularis	40.7 ± 3.26	43.0 ± 3.23	39.8 ± 7.04	47.97 ± 8.74
Max. gross photosynthesis per algal cell ($\mu l\ O_2$ cell x $10^{-6}\ h^{-1}$)	*P. porites*	18.1 ± 5.09	17.7 ± 6.11	19.9 ± 7.53	24.7 ± 3.88
	M. annularis	12.9 ± 1.09	15.5 ± 3.12	14.1 ± 3.55	16.5 ± 1.38
Alpha ($\mu lO_2\ cm^{-2}\ h^{-1}\ \mu mol\ photons^{-1}m^2sec^1$)	*P. porites*	0.237 ± 0.0555	0.245 ± 0.0597	0.240 ± 0.0337	0.231 ± 0.0412
	M. annularis	0.386 ± 0.0362	0.376 ± 0.0476	0.318 ± 0.0314	0.406 ± 0.0355
I_k ($\mu mol\ photons\ m^{-2}sec^{-1}$)	*P. porites*	274.3 ± 41.91	205.3 ± 52.46	222.8 ± 25.96	259.7 ± 113.42
	M. annularis	106.2 ± 9.43	116.1 ± 17.81	124.8 ± 13.52	117.7 + 14.75
Respiration					
Respiration rate per surface area ($\mu O_2.cm^{-2}h^{-1}$)	*P. porites*	13.5 ± 2.69	12.5 ± 3.00	12.5 ± 2.18	12.1 ± 1.78
	M. annularis	15.8 ± 1.51	16.7 ± 2.39	15.9 ± 2.06	17.9 ± 1.79
Respiration rate per protein content ($\mu O_2.mg^{-1}h^{-1}$)	*P. porites*	3.14 ± 1.204	2.75 ± 0.825	2.81 ± 0.601	2.99 ± 0.706
	M. annularis	3.92 ± 0.571	3.77 ± 0.320	3.60 ± 0.452	4.35 ± 0.405

Fig. 6.1 Plots of gross photosynthesis v. irradiance for *Porites porites* (a) and *Montastrea annularis* (b) after incubation for 30 days in photostats with four different concentrations of phosphate. Lines are derived from hyperbolic tangent function fitted to the original data. Means and error bars are omitted for clarity. n = 6 in each treatment.



difference between treatments. In all cases H_0 was found to be true: four weeks phosphate enrichment did not have a significant effect on coral photosynthesis (for $P_{gross\ max}$: $F_{3,47}$= 1.18, P>0.1; for alpha: $F_{3,47}$= 1.94, P>0.1; for I_k: $F_{3,47}$= 0.98, P>0.05). Similarly the respiration rate of the intact coral did not show any difference between treatments in both species when standardised to either surface area ($F_{3,47}$= 1.20, P>0.05) or protein content ($F_{3,38}$= 1.08, P>0.05).

6.3.3 Measurement of growth of skeleton

Weight of nubbins and explants was followed at 6 days intervals throughout the experiment: absolute weight change standardised to surface area is shown in Fig. 6.2. The daily rate of calcification is presented in Table 6.3 and Fig. 6.3.

A significant interaction was found between species and treatments in the initial ANOVA ($F_{3,49}$= 4.004, P< 0.05) so that the effect of phosphate had to be analysed separately on each species.

Table 6.3 Daily calcification rate (mg $cm^{-2}d^{-1}$) for *Porites porites* (n = 32) and *Montastrea annularis* (n= 20) after a 30 day incubation with control oligotrophic seawater and three concentrations of phosphate. Values are means ± SD; ANOVA interaction was significant so a multiple regression model was fitted separately to the data for each species. ** significant at P<0.01. See text for details of statistical analysis.

	Treatment (μM PO_4)			
	0	0.2	1	5
Porites porites	1.54 ± 0.361**	1.17 ± 0.261	1.03 ± 0.282	1.24 ± 0.078
Montastrea annularis	0.76 ± 0.144	0.87 ± 0.164	0.87 ± 0.175	0.63 ± 0.275

In *P.porites* (Fig. 6.2a) the control treatment maintained a faster growth rate throughout the experiment, nubbins living in seawater enriched with 0.2 and 1 μM PO_4 had a reduced growth rate in the last two weeks of the experiment, while the highest phosphate enrichment resulted in a consistently lower growth rate. Overall the daily growth rate decreased with increasing phosphate ($F_{3,28}$= 5.223, P< 0.01) and the lowest phosphate enrichment (0.2 μM) corresponded to the critical threshold along the phosphate concentration gradient at which the most significant effect occurred (t=2.776, P< 0.01).

The explants of *Montastrea annularis* on the other hand did not show any significant difference between treatments ($F_{3,14}$= 1.53, P> 0.05). There was a loss in weight between the weighing at

Fig. 6.2 Absolute skeletal weight increase for (a) *Porites porites* (n = 32) and (b) *Montastrea annularis* (n = 20) during a month incubation in photostat chambers with four different phosphate concentrations. Error bars are omitted for clarity.

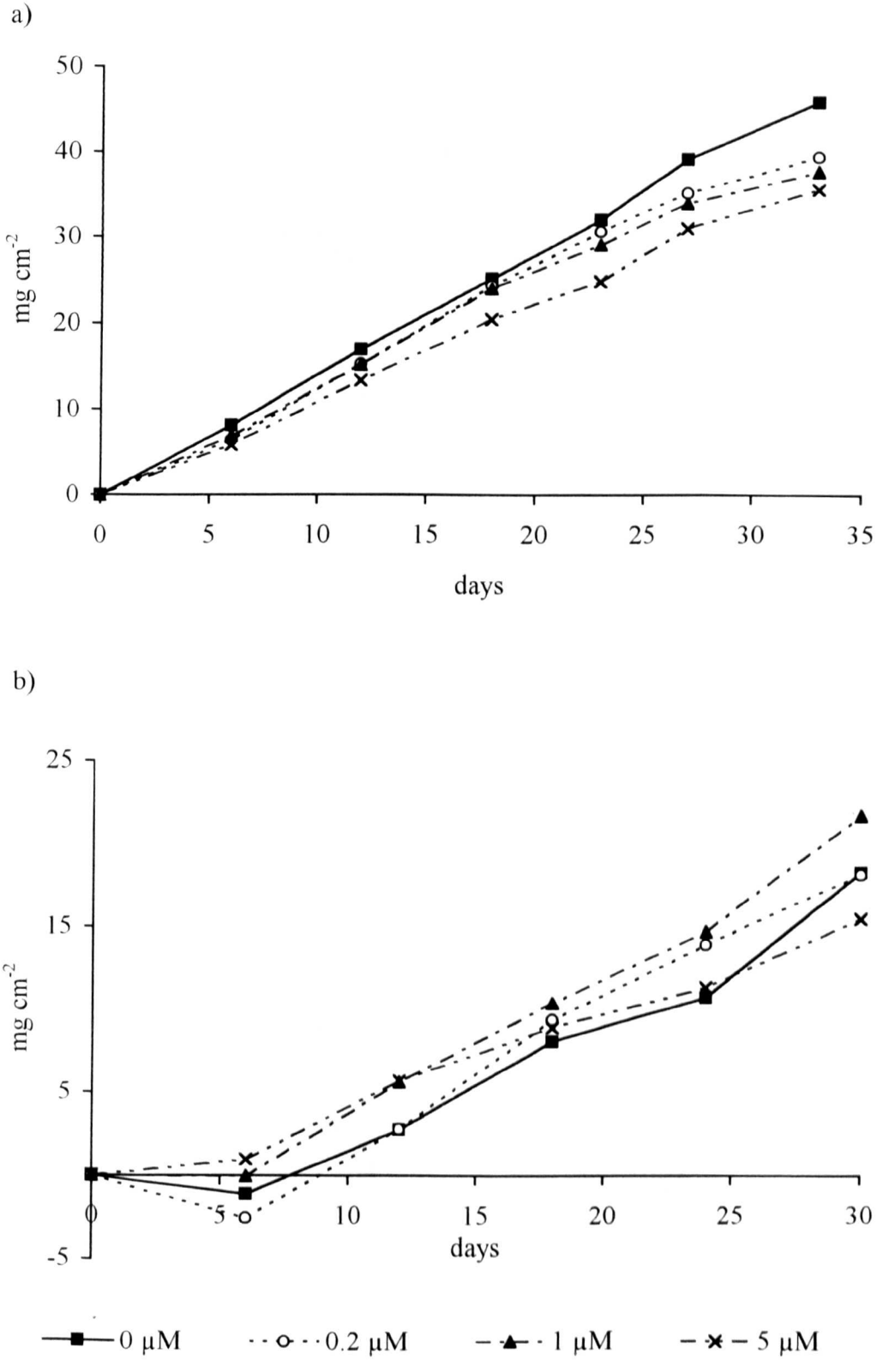


Fig. 6.3 Daily calcification rate for *Porites porites* and *Montastrea annularis* during a month incubation in photostat chambers with four different phosphate concentrations. Bars are one standard deviation.

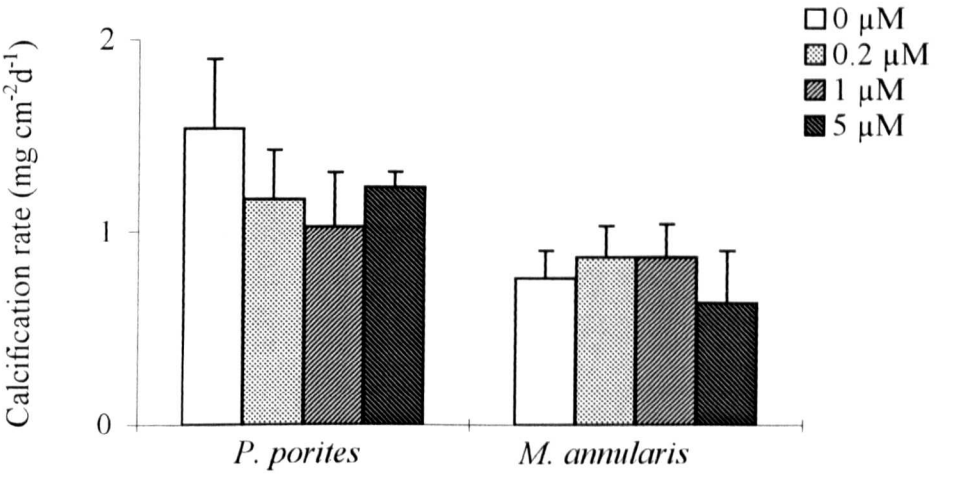


Fig. 6.4 Population density of zooxanthellae standardised to surface area in *Porites porites* and *Montastrea annularis* exposed to four different phosphate concentrations. Bars are one standard deviation. n = 5 in each treatment.

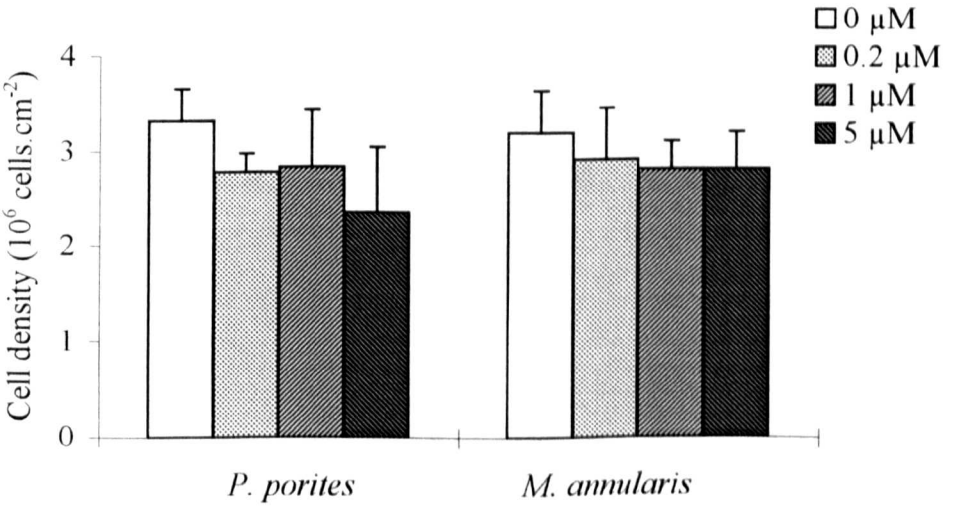


Table 6.4 Biomass characteristics for both Porites porites and Montastrea annularis after a 30 day exposure to control oligotrophic seawater and three concentrations of phosphate. Values are mean ± SD; n = 20 in each species. The data were analysed using a multiple regression model unless inappropriate (see text for explanation). Asterisks position between treatments indicates where significance first appeared. In all cases ANOVA inetraction is not significant so common multiple regression obtained by the data of the two species is used, and asterisk is shown between the rows for the two species. * significant at $P<$ 0.05; n.m.= not measured.

		Treatment (μM PO_4)			
	Species	0	0.2	1	5
Chlorophyll *a* (pg cell^{-1})	*P. porites*	5.16 ± 0.934	3.38 ± 0.287	3.64 ± 1.337	3.56 ± 1.417
	M. annularis	3.34 ± 0.575	3.46 ± 1.213	2.58 ± 0.568	2.87 ± 0.779
Chlorophyll c_2 (pg cell^{-1}) (pg cell^{-1})	*P. porites*	1.20 ± 0.202	0.67 ± 0.085	0.75 ± 0.374	0.88 ± 0.378
	M. annularis	0.99 ± 0.311	1.00 ± 0.448	0.741 ± 0.255	0.81 ± 0.300
Zooxanthella protein (pg cell^{-1})	*P. porites*	207.7 ± 37.97	165.3 ± 59.05	166.5 ± 50.46	166.7 ± 16.81
	M. annularis	177.1 ± 63.24	169.7 ± 53.74	146.6 ± 52.35	145.6 ± 31.98
Zooxanthella volume (μm^3)	*P. porites*	549 ± 46.6	541 ± 71.6	547 ± 88.3	545 ± 23.0
	M. annularis	406 ± 21.8	413 ± 38.7	423 ± 13.9	395 ± 26.0
Zooxanthellae density (x10^6 cm^{-2})	*P. porites*	3.33 ± 0.338	2.79 ± 0.193	2.84 ± 0.604	2.36 ± 0.688
	M. annularis	3.21 ± 0.437	2.93 ± 0.539	2.83 ± 0.294	2.83 ± 0.384
Zooxanthellae C content (pg C cell^{-1})	*P. porites*	185.8 ± 25.7	n.m.	n.m.	223.0 ± 44.4
	M. annularis	150.5 ± 56.7	n.m.	n.m.	154.7 ± 26.0
Coral protein (mg cm^{-2})	*P. porites*	4.84 ± 0.928	4.54 ± 0.314	4.65 ± 0.920	4.05 ± 0.804
	M. annularis	4.05 ± 0.415	4.42 ± 0.386	4.45 ± 0.638	4.16 ± 0.609
Host protein (mg cm^{-2})	*P. porites*	4.52 ± 0.732	4.09 ± 0.468	4.17 ± 0.909	3.66 ± 0.709
	M. annularis	3.43 ± 0.328	3.90 ± 0.287	4.04 ± 0.655	3.53 ± 0.292

time 0 (i.e. as soon as explants have been retrieved from the reef) and that on day 6 (i.e. the first weighing from the photostat) in all treatments. It is worth noticing that the daily calcification rate of explants in the control treatment of this experiment was lower than in the control treatment of the nitrate experiment.

6.3.4 Biometry and biomass measurements

The average values for all biomass measurements are presented in Table 6.4. Both species behaved similarly in response to enriched phosphate in all the parameters measured, i.e. no significant interaction of species and treatment occurred. Interestingly a significantly negative relationship was obtained between zooxanthellae population density and phosphate concentration ($F_{4,35}$= 3.023, P< 0.05). Between control and 5μM PO_4 the zooxanthellae population density decreased in *Porites porites* from 3.3 to 2.4 *10^6 cells cm^{-2} and in *Montastrea annularis* from 3.2 to 2.8 *10^6 cells cm^{-2} (Fig. 6.4). No other parameter shows a significant difference between phosphate treatments (P>0.05) although there was a tendency for the control corals to have the highest zooxanthellae biomass (pigment concentration, protein content per cell) and total protein content among treatments. The C content of zooxanthellae in the control corals were not significantly different from those of 5μM PO_4 treated ones.

6.4 Discussion

Nubbins of *Porites porites* and explants of *Montastrea annularis* were grown in photostat chambers for 30 days under four different phosphate regimes ranging from 0.02 to 5μM PO_4. Phosphate concentration decreased inside the chambers but the amount of phosphate lost could not be quantitatively related to coral uptake activity since some algae and bacteria were always present even though care was taken to clear chambers of all organic material every 4 days when they were washed and refilled.

6.4.1 Photosynthesis, Respiration and Biomass

Phosphate did not have any significant effect on coral photosynthesis or respiration in either species and indeed, among the host and zooxanthellae biomass parameters investigated, only the zooxanthellae population density (number of zooxanthellae per unit surface area) showed a significant difference between treatments and surprisingly, it was found to decrease with phosphate enrichment. This is the first report of a possibly antagonistic effect of phosphate on

symbiotic dinoflagellates and no clear explanation can be given. A decrease in zooxanthellae standing stock could result from either a reduction in the gross algal growth rate (μ_{GROSS} in Ch.3) or an increase in the rate of expulsion and/or digestion by the host, but unfortunately none of these parameters were measured directly. Belda *et al.* (1993a) found that the mitotic index of zooxanthellae freshly isolated from *Tridacna gigas* did not change when the clams were cultured under phosphate enrichment.

Unlike this study where algal C content was constant, Snidvongs & Kinzie (1994) working with *Pocillopora damicornis* found that the zooxanthellae from corals treated with 1.2 μM PO_4 had a significantly lower C and an even lower P content but again no information on zooxanthellae population density, growth rate or photosynthesis was given. It might be inappropriate to interpret changes in the intracellular C content of zooxanthellae as a direct result of changes in zooxanthellae physiology, because as discussed in Ch.5, carbon is preferentially translocated to the host and the regulatory mechanism of this process is still unknown. Nonetheless these authors assumed that the reduction in cellular C and P could be directly related to a reduced rate of photosynthesis under phosphate enrichment. They speculated that if calcification is reduced by phosphate then also the rate of CO_2 supplied by the following net calcification reaction (Stumm & Morgan, 1981) will be reduced:

$$Ca^{2+} + 2HCO_3^- = CaCO_3 + CO_2(aq) + H_2O$$

If the reduction in CO_2 supply by this mechanism cannot be offset by an increased influx of DIC from seawater, then photosynthesis will be limited and reduced. In this study, the rate of calcification of *P. porites* was significantly reduced by phosphate enrichment but no concomitant change in photosynthesis was observed. On the contrary, the maximum gross photosynthetic rate per algal cell tended to increase with phosphate enrichment, although the result was not significant, thus there is no evidence to suggest that the reduced zooxanthellae population density is correlated to the rate of photosynthesis or that photosynthesis is inhibited by a reduced rate of calcification.

A classification of the approaches to the study of nutrient limitation on phytoplankton was proposed by D'Elia *et al.* (1986) who defined a 'growth bioassay' as the approach in which changes in phytoplankton biomass are evaluated when nutrients are added to cultures. This approach is used routinely in algal studies and provides the best evidence for determining nutrient limitation of primary productivity (Maestrini *et al.*, 1984). Adapting this definition to zooxanthellae *in hospite*, this study on *P. porites* and *M. annularis* corresponds to a 'growth bioassay' because changes in the symbiosis biomass were evaluated in nutrient enriched cultures. Thus, if 'growth' is limited by phosphate, a positive response was expected. On the contrary zooxanthellae population density showed a negative response and all other parameters remained

constant in this study. Similar growth bioassays with *Stylophora pistillata* (Muscatine *et al.*, 1989), *Pocillopora damicornis* (Stambler *et al.*, 1991) and the giant clam *Tridacna gigas* (Belda *et al.*, 1993a; Belda & Yellowlees, 1995) showed an overall lack of response to phosphate enrichment by zooxanthellae *in hospite*. Three hypothesis can be formulated to explain a negative result in a growth bioassay in symbioses:

1- zooxanthellae *in hospite* do not experience phosphate limitation even when cultured in unenriched seawater and therefore further phosphate addition does not have any effect on biomass.

2- phosphate is not taken up by the association.

3- phosphate is taken up by the association but the host limits its supply to the zooxanthellae thereby regulating zooxanthellae growth.

There is no evidence with which to confirm the first hypothesis, while some information is available to reject it. For example, highly active acid phosphatases have been found in zooxanthellae freshly isolated from *Acropora formosa* colonies growing in oligotrophic water. These enzymes can enhance phosphate availability to the cell and are repressed by incubation of zooxanthellae in PO_4 supplemented media (Jackson *et al.*, 1989) thus are characteristic of P limitation. Zooxanthellae freshly isolated from the mantle of *Tridacna gigas* incubated for 2 months at different concentrations of phosphate (<0.1, 2, 5 and 10 μM PO_4) were found to display characteristics typical of P-limited cells independent of treatment. These included high acid phosphatase levels, high C:N:P atomic ratios and constant cell population density per gram of mantle tissue (Belda *et al.* 1993a; Belda & Yellowlees, 1995). Furthermore, cultured zooxanthellae originally isolated from *Tridacna gigas* have shown that incubation in P-rich medium results in increased growth rates *in vitro* (Belda & Yellowlees, 1995) suggesting again that P can be limiting.

The second hypothesis can be rejected because of the large amount of evidence showing phosphate uptake by intact symbiosis even at very low concentrations (Yonge & Nicholls, 1931b; Pomeroy *et al*, 1974; D'Elia, 1977; Cates & McLaughlin, 1979; Muller-Parker *et al.*, 1990). Since aposymbiotic cnidarians or symbiotic associations maintained in prolonged darkness release phosphate rather then assimilate it (Wilkerson & Kremer, 1992), zooxanthellae must play a part in phosphate uptake. The dynamics of phosphate depletion in symbiotic associations cannot be satisfied by simple diffusion and active transport is required (D'Elia, 1977). D'Elia (1977) and D'Elia *et al.* (1983) proposed a depletion-diffusion model for phosphate uptake (as well as for NH_4 and NO_3) whereby zooxanthellae deplete phosphate actively from the surrounding host tissue thus allowing for net inward diffusion from seawater into the host cytoplasm. Critics of this model (Miller & Yellowlees, 1989; Jackson & Yellowlees, 1990) have focused on the likely form and concentration of phosphate in the host cytoplasm. At the

physiological pH range (6.0-8.5) PO_4 is ionised so that transport across membranes must be carrier mediated. Although never measured directly in cnidarian host cells, the concentration of P in the cytoplasm is likely to be in the millimolar range, while for diffusion to take place, zooxanthellae uptake should reduce the internal concentrations to undetectable levels, 1000-fold lower than what is required in normal cell activity. Furthermore zooxanthellae are not embedded directly in the host cytoplasm but are in a vacuole bounded by the symbiosome membrane of animal origin (Trench, 1971; Roth, *et al.*, 1987) which provides another barrier to ion transport. Since phosphate is taken up, but zooxanthellae P limitation is unabated, Miller & Yellowlees (1989) proposed that the host must be able to regulate the availability of PO_4 (and other nutrients) to the zooxanthellae, and ultimately this mechanism could allow for host control of the symbiont growth rate. They identified the symbiosome membrane as the most likely site of host regulation. Rands *et al.* (1993) used cytochemical methods to investigate the properties of the host-symbiont interface in *Anemonia viridis*. On both zooxanthellae and symbiosome membranes, they found ATPase activity which is a basic necessity for active selective transport, and phosphatase activity which implies low ambient P concentration and thus implies that the symbiosome membrane is not freely permeable to the millimolar concentration typical of the cytoplasm. Hence they concluded that the transport of nutrients to the zooxanthellae is regulated by the symbiosome membrane rather than by the zooxanthellae demand as suggested by the depletion-diffusion hypothesis.

It has been argued that a mechanism of host regulation of zooxanthellae growth is necessary to account for the lower growth rate of zooxanthellae observed *in situ* when compared to cell cultures, and for the relatively constant zooxanthellae population density observed in many cnidarian symbioses (Porter *et al.*, 1984; Miller & Yellowlees, 1989; Falkowski *et al.*, 1993). However enrichment experiments with nitrate (see Ch.5) and with ammonia (Hoegh-Guldberg & Smith, 1989; Muscatine *et al.*,1989; Muller-Parker *et al.*, 1994a) have reported significant increases in the zooxanthellae population density. This would not be expected following the hypothesis that the host limits algal growth by regulating the flux of P to the symbionts. I suggest that if P supply is limited by the host, its advantage might not lie in regulating algal growth as much as in favouring the accumulation and thus the translocation of organic carbon. In this context it is interesting to note that mucilage and carbohydrate release by phytoplankton is strongly enhanced by P limitation (Paasche & Brubak, 1994; Myklestad, 1995).

Thus there is some evidence in support of the third hypothesis but many incongruities remain. For example, the theory of host control cannot reconcile the fact that phosphate uptake is dependent on zooxanthellae activity and yet phosphate is not made available to them, nor does it explain what is the advantage in the host actively taking up phosphate to then block its transport at the symbiosome membrane level.

6.4.2 Calcification

In vitro, the growth rate of crystals of a metastable supersaturated $CaCO_3$ solution is greatly reduced in the presence of glycerophosphate or orthophosphate and it appears that the calcium deposition in the form of apatite ($CaPO_4$) does not follow (Reddy, 1977). There is evidence that suggests a general application of this phenomenon both in vertebrate and in invertebrate calcification (see Simkiss, 1964). However the physiological mechanisms that might be involved are not understood.

Dodge *et al.* (1984a) found measurable concentrations of phosphorus in the skeletons of *Montastrea annularis*, and in some cases, the observed yearly variability in concentration was positively correlated with sewage pollution. They suggested that P trapped in the skeleton could result from the P present in the organic matrix residue or in the endolithic algae, or from actual primary deposition of $CaPO_4$. The latter possibility was rejected because the presence of apatite in corals has never been confirmed.

The early experiment by Lamberts (1974) on coral calcification indicated that coral calcification decreased with increased levels of phosphate. However the concentrations tested were very high (20, 200 and 2000 μM) and seawater pH was reduced to as low as 6.3. The effects of pH, bicarbonate availability and pCO_2 on coral calcification have not been fully understood but cannot be overlooked. For example, the daily growth rate of nubbins of *Porites compressa* decreased from 10.05 to 5.11 mg $CaCO_3$ g^{-1} skeleton d^{-1} when seawater pH was lowered from 8.0 to 7.0 (Marubini & Atkinson, in prep.). Therefore the results of Lamberts (1974) are more likely to be due to the experimental artifact of pH reduction than to the inhibitory effect of phosphate.

Thereafter investigators studied the effects of phosphate on calcification using environmentally meaningful concentrations and avoiding pH artefacts. Rasmussen (1988) found that the skeleton of *Acropora formosa* colonies grown at 1, 2 and 4 μM PO_4 appeared morphologically altered when examined under a scanning-electron microscope (SEM), with a higher proportion of voids and a thinning of skeletal walls. SEM observations have shown that a 3-month exposure to elevated phosphate produced misshapen crystals and increased cavities in the shell of *Tridacna gigas* and the shell weight was also significantly reduced. Rasmussen (1988) found evidence for a reduction in calcification in *A. formosa* by measuring the amount of strontium precipitated into the skeleton. Ferrier-Pagès *et al.* (1996) found that phosphate enrichment (2-3 μM PO_4) reduced the rate of growth of *Stylophora pistillata* measured by buoyant weighing. These results are in agreement with the negative relationship between calcification rate and phosphate concentration obtained on *P.porites* in this growth experiment.

In contrast, no reduction in the rate of calcification was found in *M. annularis*. However, the observation that the rate of calcification of controls in this experiment was lower than that of controls in the nitrate experiment (Ch. 5), and the apparent loss of weight of all explants on day 6, suggests that these were artefacts of this particular group of explants. In initial trials (2.2.3), problems during buoyant weighing were encountered when bacterial contamination within the cup resulted in bubbles, and a similar phenomenon may have been experienced here.

Stambler *et al.* (1991) found no effect of phosphate on the growth rates of *Pocillopora damicornis*, but this is the only study that measured calcification as linear extension. As pointed out by Dodge and Brass (1984), linear extension and mass accretion do not always correlate. If phosphate affects the density of the skeleton but does not alter the rate of linear extension, the ecological implications are worth investigating. Increased skeletal fragility can present a major threat to coral communities regularly threatened by bioeroders, wave damage and cyclones and such an effect could be much more detrimental for branching corals than for massive ones. If phosphate does not affect the linear extension, the host tissue and zooxanthellae growth rates (μ_{NET}) would remain unaffected.

Since the pioneer fertilisation experiment by Kinsey and Domm (1974) where both nitrogen and phosphorus levels were elevated, P has been suggested as the causal element in the reduction of calcification (Kinsey & Davies, 1979; Hawker & Connell, 1992) because it offered the certainty of a chemical explanation (Simkiss, 1964). By comparing the reduction in calcification rate obtained in this study with *Porites porites* nubbins under phosphate (25% reduction) and nitrate enrichment (40% reduction), it appears that both nutrients have the potential for long-term effects on coral communities.

Interestingly, observations on *S.pistillata* (Ferrier-Pagès *et al.*, 1996) and on *T.gigas* (Belda *et al.*, 1993) showed that the simultaneous P+N enrichment did not result in a synergistic effect. This is in agreement with the different mechanisms of inhibition of calcification postulated for each nutrient. Phosphate has a direct inhibitory effect on crystal formation thus reducing the amount of DIC fixed in skeletogenesis. Nitrogen appears to affect calcification indirectly via enhanced competition for DIC between zooxanthellae and calicoblastic cells. When both P and N are present, the increased requirements for DIC by the zooxanthellae could be balanced by the reduction in the carbon requirements of the calicoblastic cells brought about by P inhibition, and thus no synergism would be expected.

Thus, high phosphate concentration in seawater can reduce calcification in hermatypic corals and symbiotic clams, but it appears that the reduction might not be as marked as for coralline algae (Bjork *et al.*, 1995). Some physiological mechanism must exist in invertebrate calcification to minimise the purely chemical inhibitory effect of P within the tissues. Simkiss (1964) suggested that one advantage conferred to the host by the zooxanthellae is the removal of PO_4 ions before

they could reach the site of calcification. However, the evidence presented above on zooxanthellae uptake is equivocal.

This study did not find any evidence for the theory suggested by Snidvongs & Kinzie (1994) that a reduction in calcification could affect the rate of photosynthesis and growth of symbiotic zooxanthellae. A study on the flux and accumulation of phosphate using the radiochemical tracer ^{32}P to monitor the rate of incorporation of PO_4 from enriched seawater into the coral compartments of zooxanthellae, host, mucus and skeleton could succeed in elucidating much of the speculations discussed here.

Chapter 7

WATER QUALITY CHARACTERISTICS ON THE REEF AND ITS EFFECT ON THE PHYSIOLOGY OF SCLERACTINIAN CORALS.

7.1 Introduction

Experimental manipulations of nutrient levels in an otherwise constant laboratory environment have been instrumental in recognising those physiological responses of corals that are specific to nutrient enrichment. Nitrogen, both as ammonia and nitrate, has been shown to be the key limiting nutrient for zooxanthellae growth *in hospite* (see Ch.5 and 6): any change in the external nitrogen supply affects zooxanthellae population density and photosynthetic capacity and ultimately alters the balance between host and symbiont biomass, thus reducing calcification rate via endogenous carbon limitation. Phosphorus has a less obvious impact on coral biomass but has a direct inhibitory effect on calcification.

Since the ultimate goal of this research is to identify the physiological parameter that best reflects the degree of eutrophication on reefs, the next logical step is to check that the processes observed in controlled laboratory conditions apply also to corals growing *in situ*, and only as a direct response to eutrophication. Firstly, corals on the reef are able to feed opportunistically on plankton (Johannes *et al.*, 1970; Porter, 1974) so that even in the most oligotrophic seawater, the nutrients available to the algae, as by-products of host metabolism are likely to be different from that of starved corals used as laboratory controls. The degree to which feeding could alter the physiological response to external dissolved nutrient enrichment is likely to differ between species according to their dependency on autotrophy (Porter, 1976; Wellington, 1982). Secondly, areas on the reef characterised by different nutrient concentrations may also differ in a variety of other key environmental factors known to directly affect corals: water motion (Jokiel, 1978; Dennison & Barnes, 1988; Patterson *et al.*, 1991; Atkinson *et al.*, 1994), temperature (Jokiel & Coles, 1977; Coles & Jokiel, 1977; Hoegh-Guldberg & Smith, 1989; Suharsono & Brown, 1992), light intensity (Goreau & Goreau, 1959; Wethey & Porter, 1976; Falkowski & Dubinsky, 1981; Kinzie *et al.*, 1984; Muscatine *et al.*, 1984; Lesser & Schick, 1989; Achituv & Dubinsky, 1990; Gattuso *et al.*, 1993), salinity (Coffroth, 1985; Hoegh-Guldberg & Smith, 1989) and turbidity (Dodge *et al.*, 1974; Coffroth, 1985; Stafford-Smith, 1990; Hunte & Wittenberg, 1992; Wittenberg & Hunte, 1992). The degree and mode (antagonistic vs. synergistic) of interaction of environmental parameters with coral physiology are mostly unknown, but nonetheless real and some partial speculations have been put forward (Dubinsky & Jokiel, 1994).

Thus, in order to be successful, any physiological index of eutrophication has to be robust and consistently independent of any variation induced by changes in other environmental parameters.

The rate of calcification of corals has been suggested as a potential indicator of stress on reefs (Shinn, 1966; Dodge & Brass, 1984), and particularly of nutrient enrichment (Davies, 1990). It has been reported to correlate well with water quality (Dodge & Brass, 1984; Tomascik & Sander, 1985; Davies, 1990; Tomascik, 1990; Hudson *et al.*, 1994) but also with other environmental factors such as seasonality (Barnes & Crossland, 1980), light intensity (Wellington, 1982; Huston, 1985) and water motion (Dennison & Barnes, 1988). These results suggest that the observation *per se* of a reduction in calcification should not be taken as convincing evidence of nutrient enrichment unless all other environmental variables have been accounted for.

Since skeletogenesis is the product of an active process by the host, enhanced by the photosynthetic activity of its symbionts, changes in calcification might be the result of changes in the physiology of the alga-invertebrate symbiosis, and these might be measured and used as environmental indicators. Unfortunately there is no evidence to confirm this suggestion because most studies concerned with calcification *in situ* did not record any measurement of either host or zooxanthellae biomass and activity.

The present experiment is the first attempt to correlate changes in coral physiology to eutrophication in the field, by combining observations on the rate of calcification, photosynthesis, and biomass changes of both zooxanthellae and host tissue. It was carried out by comparing three groups of corals grown for one month at three locations characterised by different concentrations of nutrients on the west coast of Barbados. The aim was to determine whether changes observed under laboratory conditions could be observed under the more complex environmental regimes on the reef, and to identify the physiological parameter that would best suit the qualities required of a bio-assay for the detection of nutrient enrichment.

7.2 Methodology

7.2.1 Site choice and coral preparation

The experimental design required the identification of three sites on the West coast of Barbados, characterised by low, medium and high nutrient loading. From previous long term studies on water quality (Tomascik & Sander, 1985; Seakem, 1989; Allard, 1994), the reef at

Fig. 7.1 Aerial map of Barbados showing the position of the three experimental sites. ● Offshore (OS), ● Bellairs (BRI) and ● Spring Garden (SG).

Spring Garden (see Fig. 7.1) was known to be the most polluted: it is close to the capital and directly affected by outfalls from a rum refinery and a power plant that uses high nitrogen ground-water for its cooling system. The reef in front of the Bellairs Research Institute was chosen as the medium nutrient loading site. All inshore coastal sites have some degree of sewage input but water quality improves dramatically with distance from shore. Thus a 'low' nutrient site was identified about 1 km offshore over the submerged barrier reef where nutrient levels are close to the limit of detectability (Ott, 1975; Seakem, 1989). However at this point, the top of the reef is about 40m deep, as opposed to 2-10m of the chosen fringing reef locations. Therefore, in order to ensure that the corals received approximately similar light regimes, the racks containing nubbins and explants were suspended from buoys at 2.5m depth at all locations. The racks (with wire mesh protection) were fixed to PVC holders attached to the buoy chain as shown in Fig.7.2. Buoys were already available offshore on the barrier reef (by kind permission of the National Conservation Commission) and in front of the Institute; a further one was set up at Spring Garden.

Nubbins of *Porites porites* and explants of *Montastrea annularis*, after preparation, were left for recovery at the 'nursery' site at 2.5 m depth on the North Bellairs Reef. After approx. 20 days, they were retrieved from the reef and any encrusting algae were scraped off their tiles or caps. Corals were then buoyant weighed (2.3.1) and randomly assigned to three groups, each consisting of 10 nubbins and 7 explants. A rack with its protective wire-mesh cage was placed at each site at 2.5 m depth. Cages and trays were routinely cleaned every 6 days. Explants of *M. annularis* and nubbins of *P.porites* were placed on these racks for 26 and 31 days respectively.

7.2.2 Water quality measurements

Water samples were collected every 6 days at each site at 2.5 m depth. They were kept in a cooler while on the boat and quickly transported back to the laboratory. Samples were filtered through a GF/C glass fiber filter and immediately analysed for reactive phosphate (PO_4-P) and nitrate-nitrite-nitrogen (NO_3-NO_2-N) following the method of Strickland and Parsons (1972). One litre of sample was filtered through a pre-combusted and pre-weighed GF/C filter and dried to constant weight at 60°C for determination of the total suspended particulate matter (SPM); the filter was then combusted at 550°C for 15 min to estimate the weight of volatile particulate matter (VPM) (APHA, 1992).

Irradiance was measured *in situ* every 12 days with a light meter and a 'cosine' sensor for downwelling irradiance. It was measured inside the cage before and after cleaning and their average was expressed as % transmission of underwater surface irradiance.

Fig. 7.2 PVC holder attached to the buoy chain at the offshore site holding the coral rack in position.

7.2.3 Photosynthesis, respiration, biomass and calcification measurements

At the end of the experimental exposure at the 3 sites, the corals were collected and brought to the laboratory where measurements of photosynthesis and respiration, and subsequent biomass determinations were carried out as outlined in Ch.5 and Ch.6. Buoyant weight and surface area measurements were obtained only at the start and end of the exposure period, in order to minimise disturbance to the corals. Daily calcification rate was obtained by dividing the change in weight between initial and final measurements by the number of days of the experimental exposure.

7.2.4 Statistical methods

All data were tested for normality (one sample Kolmogorov-Smirnov test) and homogeneity of variance (Levene test).

Simultaneous analysis of the effect of site and species was performed on each physiological variable with a two-factor analysis of variance. If significant difference was obtained among the levels of a factor, then Tukey HSD test for multiple comparison was employed.

A discriminant function analysis was adopted in order to address the question of how well the three sites could be separated, given measurements on several physiological variables, rather than on one physiological variable at a time. The aim of this exploratory statistical method is to find those linear combinations of the original variables that maximise the difference between sites (Manly, 1994) and express them as linear functions (canonical discriminant functions). The number of functions obtained depends on the data set. The first function obtained reflects site differences as much as possible. The second one captures most of the difference between sites that was not displayed by the first, and so on. Thus they are uncorrelated with each other. The weight of the contribution by each original variable to each discriminant function is evaluated by correlating (Spearman's rank correlation) the physiological variables and the discriminant functions.

Thus, the discriminant functions are combined physiological variables and can be investigated in relation to light and water quality (NO_3, PO_4, SPM, VPM) in order to determine which of these environmental parameters are most responsible for the differences in coral physiology observed between sites. First, Spearman's rank correlation analysis was used to determine the relationship among the water quality parameters (NO_3, PO_4, SPM, VPM) at the three sites. They were found to be highly autocorrelated (Spearman correlation coefficient > 0.9), in agreement with the previous observations by Tomascik & Sander (1987a) and Allard (1994) who used a much larger data set including seven sites along the west coast of Barbados

monitored over several months. Such high correlation removed the possibility of investigating the effect of each water quality parameter separately. Thus, it was necessary to reduce the four water quality variables to one single linear component. This was achieved using the principal component analysis (PCA) procedure computed from correlation matrices (Manly, 1994).

A linear regression model was used to study the relationship between the discriminate functions obtained and the environmental factors reduced to the two variables of light and water quality.

7.3 Results

7.3.1 Water quality and light at the three sites

The average values of each environmental parameter are presented in Table 7.1. On average the corals at the offshore site (OS) received 58% of underwater surface illumination, those in front of the Bellairs Research Institute (BRI) were subjected to 50% while those at Spring Garden (SG) received only 31%, a significant lower irradiance than either OS or BRI ($F_{2,9}$= 10.5, p<0.01). As expected, water quality parameters showed that OS had the least amount of nutrients and particulate matter, BRI was intermediate and SG was the most polluted.

Table 7.1. Water quality characteristics (n=24) and percentage of surface photosynthetically active radiation (%surfPAR) (n=9) at the three sites during the experimental period. Values are means ±SD.

	Sites		
	OFF	BRI	SG
NO_3 (μM)	0.34 ± 0.194	0.76 ± 0.356	1.18 ± 0.598
PO_4 (μM)	0.03 ± 0.017	0.06 ± 0.025	0.06 ± 0.019
SPM (mg l^{-1})	14.9 ± 2.49	15.6 ± 3.03	16.1 ± 4.31
VPM (mg l^{-1})	1.78 ± 0.450	2.26 ± 0.452	2.31 ± 0.672
%surfPAR	58 ± 6.7	50 ± 7.1	31 ± 8.1

Using principal component analysis (PCA) these four variables were reduced to one principal component (PC1): its scores were highly correlated to each water quality parameter as shown in Table 7.2. Thus the water quality at each site could now be identified by its average PC1 score: these were -0.84 (±0.551), +0.36 (±0.557) and +0.50 (±1.311) at OS, BRI and SG respectively. Water quality offshore is significantly different from the 2 inshore sites ($F_{2,20}$=

5.35, p= 0.015) and although SG tends to score higher, the difference between BRI and SG is not significant with this relatively small sample size. PC1 scores will be identified hereafter as the 'eutrophication index' for consistence with previous work (Tomascik & Sander, 1987a; Allard, 1994).

Table 7.2 Loadings of each water quality variable on the principal component PC1, and corresponding Spearman's rank correlation coefficients (rs) for correlations between water quality variables and PC1. * significant at P<0.05 and *** significant at P<0.001

	Component Loading	r_s	P
NO_3 ($\mu mol.l^{-1}$)	0.268	0.467	*
PO_4 ($\mu mol.l^{-1}$)	0.218	0.518	*
SPM ($mg\ l^{-1}$)	0.733	0.856	***
VPM ($mg\ l^{-1}$)	0.757	0.870	***

7.3.2 Photosynthesis and respiration

Photosynthesis vs. irradiance curves were constructed for corals of both species at all sites and average values for all curve parameters are presented in Table 7.3. A two-way ANOVA was performed on each parameter and the consistent lack of interaction between species and sites was evidence that the effect of 'site' was the same for both species. The presentation of these results will focus on the differences between sites rather than between species.
Corals were found to differ significantly between sites in their maximum gross photosynthetic rate measured per unit surface area ($F_{2,30}$= 4.339, P <0.05; Tukey HSD test: P<0.05, OS=SG<BRI), but not when measured per unit zooxanthella ($F_{2,23}$= 0.985, P >0.3). The photosynthetic efficiency expressed by alpha was highly significant between sites ($F_{2,30}$= 12.24, P <0.001; Tukey HSD test: P<0.05, OS<BRI=SG). Since these corals, unlike the previous experiments in the photostat (Chs.5 and 6), were subjected to different light intensities at their respective sites during the approx. 13 hours of daylight, the integrated daily photosynthetic rate was calculated to provide a better comparative measure of the total amount of carbon fixed by these corals in the field. The maximum daily photosynthetic active radiation (PAR) experienced by the corals was estimated from a continuous recording of surface light intensity on an 'optimal' clear day (8th June 1994) and the mean percentage light transmission measured at each site. Thus, 'optimal' PAR curves were estimated (Fig.7.3) for each site. These resulted in total daily PAR estimations of 18.5, 15.5 and 9.4 photons $m^{-2}d^{-1}$ for corals at OS, BRI and SG respectively. As described in 2.3.3, the average P/I curve and

Table 7.3 Respiration and photosynthesis of *Porites porites* and *Montastrea annularis* after a month exposure at three sites along a eutrophication gradient on the west coast of Barbados. Values are mean ± SD, n=5. Data were analysed using two-way ANOVA. Interaction between species and treatment is never significant. Only significant differences between treatments are shown (P^{ϕ}, *F*-test between sites). *n.s.* not significant at $P<0.05$, * significant at $P<0.05$, ** significant at $P<0.01$, *** significant at $P<0.001$. Groups are significantly different (Tukey HSD, $P<0.05$) when identified by different letters.

Parameter	P^{ϕ}	Species	Sites		
			OS	BRI	SG
Photosynthesis					
Max. gross photosynthesis per surface area ($\mu lO_2\,cm^{-2}\,h^{-1}$)	*	*P. porites*	52.9 ± 10.87 } a	74.8 ± 16.65 } b	61.1 ± 11.95 } a
		M. annularis	62.6 ± 17.17 }	70.0 ± 14.30 }	54.7 ± 11.43 }
Max. gross photosynthesis per algal cell (μlO_2 cell x $10^{-6}\,h^{-1}$)	*n.s.*	*P. porites*	17.7 ± 3.69	17.6 ± 4.51	16.4 ± 2.52
		M. annularis	10.9 ± 1.68	10.4 ± 1.72	8.7 ± 1.90
Alpha ($\mu lO_2\,cm^{-2}\,h^{-1}$ µmol photons$^{-1}m^2s^1$)	***	*P. porites*	0.237 ± 0.0135 } a	0.326 ± 0.0636 } b	0.269 ± 0.0391 } b
		M. annularis	0.392 ± 0.0260 }	0.484 ± 0.0830 }	0.505 ± 0.0135 }
I_k (µmol photons $m^{-2}s^{-1}$)	*n.s.*	*P. porites*	223.7 ± 46.04	239.2 ± 84.49	229.7 ± 49.04
		M. annularis	161.0 ± 48.6	149.7 ± 51.96	108.5 ± 22.73
Daily integrated gross photosynthesis ($\mu lO_2\,cm^{-2}\,d^{-1}$)	***	*P. porites*	519.6 ± 88.41 } a	685.1 ± 113.56 } b	472.1 ± 65.43 } a
		M. annularis	651.8 ± 148.6 }	717.9 ± 120.2 }	542.1 ± 88.38 }
Respiration					
Respiration rate per surface area ($\mu lO_2\,cm^{-2}\,h^{-1}$)	**	*P. porites*	9.05 ± 1.74 } a	12.4 ± 2.68 } b	8.66 ± 1.85 } a
		M. annularis	16.8 ± 2.86 }	18.3 ± 2.46 }	15.6 ± 2.52 }
Respiration rate per protein content ($\mu lO_2\ mg^{-1}\,h^{-1}$)	*	*P. porites*	2.20 ± 0.496 } a	2.77 ± 0.605 } b	1.92 ± 0.356 } a
		M. annularis	2.21 ± 0.277 }	2.22 ± 0.359 }	1.78 ± 0.452 }

PAR at each site were integrated to obtain the photosynthetic curves for both *P. porites* and *M. annularis* shown in Fig. 7.4. The total daily gross oxygen production data are shown in Table 7.3. A two-way analysis of variance found that corals at BRI had a significantly higher daily gross photosynthetic rate than those at either OS or SG ($F_{2,30}$= 9.934, P <0.001; Tukey HSD test: P <0.05, OS=SG<BRI) in both species. The difference between species was also significant ($F_{1,30}$= 4.779, P <0.05) but less strongly then the difference between sites. No interaction was found between sites and species. Corals at BRI were also characterised by a significantly higher rate of respiration when expressed per surface area ($F_{2,30}$= 5.94, P <0.01) or per protein content ($F_{2,23}$= 5.074, P <0.05) but no difference was found between OS and SG.

7.3.3 Measurement of skeletal growth

The daily calcification rate standardised to surface area (Table 7.4) was found to be significantly different between species ($F_{1,45}$= 64.03, P <0.001) and between sites ($F_{2,45}$= 6.372, P <0.01). There was no significant interaction between species and sites, and the post-hoc multiple comparison test identified the growth rate of corals at BRI as significantly higher than the rate attained both at OS and SG for both species.

Table 7.4 Daily calcification rate (mg $cm^{-2}d^{-1}$) for *Porites porites* and *Montastrea annularis* cultured for 31 and 26 days, respectively, at three sites along a eutrophication gradient on the west coast of Barbados. Values are means ± SD. In the two-way ANOVA, interaction between species and treatments was not significant. Only significance between treatments is shown. (P^{ϕ}, F-test between sites). ** significant at P <0.01. Groups are significantly different (Tukey HSD, P<0.05) when identified by different letters.

Species	P^{ϕ}	Sites		
		OS	BRI	SG
P. porites (n = 30)	**	1.91 ± 0.59 } a	2.40 ± 0.22 } b	1.74 ± 0.42 } a
M. annularis (n=20)		1.14 ± 0.178 }	1.30 ± 0.137 }	1.078 ± 0.212 }

Fig.7.3 Photosynthetically active radiation (PAR) at three study sites at 2.5m depth on the West coast of Barbados. Estimated from continuous recordings of surface PAR on an 'ideal' day (the 8th June 1994), and from the average percentage transmission obtained at each site during the experimental period.

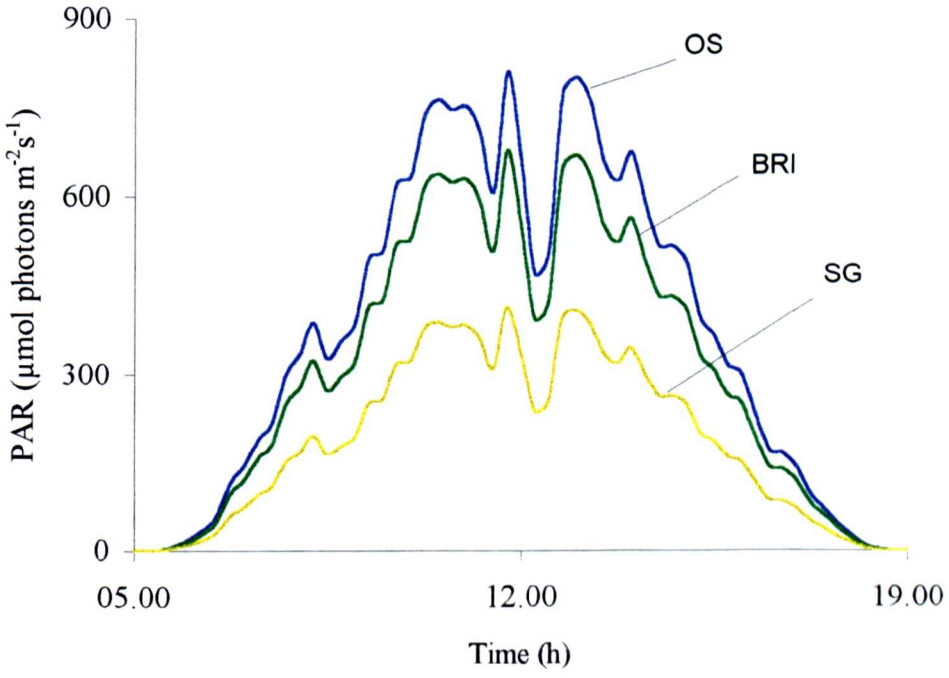
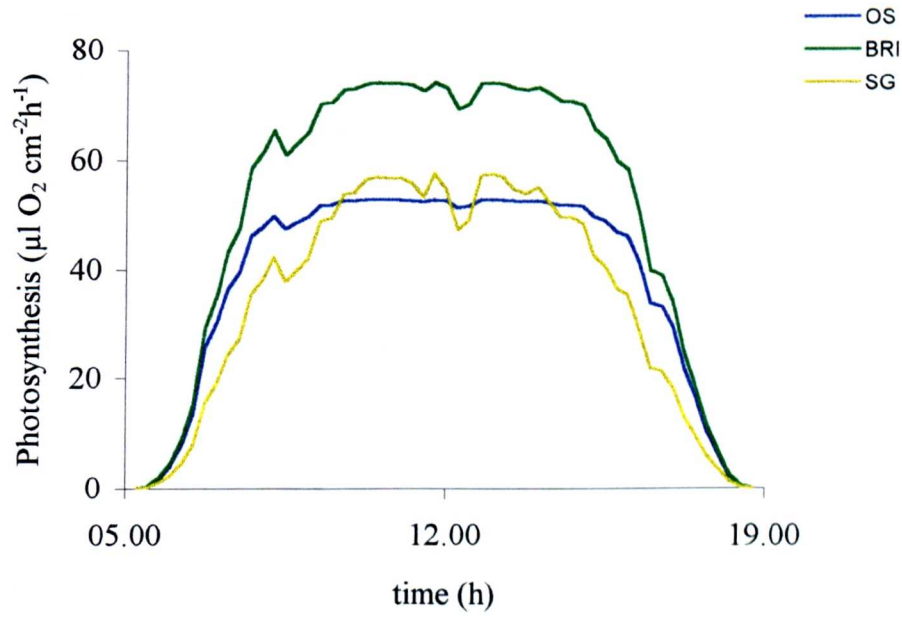
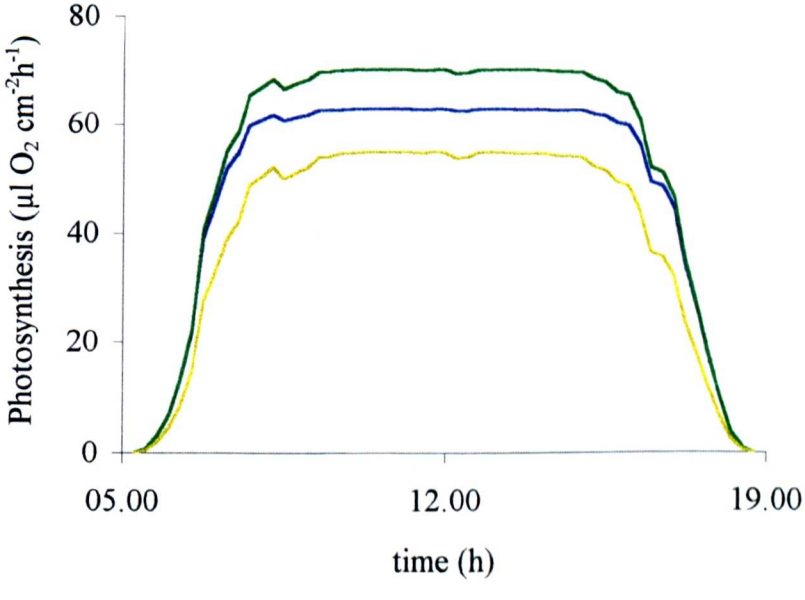


Fig. 7.4 Gross photosynthesis for *Porites porites* and *Montastrea annularis* at the three study sites as estimated using the PAR for an 'ideal' day shown in Fig. 7.3.

a) *Porites porites*



b) *Montastrea annularis*



7.3.4 Carbon limitation test

Following from ch5, the ratio of total net carbon fixed during daylight hours to carbon used daily in calcification ($dayP_{net}^{c}$:Calc) is worth investigating in order to assess if competition for carbon between zooxanthellae and calicoblastic cells occurred during this field experiment. The transformations of oxygen flux data and skeletal weight into C units was carried out as described in 5.4.4, and the results are shown in Table 7.5. Ratios of $dayP_{net}^{c}$:Calc are significantly different between species ($F_{1,30}$= 24.721, P <0.001) but constant across sites ($F_{2,30}$= 0.949, P >0.05). Data from Ch.5 suggested that carbon limitation of skeletogenesis induced by competition with zooxanthellae photosynthesis can be implied only if:

'$dayP_{net}^{c}$:Calc' ≥2.4 in *P. porites* and '$dayP_{net}^{c}$:Calc' ≥3.6 in *M. annularis*

Hence in this reef experiment, there is no evidence for this C limitation mechanism occurring. In fact, what changed between sites is the total carbon flux into the system (P_{net} + calcification). Corals from BRI had a significantly higher DIC flux than those from the other sites ($F_{2,30}$= 12.797, P <0.001).

Table 7.5 Average values for the 'Carbon limitation' test for nubbins of *Porites porites* and explants of *Montastrea annularis* exposed to different environmental conditions at three sites on the west coast of Barbados along a eutrophication gradient. Values are μgC $cm^{-2}d^{-1}$.

	OS	BRI	SG
Porites porites			
Daily net photosynthesis	215	280	192
Daily calcification	229	288	208
DIC flux	444	568	400
$dayP_{net}^{c}$:Calc	**0.94**	**0.97**	**0.92**
Montastrea annularis			
Daily net photosynthesis	232	257	182
Daily calcification	137	156	129
DIC flux	369	413	311
$dayP_{net}^{c}$:Calc	**1.69**	**1.65**	**1.41**

A more detailed discussion of the carbon limitation in hermatypic corals will be developed in Appendix 1.

7.3.5 Coral and zooxanthellae biomass

Average values for biomass parameters are presented in Table 7.6 for each site and species. In all cases a two-way ANOVA was used and since the interaction term between species and sites was never significant, it was accepted that site induced the same response in both coral species. Therefore I shall concentrate on the comparisons between sites.

A significant difference between sites was found in the protein content of the coral tissue standardised to surface area ($F_{2,29}$= 3.35, P <0.05), but not in the protein content of the host component only ($F_{2,29}$= 2.33, P >0.1). Thus, the difference in coral protein must be attributed to the difference in zooxanthellae biomass: the protein content of algal cells was found to be constant ($F_{2,29}$= 1.02, P >0.1), but the zooxanthellae population density was significantly different between sites ($F_{2,24}$= 3.22, P <0.05; Tukey HSD test: P<0.05, OS<BRI).

The chlorophyll*a* and chlorophyllc_2 content per zooxanthella was highly augmented in both inshore sites in comparison to the low nutrient offshore one (chl*a*: $F_{2,24}$= 10.20, P <0.001 and chlc_2: $F_{2,24}$= 11.17, P <0.001).

The volume of the zooxanthellae and the content of C and N did not vary significantly between sites (Table 7.7), however N content did show an increasing trend from the offshore to the most polluted SG site. A significant result was obtained when the elemental composition of the algae was expressed as C:N ratio ($F_{2,18}$= 6.67, P <0.01) with the highest ratio occurring at OS. The difference in this ratio is mainly due to the change in the proportion of nitrogen rather than a change in carbon.

Table 7.7 Elemental characteristics of zooxanthellae isolated from *Porites porites* and *Montastrea annularis* after a 31 and 27 day exposure respectively to three sites on the west coast of Barbados along a eutrophication gradient. Values are means ± SD; n = 4 in each treatment and species. In the two-way ANOVA, interaction between species and treatments was not significant. Only significance between treatments is shown. (P^{ϕ}, F-test between sites). ** significant at P <0.01. Groups are significantly different (Tukey HSD, P<0.05) when identified by different letters.

	P^{ϕ}	Species	Site		
			OS	BRI	SG
C content (pg C cell^{-1})		*P. porites*	126.7 ± 7.19	129.6 ± 20.13	126.6 ± 7.62
		M. annularis	156.3 ± 14.18	141.3 ± 42.50	162.7 ± 71.93
N content (pg C cell^{-1})		*P. porites*	25.3 ± 3.252	26.8 ± 3.907	28.6 ± 2.292
		M. annularis	22.8 ± 2.282	23.7 ± 7.985	29.3 ± 13.161
C:N ratio	**	*P. porites*	5.05 ± 0.535 } a	4.83 ± 0.31	4.43 ± 0.223 } b
		M. annularis	6.85 ± 0.367 }	6.01 ± 0.704	5.56 ± 0.767 }

Table 7.6 Biomass characteristics for both *Porites porites* and *Montastrea annularis* after a 31 and 27 day exposure respecively to three sites on the west coast of Barbados along a eutrophication gradient. Values are mean ± SD, n=5. Data were analysed using two-way ANOVA. Interaction between species and treatment is never significant. Only significant differences between treatments are shown (P^{ϕ}, *F*-test between sites). *n.s.* not significant at $P<0.05$, * significant at $P<0.05$, ** significant at $P<0.01$, *** significant at $P<0.001$. Groups are significantly different (Tukey HSD, $P<0.05$) when identified by different letters.

Parameter	P^{ϕ}	Species	Site		
			OS	BRI	SG
chlorophyll *a* (pg cell^{-1})	***	*P. porites*	2.92 ± 0.354 } a	4.07 ± 0.688 } b	3.48 ± 0.528 } b
		M. annularis	2.58 ± 0.238 }	3.37 ± 0.834 }	3.58 ± 0.245 }
chlorophyll c_2 (pg cell^{-1}) (pg cell^{-1})	***	*P. porites*	0.633 ± 0.1402 } a	0.986 ± 0.111 } b	0.881 ± 0.203 } b
		M. annularis	0.584 ± 0.0678 }	0.872 ± 0.338 }	1.028 ± 0.0847 }
zooxanthella protein (pg cell^{-1}) (pg cell^{-1})	*n.s.*	*P. porites*	165.7 ± 12.14	179.7 ± 24.01	165.8 ± 10.17
		M. annularis	150.9 ± 17.64	151.3 ± 30.58	182.4 ± 40.32
zooxanthella volume (μm^3)	*n.s.*	*P. porites*	479 ± 36.5	523 ± 36.8	496 ± 25.6
		M. annularis	427 ± 25.5	422 ± 29.5	410 ± 42.3
zooxanthellae density (*10^6 cm^{-2})	*	*P. porites*	3.26 ± 0.396 } a	4.23 ± 0.849 } b	3.88 ± 0.335
		M. annularis	5.60 ± 1.390 }	6.47 ± 0.868 }	5.98 ± 0.544
coral protein (mg cm^{-2})	*	*P. porites*	4.41 ± 0.466 } a	4.58 ± 0.237 } b	4.80 ± 0.239 } b
		M. annularis	7.38 ± 1.066 }	8.46 ± 0.553 }	8.55 ± 1.132 }
host protein (mg cm^{-2})	*n.s.*	*P. porites*	3.87 ± 0.448	3.81 ± 0.239	4.16 ± 0.282
		M. annularis	6.54 ± 0.867	7.49 ± 0.617	7.47 ± 1.047

7.3.6 Effect of eutrophication and light on coral physiology

Since it has been established that corals differ between sites in various physiological responses, and differences between species did not interact with the effect of site on the physiological response, a holistic approach to the same problem was taken in order to explore the data further. Fig. 7.5 summarises all significant differences between sites as indicated from the ANOVAs above. A pattern emerged from this: corals grown offshore were consistently low in all parameters (accepting that a high C:N ratio corresponds to a low N content), those at Bellairs were consistently high but those grown at Spring Garden had both high and low responses depending upon the parameter measured.

Discriminant function analysis on several of these responses was therefore employed to search for the best combination of the original physiological variables that would maximise the difference between sites. The discriminant function analysis performed using the parameters listed in Table 7.9 was successful (Wilks' Lamda: $F_{16,38}$=4.5399, P<0.001) and resulted in two functions with significant discriminating power. The first function was able to account for 76.2% of the variance in the population and the second for the remaining 23.8 %. From each function, the discriminant scores of each coral were determined. Thus, the eight physiological variables used in this multivariate statistical method, were condensed into two variables (DF1 and DF2) with a value for each coral (Table 7.8). DF1 and DF2 were then tested with ANOVA followed by post-hoc Tukey test to determine differences between sites. The first was found to discriminant between OS and the inshore sites significantly ($F_{2,26}$= 41.91, P <0.001; Tukey HSD test: P<0.05, OS<BRI=SG). The second function could not discriminate significantly between OS and BRI but succeeded in separating these two sites from the most polluted one ($F_{2,26}$= 13.09, P <0.001; Tukey HSD test: P<0.05, SG<OS=BRI).

Table 7.8 Scores calculated for each coral (both species are combined) from two significant discriminant functions (DF1 and DF2) are grouped by site. n=10 at each site. A one-way ANOVA was used. *** significant at P<0.001. Groups are significantly different (Tukey HSD, P<0.05) when identified by different letters.

Parameter	P	Values	Site		
			OS	BRI	SG
DF1	***	mean	-2.334 a	+1.406 b	+1.031 b
		SD	0.5348	1.2437	1.0900
DF2	***	mean	+0.117 a	+1.048 a	-1.294 b
		SD	0.9908	1.1555	0.8023

Fig. 7.5 Summary diagram of Tukey's HSD tests for the physiological characters that were found to be significantly different between sites for both *Porites porites* and *Montastrea annularis* after two-way ANOVAs. The position of the line identifies similarities and differences between sites for each parameter. Dotted lines refer to non significant cases.

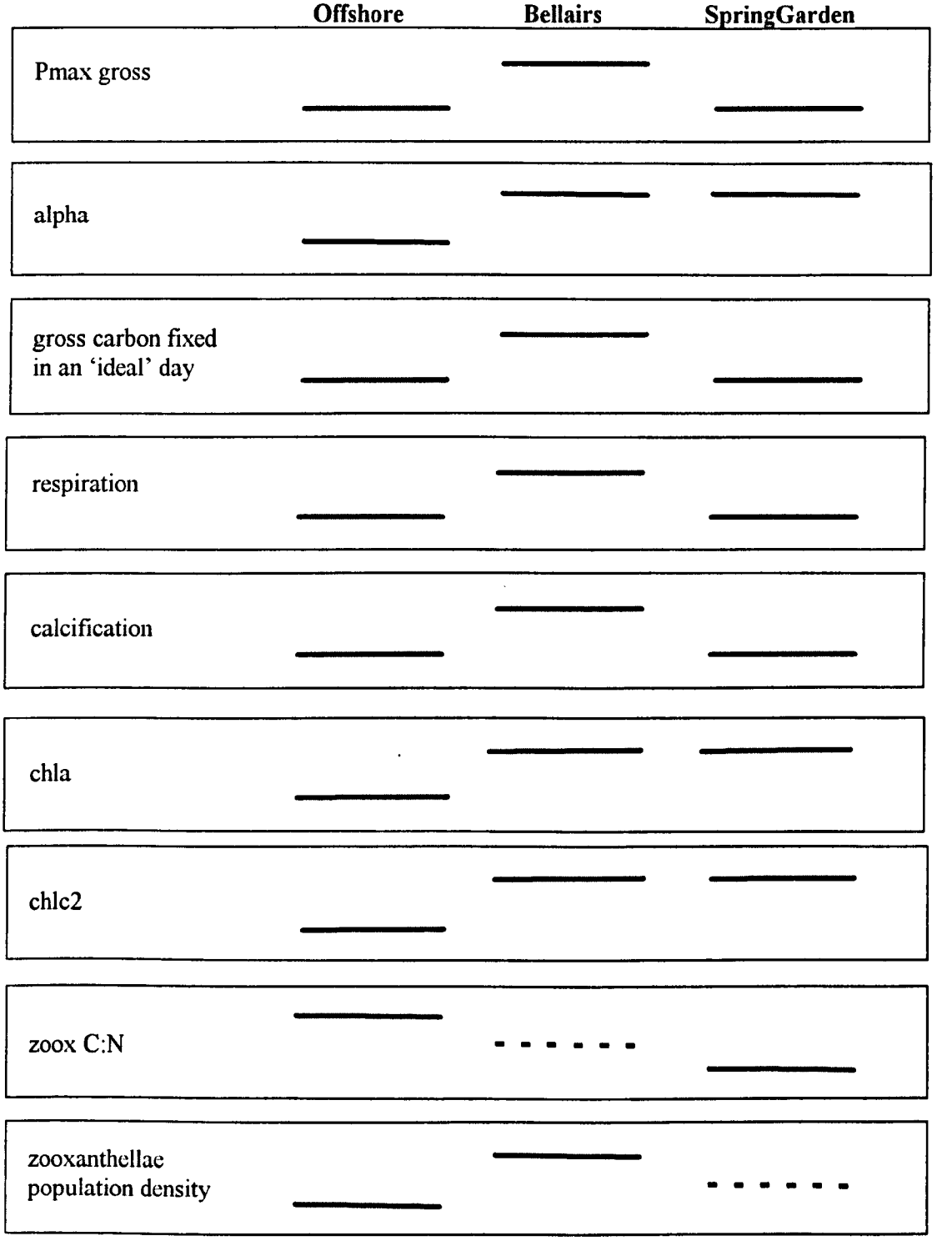
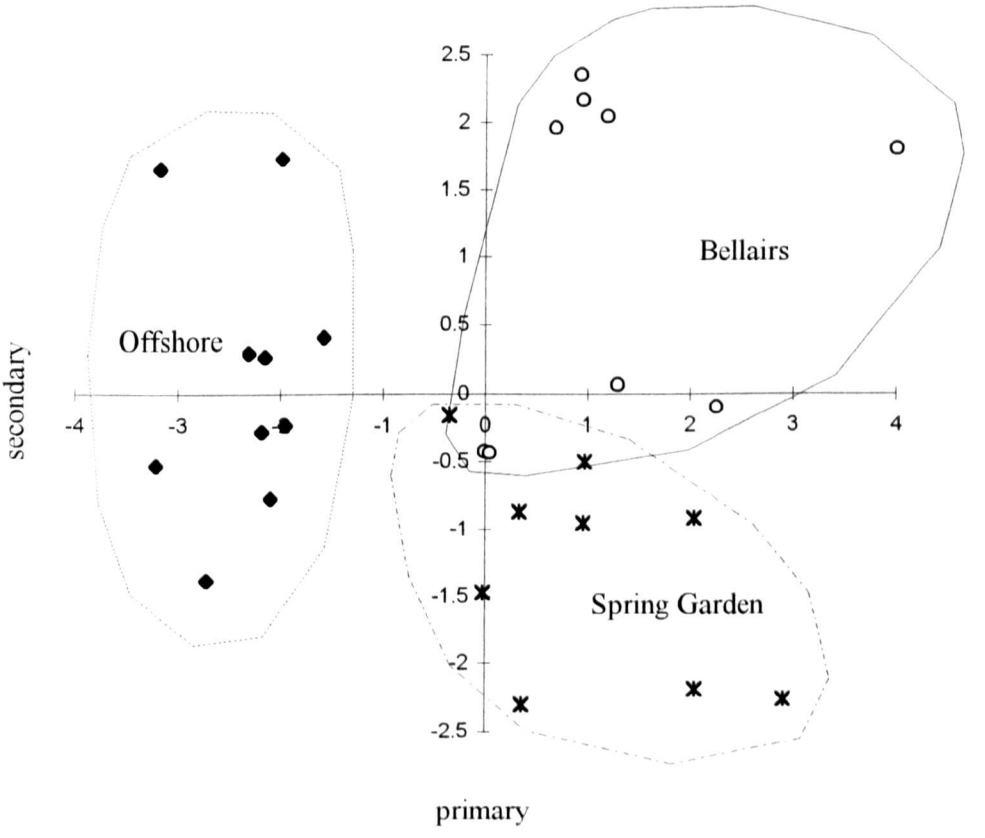


Fig. 7.6 Plot of first discriminant scores (x-axis, primary) vs. second discriminant scores (y-axis, secondary). Corals of both species belonging to each site are encircled to enphasise the degree of separation obtained.



The relative importance of each original physiological variable within the two discriminant functions can be estimated by observing the correlations between them as shown in Table 7.9. Chlorophyll*a* and chlorophyllc_2 contribute largely to the first function, while daily carbon fixed is the most heavily weighted parameter on the second function.

Plots of DF1 against DF2 (Fig.7.6) showed that both nubbins of *P. porites* and explants of *M.annularis* fell into three discrete groups corresponding to their site and lacking overlapping. To investigate the relationship between the new physiological variables (DF1 and DF2) and the environmental parameters (light and eutrophication index) as well as the effect of belonging to different species, a linear regression model was fitted. It was found that 76% of the variance in DF1 (R^2=0.763) could be explained by the eutrophication index alone (T=6.639, P<0.001) and neither light (T=1.67, P>0.5) nor species (T=0.069, P>0.9) were found to be significant. On the other hand, DF2 was highly dependent (R^2=0.502) on light (T=5.031, P<0.001) and also on the eutrophication index (T=3.524, P<0.01); species did not have any effect (T=0.269, P>0.9).

Table 7.9
Spearman's rank correlation coefficients for correlations between discriminant functions (DF1 and DF2) and original variables. * significant at P<0.05, ** significant at P<0.01 and *** significant at P<0.001.

Parameters	DF1		DF2	
chlorophyll a	0.495	**	-0.127	
chlorophyll c2	0.457	**	0.078	
alpha	0.252	*	0.152	
coral protein	0.081		0.014	
daily gross C fixed	0.055		0.770	***
daily C respired	0.081		0.447	*
calcification	0.079		0.181	
zooxanthellae density	0.137		0.160	

7.4 Discussion

Budd Foster (1980) investigated the degree of plasticity in skeletal characters in colonies of *Montastrea annularis* and *Siderastrea siderea* collected at five environmentally distinct localities within a limited geographical area and concluded that both species were capable of responding morphologically to the environment. Localities differed in terms of water velocity, sedimentation rate, light intensity and 'food supply' and both coral species were found to possess site-specific skeletal characters, such as growth rate, corallite dimensions and dissepiment thickness. In the

present study the physiological plasticity of corals in relation to environmental variability was investigated.

7.4.1 Photosynthesis, respiration and biomass measurements

The three experimental sites were located on the west coast of Barbados (Fig. 7.1), the leeward side of the island, but during the experimental period they were affected by unusually strong south-westerly currents and heavy swells. Water velocity was not measured directly at each site but it was high and consistently so at all sites because the coastal profile, void of bays or islands, does not offer any protection from south-westerly or westerly swells. Salinity and temperature had been previously monitored and found to be approximately constant (Ott, 1975; Tomascik & Sander, 1985; Seakem, 1989). Therefore the sites differed mainly in terms of light intensity, nutrient concentration (NO_3, PO_4) and turbidity (SPM, VPM).

Light intensity was highest at OS and BRI and significantly lower at SG. In this experiment, the water quality parameters (NO_3, PO_4, SPM and VPM) were highly correlated, and therefore they were combined into a single component identified as the 'eutrophication' index (PC1 in principle component analysis), in accordance with previous work (Tomascik & Sander, 1985; Allard, 1994). The offshore site had the lowest 'eutrophication' score and was significantly different from both inshore sites. Although the score at SG was higher than at BRI, the difference was not significant.

Corals exposed for one month to these differing environmental conditions were significantly different between sites in many physiological parameters.

Because of the autotrophic nature of the symbiosis, the effect of light on coral physiology is pivotal and multifaceted, and thus it will be discussed first. Corals were held in shallow water at 2.5 m depth at all sites, but the amount of light they received varied according to the degree of attenuation produced by suspended matter at each site. Thus, light intensity was significantly higher at OS and BRI than at SG. Reduced light availability is known to produce a photo-adaptive response in zooxanthellae to maximise light harvesting. This mainly consists in an increased production of photosynthetic pigments per cell (Falkowski & Dubinsky, 1981; McCloskey & Muscatine, 1984; Porter *et al.*, 1984;) which is reflected in an increased photosynthetic efficiency (alpha) per unit surface area so that the coral is able to attain a higher photosynthetic rate at sub-saturating irradiances (Chalker *et al.*, 1983; Porter *et al.*, 1984). Photo-adaptation can compensate for the reduction in photosynthesis due to decreased irradiance only up to a point: if light is reduced further the net result will be a decrease in gross photosynthetic rate. The effect of this on the whole symbiosis has been assessed in terms of carbon budgets by Muscatine *et al.* (1984): zooxanthellae in light-adapted colonies of *Stylophora*

pistillata translocate five times as much carbon as those of shade corals and contribute to as much as 143% of the respiratory requirements of the host against only 58% satisfied in shade corals.

Thus, if light intensity were the only parameter affecting the corals at the three sites, one would expect to find a strong correlation between light intensity and chlorophyll content, alpha and daily gross photosynthetic rate. In particular, similarities would be expected between corals at OS and BRI where the light intensity is not significantly different, while significant differences would be expected between these and corals from SG where light intensity is significantly reduced by approx. 40%. While a comparison between OS and SG does fit the photo-adaptation hypothesis, zooxanthellae of *Porites porites* and *Montastrea annularis* from BRI do not. The light regime is high at BRI but the chlorophyll content and the photosynthetic efficiency (alpha) is significantly increased in comparison with OS and reaches levels as high as at SG. Therefore environmental factors other than light appear to be also influencing the physiology of the symbiosis.

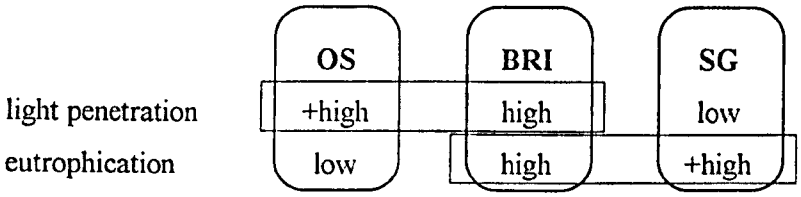
The other measured factors were nutrient concentration and suspended particulate matter so these will be discussed in turn. Corals growing in oligotrophic conditions have been found to experience nitrogen limitation which can be removed by inorganic nitrogen enrichment of seawater and to a lesser extent by increased host heterotrophic feeding. Nitrogen enrichment results primarily in changes of the zooxanthellae biomass: zooxanthellae population density and chlorophyll content per zooxanthella in particular have been shown to increase significantly (Muscatine *et al.*, 1989; Hoegh-Guldberg & Smith, 1989; Dubinsky *et al.*, 1990; Muller-Parker *et al.*, 1994b). A change in the elemental composition of the algae has also been reported (Muller-Parker, 1994a; Snidvongs & Kinzie, 1994) and an increase in the N content per cell and a corresponding reduction in the C:N ratio are characteristic of N enrichment. Ultimately this results in an increase in the rate of gross photosynthesis per unit surface area. A detailed analysis of the effects of N on zooxanthellae and host biomass is discussed in Ch.5.

Thus, if corals at the three sites were affected only by the nitrogen concentration in the surrounding water, one would expect the zooxanthellae from corals at OS to express all the characteristics of nitrogen limitation and to differ significantly in these characteristics from zooxanthellae from corals at BRI and SG. The data in this study fit this expectation. However, one would also expect the gross photosynthetic carbon fixation to be low at OS and highest at SG but this is not reflected in the data: the highest amount of carbon fixation occurs at the intermediate site (BRI) and corals from OS and SG do not show a significant difference in relation to this parameter.

Following the results from the experiment presented in Ch.6, no significant change in zooxanthellae or host biomass was expected in relation to the difference in phosphate concentration between sites.

Suspended particulate matter (SPM) plays a complex role in affecting coral physiology. It will reduce water transparency and hence light transmission, producing the same physiological response previously ascribed to low light intensity such as reduced calcification (Dodge *et al.*, 1974; Hudson, 1981; Szmant-Froelich *et al.*, 1981) and zooxanthellae photoadaptation (Edmunds & Davies, 1989). It might constitute an important food source on reefs especially if containing a high proportion of organic particulate matter (VPM) (Lewis & Price, 1975). It might result in a considerable energy cost to the host which, to maintain effective sediment rejection, might divert energy from active growth processes (Bak, 1978; Dallmeyer *et al.*, 1982). However this latter hypothesis has never been experimentally tested and appears questionable in view of the amount of energy stored as lipids found in corals even in areas of high sedimentation (Harland *et al.*, 1992). Overall, studies on the effects of SPM on hermatypic corals have focused on calcification, mucus production and survival (Dodge *et al.*, 1974; Bak, 1978; Rogers, 1983; Dodge & Brass, 1984; Kendall *et al.*, 1985; Stafford-Smith, 1992; Riegl & Branch, 1995), but the sub-lethal effects on the zooxanthellae photosynthesis and biomass characteristics have not yet been fully investigated. Due to the lack of information on the effects of SPM, and to the strong correlation in this study between SPM (and VPM) and nutrient concentration, differences between sites will be discussed the effects of eutrophication in terms of nutrient effects.

On their own, neither light nor eutrophication are capable of setting each site apart, but when in combination, each site is qualitatively different as shown in the following diagram:



The different combinations of light and eutrophication at each site are reflected in the physiology of the corals. Zooxanthellae from both *Porites porites* and *Montastrea annularis* at the offshore site showed characteristic features of nitrogen limitation and high light: i.e. low zooxanthellae population density, low photosynthetic pigment content per cell, high C:N ratio, low alpha. Zooxanthellae from the two inshore sites are both eutrophic but differ in light intensity. At BRI the combination of higher nutrients and high light resulted in higher rates of photosynthesis enhanced by increased zooxanthellae population density and photosynthetic pigments per cell. At SG on the other hand the reduction in light offsets the nutrient enhancement of zooxanthellae biomass and daily gross photosynthesis is reduced.

7.4.2 Calcification

In situ measurements of calcification have shown that growth rate is affected by turbidity and eutrophication (Budd Foster, 1980; Dodge & Brass, 1984; Tomascik & Sander, 1985).
In their 1981-1982 study, Tomascik & Sander (1985) identified a gradient of eutrophication along the west coast of Barbados, and found this gradient to correlate with the linear extension rate of colonies of *Montastrea annularis*. In particular, the growth rate at SG was depressed and that at BRI intermediate when compared to less eutrophic sites. Also Davies (1990) chose three fringing reefs on the eutrophication gradient along the west coast of Barbados as study sites for a short-term (3-6 d) investigation. While no difference in growth (measured by buoyant weighing) could be detected in *P. porites*, growth rate of *M. annularis* was negatively correlated to eutrophication. Further studies have shown that this eutrophication gradient persists and is positively correlated with reef degradation (Seakem, 1989; Allard, 1994).
It was therefore unexpected to find in this study that, in both *Porites porites* and *Montastrea annularis*, no linear correlation existed between eutrophication and skeletal growth. The maximum rate of calcification was attained at the intermediate BRI site and corals offshore in the most oligotrophic conditions grew at a rate not significantly different from those in the most polluted site.
However, Tomascik and Sander (1985) investigated the relationship between eutrophication and calcification further by combining data from their 1982 study with those of a decade earlier and used published estimates of SPM levels as an estimate of eutrophication. In 1972, SPM levels were much lower along the coast but linear skeletal extension was nevertheless reduced. Hence the overall relationship found was not linear but followed the single-humped pattern of a second-order polynomial, a curve similar to that found in the present study. They suggested that SPM has a positive effect as a 'food' source and this enhances calcification until a maximum is reached after which the decrease in light offsets the benefits of feeding.
The present results, comparing the calcification rate between sites, can be discussed in terms of the combined effects of light and eutrophication.
As discussed in Ch1, in addition to the effects on the photosynthetic rate, light has also a crucial role in calcification. It has been suggested that, on the one hand, photosynthesis provides transferable products used as an energy source for the active processes of calcification, and on the other, by removing CO_2, it produces a shift in intracellular pH towards a higher value more favourable to carbonate deposition and crystallisation (Goreau, 1963; Pearse & Muscatine, 1971; Vandermeulen *et al.*, 1972; Chalker, 1975; Johnston, 1980; Gladfelter, 1983; Barnes & Chalker, 1990). Indeed, coral calcification measured as linear extension has been negatively correlated with light intensity along depth gradients (Houston, 1985; Logan & Tomascik, 1991). In this

experiment, daily gross photosynthetic production was highest at BRI and low both at OS and SG, and thus the rate of mass accretion correlates with photosynthesis.

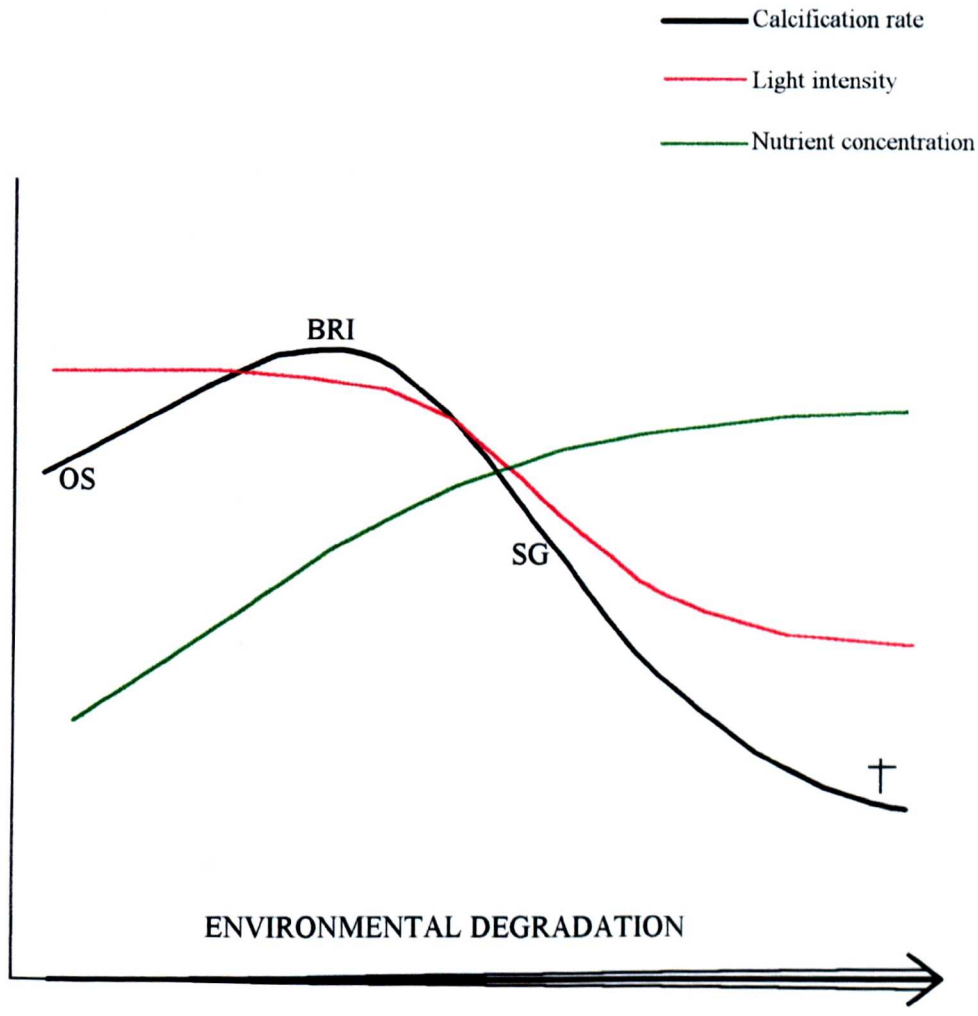
Nutrient concentration in seawater is also expected to influence the process of skeletogenesis: phosphorus acts directly on calcification by inhibiting the formation of calcium carbonate crystals (see Ch. 6), and nitrogen, (via its enhancement effect on the symbiont population), might reduce $CaCO_3$ deposition by limiting the endogenous availability of inorganic carbon to the calicoblastic cells (see Ch. 5).

Phosphate offers a direct chemical explanation of reduced calcification, but the growth data do not follow the expected pattern if phosphate were the only factor influencing calcification: between OS and SG there is a 2-fold increase in P and calcification is the same, between OS and BRI there is again a 2-fold increase in P but calcification increases by approx. 20%, while at BRI and SG the same average P concentration corresponds to a 30% decrease in calcification. Thus, the potential effect of P in poisoning crystal formation on these reefs is confounded by changes in calcification due to other parameters.

Nitrogen was identified as the most important factor among those water quality parameters affecting the zooxanthellae biomass and photosynthetic activity between sites, and therefore its effect was expected to be similarly important in calcification. In the laboratory experiments conducted in a photostat (Ch.5), it was suggested that high levels of nitrogen resulted in the 'endogenous carbon limitation of calcification'. Following this model, the rate of calcification is reduced because the enlarged population of zooxanthellae removed the limited supply of DIC for their use in photosynthesis, thereby limiting the DIC available to the calicoblastic cells. However, as discussed in Appendix 1, endogenous carbon limitation can take place only when the endogenous requirements for DIC exceed the rate of its supply. Corals in this field experiment were exposed to a water motion 60% higher than in the photostat and this might correspond to a rate of DIC diffusion roughly 60% higher than in the photostat. Possibly, the enhanced rate of DIC influx on the reef was sufficient to balance the higher photosynthetic requirements of corals in the nitrogen enriched sites and therefore competition between photosynthesis and calcification could not take place. In fact, the flux of DIC changed between sites, and was highest at BRI, where gross photosynthesis was maximal. The fact that the ratio of '$dayP_{net}^{c}$:Calc' was constant at all sites and lower than in the photostat experiment, suggests that this reasoning is correct.

In conclusion, I suggest that in high energy locations on the reef, the relationship between calcification and environmental degradation (a term used here to combine the two environmental parameters of light and eutrophication) is not unidirectional, but follows a single-humped curve (Fig. 7.7).

Fig. 7.7 Theoretical relationship between the calcification rate of hermatypic corals and the changes in nutrient concentration and light penetration occurring along a gradient of environmental degradation.



This pattern can be interpreted as follows:

1. in pristine conditions (high light intensity and oligotrophic water), calcification and photosynthesis are strongly correlated (OS).
2. as environmental degradation starts, nutrient enrichment enhances the zooxanthellae population density and the daily photosynthetic rate per surface area increases with a corresponding increase in calcification (BRI)
3. as degradation proceeds, nutrient enrichment is accompanied by turbidity and light reduction, and photosynthesis and calcification are reduced again (SG)
4. in the worst case scenario, turbidity is so extreme that active rejection by polyps drains energetic resources, and without success, corals perish under persisting sediment loads (Rogers, 1983; Hallock & Schlager, 1986; Stafford-Smith, 1992).

7.4.3 Towards the development of an early-warning bio-assay for the identification of nutrient enrichment on coral reefs

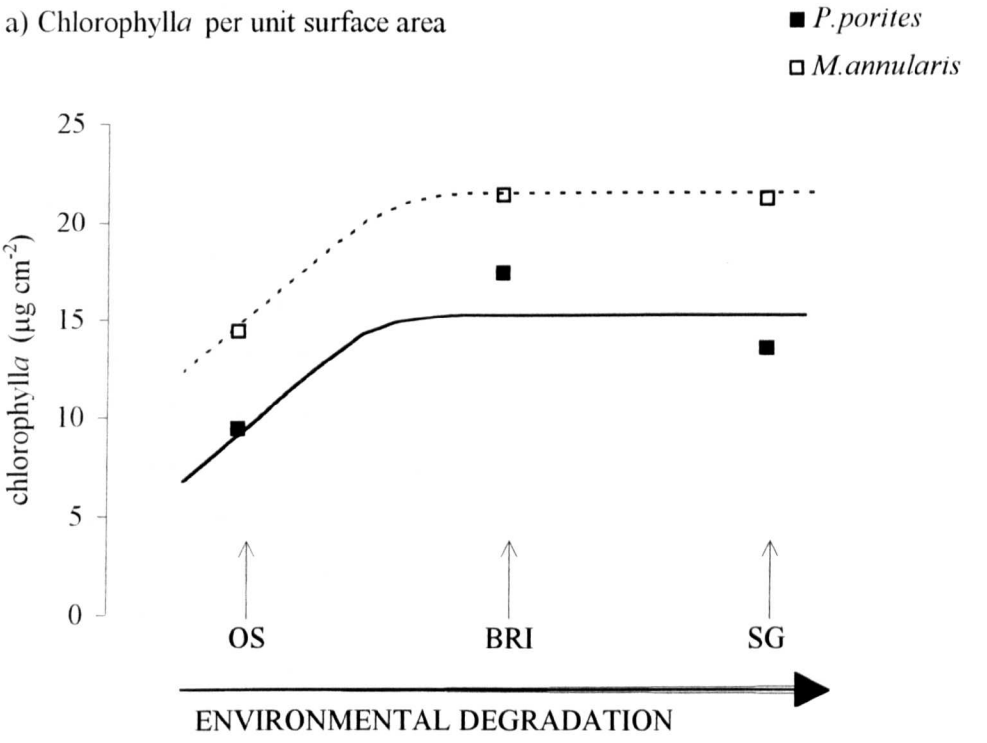
The use of discriminant function analysis enabled comparison of the physiology of corals belonging to all three sites simultaneously. This was very successful in identifying two functions which, plotted against each other (Fig. 7.6), were able to significantly discriminate between each site.

The first discriminant function, hereafter called 'primary' function, was found to be strongly correlated to zooxanthellae parameters and especially with chl*a* and chlc_2 content: a change in these parameters can result from either increased nitrogen availability or decreased light intensity. However, when this function was regressed on the two environmental components, the eutrophication index alone was found to be able to explain 76% of its variation, and light intensity was not significant This is interpreted as strong evidence that the change in photosynthetic pigments was induced by nutrient enrichment. Photosynthetic pigment content is recognised here as the 'primary' character which is most sensitive to eutrophication. The relationship of 'primary' characters to environmental degradation has been plotted in Fig. 7.8(a) using the data for both *Porites porites* and *Montastrea annularis* at the three sites. Chlorophyll*a* content per surface area (thus including both zooxanthellae population density and pigment content per cell in one variable) was the chosen 'primary' character. It was found to be unidirectionally related to environmental degradation, that is to say that the concentration of photosynthetic pigments increases with nutrient concentration, and remains high as eutrophication progresses.

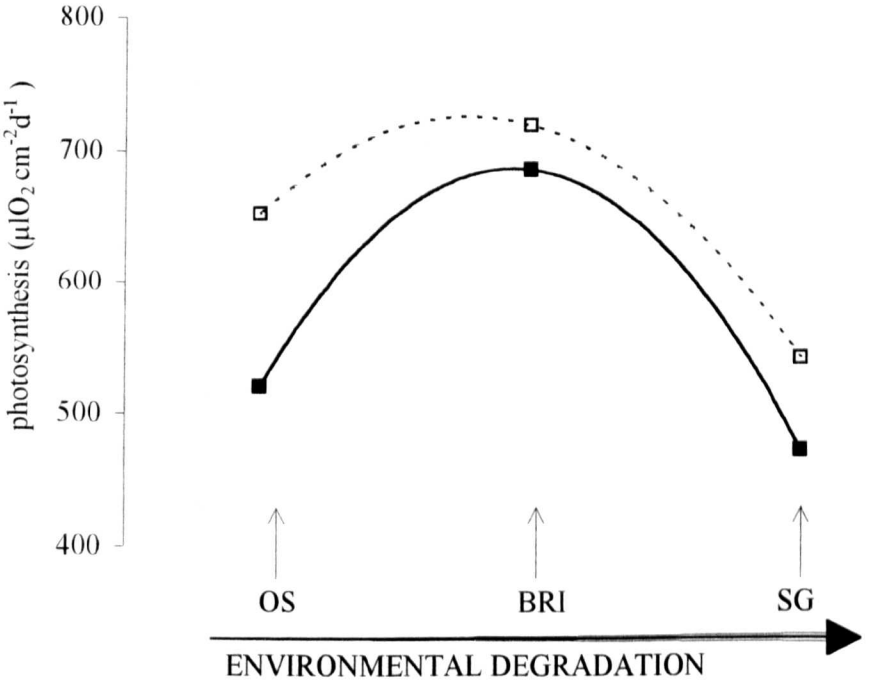
Daily gross photosynthesis was the parameter most highly correlated to the second discriminant function, and was therefore identified as the most sensitive 'secondary' character. The second

Fig. 7.8 Response of 'primary' (chlorophyll*a* content) and 'secondary' (daily gross photosynthesis) characters of *Porites porites* and *Montastrea annularis* to environmental degradation. The three experimental sites were OS (offshore), BRI (Bellairs reef) and SG (Spring Garden).

a) Chlorophyll*a* per unit surface area



b) Daily gross photosynthesis



discriminant function was regressed significantly on both environmental parameters. In fact as detailed above, photosynthesis per unit surface area is a function of light energy, but also of zooxanthellae photo-efficiency and of zooxanthellae population density, both of which are enhanced by nutrient concentration.

The relationship of a 'secondary' character to environmental degradation is shown in Fig. 7.8(b), by plotting the daily gross photosynthesis of both nubbins and explants from all sites. It follows the single-humped curve previously described for calcification.

Thus the data in this study suggest that a bio-assay based on 'primary' characters will be able to identify the onset of nutrient enrichment. This point along the degradation gradient might not be associated with any negative effect to the 'health' of coral colonies (as in this study for example). Corals at BRI showed significant changes in their physiology compared to corals grown in the oligotrophic waters offshore, but there was no evidence to suggest that their 'health' status was reduced. On the contrary, their skeletal growth and photosynthetic capacity were enhanced. A bio-assay based exclusively on 'secondary' characters might produce conflicting results because oligotrophic and highly eutrophic conditions produce a similar response. A combination of both 'primary' and 'secondary' characters would produce a bio-assay to identify both the onset of nutrient enrichment and the point along the degradation gradient at which conditions inhibit coral growth and thus coral 'health'.

This reasoning has to be taken a step further because the ultimate goal of the bio-assay is to identify the 'health' status of the reef and not only the 'health' status of hermatypic coral colonies. To this aim it is necessary to investigate how 'primary' and 'secondary' characters of hermatypic corals relate to the 'health' of the reef community (expressed in terms of coral diversity, % coral cover, % algal cover etc.). This step in the investigation was not carried out but some insight can be obtained from previous studies. Tomascik & Sander (1987a), Wittenberg & Hunte (1992), Allard (1994) established that coral abundance and diversity along the fringing reefs on the west coast of Barbados are inversely proportional to the eutrophication gradient. They reported that the reef at BRI is in sub-optimal condition, and that at SG is the worst impacted reef with a coral cover of about 10% and a very reduced coral diversity. This suggests that the 'health' status of the reef can be affected by eutrophication at a degree lower than that required to produce a reduction in the 'health' status ('secondary' parameters) of adult corals. The obvious reason for this relates to the fact that the health of a reef does not depend only on well established colonies but on the continual recruitment of new individuals. Corals in the settlement and juvenile stages are particularly sensitive to nutrient enrichment because of the competition from benthic macroalgae (Hunte & Wittenberg, 1992; Wittenberg & Hunte, 1992). Reefs in Barbados suffered acute damage in 1980 from hurricane Allen which was reported to cause major changes in coral abundance (Mah & Stearn, 1986). Recovery has been impaired by

the growth of macroalgae favoured by chronic coastal eutrophication and by the die-off of the herbivore *Diadema antillarum* (Allard, 1994).

Hence, a highly effective bioassay technique should result from further research specifically focused on 'primary' parameters because they are earlier indicators of potential damage. In particular chlorophyll content per surface area should be considered: this measure is easy to carry out and standardised to surface area it eliminates the problems of zooxanthellae counts, increasing sample size and accuracy. However, chlorophyll content is also the first response to change with light reduction: it is imperative that sites are compared across depth in order to identify changes due to eutrophication.

Chapter 8

DISCUSSION

8.1 Introduction

In light of the economic and social value of reefs, their 'health' status is critical to any management strategy committed to the sustainable development of coastal resources. In order to monitor the fitness of the ecosystem and any changes over time, the present need of coastal managers is for an early-warning system which can indicate the onset of a stress response before reef deterioration becomes obvious and while the potential for recovery still exists. As yet, there is no scientific tool able to satisfy these needs (Davies & Brown, 1992). The common monitoring technique whereby a coral community is surveyed and assessed in terms of coral diversity or percentage coral cover can only measure changes when they are already evident at the community level. This technique is useful in estimating damage, but not in trying to prevent it or in trying to identify the stress factors that caused it. Instead, Brown & Howard (1985) and Brown (1988) emphasised the potential of bio-assays, and in particular the use of specific physiological responses of hermatypic corals, as an ecotoxicological tool for monitoring reef health.
In this context, the physiological processes of photosynthesis, respiration and skeletal growth are especially attractive because they constitute the major components of the primary productivity and of the structural accretion of many reefs. Thus a bio-assay based on these processes could identify the presence of a stress factor while providing information necessary to model whole reef responses.

Nutrient enrichment of tropical coastal waters resulting from anthropogenic activities constitutes a long-term threat for the biodiversity of coral reefs world-wide. This thesis has been devoted to understanding the physiological responses of hermatypic corals that are specific to nutrient enrichment.
Photosynthesis, respiration and skeletal growth are the major components of the carbon economy of corals and can be measured in terms of a 24-hour carbon budget (Davies, 1984; Edmunds & Davies, 1989; Muscatine, 1980; Muscatine *et al.*, 1984). The budget methodology was therefore adopted throughout this thesis to investigate the way in which nutrients affect the essential processes of coral physiology in a manner that has direct ecological significance. It was hoped that this holistic approach would highlight the physiological parameters that have a high sensitivity to nutrient enrichment and therefore a particularly high potential as a monitoring tool.

Hence this thesis addressed four main areas:

1. A methodological development of procedures for culturing corals in laboratory conditions, and a review and development of procedures for carbon budgets

2. A laboratory investigation of the effects of nitrate and phosphate as single factors affecting the components of the carbon budget

3. An analysis of the effects of a eutrophication gradient on the reef where the effects of nutrient enrichment can be masked by changes in other environmental variables

4. An evaluation of possible bio-assays for nutrient enrichment

These will be reviewed in turn in this Discussion.

8.2 Methodological developments

8.2.1 Laboratory culture methods

Nubbins and explants of hermatypic corals (Davies, 1984; Davies, 1995) were chosen as the coral preparations most amenable to experimental work. They have a small and consistent size which greatly simplifies the associated experimental instrumentation and handling, and reduces the total amount of coral removed from the reef. At the same time they appear to reflect the physiological behaviour of the intact colony (Davies, 1989). The common shallow reef branching coral *Porites porites* and massive coral *Montastrea annularis* were chosen for this study.

A laboratory apparatus (photostat chamber) was constructed to grow nubbins of *Porites porites* and explants of *Montastrea annularis* under the controlled conditions that are necessary for experiments designed to test the effects of a particular factor in a controlled environment. The apparatus consisted of a 7l glass chamber, fitted with coral trays and a specially designed air line system that produced a strong and turbulent water motion independent of the rate of water inflow. A peristaltic pump ensured a daily turnover of water. Chambers were placed in a constant temperature water-bath under artificial lighting provided by metal halide lamps. In these conditions corals were cultured for at least nine weeks (the longest pre-experimental trial) without a supply of zooplankton and no sign of tissue damage or bleaching was observed. Corals grown in the photostat were compared to corals on an exposed reef. The two locations differed in many environmental parameters (water velocity, light regime, plankton availability) and the physiology of the corals was also found to differ. Laboratory reared corals had a lower calcification rate (for both species) and, in the case of *Montastrea annularis*, a lower photosynthetic rate. Even though these differences were statistically significant, their magnitude was comparable to significant differences measured under different conditions on reefs (Barnes & Chalker, 1990; Edmunds & Davies, 1986). Therefore the success of the photostat as an experimental growth chamber was

not undermined, but scope for improvement is still present. In particular, different light sources (Jokiel pers. comm.) could be tested. Corals were not supplied with zooplankton in this study because of the potential confounding effect of heterotrophic feeding on testing the effects of inorganic nutrients (Muscatine *et al.*, 1989). However feeding might be advantageous to the laboratory culture of corals and more so to those corals, such as *Montastrea annularis*, that are thought to rely more on heterotrophic feeding (Porter, 1976).

The photostat provides controlled conditions for experimental work with corals, and has therefore succeeded in abolishing the requirement of previous studies (Crossland *et al.*, 1980; Szmant-Froelich *et al.*, 1981; Hoegh-Guldberg & Smith, 1989; Stambler *et al.*, 1994) for a fast flow-through system and thus for a large and continuous supply of seawater. This is an important development towards the use of nubbins and explants for ecotoxicological or pathobiological studies.

The 'mesocosm' system developed by Jaubert (1989) is a quality-controlled closed system aquarium that can mimic a natural reef environment. However the maintenance of the water quality is based on an integrated biological filter which may make it less suitable than the present photostat system for studies with toxic pollutants.

8.2.2 Carbon budget methodology

Carbon and energy budgets have been used to compare the physiological condition of hermatypic corals in relation to *in situ* irradiance and sediment regimes following the two methodologies developed by Muscatine and colleagues (Muscatine, 1980; Porter, 1980; Muscatine, *et al.* 1981; Muscatine, *et al.*, 1983) and Davies and colleagues (Davies, 1984, 1991; Edmunds & Davies, 1986, 1989; Al-Sofyani, 1991; Harland, 1992). These methodologies were compared using nubbins of *Porites porites* in order to develop an improved carbon budget methodology (Chs. 2 and 3). In both cases, gross photosynthesis is assumed to be the only source of fixed carbon to the symbiotic association which is then used for the respiration and growth of both zooxanthellae and host. The two methodologies were found to differ markedly in the technique used to measure zooxanthellae respiration and zooxanthellae growth.

Muscatine's method to estimate the amount of carbon respired by the zooxanthellae ($R_{Z.MUSCATINE}$) is based on the untested assumption that respiration of both zooxanthellae and host is directly proportional to their relative biomass (or protein content). Davies' method ($R_{Z.DAVIES}$) on the other hand, measured the rate of respiration of freshly isolated zooxanthellae (f.i.z.) making the assumption that f.i.z. respiration *in vitro* is the same as that *in situ*. Thus both methods are somewhat unsatisfactory. However when compared, the difference was only two-fold ($R_{Z.MUSCATINE}$ = 33.1 μgC $cm^{-2}d^{-1}$ vs. $R_{Z.DAVIES}$ = 18.1 μgC $cm^{-2}d^{-1}$) and overall it accounted for

only 3% of the total gross photosynthetic input, thus $R_{Z,MUSCATINE}$ was chosen in the new integrated methodology because it is the easier method to use.

The rate of zooxanthellae growth is measured differently too. On the one hand, Muscatine's method determines zooxanthellae growth from the mitotic index of zooxanthellae, and therefore it measures the rate of gross growth (μ_{GROSS}). The calculation of μ_{GROSS} might be prone to error because it involves the estimation of the duration of cell division (t_d). Since t_d cannot easily be measured in asynchronously dividing cells (which is commonly the case in zooxanthellae), it has been assumed to have a duration of 11h, as determined for zooxanthellae from the jellyfish *Mastigias* sp. (Wilkerson *et al.*, 1983). On the other hand, Davies' method determines the growth rate of zooxanthellae from the increase in surface area, assuming that zooxanthellae population density is constant, and therefore it measures the rate of net growth (μ_{NET}). The rates produced following these two methods resulted in a 25-fold difference (μ_{GROSS} = 26.3 μgC $cm^{-2}d^{-1}$ vs. μ_{NET} = 1.6 μgC $cm^{-2}d^{-1}$). This difference can be reconciled only by introducing the component of zooxanthellae loss. This can occur by expulsion, but reported rates of expulsion can account for only a small proportion of cells (Hoegh-Guldberg *et al.*, 1987; Stimson & Kinzie, 1991). Zooxanthellae loss by digestion by the host becomes a necessary component of coral carbon flux. Host digestion has been suggested since Boschma (1925), and its study should be fully addressed in future research. This component is important in affecting the total form of the carbon budgets because the carbon content of digested zooxanthellae has to be an intrinsic part of the total translocation of fixed carbon passing from the symbiont to the host. Thus Muscatine's budget underestimated translocation by the amount of digestion, while Davies' overestimated it by the amount of carbon lost in expelled zooxanthellae. In the new methodology digestion is an integral part of translocation. Translocation is then identified as total translocation (TT) to stress the difference from previous budgets.

A succinct form of the new carbon budget methodology was then drawn to investigate the effects of nutrient enrichment in laboratory and reef experiments. Its potential in identifying physiological responses to nutrient enrichment will be discussed in section 8.5.

8.3 Effects of nitrate and phosphate in the photostat

The photostat was used to grow the corals in four different concentrations of nitrate (Ch.5) and of phosphate (Ch.6).

Studies on the effects of nitrogen on hermatypic corals have centred on growth experiments using elevated levels of ammonia, and were pivotal in accumulating evidence for nitrogen limitation of

zooxanthellae *in hospite* of corals living in oligotrophic conditions. Experimental increase in the concentration of ammonia in seawater produced marked increases in the gross photosynthesis per surface area, in the nitrogen, protein and chlorophyll content per cell, and in the zooxanthellae population density (Hoegh-Guldberg & Smith, 1989; Muscatine *et al.* 1989; Dubinsky *et al.*, 1990; Muller-Parker *et al.*, 1994a,b; Snidvongs & Kinzie, 1994). However, since nitrogen in seawater accumulates more readily in the form of nitrate, and nitrate uptake by corals was a controversial issue (Franzisket, 1974; Cates & McLaughlin, 1979; Muscatine *et al.*, 1984; Miller & Yellowlees, 1989; Bythell, 1990), for this study it was thought ecologically relevant to test the physiological response of corals to this oxidised form of nitrogen. The observed response was very similar to that presented above for ammonia enrichment, thus adding more evidence to the hypothesis that zooxanthellae growth and biomass is limited in oligotrophic environments by the availability of nitrogen.

Falkowski *et al.* (1993) proposed that the increased growth of zooxanthellae under nitrogen enrichment could result in a reduction of the total amount of translocate from the zooxanthellae to the host as a larger proportion of photosynthate would be required in cellular growth. Ultimately, this could impair the long-term survival of the host. In the current study no evidence could be found to support this hypothesis. Carbon budgets were constructed for both *Porites porites* and *Montastrea annularis* at the end of the nitrate experiment (see Tables 8.1 and 8.2). The growth rate of zooxanthellae increased with nitrate, but the proportion of photosynthate required in zooxanthellae growth was constantly small (3-4%), and the proportion translocated remained approximately 90% in all treatments. This is because the photosynthetic rate was also increased as a result of the higher zooxanthellae population density brought about by elevated nitrate levels, thus the increased rate of photosynthesis per surface area could balance the small increment in carbon required by the higher zooxanthellae growth rate.

Experiments on *Stylophora pistillata* by Hoegh-Guldberg & Smith (1989) and Dubinsky *et al.* (1990) found a reduction in the photosynthetic rate per cell when ammonium enrichment had brought about a 3-fold increment in zooxanthellae densities from 0.55 to 1.49 ($*10^6$ cells mg protein^{-1}) and from 0.6 to 1.65 ($*10^6$ cells cm^{-2}) respectively. The authors suggested that the inverse correlation between zooxanthellae density and the rate of photosynthesis per cell could be evidence for competition among the algae for CO_2. In the same study, Hoegh-Guldberg & Smith (1989) found that the rate of photosynthesis per cell was constant in *Seriatopora hystrix* where the zooxanthellae population density under ammonium increased only from 2.11 to 2.78 $*10^6$ cells mg protein^{-1}. The effect of nitrate on *Porites porites* and *Montastrea annularis* in the present study resembles the case of *S.hystrix* because the increase in zooxanthellae from 2.9 to 3.9 ($*10^6$ cells cm^{-2}) and from 3.9 to 4.8 ($*10^6$ cells cm^{-2}) for *P. porites* and *M. annularis* respectively brought about by nitrate did not produce any change in the photosynthetic rate per cell.

However the possibility of inorganic carbon limitation was discussed in this study in relation to calcification. An inverse relationship was found between nitrate concentration and rate of calcification measured as mass accretion by buoyant weighing. A similar reduction in calcification was previously reported with *Pocillopora damicornis* and *Stylophora pistillata* following ammonia enrichment (Stambler *et al.*, 1991; Stimson, 1992; Ferrier-Pagès *et al.*, 1996). Since measurements of both photosynthesis and calcification were available in the current study, it was possible to compare the inorganic carbon requirements of zooxanthellae and of calicoblastic cells in relation to nitrate enrichment. It was found that the amount of carbon fixed by the corals (net photosynthesis + calcification) did not change between treatments, but the proportion of carbon allocated to photosynthesis increased with nitrate to the detriment of calcification. This finding led to the formulation of an endogenous carbon limitation of calcification. Since photosynthesis and calcification are two processes drawing on the same pool of dissolved inorganic carbon (DIC), the enhancement of one could result in the depression of the other, whenever the total rate of DIC demand was higher than the rate of supply. The fact that DIC might be in limiting supply has been frequently proposed (Burris *et al.*, 1983; Dennison & Barnes, 1988; Muscatine *et al.*, 1989; Weis *et al.*, 1989; Newton & Atkinson, 1991; Patterson *et al.*, 1991; Harland & Davies, 1995; Al-Moghrabi *et al.*, in press). Zooxanthellae were thought to possess a competitive advantage on the calicoblastic cells because of their position in the gastrodermal cells closer to the DIC pool of seawater. A study investigating this hypothesis is called for (see Appendix 1).

The phosphate enrichment experiment produced a significant inverse relationship between enrichment and skeletal growth. This result was in agreement with the coral growth studies by Rasmussen (1988) and Ferrier-Pagès *et al.* (1996). In all cases, the known properties of phosphate as an inhibitor of the formation of calcium carbonate crystals (Simkiss, 1964; Reddy, 1977) were postulated to explain the observed reduction in the rate of calcification.
Among the other components of the carbon budgets, no other change was measured between corals grown in oligotrophic or phosphate enriched sea water, with the exception of a reduction in the zooxanthellae population density for which I am unable to give an explanation. This overall lack of response by symbiotic associations to phosphate has been reported previously for *Stylophora pistillata* (Muscatine *et al.*, 1989), *Pocillopora damicornis* (Stambler *et al.*, 1991) and *Tridacna gigas* (Belda *et al.*, 1993). Many ambiguities remain unresolved. Phosphate is readily taken up by symbiotic cnidarians (Yonge & Nicholls, 1931b,c; D'Elia, 1977; Cates & McLaughlin, 1979; Wilkerson & Kremer, 1992) and due to the ionic form at sea water pH, phosphate must be actively taken up by the host. However, zooxanthellae *in hospite* show characteristics of P-limitation even when the host is exposed to elevated concentrations, and this

suggests that the host might be involved in limiting the supply of P to the zooxanthellae (Miller & Yellowlees, 1989; Belda *et al.*, 1993; Rands *et al.*, 1993; Belda & Yellowlees, 1995).

8.4 Effects of a eutrophication gradient on the reef

The experiment on the reef (Ch.7) was conducted to test if differences in the water quality along a eutrophication gradient are mirrored by differences in the physiological response of corals and, if so, to test if the results from the laboratory experiment can be extrapolated to the more complex conditions on the reef.

The results were important in highlighting the plasticity of coral physiology. After just one month exposure, corals growing offshore at the oligotrophic site (low nutrient, high light) showed characteristics indicating nitrogen limitation and adaptation to high light such as low chlorophyll content, high C:N ratio, low photosynthetic efficiency, and low zooxanthellae population density. Corals at the intermediate site (high nutrients, high light) had the highest rate of photosynthesis and calcification as well as zooxanthellae biomass characteristic of nitrogen enrichment. Corals at the most polluted site (high nutrient, low light) suffered from the combined effect of light reduction and nitrogen enrichment and had high photosynthetic efficiency and high chlorophyll content per zooxanthellae but fixed a smaller amount of carbon in photosynthesis.

Thus the results from the photostat experiments on nitrate in combination with the literature on ammonia and on the photo-adaptation processes of zooxanthellae *in hospite* were used to interpret the changes found on the reef to the differences in water quality and light regimes experienced at the three sites.

The photostat system offers the possibility of testing the combined effect of nitrogen concentration and light intensity on corals, by experimentally changing these parameters under controlled laboratory conditions. This opportunity should be exploited further in future experiments since understanding how parameters interact is an important prerequisite when trying to extrapolate from experimental to field conditions.

Using discriminate function analysis, it was found that the physiological parameters that differed between sites could be divided into 'primary' and 'secondary' factors. Photosynthetic pigment content and photosynthetic efficiency were 'primary' characters found to respond closely and unidirectionally to the eutrophication gradient. Gross photosynthesis, calcification and respiration were 'secondary' characters positively correlated to eutrophication and inversely correlated to light. Thus, along an environmental degradation gradient (including changes both in water quality and light), secondary characters were found to display a single-humped curve. Hence the rate of calcification at the least and at the most eutrophic sites were not significantly different.

This was unexpected in view of the laboratory experiments and the large number of reported cases correlating water quality to skeletal growth. The most important implication of this is that it cautions against the use of skeletal growth as a quick bio-assay for the identification of nutrient enrichment. The process of skeletogenesis integrates a variety of physiological pathways and is dependent upon many environmental factors including *in situ* irradiance and water motion. Thus, although N and P enrichment are known to reduce calcification in some cases, a lower calcification rate *per se* cannot be taken to imply nutrient enrichment.

8.5 Carbon budgets

Carbon budgets were constructed for corals from the nitrate (all treatments), phosphate (control and 5μM PO_4) and reef (all sites) experiments using the new methodology developed in Ch.3 and summarised in section 3.5. These are presented in Tables 8.1 and 8.2. Carbon budgets combine a lot of information and present it in a distilled form. The most remarkable feature of these budgets is the overall lack of variability between treatments. In all cases zooxanthellae use about 10% of the carbon fixed in photosynthesis for their respiration and growth (μ_{NET}), leaving 90% available for translocation. In all cases, the amount of translocate is able to satisfy and exceed the C requirement for respiration and growth of the host. Such high degree of phototrophy agrees with previous measurements for *P.porites* (Edmunds & Davies, 1986, 1989). Budgets for *Stylophora pistillata* obtained from different sites in the Red Sea showed that the respiratory carbon requirements were not met by translocated photosynthate only when corals were subjected to very low light regimes, due to shade (Muscatine *et al.*, 1984) or depth (McCloskey & Muscatine, 1984), and thus photosynthesis was reduced. In my experiments, both in the photostat and in the field, corals were subjected to very high irradiance regimes characteristic of the shallow fringing reefs. The estimation of photosynthesis in the budgets for the reef experiment were calculated for an 'optimal' clear day (because no continuous light recording was available over the entire duration of the reef experiment) thus introducing a potential over-estimation.

No evidence for any negative effect of nitrogen on the corals can be seen from the budgets, even when the rate of skeletal growth was significantly reduced. To identify effects brought about by nutrient enrichment it appears preferable to focus specifically on one component of the budget *per se* (for example zooxanthellae density and growth which have shown to respond readily to nitrogen supply) rather than measuring it as part of the budget. The main reason for this is that in comparison to photosynthesis and respiration, all other components are small and therefore, when expressed as a percentage of photosynthesis, their variability between treatments is minimised to the point of non-existence.

Fig. 8.1 *Porites porites.* Partitioning of the 24h energy budget into zooxanthellae and host components for corals in the nitrate (Ch.5 -all treatments), phosphate (Ch.6 -control and 5μM PO_4) and reef (Ch.7 -all sites) experiments. The budgets were estimated for a 10 cm^2 coral tip and expressed both as absolute carbon (mgC $10cm^{-2}d^{-1}$) and as a percentage of the gross photosynthetic input. See Ch.3, section 3.5.1 for details of methodologies adopted.

		Nitrate (μM)				Phosphate (μM)		Reef		
mgC $10cm^{-2}d^{-1}$		0	1	5	20	0	5	OFF	BRI	SG
Zoox	Photosynthesis (gross)	2.45	2.52	2.79	2.46	3.39	3.00	2.80	3.72	2.54
	Respiration	0.19	0.15	0.21	0.23	0.28	0.17	0.16	0.30	0.17
	Growth (μ_{NET})	0.02	0.01	0.01	0.01	0.03	0.02	0.03	0.05	0.03
	Expulsion	0.06	0.05	0.07	0.08	0.08	0.06	0.05	0.07	0.06
	Translocation (TT)	2.18	2.31	2.49	2.14	2.99	2.75	2.56	3.30	2.28
Host	Respiration	1.02	0.98	0.85	0.72	1.22	1.17	0.85	1.08	0.80
	Growth	0.08	0.06	0.04	0.05	0.09	0.08	1.15	1.45	1.05
	loss	1.08	1.27	1.60	1.38	1.69	1.49	0.56	0.77	0.44
%										
Zoox	Photosynthesis (gross)	100	100	100	100	100	100	100	100	100
	Respiration	7.6	5.9	7.5	9.2	8.2	5.6	5.7	8.0	6.6
	Growth (μ_{NET})	0.83	0.45	0.46	0.60	0.99	0.70	1.01	1.29	1.18
	Expulsion	2.5	1.8	2.6	3.3	2.4	2.1	2.0	2.0	2.5
	Translocation (TT)	89.1	91.8	89.4	86.9	88.4	91.6	91.3	88.7	89.7
Host	Respiration	41.7	39.0	30.6	29.1	36.0	39.1	30.2	29.1	31.3
	Growth	3.3	2.5	1.4	1.8	2.6	2.7	41.1	38.9	41.3
	loss	44.0	50.3	57.4	56.0	49.8	49.8	20.0	20.8	17.2

Fig. 8.2 *Montastrea annularis* . Partitioning of the 24h energy budget into zooxanthellae and host components for corals in the nitrate (Ch.5 -all treatments), phosphate (Ch.6 -control and 5μM PO_4) and reef (Ch.7 -all sites) experiments. The budgets were estimated for a 10 cm^2 coral head with a perfectly hemispherical shape and expressed both as absolute carbon (mgC $10cm^{-2}d^{-1}$) and as a percentage of the gross photosynthetic input. See Ch.3, section 3.5.1 for details of methodologies adopted.

		Nitrate (μM)				Phosphate (μM)		Reef		
mgC $10cm^{-2}d^{-1}$		0	1	5	20	0	5	OFF	BRI	SG
Zoox	Photosynthesis (gross)	2.65	2.56	3.20	3.38	2.80	3.26	3.52	3.87	2.92
	Respiration	0.19	0.23	0.26	0.44	0.32	0.26	0.28	0.31	0.29
	Growth (μ_{NET})	0.02	0.01	0.01	0.02	0.02	0.01	0.04	0.05	0.04
	Expulsion	0.05	0.06	0.08	0.11	0.07	0.06	0.11	0.12	0.13
	Translocation (TT)	2.39	2.26	2.86	2.82	2.39	2.93	3.09	3.40	2.47
Host	Respiration	1.45	1.27	1.34	1.22	1.43	1.73	1.59	1.73	1.45
	Growth	0.14	0.08	0.06	0.07	0.12	0.09	0.30	0.35	0.25
	loss	0.80	0.90	1.46	1.53	0.84	1.12	1.20	1.33	0.77
%										
Zoox	Photosynthesis (gross)	100	100	100	100	100	100	100	100	100
	Respiration	7.1	9.1	8.0	13.0	11.4	7.8	7.9	7.9	9.8
	Growth (μ_{NET})	0.69	0.37	0.30	0.47	0.54	0.34	1.08	1.16	1.36
	Expulsion	2.1	2.4	2.3	3.1	2.4	1.9	3.2	3.1	4.3
	Translocation (TT)	90.2	88.2	89.3	83.4	85.6	90.0	87.8	87.9	84.5
Host	Respiration	54.8	49.8	41.8	36.2	51.2	53.0	45.2	44.7	49.5
	Growth	5.3	3.3	2.0	2.0	4.2	2.7	8.5	8.9	8.5
	loss	30.1	35.1	45.5	45.2	30.1	34.2	34.2	34.2	26.4

Table 8.3 Mean values for the ratio of daily gross photosynthesis to coral respiration ($dayP_{gross}/24hR_c$) for *Porites porites* and *Montastrea annularis* in the nitrate, phosphate and reef experiments. Daily gross photosynthesis is calculated from the photosynthesis vs. irradiance curves obtained in a closed chamber respirometer in combination with the daily light intensity curve. In the photostat experiments light intensity was fixed for 13h at 250 μmol photons $m^{-2}s^{-1}$. On the reef, $dayP_{gross}$ was estimated for a 'optimal' clear day, using the recording of surface light intensity on the 8th June 1994 and the mean percentage transmission measured at each site during the experimental period.

Experiment	Treatment	Species	
		P. porites	*M. annularis*
NO_3 (μM)	0	2.03	1.61
	1	2.23	1.69
	5	2.62	2.00
	20	2.61	2.03
PO_4 (μM)	0	2.26	1.59
	5	2.23	1.64
REEF	OS	2.78	1.88
	BRI	2.70	1.90
	SG	2.64	1.68

Hence in all experimental treatments, photosynthesis (dayP_{gross}) provided more carbon than the coral used daily. Respiration alone (by the coral as a whole, 24hR_c) could account for most (>90%) of the expenditure of carbon. Therefore it appears that the much simpler measurement of the ratio of dayP_{gross}/24hR_c (see Table 8.3) is an appropriate simplification of the entire carbon budget calculation.

I feel it is now appropriate to digress and return to the concept of CZAR. This is the integrated measure of autotrophy which Muscatine and co-workers developed in order to specifically improve on the simple dayP_{gross}/24hR_c ratio. Thus, rather than a simple oxygen flux measurement, the following equation was introduced:

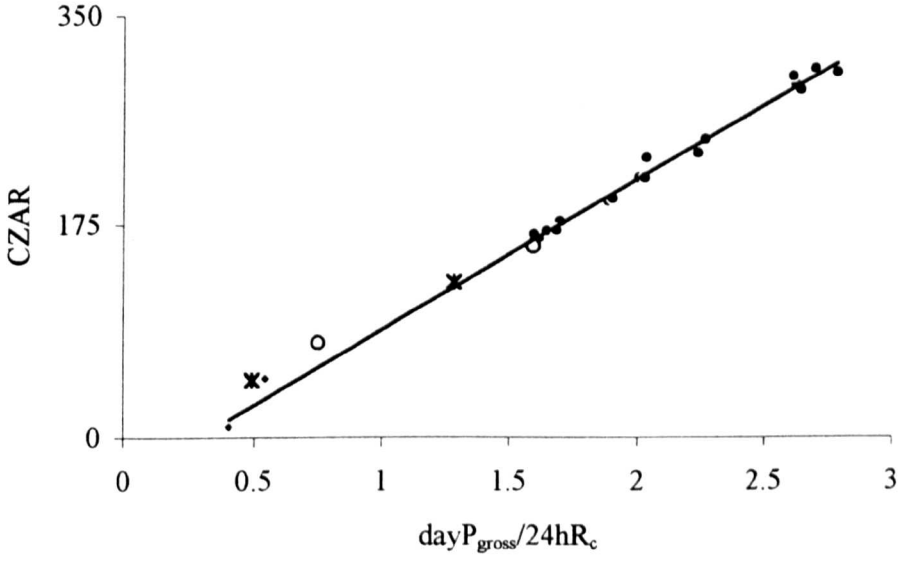
$$CZAR = \frac{\left[\left[(F/24Rc)+1\right]-\left[(1-B)(Tday/24)(PQz*RQc)\right]\right]*\left(\frac{\mu_c-\mu}{\mu_c}*100\right)}{B(PQz*RQc)}$$

which can be reduced to the equation presented in section 3.1.2:

$$CZAR = \frac{P_Z * T}{R_A}$$

As discussed in depth in Ch.3, this measurement is based on untested assumptions and on the problematic estimation of t_d. Furthermore, the methodology to calculate the above is presented in such a convoluted manner to often confuse the reader and errors in the measurement of CZAR have appeared in the literature: R_Z (subtracted from Pgross in the estimation of P_Z net) has been calculated for daylight hours only rather than for 24h (Muscatine et al., 1984) and P_Z net has been mistaken with P_C net (Steen & Muscatine, 1984) with incorrect results.
The relationship between CZAR and dayP_{gross}/24hR_c was investigated further with all the data obtained in this study, for both nubbins and explants (Fig. 8.1). A linear regression model showed that 98% of the variation in CZAR can be explained by dayP_{gross}/24hR_c. On the same graph, published data from Muscatine et al. (1984), McCloskey & Muscatine (1984) and Verde & McCLoskey (1996b) were plotted. Even though these data were measured somehow differently (they use PQ of 1.1 and RQ of 0.8 and overestimate translocation by the amount carbon in digested zooxanthellae) they fit within the 95% confidence limits of the previous regression. This is overwhelming evidence that CZAR does not succeed in improving on the dayP_{gross}/24hR_c ratio. Interestingly, this implies that even under different regimes (nutrient supply, light, reef and photostat) and in different symbiotic cnidarians, the amount of carbon fixed that is not translocated to the host (i.e. zooxanthellae respiration, net zooxanthellae growth and zooxanthellae expulsion) is always a much smaller proportion then that translocated. Therefore any change in Pgross has a much stronger effect on CZAR then any change in zooxanthellae biomass. This point was well exemplified by Muscatine et al. (1984) comparing light- and

Fig. 8.1 Relationship between CZAR and $dayP_{gross}/24hR_c$ in symbiotic anthozoans from different studies.



Data points from:

- *Porites porites* and *Montastrea annularis* (this study)
- ✱ *Stylophora pistillata* (Muscatine et al., 1984)
- o *Stylophora pistillata* (McCloskey & Muscatine, 1984)
- · *Anthopleura elegantissima* (Verde & McCloskey, 1996)

shade-adapted corals. The difference in Pgross between light- and shade-adapted *Stylophora pistillata* was five-fold and brought about by a much reduced irradiance at the shaded site: accordingly the difference in CZAR was five-fold too. Even though a two-fold change in carbon used in algal gross growth and algal respiration occurred, this accounted for only 1% of total carbon fixed.
Thus we can rely on the dayP_{gross}/24hR_c ratio as an appropriate measure of autotrophy.

Porter (1976) suggested that autotrophy in corals is inversely related to polyp size. The data in this study fit Porter's model. The branching coral *Porites porites* has a ratio of dayP_{gross}/24hR_c between 2.0 and 2.8, depending on treatment, while the massive coral *Montastrea annularis*, a common species down to 50m. depths, has a ratio between 1.6 and 2.0.
For both coral species, the ratio of dayP_{gross}/24hR_c shows a positive trend with nitrate enrichment, but not with phosphate enrichment. The reef experiment shows that the least and the most polluted sites have the same ratio, somewhat lower than that for the intermediate site. Thus dayP_{gross}/24hR_c correlates well with changes in photosynthesis, and can therefore be added to the list of 'secondary' characters. These are the physiological characters that are affected both by the reduction in light and the increase in nutrients along a gradient of environmental degradation, as discussed in section 8.4.

8.6 Evaluation of a bio-assay

One aim of this study was to identify a physiological parameter that could be used as an early-warning bio-assay by reef managers in order to detect nutrient enrichment on coral reefs before any negative impact has become evident at the reef community level. Ideally the response of the bioassay to nutrient stress must be measurable at a lower concentration, or after a shorter exposure than that required to damage the health of the community. To this aim, I chose to focus on the measurement of the organic (photosynthesis) and inorganic (calcification) productivity of corals, and thus on carbon budgets. This choice followed the reasoning that any bioassay based on these processes would have the added advantage of providing direct information on the physiological condition of corals. Such information could then be used to extrapolate the results at the ecosystem level, and to model the response of the coral reef as a whole to nutrient enrichment.
During the course of this study it became apparent that the initial choice of measurements was not ideal in the case of nutrient pollution. Firstly, it was shown that the construction of complete carbon budgets was superfluous in measuring the health of a coral in relation to nutrient

enrichment, because the same result could be obtained from the ratio dayP_{gross}/24hR_c. The latter is a much easier, faster and cheaper measurement to make, and is therefore more amenable to monitoring studies. Moreover, the reef experiment highlighted the problem of using measures of photosynthesis or calcification as a bio-assay for nutrient pollution. These processes in fact were found to belong to a group of 'secondary' characters which related to environmental degradation by a single-humped curve. The shape of this relationship implies that measurements of photosynthesis and calcification cannot be relied upon to discriminate between oligotrophic and heavily eutrophic sites. Due to its critical implications, it is necessary to further investigate the observed single-humped curve relationship for a larger number of hermatypic corals under a wider spectrum of water quality conditions.

On the other hand, this study was successful in identifying a set of 'primary' physiological characters that were linearly related to nutrient pollution. Thus their measurement can be adopted as a bio-assay to discriminate between oligotrophic and nutrient enriched sites. In particular, the measurement of chlorophyll content per surface area (rather than per cell) was suggested as a most promising bio-assay because it integrates both the change in pigment and in zooxanthellae population density. This would avoid the time consuming process of counting cell density, thus allowing for much larger sample sizes. Photosynthetic pigments change in concentration depending upon light intensity, which in turn is a function of depth or eutrophication; if depth is accounted for, changes in pigments will relate directly to nutrient levels. Overall this parameter is relatively easy, cheap and fast to measure since it does not require the preparation of nubbins and explants. A small tissue sample (1 cm^2) is sufficient for analysis and therefore damage to colonies can be small.

It is therefore suggested that monitoring the chlorophyll content of corals would succeed in identifying changes in the water quality over a reef. There are at least two important advantages in monitoring the chlorophyll content of corals (i.e. a sensitive response of corals to nutrient enrichment) rather than the nutrient concentration in the water column. Firstly, pigment concentration is an integrated response to the average nutrient availability, thus it minimises the likely fluctuation in nutrient concentration that can be encountered when sampling water quality. Secondly, the concentration of nutrients in the water column can be a misleading measurement; if a reef community is readily taking up nutrients, the concentration can remain low even though the total flux has markedly increased. In contrast, a change in pigment concentration ensures that the change in water quality, however small, has a measurable effect..

In order to offer the measurement of chlorophyll as a viable bio-assay it is necessary to carry out a larger-scale survey to test and ensure that the variability in pigment content in corals sampled from the same depth within oligotrophic sites is significantly less than the variability in pigment

content encountered when corals from the same depth are sampled between oligotrophic and eutrophic sites.

It is also important to recognise the limits of a bio-assay based on the pigment content of corals. For example, on its own this measurement cannot detect when a nutrient concentration is detrimental to the coral colony. To do that, this bio-assay will have to be used in combination with other measurements either directly related to coral 'health' as, for example, a measure of stress proteins or related to coral growth, such as the rate of calcification (as discussed in section 7.4.3). However, the physiology of corals is complex, and the relationship between two processes (such as the photosynthetic pigments and the calcification rate) in their response to nutrient enrichment can be highly variable. For example, chlorophyll content and calcification rate were inversely related in the nitrate enrichment experiment in the photostat, but linearly related at the two sites with the same light regime in the field experiment.

Moreover, the ultimate goal of reef managers is to detect the presence of nutrient enrichment before the health of the reef (rather than that of a coral colony) has deteriorated. In fact, the health status of a reef does not automatically mirror the health status of a coral. For example, it is feasible that an increase in nutrients detectable in the pigment concentration of the coral would not produce a stress response by that coral, but would be sufficient in enhancing the growth rate of benthic algae. In turn, the increase in benthic algae can bring about a reduction in the recruitment rate of coral planulae. If the reef has recently suffered acute damage, such as after a hurricane, the impaired recovery potential brought about by the increase in nutrients might succeed in lowering the overall 'health' of the community.

Even though at the community level the relationship between nutrients and reef health is unquestionably complex, it is worth investigating how the chlorophyll content of corals relates to measures of community health such as percentage coral cover, species diversity or coral recruitment rates. For the reasons given above, the measure of chlorophyll content is likely to be a more efficient measure of nutrient availability than the direct measurement of nutrient concentration. A survey encompassing reefs that are spatially distant but structurally similar should be carried out to establish how chlorophyll content in corals relates to community 'health'. Only then it will be possible to prove if the chlorophyll content of corals can be used not only as a tool to detect changes in water quality but also as an efficient bio-assay to monitor the 'health' of a coral reef in relation to nutrient enrichment.

Appendix 1

CARBON LIMITATION

This is not a thorough review of the likelihood of dissolved inorganic carbon (DIC) limiting essential physiological processes of hermatypic corals such as photosynthesis or calcification, nor a review of the possible mechanisms involved. Rather I aim to summarise here the largely hypothetical logic on which the theory for the endogenous carbon limitation of calcification described in Ch.5 is based. This should be read as a compendium to the rest of the thesis, thus no reference is used to justify the statements made.

The ultimate goal of this writing is to show how the differing results obtained from photostat experiments (nitrate enrichment produced a reduction in the rate of calcification), reef study (nutrient concentration can increase calcification if light penetration is not affected) and literature (inverse correlation between zooxanthellae population density and photosynthetic rate per cell) are not contradicting results but are different cases of the same theory. It is hoped that this summary can be used in the future as a basis for an experiment to test directly the theory of endogenous carbon limitation of calcification.

Definitions used:

- **DIC demand = rate at which DIC is required by the symbiosis to satisfy photosynthesis and calcification**

  It depends largely on light and on the biomass of zooxanthellae (which in turn can be enhanced by nitrogen enrichment) because of these two factors directly affect the rate of photosynthesis.

- **DIC supply = rate at which DIC is made available to the symbiosis for both photosynthesis and calcification**

  It depends on physical (thickness of the boundary layers, shape of the coral) and biological factors (active transport of bicarbonate, carbonic anhydrase activity) but in this speculation, DIC supply is made to depend exclusively on water velocity due to its effect on the thickness of the boundary layers.

- **DIC flux = amount of DIC entering the symbiosis**

  It depends on both DIC supply and DIC demand, and will be shared between photosynthesis and calcification. In the optimal environmental conditions for coral growth (high light, high water velocity, low nutrients), photosynthesis and calcification are assumed to be positively correlated to each other. An increase in photosynthesis will

result in an increase in calcification, so that the ratio of DIC used in photosynthesis and calcification is constant and characteristic of each coral species.

The relationship of DIC flux to DIC demand and DIC supply can be hypothesised for each of the following conditions:

- **DIC supply > DIC demand** (Fig. A1.1)

When the rate of supply is faster than the rate of demand, the flux of DIC will depend only on demand. It will increase until demand will become limited by the physiological constraints. The proportional share of DIC by photosynthesis and calcification will remain constant as demand is increased.

- **DIC supply < DIC demand**

When the rate of supply is slower than the rate of demand, carbon will be limiting. Two conditions can fit this case:

DIC supply < **DIC demand = constant** (Fig. A1.2a)

When rate of demand is constant (as well as higher than supply), the flux of DIC will depend only on the rate of DIC supply which can be increased by water velocity (hence this variable on the x-axis) up to a physical maximum. Again photosynthesis and calcification will share the available DIC proportionally.

DIC supply = constant < DIC demand (Fig. A1.2b)

When the rate of supply is constant (e.g. constant water velocity and DBL) as well as lower than demand, the DIC flux will also have to be constant. Under these conditions, competition between zooxanthella and calicoblastic cells ensues as soon as demand is increased (for example by and increment in zooxanthellae population density caused by nitrogen enrichment). Zooxanthellae, because of their position closer to sea water, have an advantage on calicoblastic cells. Thus they scavenge DIC more efficiently and reduce the rate of calcification proportionally (period marked by arrow 1). This specific condition, hypothesised in Ch.5, was identified as 'endogenous carbon limitation of calcification'.

If photosynthetic demand for carbon increases further once the minimum rate of calcification is attained, competition between zooxanthellae will take place to the detriment of the rate of photosynthesis per cell (period marked by arrow 2).

In Fig. A2, some of the data obtained in this thesis with nubbins of *Porites porites* under different experimental conditions have been superimposed on the models described.

1) On the reef, in the experiment presented in Ch.7, water velocity was high. DIC demand (photosynthesis + calcification) increased from the oligotrophic site (OS) to the intermediate site (BRI). This resulted in an increase of the total flux of DIC into the symbiosis, and the ratio of carbon used in photosynthesis and calcification was constant.

2a) Corals in the oligotrophic treatment in the photostat (for example during the control treatment during the nitrate experiment of Ch.5) and in the reef experiment (site OS in Ch.7) can be assumed to have the same DIC demand. However, in the photostat water motion is 37% that found on the reef. Thus these two groups of corals fit on two different points on the x-axis (DIC supply, water velocity). The total flux of DIC into the symbiosis was constant, as was the ratio of carbon used in photosynthesis and calcification.

2b) The endogenous carbon limitation of calcification was identified in the photostat experiment when corals were incubated with elevated levels of nitrate. While the total DIC flux was constant, the proportional share by photosynthesis and calcification changed to the detriment of the latter.

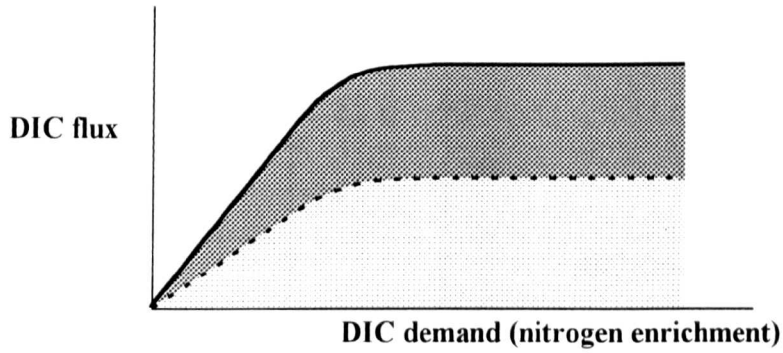
I believe it is very important to test these hypotheses. The interaction between water velocity and nitrogen enrichment could be tested with a factorial designed experiment, using flumes to estimate water velocity correctly.

Future research should then focus on how commonly water velocity on reefs drops to levels that fit the model of endogenous carbon limitation of calcification (2b). These will be the environments at greatest risk from nitrogen enrichment damage.

Fig. A1 The hypothesised relationships between DIC flux and DIC supply and demand.

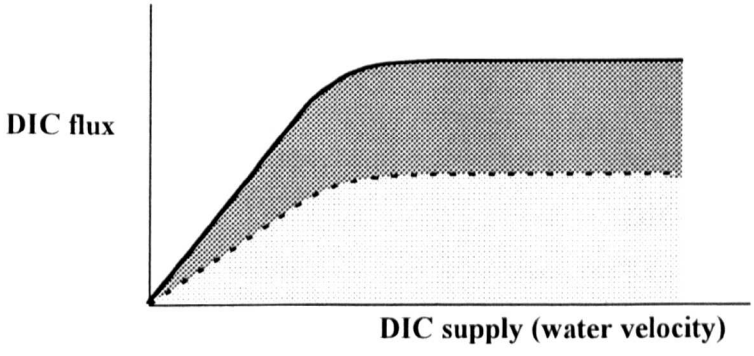
Proportion of DIC flux used in photosyntheis ▒ and calcification ⁘

1) DIC supply > DIC demand



2) DIC supply < DIC demand

2a) DIC demand = constant



2b) DIC supply = constant

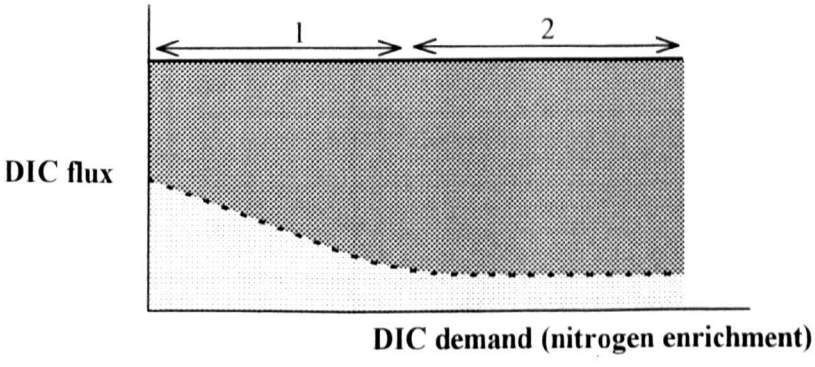
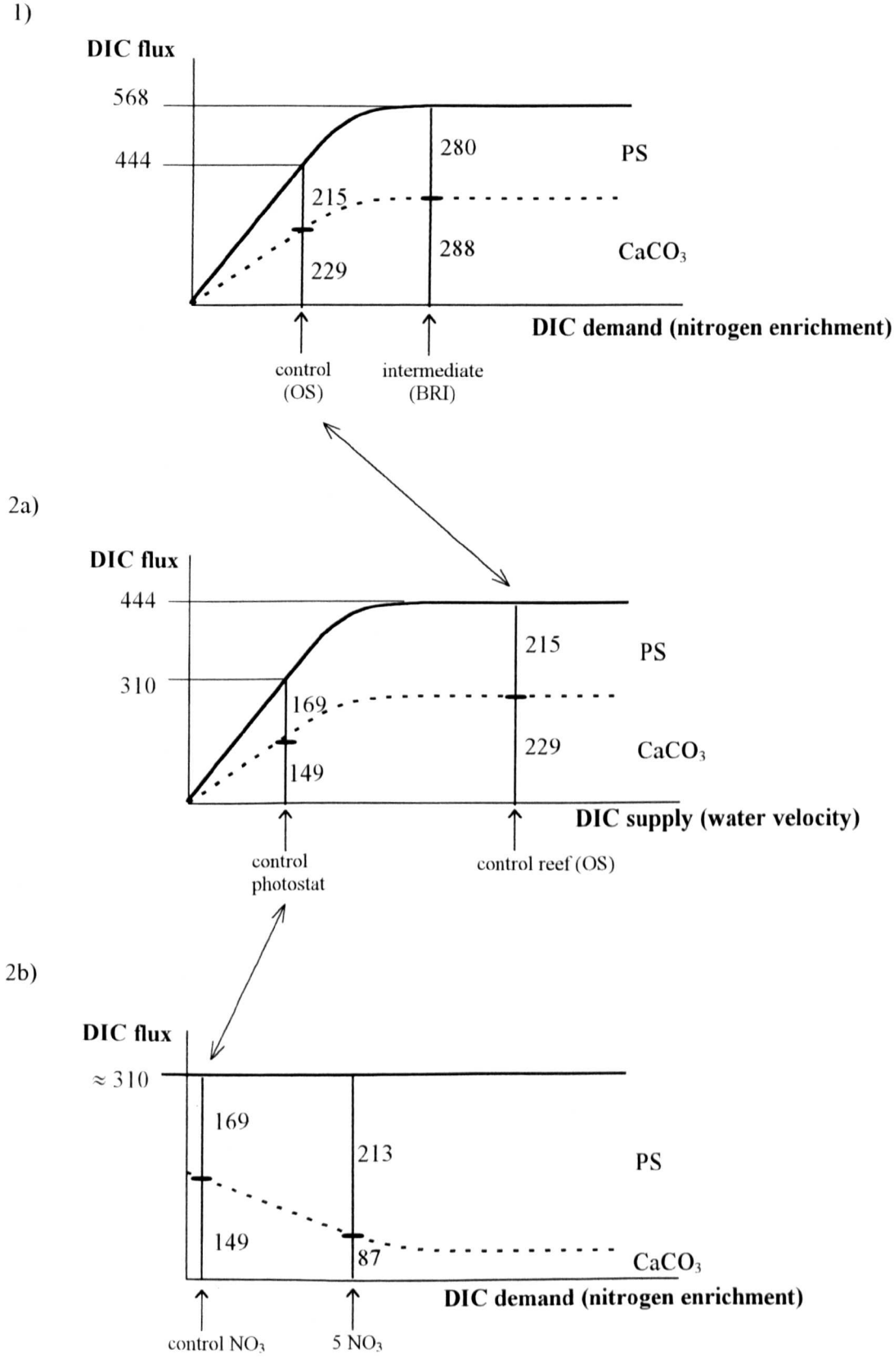


Fig. A.2 DIC flux and its partition into photosynthesis (PS) and calcification ($CaCO_3$) as measured in nubbins of *Porites porites*.



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