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Effects of marine bacteria on axenic, algal-fed (Isochrysis galbana) larvae of the oyster Crassostrea gigas were studied. Repeated enhancement of larval survival and growth was found in axenic cultures inoculated with bacteria strain CA2, compared with that of larvae fed algae alone. Larval populations fed only algae consisted of high proportions of slow growing larvae, which were not present in cultures inoculated with strain CA2, suggesting that CA2 bacteria make a nutritional contribution to oyster larvae. Addition of CA2 bacteria to cultures of non-axenic larvae fed I. galbana, I. aff. galbana (clone T-ISO) or Pseudoisochrysis paradoxa (clone VA-12) enhanced larval growth, the proportion of larvae that set and the subsequent size of spat.

Free-swimming, DAPI-stained CA2 cells were readily

ingested and accumulated in the digestive systems of oyster larvae. Ingestion of bacterial ^{14}C was significant at bacterial concentrations higher than $5x10^6$ cells ml^{-1}. Experimental methods that reduced label recycling in ^{14}C-feeding trials permitted measurement of larval retention efficiencies for bacterial and algal (I. galbana) carbon. Straight-hinged oyster larvae fed CA2 bacteria at $1.5x10^7$ cells ml^{-1} in "pulse-chase" ^{14}C-feeding experiments could potentially meet 146% of their carbon metabolic requirements with retained bacterial carbon. This bacterial carbon equalled 46% of the carbon retained by straight-hinged larvae fed a similar amount of carbon in the form of algae (I. galbana at 54 cells μl^{-1}). The potential contribution of bacterial carbon towards the metabolic carbon requirements of oyster larvae decreased as larvae grew in size. This is the first reported study of the contribution of bacteria to the carbon requirements of bivalve larvae.

Beneficial Effects of Bacteria on the Culture of Larvae of the Pacific Oyster Crassostrea Gigas (Thunberg)

by

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Typed by researcher for Philippe Douillet

To my parents and sister

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Beneficial Effects of Bacteria on the Culture of Larvae of the Pacific Oyster Crassostrea Gigas (Thunberg)

GENERAL INTRODUCTION

When the dutch lens maker Antonie van Leeuwenhoek (1632-1723) saw microbes for the first time, he opened a field of research that has never ceased to puzzle and amaze naturalists and scientists. The italian Lazzarro Spallanzani (1729-1799) then presented experimental evidence against spontaneous generation by showing that microorganisms were the cause of decomposition of organic matter, which was then confirmed by the findings of the french Louis Pasteur (1822-1895). This researcher demonstrated that microbes were the cause of fermentation and disease. A russian scientist, Sergei Winogradsky (≈1890) went on to show the environmental importance of microbes by discovering several bacteria-mediated reactions, such as nitrification, oxidation of H_2S and sulfur and anaerobic nitrogen fixation, among others. This researcher also advanced the concept of chemoautotrophy. Since then, contributions from many scientists have shown bacteria to be indispensable in cycling of nutrients in natural and artificial environments.

Naturally-occurring bacteria live in mixed bacterial

communities made up of metabolically different strains which affect one another through complex interactions (Bull and Slater, 1982). Furthermore, bacteria have been called adaptable chimaeras whose metabolic plasticity results from widespread transfer of information through plasmids and prophages (Sonea, 1988). Metabolic diversity and plasticity of ubiquitous and abundant microbes are likely to influence the physico-chemical and biochemical characteristics of many habitats.

Recent studies have revealed the importance of small sized organisms in aquatic ecosystems. Picoplankton (0.2-2 μm) have been shown to represent up to 90% of the phytoplankton biomass and has been estimated to carry out up to 80% of the inorganic carbon fixation in oceanic waters (Li et al., 1983). Bacteria may constitute over 25% of the heterotrophic carbon biomass in the marine environment (Williams, 1984) and estimates indicate that between 10 to 50% of the organic carbon fixed by primary producers might pass through a "bacterial loop" (Fuhrman and Azam, 1982). Controversy exists over whether or not microbial food webs act as "links" or "sinks" in the exchange of carbon and energy with higher trophic levels (Ducklow et al., 1986). Because of their abundance and

wide-ranging activities in aquatic systems, bacteria are likely to play a major role in the development of populations of organisms in the marine environment.

The objectives of this study were to:

a) determine the effect of bacteria (single strains and natural communities) on the survival and growth of axenic larvae of the Pacific Oyster Crassostrea gigas (Thunberg).

b) select bacteria strains showing consistent beneficial effects upon axenic oyster larvae in repeated experiments.

c) determine if the beneficial effects of bacteria strains upon oyster larvae were also evident in the presence of other microbial contaminants.

d) determine the nature of mechanisms by which bacteria enhance oyster cultures.

CHAPTER 1

Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae

ABSTRACT

A study of the effects of marine bacteria on bivalve larvae was undertaken whereby bacteria-free oyster larvae (Crassostrea gigas) were cultured under aseptic conditions using axenic algae (Isochrysis galbana) and inoculations of isolated strains of bacteria. Twenty-one bacteria strains were tested and most were detrimental to larval survival and growth. However, additions of strain CA2 consistently enhanced larval survival (21-22%) and growth (16-21%) compared with that of controls fed algae alone. These control cultures fed only algae were characterized by high proportions of slow growing larvae which were less evident in cultures inoculated with strain CA2, suggesting that CA2 bacteria may make a nutritional contribution to the growth of larvae. Larval growth improvement was not caused by bacterial enhancement of algal growth, and consequently food availability, because I. galbana did not grow under the light intensities used for larval culture. Naturally occurring microflora from Yaquina Bay, Oregon, were found to depress survival or growth of larvae fed live algae.

INTRODUCTION

A common observation in experiments with bivalve larvae is substantial variation in larval survival and growth (Davis, 1953; Loosanoff, 1954; Walne, 1956). Twenty-five percent of the variability in growth of a single population of mussel larvae was determined to be due to genetic factors (Innes and Haley, 1977), and genetics accounted for 25 to 50% of growth variability among different populations of larval Crassostrea virginica (Newkirk et al., 1977). Similarly, a significant proportion of the variability in survival of C. gigas larvae was attributed to genetic factors (Lannan, 1980). Exogenous factors such as temperature (Loosanoff, 1959), salinity (Bayne, 1965), pH (Calabrese and Davis, 1970), food quantity (Walne, 1965), food quality (Davis, 1953), age of algal food (Dupuy, 1975), larval concentration (Loosanoff et al., 1953), size of container (Dupuy, 1975), silt (Davis and Hidu, 1969), algal exudates of unfavorable algal species (Bayne, 1965), water quality (Millar and Scott, 1967) and toxicants (Walne, 1970) have been found to contribute significantly to variability in larval growth. Nonetheless, variation in growth among different cultures of larvae obtained from the same parents and grown under identical conditions of temperature, salinity and ration has been commonly reported (Bayne, 1983).

The role of bacteria as beneficial or harmful agents in the culture of bivalve larvae has been the subject of many investigations, but as results indicate, this role has not been fully evaluated. Thirteen different isolates of marine bacteria did not support growth of oyster larvae when fed to larvae as the sole source of particulate food (Davis, 1950; 1953). High bacteria densities in bivalve larvae cultures are generally considered deleterious to larvae (Walne, 1956a; 1956b; 1958), and even innocuous bacteria in large numbers have been reported to depress the rate of algal ingestion (Ukeles and Sweeney, 1969). Numerous publications have reported the ability of some bacterial strains to invade larvae, to produce toxins, or both (Guillard, 1959; Tubiash et al., 1965; Tubiash et al., 1970; Brown, 1973; Di Salvo, 1978; Elston et al,. 1981; Nottage and Birkbeck, 1986). In contrast, there have been reports in which bacteria were implicated as a food source for bivalve larvae (Carriker, 1956; Hidu and Tubiash, 1963) or improved growth of larvae fed algae (Martin and Mengus, 1977; Beese, in Prieur et al., 1990).

The use of axenic organisms has proven indispensable to ascertain the nutritional requirements of invertebrates without interference from microbial contaminants (Singh and Brown, 1957; Provasoli and Shiraishi, 1959; Akov, 1962). Furthermore, undefined microbial contaminants must be

eliminated in order to study the possible effects that a bacterial strain might have on the culture of an organism. This approach was used to study the effects of several bacterial strains on cultures of the protozoan Amoeba nitrophila (Frosch, 1897 in Luck et al., 1931); the cladoceran Moina macrocopa (Stuart et al., 1931) and larvae of the clam Mercenaria mercenaria (Guillard, 1959). In the present study, axenic larval Crassostrea gigas, obtained without the use of antibiotics, were used in a series of experiments designed to determine if selected strains of marine bacteria can consistently improve survival and growth of algal-fed oyster larvae.

METHODS

Bacteria-free oyster larvae were obtained according to the method described by Langdon (1983). Adult oysters Crassostrea gigas were held at 18° C in a recirculating seawater system for a period of 4 to 6 weeks, depending on the initial reproductive condition of the broodstock. After this conditioning period, oysters were opened and shucked. Using aseptic techniques in a laminar-flow hood, the external surface of the gonads of each oyster was disinfected with a 1% solution of sodium hypochlorite. A small incision was made through the surface of the gonads with a heat-sterilized scalpel, and gametes from each oyster were removed with sterile Pasteur pipettes and transferred to separate sterile flasks containing 0.2 μm-filtered, autoclaved seawater (FSSW). Eggs were fertilized by the addition of a few drops of sperm suspension and then transferred at a density of 100 ml^{-1} to Erlenmeyer flasks containing FSSW. Eggs were incubated on an orbital shaker at 25° C for 48 h. As soon as trocophore larvae developed into veligers (straight-hinged larvae), samples of larvae were added to 1/10 recommended concentration of Difco Marine Broth 2216 (3.74 g l^{-1}, salinity 30 ppt) and incubated for a month at 25° C under aerobic or anaerobic conditions (BBL GasPack Pouch). Axenicity of larvae was also determined by epifluorescence

microscopy using 4'6-diamidino-2-phenylindole (DAPI) staining techniques (Porter and Feig, 1980). Remaining larvae were starved at 5° C for 5 days while testing for axenicity of broth incubations of larval samples. Larvae from cultures showing no evidence of microbial contamination were transferred at a density of 5 ml^{-1} to 250 ml Erlenmeyer flasks, each containing 150 ml of FSSW, closed with cotton plugs and capped with aluminum foil. Shell lengths of 100 randomly selected larvae were measured with an optical micrometer fitted to a compound microscope, or using an image analysis system (Zeiss Videoplan 2).

Marine bacteria strains were isolated from cultures of algae or oyster larvae at the Whiskey Creek Hatchery, in Netarts Bay, Oregon. Other bacteria were isolated from either the guts of adult oysters or from incubations of protein capsules (Langdon, 1989) suspended in unfiltered seawater. Pure bacterial strains were obtained following the dilution method of Rodina (1972). Strains were grown at 25° C on Marine Agar 2216 or Brain Heart Infusion Agar (Difco). Bacteria grown on solid media for 3-5 days were re-suspended for 24 h in FSSW; then, washed by centrifugation at 20,000 x g for 10 minutes and re-suspended in FSSW.

Strains were added to larval cultures at

concentrations of 10^5-10^6 cells ml^{-1}. Cell concentrations were estimated by reference to an equation derived by relating the spectrophotometric absorbance (600 nm) of several bacterial strains to bacterial concentration, the latter determined by direct counts with DAPI-staining methods (Porter and Feig, 1980). Similar equations were developed and used for each strain tested in all subsequent experiments to Experiment 2.

Axenic Isochrysis galbana Parke (clone ISO) was obtained from the Culture Collection of Marine Phytoplankton (Maine). Algal cultures were grown at 20° C in 200 ml f/2 medium (Guillard and Ryther, 1962) illuminated by 1000-1500 lux of cool white fluorescent light under a 12 h light / 12 h dark photoperiod. Axenicity of algae was determined according to the methods described above for larvae.

All glassware was washed in 10% nitric acid, rinsed 7 times with distilled water and baked overnight at 450° C. Disodium ethylenediamine-tetraacetate (EDTA) was added at a final concentration of 1 ppm to all seawater in order to improve water quality (Utting and Helm, 1985). Salinity of seawater after sterilization varied between 28 and 31 ppt. Heat sterilization was carried out for 15 minutes at 121° C and 1.06 kg cm^2 pressure.

LARVAE FED ON LIVE ALGAE AND BACTERIA.

Twenty-one marine bacteria isolates were tested in three culture experiments for their effects on survival and growth of larvae fed axenic Isochrysis galbana. In Experiment 1, 7 microbial isolates from the Whiskey Creek Hatchery (H1-H7) and 5 isolates from the guts of adult oysters (G1-G5) were tested for their effects on axenic cultures of oyster larvae fed on live algae. Control treatments were either larvae fed only algae or starved larvae.

Two strains (H6,H7) that showed beneficial effects on larval cultures in Experiment 1 were tested again in Experiment 2. Other bacteria screened in the second experiment were an additional five strains isolated from the Whiskey Creek Hatchery (H8-H12), one strain isolated from the gut of an adult oyster (G6) and three strains isolated from protein capsules incubated in seawater (CA1-CA3). Control treatments included starved larvae and larvae fed only algae. A third control treatment (SW) consisted of cultures of larvae inoculated at the beginning of the experiment with naturally occurring bacteria present in 5 ml samples of 1 μm-filtered seawater collected from Yaquina Bay, Oregon. The larvae in the third control treatment were fed axenic algae every other day. Experiments 1 and 2 were carried out with 4 replicates per treatment.

Strains found beneficial for larvae in Experiment 2 (H7,CA2) were tested in Experiment 3. Similar control treatments were included as those described for Experiment 2. Experiment 3 was carried out with 8 replicates per treatment.

Cultures of bacteria-free oyster larvae were inoculated once at the beginning of each experiment with bacteria strains. Bacteria-free algal cells, harvested from cultures in exponential growth phase, were added to larval cultures every two days. Seawater of the larval cultures was not renewed during the culture period. Concentrations of algal cells in each larval culture flask were estimated prior to each feeding. A 2 ml sample of the larval culture medium was aseptically removed from each flask, using a pipet with its end covered by a 64 μm Nitex screen to prevent removal of larvae. Algal cells were preserved with formalin, concentrated by centrifugation and re-suspended in 100 μl of 0.2 μm-filtered seawater. Algal concentrations in the samples were then determined using a hemocytometer. Algae were then added to larval culture flasks to provide cell concentrations at pre-determined levels. Algal cell concentrations were increased by 15,000 cells ml^{-1}, from 40,000 to 100,000 cells ml^{-1}, over a 10 day culture period. To provide uniform food quality during the experiments, algae from a single culture were added to all larval

cultures receiving an algal diet at each feeding period.

Larval culture flasks were randomly placed on orbital shakers in a temperature-controlled room at 25° C. Larval cultures were exposed to a light intensity of 50-70 lux for 12 h each day. No algal growth resulted at this low light intensity. After 10 days of culture, samples of water were aseptically withdrawn from flasks containing starved larvae or larvae fed only axenic algae and analyzed for microbial contamination as described above. Experimental data was not considered for analysis unless these control treatments were bacteria-free at the end of the 10 day culture period.

BACTERIA-ALGAE INTERACTIONS

In order to determine possible effects of CA2 bacteria on the growth of axenic Isochrysis galbana in larval cultures, algal cells were initially suspended at a concentration of 40,000 ml^{-1} in f/2 medium and then subsequently subdivided in sixteen 250 ml Erlenmeyer flasks. CA2 cells were added at 10^5 cells ml^{-1} (final concentration) to eight flasks while FSSW was added to the other eight flasks in order to maintain similar initial algal concentrations in all flasks. The final volume of each algal culture was 200 ml. Four algal cultures inoculated with bacteria and four cultures that received only FSSW were placed in conditions found satisfactory for

growth of I. galbana (1000-1500 lux and 20° C), while the remaining algal cultures were exposed to culture conditions used for larval culture (50-70 lux and 25° C). Algal cultures were placed on orbital shakers and incubated for three weeks. Every second day, 10 ml samples were aseptically removed from each algal culture, and algal concentrations determined using a Coulter Counter (Model ZB1).

LARVAE FED ON DEAD ALGAE AND BACTERIA

Interactions between strain CA2 and living Isochrysis galbana that could result in modification of algal food quality were not addressed in previous experiments. To determine whether or not bacteria could enhance cultures of larvae fed on non-living diets, live I. galbana was replaced with dead algae. In Experiment 4, known concentrations of axenic I. galbana were frozen at -5° C. Freezing and thawing broke the cell walls and membranes of algal cells. Larvae were fed dead freeze-killed algae (FA) every two days according to the same methods used with live algae. One group of larval cultures fed FA was maintained bacteria-free, two groups were inoculated at the beginning of the experiment with strain H6 at 10^5 cells ml^{-1} (final concentration) or with an inoculum of naturally occurring bacteria (SW) present in 5 ml samples of 1 μm-filtered seawater collected from Yaquina Bay, Oregon, and containing

10^5-10^6 cells ml^{-1}. Other cultures received additions of strain H6 (at 10^5 cells ml^{-1}, final concentration) alone or naturally occurring bacteria (SW) (5 ml of 1 μm-filtered seawater) alone every other day of the experiment. Control treatments included starved larvae and larvae fed every second day on live axenic I. galbana. Culture conditions and sample treatments were similar to those of experiments carried out with live algae. Four replicates were tested per treatment.

A second method used to kill algal cells consisted of ^{60}Co-irradiation (5 megarads) performed at the Radiation Center at Oregon State University. Non-viability of irradiated algae (IA) was evident by lack of growth of cells in f/2 medium at 20° C under 1000-1500 lux of fluorescent light emitted 12 h a day. Furthermore, the irradiation process destroyed contaminants as demonstrated by incubations at 25° C of irradiated algae in 1/10 diluted Marine Broth 2216 (3.74 g l^{-1}, salinity of 30 ppt) under aerobic or anaerobic conditions (BBL GasPack Pouch). Integrity of irradiated algal cells was verified by microscopic examination. Cell volumes of irradiated and non-irradiated algae from 7 different cultures were determined using a Coulter Counter (Model ZB1) in conjunction with a Coulter Channelyser (Model 256). Latex beads of different diameters (2.17 μm [Interfacial Dynamics

Corporation], 3.43 μm and 4.54 μm [Polysciences]) were used for volume calibration of the Coulter Channelyser.

To make sure that IA were acceptable to larvae as a food source, grazing rates of larvae fed on either IA or live Isochrysis galbana were compared. Methods used for this evaluation are presented in Appendix I. Larval ingestion rates for live and ^{60}Co-irradiated algae (Table I; Appendix I) were compared using a 2 sample t-test, after verifying homocedasticity by Cochran's test for homogeneity of variances, at the 0.05 level of probability.

In Experiment 5, oyster larvae were fed IA every second day according to the same methods employed with live algae in Experiments 1 to 3. Three groups of larval cultures were fed IA. One group was maintained bacteria-free, while the two others were inoculated at the beginning of the experiment with strains H7 or CA2. Control treatments included starved larvae or larvae fed every 2 days on live axenic Isochrysis galbana. Eight replicates were tested per treatment. Larval survival and growth were determined as described below.

DATA COLLECTION AND ANALYSIS

At the end of each experiment, larvae were carefully transferred to scintillation vials containing buffered

formaldehyde (2% final concentration, pH = 8). Rose of Bengal was added to stain larval tissues in order to differentiate larvae that were alive from empty shells when sampled at the end of each experiment. The whole larval population in each flask was counted using a dissecting microscope and the shell lengths of 100 randomly selected larvae were measured with an optical micrometer fitted to a compound microscope, or using an image analysis system (Zeiss Videoplan 2). Survival and growth data were transformed to satisfy assumptions of ANOVA. Survival data were transformed as:

$$\arcsin\left(\sqrt{\text{percent survival } 100^{-1}}\right)$$

Growth data were transformed as:

$$\arcsin\left(\sqrt{(\ln L_t - \ln L_0)\, t^{-1}}\right)$$

where;

L_t = final mean shell length (μm)

L_0 = initial mean shell length (μm)

t = culture period (10 days)

Transformations were successful in reducing heterocedasticity of survival data but not of growth data (Cochran's test for heterogeneity of variances, at the 0.05 level of probability). Treatment effects on larval survival were tested with one-way ANOVA. Where significant

differences were indicated, Tukey's honestly significant difference test (T-HSD) was applied to determine the statistical significance of differences among individual treatments, at the 0.05 level of probability. Treatment effects on larval growth were analyzed using the Kruskal-Wallis test (KW). Differences among individual treatments were determined by means of the Games and Howell test (G&H) of equality of means with heterogeneous variances (Sokal and Rohlf, 1981), at the 0.05 level of probability. All test were performed with the computer program Statistix (NH Analytical Software) except for the Games and Howell test which was carried out using the program Biom (Rohlf, 1982).

Size frequency distributions of populations of larvae fed on algae were compared between cultures kept bacteria-free and those inoculated with CA2 bacteria in Experiments 2 and 3. Skewness coefficients (g1; Sokal and Rohlf, 1981) of larval populations from each replicate flask were calculated and used to compare larval size frequency distributions. A normal size distribution would have a g1 coefficient equal to 0. A skewness coefficient higher than 0 indicates positive skewness of the size distribution (higher proportion of small-sized individuals), while a skewness coefficient smaller than 0 indicates negative skewness of the size distribution. After confirmation of homocedasticity of g1 values by Cochran's test at the 0.05

probability level, data were analyzed by two-way ANOVA with treatment (algae, algae + CA2) and experiment as factors. As dictated by the results of ANOVA, appropriate multiple comparisons of means were conducted at the 0.05 level of probability using the Student-Newman-Keuls procedure (SNK), controlling for experiment-wide error (Underwood,1981)

CRYOPRESERVATION OF BACTERIA

Bacteria have been described as adaptable chimaeras whose metabolic plasticity results from widespread transfer of genetic information though plasmids or prophages (Sonea, 1988). This evolutionary strategy for adaptation to changing environments may result in the loss of beneficial characteristics of selected bacteria strains. In order to reduce the possibility of changes in bacteria characteristics between successive experiments, selected strains were cryopreserved at -70° C in 10% (V/V) glycerol in sterile 1/10 diluted Marine Broth 2216.

IDENTIFICATION OF STRAIN CA2

Identification of bacteria strain CA2 was based on Bergey's Manual of Systematic Bacteriology (Holt, 1984). Methodology used for different procedures followed the Manual of Methods for General Bacteriology (Gerhardt et al, 1981). Exponentially growing cells cultured on Marine agar 2216 were used for all tests performed at the Hatfield

Marine Science Center, Newport, Oregon. Cells were Gram stained. Motility was determined by observations of wet mounts with light microscopy. Oxidase activity was determined by spreading CA2 cells with sterile cotton swabs over Pathotec Cytochrome Oxidase Test Strips (General Diagnostics), which contained a derivative of dimethyl-p-phenylenediamine and α-naphthol. Cultures of CA2 cells were flooded with 3% hydrogen peroxide for catalase testing. Oxidation and fermentation of glucose was assayed using the modified O-F medium of Leifson (1963). Utilization of inorganic sources of nitrogen was evaluated by culturing CA2 cells on media prepared with NH_4Cl or $NaNO_3$ (0.5 g l^{-1}), glucose (0.1 g l^{-1}), Na_2HPO_4 (0.1 g l^{-1}), $FePO_4$ (0.004 g l^{-1}) and 1 ml l^{-1} of f/2 vitamin mix (Guillard and Ryther, 1962). Culture media used as controls were prepared by replacing $NaNO_3$ or NH_4Cl by peptone or tryptone (Difco) at 0.5 g l^{-1}. Anaerobic growth was determined by transferring CA2 cells either onto solid media in Petri dishes or into 25 ml 1/10 diluted Marine Broth 2216 (3.74 g l^{-1}; salinity 30 ppt) contained in 50 ml Erlenmeyer flasks, placing these cultures in Anaerobic GasPaks (BBL), and incubating the cells at 20° C for up to one month.

The following tests were carried out by Dr. Ronald Weiner (University of Maryland at College Park). Methodology followed the Manual of Methods for General

Bacteriology (Gerhardt et al., 1981). Salt requirements were evaluated by culturing CA2 cells in Tryptic Soy Agar (TSA) prepared at different salt concentrations by additions of NaCl at 1% increments up to 10% concentration. Presumptive evidence of anaerobic growth and motility were obtained by observing the pattern of growth of CA2 cells after inoculating a tube containing semisolid Tryptic Soy Broth enriched with 0.8% agar and 1% NaCl using a straight needle. Flagella staining was carried out by the Leifson method (Gerhardt et al., 1981). Synthesis of exopolysaccharides was evaluated by the Phenol-Sulfuric Acid reaction (Gerhardt et al., 1981). The mole percent guanine plus cytosine (mol% G + C) in extracted deoxyribonucleic acid (DNA) was determined by the thermal melting (denaturation) methods of Marmur and Doty (1962) using a Gilford UV programmable spectrophotometer. Antibodies of 20 different bacteria strains belonging to the Alteromonas/Shewanella group were tested for reaction with exopolysaccharides of CA2 cells. Fatty acids were saponified, methylated and analyzed by gas chromatography (Five Star Laboratories, Connecticut). A second fatty acid analysis of strain CA2 was carried out by Dr. Fred Singleton (Center for Marine Biotechnology, University of Maryland) for comparison with an Alteromonas data base.

RESULTS

LARVAE FED ON LIVE ALGAE AND BACTERIA.

Single additions of marine bacteria isolates to oyster larvae cultures significantly affected larval survival (ANOVA, $p<0.01$; Appendix II) and growth (KW, $p<0.01$) after 10 days of culture in all experiments (Figs. 1, 2, 3). Microbes tested can be divided into categories depending on their effects upon oyster larvae: adverse, neutral or beneficial. Bacteria belonging to the last category were tested further and their effects upon oyster larvae were divided into either variable or consistently beneficial.

a) Adverse strains. Strains G1, G2 and G4 adversely affected larval survival (T-HSD, $p<0.05$), while strains G1, G2, G4, G5, H8, H10 adversely affected larval growth (G&H, $p<0.05$). Bacteria present in 5 ml aliquots of 1 μm-filtered seawater depressed larval survival (T-HSD, $p<0.05$) in Experiment 2 and larval growth (G&H, $p<0.05$) in Experiment 3.

b) Neutral strains. A large proportion of strains (H1, H2, H3, H4, H5, H9, H11, H12, G3, CA1, CA3) added to cultures of oyster larvae did not result in significant enhancement or depression of larval survival (T-HSD, $p>0.05$) or growth (G&H, $p>0.05$) compared with cultures fed algae alone.

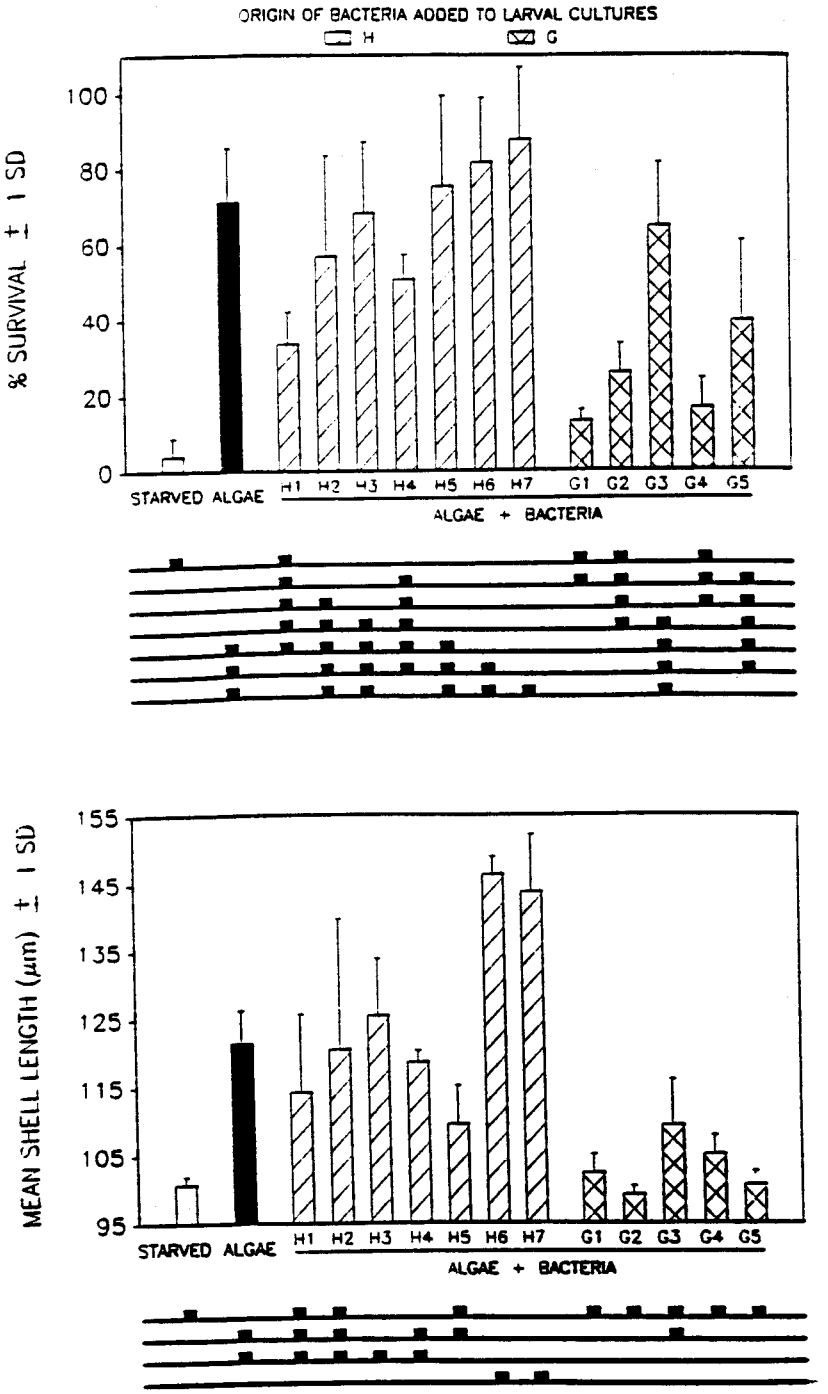

Fig.1. Experiment 1. Survival and growth of oyster larvae after 10 days of culture on axenic Isochrysis galbana supplemented with different bacteria strains. Tested bacteria were isolated from the Whiskey Creek Hatchery, Oregon (H) or from the guts of adult oysters (G). Control treatments were starved or fed axenic I. galbana. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below the histograms of survival and growth, respectively. Squares on the same horizontal line indicate that mean values are not different at the 0.05% level of significance.

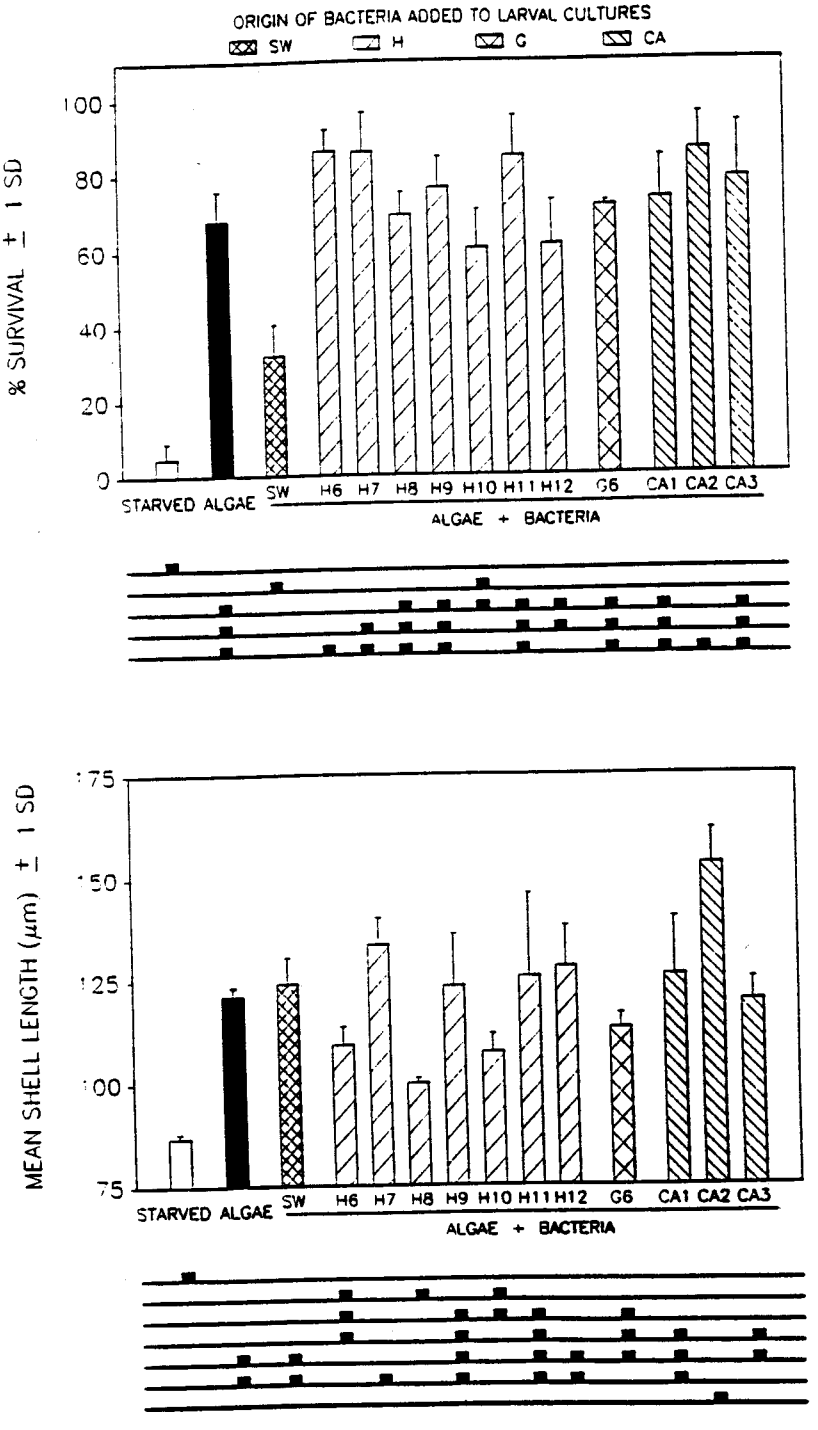

Fig.2. Experiment 2. Survival and growth of oyster larvae after 10 days of culture on axenic Isochrysis galbana supplemented with different bacteria strains. Tested bacteria were isolated from the Whiskey Creek Hatchery, Oregon (H), from the guts of adult oysters (G), or from incubations of protein capsules in seawater (CA). Naturally-occurring bacteria present in 1μm-filtered seawater (SW) were added in a control treatment. Other control treatments included larvae fed axenic I. galbana or starved. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below the histograms of survival and growth, respectively. Squares on the same horizontal line indicate that the mean values are not different at the 0.05% level of significance.

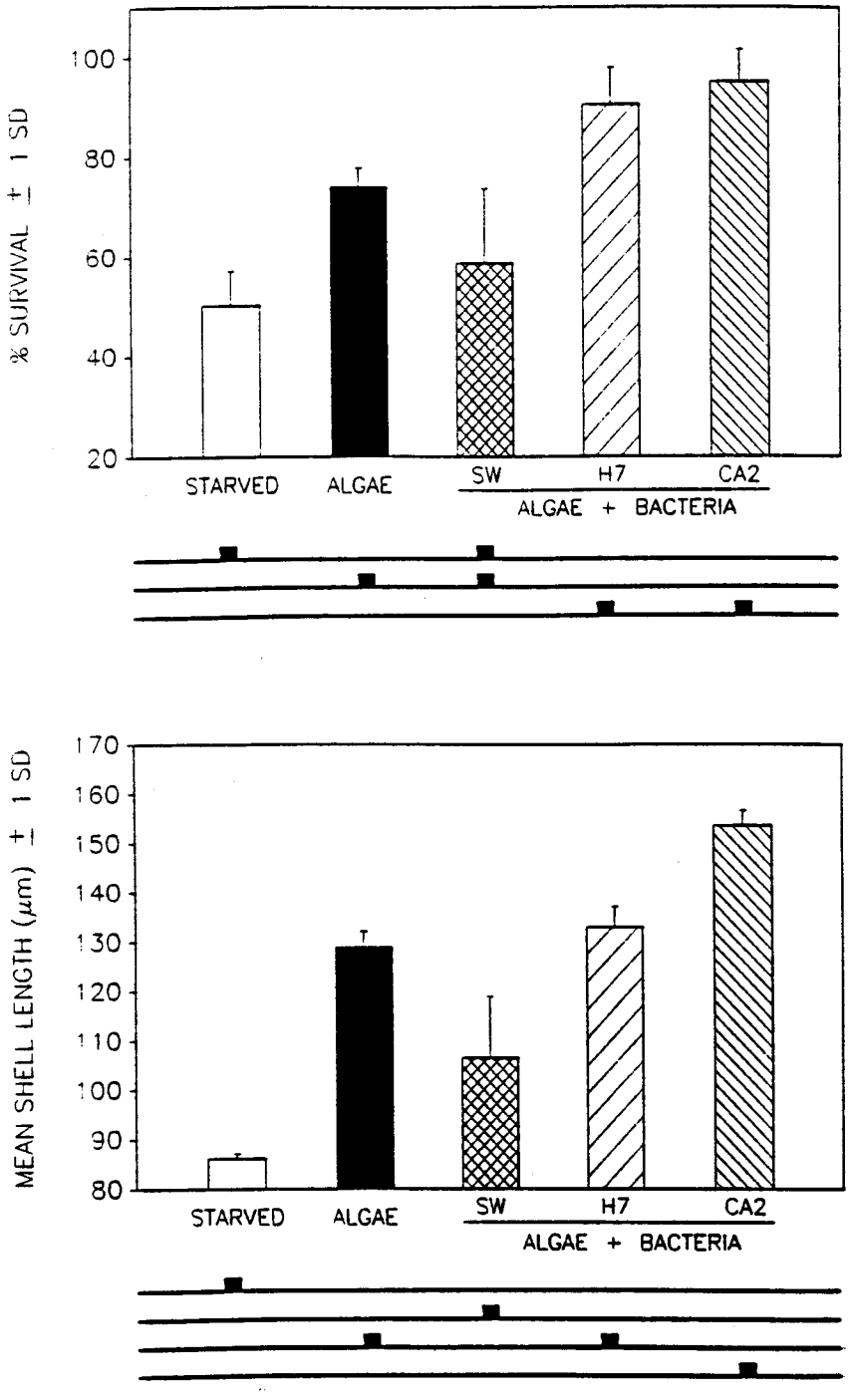

Fig.3. Experiment 3. Survival and growth of oyster larvae after 10 days of culture on axenic Isochrysis galbana supplemented with different bacteria strains. Tested bacteria were isolated from the Whiskey Creek Hatchery, Oregon (H) or from incubations of protein capsules in seawater (CA). Naturally-occurring bacteria present in 1 μm-filtered seawater (SW) were added in a control treatment. Other control treatments included larvae fed axenic I. galbana or starved. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below the histograms of survival and growth, respectively. Squares on same horizontal line indicate that the mean values are not different at the 0.05% level of significance.

c) Variable strains. Addition of strains H6 and H7 to larval cultures resulted in inconsistent improvements of larval growth among experiments. Larval growth was enhanced (G&H, $p<0.05$) in cultures inoculated with strains H6 and H7 in Experiment 1. In contrast, larval growth enhancement with strain H7 was found to be statistically insignificant in Experiments 2 and 3 (G&H, $p>0.05$). Surprisingly, larval growth was depressed (G&H, $p<0.05$) by addition of strain H6 to larval cultures in Experiment 2.

d) Beneficial strains. In both experiments 2 and 3, larvae grown in cultures inoculated with strain CA2 were significantly larger in shell length than control larvae fed only axenic algae (G&H, $p<0.05$). Larval survival was enhanced in cultures inoculated with strain H7 and CA2, but this enhancement was only found statistically significant in Experiment 3 (T-HSD, $p<0.05$).

A high proportion of non-growing larvae were present after 10 days of culture in all populations fed axenic algae alone. In contrast, cultures of larvae inoculated with CA2 bacteria and fed algae consisted of a smaller proportion of slow-growing larvae than that in cultures of larvae fed algae alone (Fig.4; Tables 1 and 2). Analysis of variance indicated a significant interaction

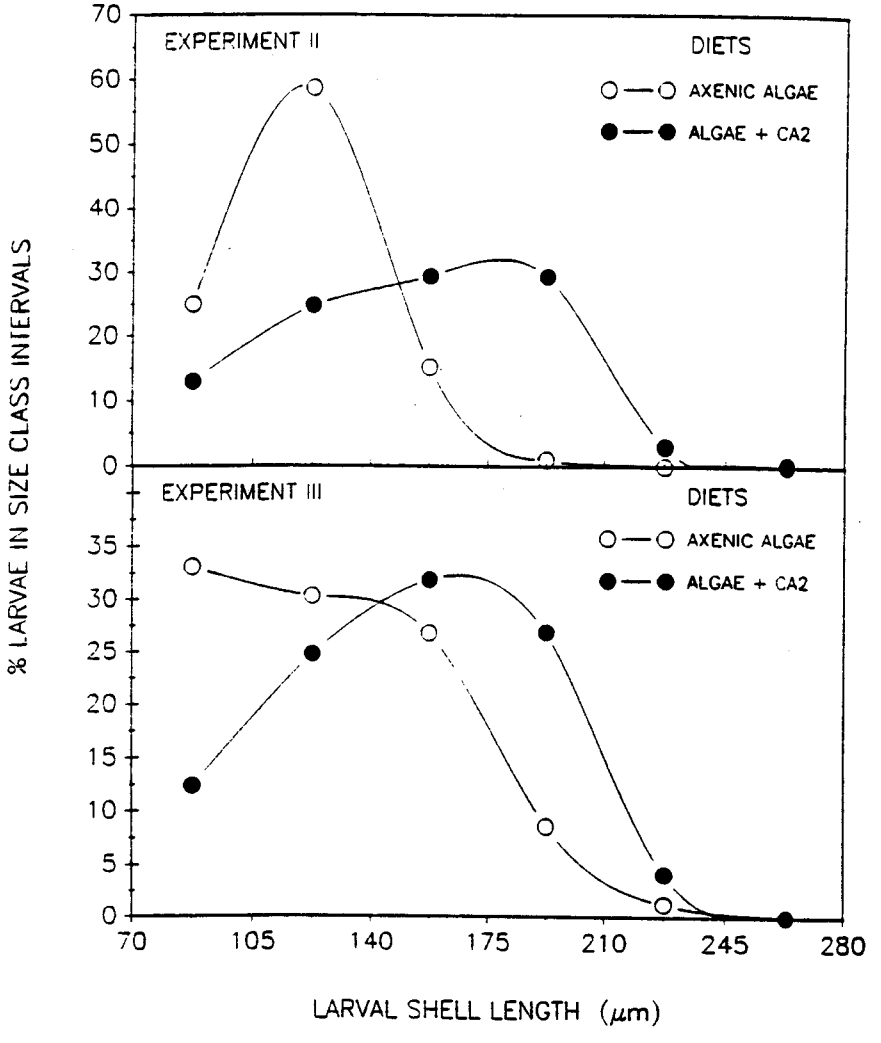

Fig.4. Size frequency distributions of larvae fed on Isochrysis galbana with or without addition of CA2 cells. Points represent percent larvae for each shell length interval of 30 μm. Lines used for illustrative purposes only. Data from Experiment 2 (n=400) and 3 (n = 800) for each treatment.

Table 1. Skewness coefficients (g1) from size frequency distributions of populations of larvae cultured with either axenic Isochrysis galbana (ISO) alone or I. galbana plus CA2 bacteria in Experiments 2 and 3.

Experiment	Diet	Average skewness (g1) of populations ± 1 S.D.	
2	ISO	0.7906 ± 0.2134	(n=4)
2	ISO + CA2	-0.0605 ± 0.2235	(n=4)
3	ISO	0.3801 ± 0.1720	(n=8)
3	ISO + CA2	-0.0466 ± 0.2910	(n=8)

Table 2. Two-way analysis of variance of skewness coefficients (g1) for size frequency distributions of populations of larvae cultured either with Isochrysis galbana alone or I. galbana plus CA2 bacteria in Experiments 2 and 3.

Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	1	0.31468	0.31468	5.79	0.0259
CA2 addition	(B)	1	3.2656	3.2656	60.12	0.0000
Interactions	(A*B)	1	0.36039	0.36039	6.63	0.0180
Replicates	(C)					
Residual	(A*B*C)	20	1.0864	0.05432		
Total		23	5.0271			

between treatment and experiment factors. In both Experiments 2 and 3, skewness coefficients for populations

of larvae fed axenic algae alone were significantly larger (SNK, $p<0.05$) that those for populations of larvae fed algae and inoculated with CA2 bacteria. The difference between skewness coefficients of treatments in Experiment 2 was larger than in Experiment 3, explaining the significant interaction determined by the two-way ANOVA test.

BACTERIA-ALGAE INTERACTION

Cells of Isochrysis galbana did not grow with or without inoculations of CA2 bacteria under the conditions used to culture larvae (Fig. 5). Growth of algae under favorable light intensity (1000-1500 lux) and temperature (20° C) was not affected by the presence or absence of CA2 cells in the culture medium.

LARVAE FED ON DEAD ALGAE AND BACTERIA

Significant differences among treatments in Experiments 4 and 5 were determined for larval survival (ANOVA, $p<0.01$; Appendix II) and growth (KW, $p<0.01$). Survival of larvae cultured on axenic FA or IA alone was significantly lower (T-HSD, $p<0.05$) than that of larvae cultured on live axenic algae alone (Figs. 6, 7). However, survival of larvae fed FA or IA was higher (T-HSD, $p<0.05$) than that of starved larvae. In contrast, no significant

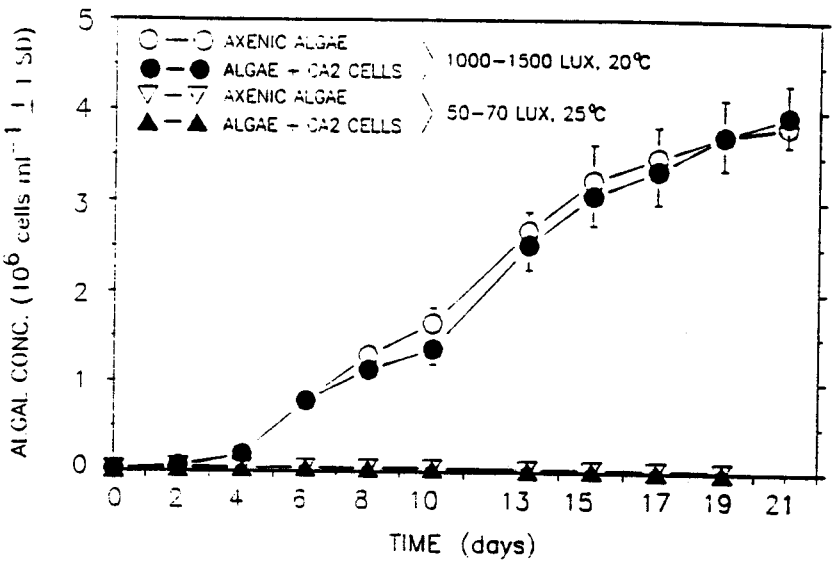

Fig.5. Effects of CA2 bacteria on growth of Isochrysis galbana under conditions used to raise larvae (50-70 lux; 25° C) or under conditions found optimal for algal growth (1000-1500 lux; 20° C).

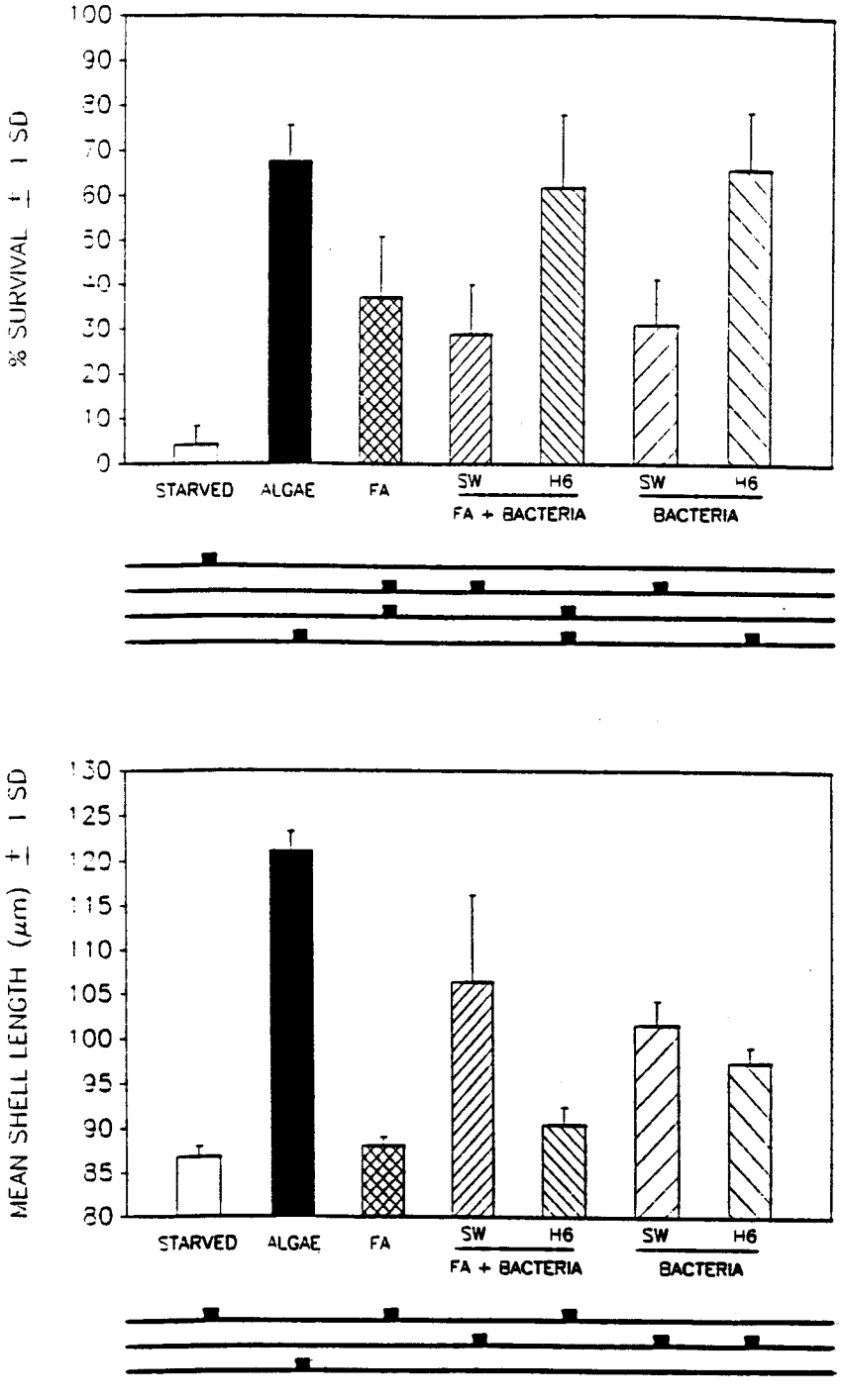

Fig.6. Experiment 4. Survival and growth of oyster larvae after 10 days of culture when fed on a diet of either bacteria alone (strain H6, naturally-occurring bacteria present in 1μm-filtered seawater (SW)) or freeze-killed Isochrysis galbana (FA) with or without supplements of bacteria (H6 or SW). Control treatments were starved or fed axenic I. galbana. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below survival and growth histograms, respectively. Squares on same horizontal line indicate that mean values are not different at the 0.05% level of significance.

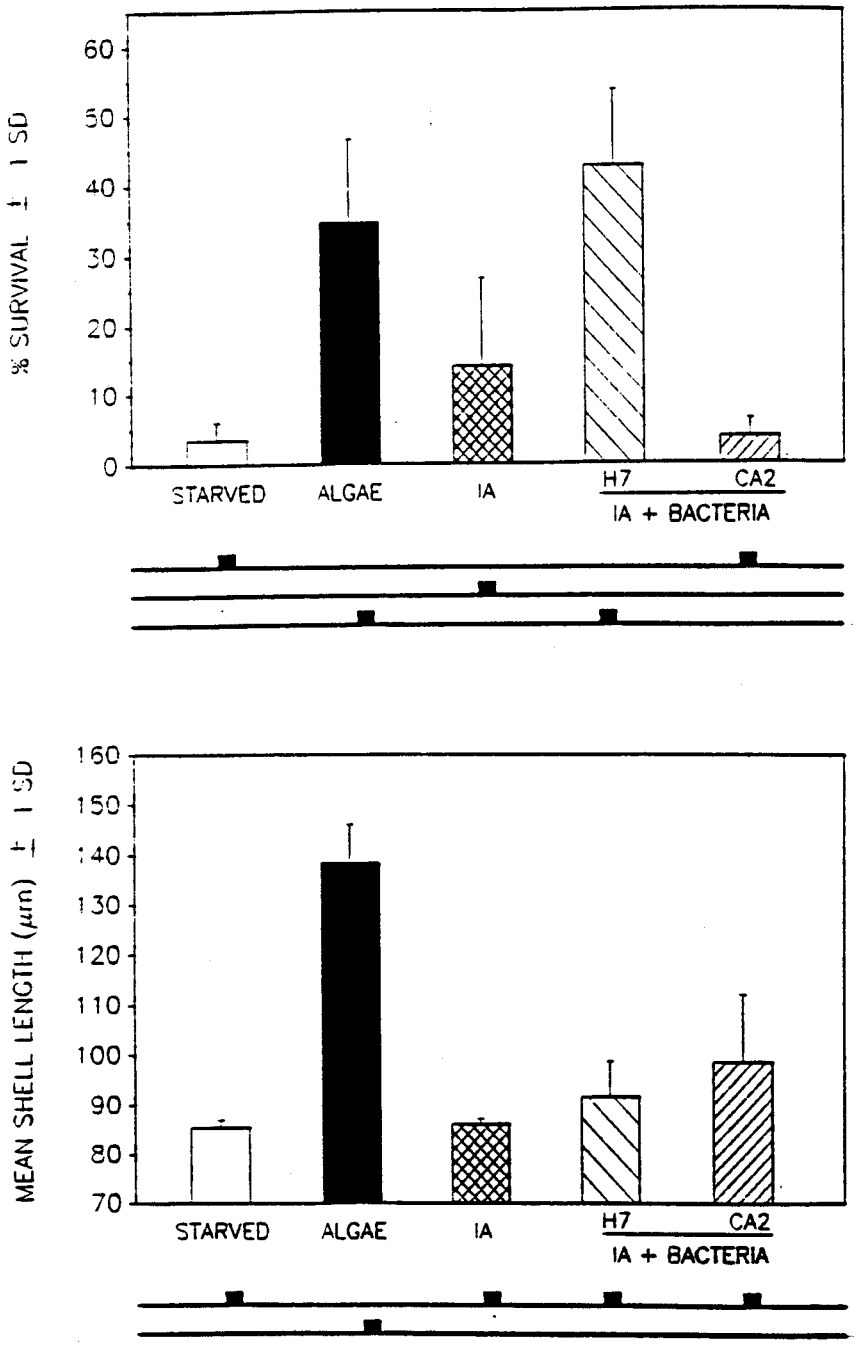

Fig.7. Experiment 5. Survival and growth of oyster larvae after 10 days of culture on ^{60}Co-irradiated Isochrysis galbana (IA) with or without supplements of H7 and CA2 bacteria. Control treatments were starved or fed axenic I. galbana. Results of Tukey's HSD pairwise comparisons and Games and Howell's tests are displayed below survival and growth histograms, respectively. Squares on horizontal line indicate that mean values are not different at the 0.05% level of significance.

differences in larval survival were detected between cultures fed live algae and cultures fed FA or IA inoculated with strains H6 and H7, respectively (T-HSD, $p>0.05$). Survival of larvae fed every two days on bacteria H6 alone was not significantly different (T-HSD, $p>0.05$) from that of larvae fed live algae, and was significantly higher (T-HSD, $p<0.05$) than that of starved larvae. Larvae from cultures inoculated every two days with 5 ml of 1 μm-filtered seawater (SW) also showed higher survival (T-HSD, $p<0.05$) than that of starved larvae.

Larvae fed on FA or IA were significantly smaller than larvae fed on live axenic algae (G&H, $p<0.05$), and were not different from the size of starved larvae (G&H, $p>0.05$) at the end of the experiment. Additions of single bacteria strains to cultures of larvae fed FA or IA did not improve larval growth compared to larvae fed FA or IA alone (G&H, $p>0.05$). In contrast, growth of larvae fed FA inoculated with 5 ml of 1 μm-filtered seawater was significantly enhanced (G&H, $p<0.05$) compared to that of larvae either fed FA alone or starved larvae. Similarly, additions every two days of 5 ml of 1 μm-filtered seawater or strain H6 alone to larval cultures significantly enhanced growth of larvae (G&H, $p<0.05$) compared to that of starved larvae.

Poor growth of larvae fed FA may have been due to the

rupture of the freeze-killed algal cells. ^{60}Co-irradiation did not affect the integrity of algal cells but reduced their volume from 44.4 ± 1.92 μm^3 to 26.3 ± 0.59 μm^3 ($\bar{x}$ ± 1 SD). A high proportion of irradiated cells remained intact while in suspension in seawater, as demonstrated by the small decrease in cell concentration in control flasks from 59,043 ± 1,119 cells ml^{-1} to 58,539 ± 1,505 cells ml^{-1} ($\bar{x}$ ± 1 SD) in 105 minutes compared with a decrease in the concentration of live algae from 59,089 ± 754 cells ml^{-1} to 53,328 ± 1,777 cells ml^{-1} over the same time period. IA cells were ingested by oyster larvae at rates significantly (2 sample t-test, $p<0.01$) greater than that for live cells (Appendix I).

IDENTIFICATION OF STRAIN CA2

Stain CA2 was presumptively identified as Alteromonas sp. on the basis of the following characteristics:

- Gram negative rod
- Aerobic
- Oxidase positive
- Requires 250 nM salt
- Motile with polar flagella
- Exopolysaccharide synthesis
- G + C 43% (T_m)

However, Dr. Fred Singleton's analysis of fatty acids revealed a very unusual fatty acid profile with a high proportion of C-14, C-15 fatty acids. This fatty acid profile is not characteristic of the genera Alteromonas.

Further characteristics of strain CA2 include yellow pigment production, oxidation and fermentation of glucose but no gas production, and inability to utilize inorganic sources of nitrogen, such as NH_4Cl or $NaNO_3$ for growth. Catalase was weakly positive. Less than 5% of the fatty acids of CA2 have chain lengths of more than 17 carbon atoms (Table 3). The exopolysaccharides of CA2 bacteria did not react with antibodies to 20 species of Alteromonas.

Table 3. Fatty acid composition of CA2 bacteria (Five Star Laboratories)

Fatty acid	% composition
11:0	2.35
12:0	2.86
13:0 ISO	11.39
13:0	7.56
13:0 ISO 3OH	7.96
14:0	2.11
15:0	12.14
15:0 ISO	10.47
15:1 A	3.02
15:1 B	2.92
16:0	4.63
16:1 CIS 9	11.72
17:1 B	13.22

Sum of unidentified compounds account for 7.65% of total fatty acid composition.

DISCUSSION

Axenic larval Crassostrea gigas were used to determine the effects of additions of single bacterial strains on the survival and growth of larvae cultured with algae. Bacteria can be categorized into adverse, neutral or beneficial groups, depending on their effects upon oyster larvae. Furthermore, bacteria found beneficial in one experiment were re-tested in subsequent experiments and could be further categorized as either variable or consistently beneficial strains. Additions of strain CA2 to larval cultures resulted in consistent enhancement of larval survival (21-22%) and growth (16-21%) compared with that of larvae fed on algae alone.

Specificity of bacteria strains as food for grazers has frequently been reported (Frosch, 1897 in Luck et. al., 1931; Stuart et.al., 1931; Curds and Vandyke, 1966). Furthermore, Curds and VanDyke (1966) found that the effects of one bacteria strain on ciliate cultures differed according to the species of ciliate tested; for example, one bacteria strain was found to be either slightly toxic, unfavorable or favorable according to the ciliate species tested. In contrast, a single bacteria strain (PM-4) was found to promote growth of both shrimp (Penaeus monodon) and crab (Portunus tridentatus) larvae (Maeda, 1988; Maeda and Nogami, 1989). Consequently, no generalization can be

made on the beneficial effects of specific bacteria strains without testing the strain with each target species.

Bacteria may be directly used as a food item by oyster larvae (see Chapter 3). Starved axenic oyster larvae showed poor survival and did not grow after 10 days of culture. In contrast, larvae in cultures inoculated with single bacterial strains or mixtures of naturally-occuring marine bacteria showed higher survival than that of starved larvae, but poor growth rates compared to that of larvae fed on algal diets. Consequently, tested bacteria strains did not provide all the nutritional requirements for larvae, but appeared to at least partially satisfy larval metabolic requirements, as demonstrated by the beneficial effects of bacteria on larval survival and growth. Beese (in Prieur et. al., 1990) determined that xenic, starved larval Crassostrea gigas grew 60% in seven days of culture, whereas no growth was observed with starved axenic larvae. The ability of starved xenic bivalve larvae to grow has been determined to be greater for larvae of the mussel Mytilus edulis than for larval C. gigas (His et al., 1989). However, bacteria lack long-chain polyunsaturated fatty acids (PUFA) (Kates, 1964; Perry et al., 1979) and sterols (Lehninger, 1975) which may be both essential for growth of marine bivalves (Trider and Castell, 1980; Langdon and Waldock, 1981). This lack of essential nutrients could

explain why larvae grew poorly on a diet of bacteria alone compared with growth of larvae fed on algae.

Size frequency distributions of bacteria-free oyster larvae cultured for 10 days on axenic live algae were always positively skewed due to a high proportion of non-growing individuals. Algae were always present in cultures at satisfactory concentrations for larval growth (Breese and Malouf, 1975); therefore, the poor growth of some larvae in populations could not be due to insufficient algal food. In contrast, additions of CA2 bacteria to cultures of algae-fed larvae consistently normalized larval size frequency distributions. Larval survival was equal (Experiment 2; fig. 2) or higher (Experiment 3; fig. 3) in cultures inoculated with strain CA2 than in cultures fed algae alone; therefore, changes in size frequency distributions were not due selective death of slow growing larvae in bacterized cultures. Instead, it would seem that additions of strain CA2 to larvae fed on algae shifted larval size frequency distributions by promoting growth of larvae that would grow poorly on an algal diet alone. This result suggests that some oyster larvae in cultured populations require supplements of bacteria in order to grow and that an algal diet of Isochrysis galbana alone is not sufficient to meet their nutritional requirements.

Inability of a single algal food species to support larval growth rates comparable to those obtained when larvae are fed on mixtures of algal species suggests that diets of single algal species can be nutritionally inadequate for maximum larval growth (Davis and Guillard, 1958, Walne, 1970). Microbes could provide dietary micronutrients, such as vitamins (Kutsky, 1981) or other growth factors that could be deficient in algal diets. Vitamin deficiencies in the media used to culture axenic Artemia have resulted in arrest of growth and early mortality of the crustacean (Provasoli and D'Agostino, 1962). Vitamin supplements increased the growth rate of larval Crassostrea virginica, both when given alone or in combination with Chlorella (Davis and Chanley, 1956). The high nutritional value of bacteria is indicated by bacteria supplements improving the quality of algae or dried diets of different chemical composition which alone did not support growth of crustaceans (Provasoli et al., 1959; Douillet, 1985).

Bacterial enhancement of larval cultures may also have been due to other mechanisms apart from bacterivory. Oyster larvae were grown for 10 days without changing the culture medium, thus metabolites excreted by bivalves (Cockcroft, 1990) and algae (Hellebust, 1974) would accumulate in the larval cultures. It is conceivable that strain CA2 enhanced

larval cultures by removing toxic metabolites. This may have stimulated larval growth, as well as normalized the size frequency distribution by promoting the growth of larvae that were more sensitive than others to the adverse growth effects of metabolites.

Bacteria could also have acted as a symbiont for larvae by contributing to the larva's protein nutrition by means of nitrogen fixation (Benemann, 1973; Carpenter and Culliney, 1975; Guerinot and Patriquin, 1981), or by aiding in the digestion and assimilation of ingested algae. The bacterial flora of bivalve larvae has been found to consist of a high proportion of strains that produce extracellular enzymes, such as proteases and lipases (Prieur, 1982).

Bacteria strain CA2 did not indirectly affect larvae by increasing algal growth and food availability in larval cultures because no enhanced algal growth occurred in the presence of CA2 bacteria.

Larvae did not grow when fed on freeze-killed or ^{60}Co-irradiated Isochrysis galbana, and additions of bacteria did not significantly improve growth of larvae fed on either of the two killed algal diets. ^{60}Co-irradiated algal cells were grazed by larvae at rates that were significantly higher than those for live algal cells. This

suggests that the poor growth of larvae fed on killed algal diets was not due to a lack of available particulate matter, rather it is likely that essential nutrients for oyster larvae were either destroyed or lost from killed algal cells. Supplements of bacteria strains or mixtures of naturally occurring bacteria did not overcome these possible nutritional deficiencies of the killed algal diets.

The ability of larvae of some bivalve species to utilize dead algae as food under xenic conditions has been well documented. Larvae of the mussel Mytilus galloprovincialis grew at similar rates when fed on either live or frozen Monochrysis lutherii (Masson, 1977). Chanley and Normandin (1967) reported comparable growth and survival of larvae of the clam Mercenaria mercenaria when fed on either live or frozen cells of Isochrysis galbana. However, different species of bivalves appear to have different nutritional requirements, as indicated by the findings of Loosanoff (1954) on the ability of M. mercenaria larvae to utilize a greater variety of natural foods than larvae of the American oyster Crassostrea virginica. Larvae of M. mercenaria grew when fed on a diet of lyophilized I. galbana (Hidu and Ukeles, 1962) or frozen I. galbana (Chanley and Normandin, 1967), whereas larval C. virginica did not grow when fed on either of these non-

living diets. Failure of larval Crassostrea gigas to grow when fed on dead algae impeded evaluation of the direct nutritional contribution by bacteria under conditions were potential bacteria-algal interactions were eliminated by the use of killed rather than living algal cells.

In summary, bacteria added as single strains or as natural communities were found to be major sources of variation in cultures of Crassostrea gigas larvae. Selection of a consistently beneficial bacteria (strain CA2) for bivalve larval culture offers a valuable tool for research on the role of bacteria in the nutrition and culture of marine invertebrates. In addition, the use of beneficial microbes in aquaculture may contribute towards reducing undesirable variation in cultures success.

CHAPTER 2

Enhancement of cultures of larval Pacific oysters (*Crassostrea gigas* Thunberg) by addition of a marine bacteria strain.

ABSTRACT

Additions of bacteria strain CA2 as a food supplement to xenic larval cultures of the oyster Crassostrea gigas consistently enhanced growth of larvae during different seasons of the year. Bacterial enhancement of larval growth occurred when either Isochrysis galbana (ISO), I. aff. galbana (T-ISO) or Pseudoisochrysis paradoxa (VA-12) were used as algal foods. Additions of CA2 bacteria at 10^5 cells ml^{-1} to cultures of algal-fed larvae increased larval growth, the proportion of larvae that set to produce spat, and the subsequent size of spat. A lower proportion of slow-growing larvae in populations receiving additions of CA2 bacteria compared with populations of larvae fed only algae, suggests a bacterial nutritional contribution to larval growth. Manipulation of bacterial populations present in bivalve larval cultures is a potentially useful strategy for the enhancement of oyster production.

INTRODUCTION

The Pacific oyster Crassostrea gigas has become one of the most important bivalve species cultured on the Pacific coast of North America. The Pacific Coast oyster industry largely depends on the production of larvae from hatcheries. However, culture of bivalve larvae is characterized by high variability in larval survival and growth (Davis, 1953; Loosanoff, 1954; Walne, 1956b), and by considerable variation in the proportion of larvae which successfully metamorphose (Walne, 1958). Genetic sources of variation have been determined to be significant for oyster larval survival (Lannan, 1980) and growth (Newkirk et al., 1977). Non-genetic sources of variation in larval cultures include larval density (Loosanoff et al., 1953) size of container (Dupuy, 1975), algal exudates of unsatisfactory algal species (Bayne, 1965), age of algal food (Dupuy, 1975); quality and quantity of algal food, temperature, pH, silt and toxicants (see review Bayne, 1983). Bacteria have been suggested to play an important part in influencing larval culture success by many researchers, including Guillard (1957), Walne (1958, 1966) and Prieur (1982).

Sources of bacteria in larval cultures of bivalves include seawater, algal foods, and to a lesser extent, gametes from adult oysters, and airborne contamination

(Walne, 1966; Prieur, 1981). Bacterial growth is stimulated by high concentrations of organic nutrients available in cultures of bivalve larvae (Manahan and Stephens, 1983) and by confinement of water in batch culture techniques (Zobell and Anderson, 1936). Water treatments such as filtration, ultraviolet radiation and heating modify the generic composition of the microflora, but do not generally cause permanent changes in bacteria concentrations in larval cultures (Murchelano et al., 1975; Prieur and Carval, 1979). Current hatchery management practices aimed at controlling opportunistic pathogens include maintenance of pathogen-free algal stocks, use of pathogen-free seawater (filtration, UV treatment), frequent water changes, application of hygienic practices and disinfection of equipment (Elston, 1984).

Bacteria belonging to the genus Pseudomonas and Vibrio have been identified as the most common epizootic agents in cultures of bivalve mollusks (Tubiash et al., 1970; Brown, 1973). The digestive systems of oyster larvae seem to provide suitable conditions for growth of Vibrio (Prieur, 1981), which could lead to increases in the proportion of bacteria of this genus in the culture environment (Kogure et al., 1980). Therefore, conditions optimal for opportunistic, undesirable bacteria strains are commonly provided in bivalve hatcheries.

Attempts have been made to enhance larval cultures of bivalves by modifying the microbial flora. Antibiotics (Combistrep) were added to larval cultures to repress pathogenic strains, while favoring growth of "beneficial" bacteria (Hidu and Tubiash, 1963). However, additions of antibiotics to cultures of bivalve larvae by other researchers have resulted in variable and even adverse effects on larval survival and growth (Le Pennec and Prieur, 1977). Furthermore, resistant strains of bacteria are created by systematic use of antibiotics (Jeanthon et al., 1988).

Another approach to modify the microbial flora of bivalve cultures has involved inoculations of a single "beneficial" bacterial strain to cultures of bivalve larvae (Martin and Mengus, 1977). Unfortunately, Martin and Mengus (1977) reported that the beneficial effects of the added bacteria strain were not consistent in repeated experiments.

In order to determine the effects of a specific bacterial strain on a cultured organism, other microbes should be excluded from the culture system in order to avoid microbial interactions (Bull and Slater, 1982) that may have a direct effect on the system. Additions of a bacterium (strain CA2) to axenic cultures of larval

Crassostrea gigas fed axenic Isochrysis galbana enhanced survival and growth of larvae in repeated experiments (Chapter 1). In contrast, naturally occurring microflora present in seawater had adverse effects on the survival and growth of algal-fed oyster larvae cultured under aseptic conditions (Chapter 1). The objectives of this research were to determine if enhancement of oyster larvae cultures resulting from additions of CA2 bacteria was affected by: a) the presence of naturally occurring bacteria present in hatchery culture conditions at different seasons of the year, and b) the algal food species used to culture larvae.

METHODS

CULTURE TECHNIQUES

Oyster larvae were obtained by either spawning conditioned broodstock oysters by thermal stimulation (cyclic temperature changes between 25 and 30°C; Breese and Malouf, 1975) or by stripping broodstock oysters (Loosanoff and Davis, 1963). Spawning was carried out at the Whiskey Creek Hatchery at Netarts Bay, Oregon, or at the Hatfield Marine Science Center in Newport, Oregon. Larvae were raised using traditional hatchery methods (Breese and Malouf, 1975). Culture seawater was replaced every second day using sand-filtered seawater from Yaquina Bay, Oregon (salinity 28 to 32 ppt, temperature 25° C). Larvae were collected on a 65 μm Nitex screen at each water change, in order to retain larvae of all sizes in the cultured populations.

Larvae were raised in glass (Pyrex) beakers containing 1 l of seawater. Initial larval density was 5 ml^{-1} except in Experiment 4 where the initial density was 10 larvae ml^{-1}. Cultures were agitated on an orbital shaker in a temperature-controlled room at 25° C. The beakers were illuminated with cool-white fluorescent light at an intensity of 50-70 lux, under a 12 h light / 12 h dark photoperiod. Seawater for each water change was sand-

filtered and brought to culture temperature by overnight storage in in 100 l "Nalgene" tanks held at 25° C.

DIETS

The three phytoplankton species used as food in the culture experiments were Isochrysis galbana Parke (clone ISO), Isochrysis aff. galbana (clone T-ISO) and Pseudoisochrysis paradoxa (clone VA-12). Algal clones were obtained from the Culture Collection of Marine Phytoplankton (Maine). Algal cultures were grown in 100 l "Nalgene" tanks or in 20 l glass flasks in f/2 medium (Guillard and Ryther, 1962). Cultures were constantly illuminated with cool-white fluorescent light at an intensity of 800-1500 lux and at a temperature of 18-20° C. Algal cultures growing in exponential phase were harvested and added as food for larvae at each water change. The algal ration added to larvae at the beginning of the culture period was 40,000 cells ml^{-1} (Nascimento, 1980). This ration was increased by 15,000 cells ml^{-1} at each feeding over the first 10 days of culture. From the tenth to the thirtieth day of culture, the algal ration was maintained at 100,000 cells ml^{-1}. Algal concentrations were determined with a Coulter Counter (Model ZB1) used in conjunction with a Coulter Channelyser (Model 256).

A bacterium (strain CA2) found to enhance survival and

growth of axenic oyster larvae fed axenic Isochrysis galbana (Chapter 1) was selected for testing with cultures of oyster larvae in the presence of naturally occurring microbial contaminants. CA2 bacteria was obtained from stocks cryopreserved at -70° C in 10% (V/V) glycerol in sterile 1/10 diluted Marine Broth 2216 (Chapter 1). Bacteria were thawed and cultured at 20-25° C on Marine Agar 2216 for 3-4 days, re-suspended for 24 hours in 0.2 μm-filtered and autoclaved seawater (FSSW), then centrifuged at 20,000 x g for 10 minutes. The centrifuged bacteria were finally re-suspended in 35 ml FSSW in order to obtain a concentrated stock suspension for feeding larvae. Densities of CA2 bacteria were estimated by measuring absorbances at 600 nm, using a Beckman DU-6 spectrophotometer. Cell concentrations were then calculated from absorbance values, using an equation previously established for strain CA2, relating absorbance to bacteria concentration:

$$\text{CA2 cells ml}^{-1} = (2.034\text{x}10^{9} \times \text{absorbance}) + 8.129\text{x}10^{5} \quad (r = 0.99)$$

CA2 cells were added to larval cultures after each water change. The bacterial inoculum for each treatment was not varied during the larval culture period.

EXPERIMENTAL DESIGN

The effects of different concentrations of CA2 cells on survival and growth of oyster larvae were evaluated in Experiments 1 and 2 which were carried out at 25° and 20° C, respectively. CA2 bacteria were added at four concentrations (10^4, 10^5, 10^6, 10^7 cells ml^{-1}) to cultures of oyster larvae fed Isochrysis galbana. Algae and CA2 bacteria were added every second day after replacing the culture seawater. Control treatments included starved larvae and larvae fed only algae. Experiments 1 and 2 were carried out from October 16 to November 15 1987 and from February 3 to March 5 1988, respectively.

Repeatable enhancement of oyster larvae cultures by the addition of CA2 bacteria was tested at two additional times of the year in Experiments 3 and 4, carried out from June 10 to June 20 and from October 17 to October 27 1988, respectively. Larval cultures fed Isochrysis galbana were supplemented with CA2 bacteria at the optimal cell concentration determined in Experiments 1 and 2 (10^5 cells ml^{-1}). Control larval cultures were starved or were fed only algae.

Enhancement of oyster larvae production by additions of CA2 bacteria to larvae fed on different algal species was determined in Experiment 5. Cultures of larvae fed T-

ISO or VA-12 received additions of 10^5 CA2 cells ml^{-1} (final concentration) after every water change. Control cultures included starved larvae, larvae fed only algae or larvae fed only CA2 bacteria every two days. Four replicate cultures were tested per treatment in all experiments. Experiment 5 was carried out from January 21 to February 20, 1989.

DATA COLLECTION AND ANALYSIS

At the beginning of each experiment (day 0), shell lengths of 30 randomly selected veliger larvae (straight-hinged stage) were determined by measuring the distance from the anterior to the posterior edges of the shells with a image analyzer (Zeiss Videoplan 2). After 10 days culture, the contents of each culture beaker were poured through a 64 μm Nitex screen and the screened larvae were then re-suspended in 25 ml FSSW. The larval suspension was thoroughly stirred with a perforated plastic plunger to ensure uniform distribution of larvae and four samples of 0.5 ml were withdrawn and stored in scintillation vials containing 100 μl of 10% buffered formalin (pH=8). Rose of Bengal was added to stain larval tissues, permitting visual separation of empty shells from shells that contained healthy larval tissue at the sampling time. Larvae in each sample were counted and the shell lengths of 30 to 50 randomly selected larvae were determined as described

above.

Survival and growth data were transformed where necessary to satisfy assumptions of ANOVA. Survival data was transformed as:

$$\arcsin\left(\sqrt{\text{percent survival } 100^{-1}}\right)$$

Growth data was transformed as:

$$\arcsin\left(\sqrt{(\ln L_t - \ln L_0)\, t^{-1}}\right)$$

where;

L_t = final mean shell length (μm)
L_0 = initial mean shell length (μm)
t = culture period (10 days)

Survival and growth of larvae under the different treatments were analyzed using one or two-way ANOVA. Where significant differences were indicated among treatment effects, Tukey's honestly significant difference test (T-HSD) was applied to determine the statistical significance of differences among individual treatments, at the 0.05 level of probability. Data that were heterocedastic after transformation (Cochran's test for homogeneity of variances, at the 0.05 level of probability) were analyzed by the Kruskal-Wallis test, at the 0.05 level of probability. Differences among individual treatment effects

were determined by the Games and Howell range test (G&H; Sokal and Rohlf, 1981) for equality of means with heterogeneous variances, using the computer program Biom (Rohlf, 1982). All other statistical tests were performed with the computer program Statistix (NH Analytical Software).

Additions of CA2 bacteria to cultures of bacteria-free oyster larvae fed axenic Isochrysis galbana resulted in growth enhancement of slow-growing individuals and consequently affected the shape of larval size frequency distributions (Chapter 1). To evaluate if this effect also occurred in the presence of microbial contaminants, skewness coefficients (g1; Sokal and Rohlf, 1981) were calculated for size frequency distributions from each larval population fed algae alone or algae supplemented with CA2 bacteria. These coefficients were analyzed by one or two-way ANOVA, after confirmation of parametric assumptions.

After sampling larvae from 10 day-old cultures, remaining larvae were cultured for an additional twenty days. Clean, aged scallop shells were added as cultch to each culture flask as soon as larvae developed eye-spots. After a total of 30 days culture, spat set on cultch and glass surfaces of culture containers were counted and their

shell lengths measured from the anterior to the posterior edges of their shells, using an eye-piece micrometer fitted in a dissecting microscope. Statistical differences in larvae set and shell length of spat among treatments were tested using one-way ANOVA, following confirmation of homogeneity of variances with Cochran's test, at the 0.05 level of probability. Where significant differences were indicated, treatment means were compared by Tukey's HSD (T-HSD) test, at the 0.05 probability level.

RESULTS

Larval survival and growth were significantly affected (ANOVA, $p<0.01$) by supplemental feeding with different concentrations of CA2 bacteria (Appendix III). Additions of CA2 cells to cultures of oyster larvae at 10^7 cells ml^{-1} depressed (T-HSD, $p<0.05$) both survival and growth compared to that of larvae fed on Isochrysis galbana alone (Fig.8). Bacteria supplied at the three lower concentrations (10^4, 10^5 and 10^6 cells ml^{-1}) did not affect larval survival (T-HSD, $p>0.05$), but did enhance larval growth (T-HSD, $p<0.05$). The number of larvae that successfully set and metamorphosed and subsequent shell lengths of the spat after 30 days culture were significantly different (ANOVA, $p=0.02$; $p<0.01$, respectively) among treatments. However, numbers of spat from larvae fed only algae were not significantly different from those from larvae fed algae and CA2 bacteria (Fig.9). Shell lengths of spat were significantly larger (T-HSD, $p<0.05$) in cultures receiving 10^4 and 10^5 CA2 cells ml^{-1} than for spat fed only algae.

Experiment 1 was repeated at a temperature of 20° C instead of a culture temperature of 25° C used in all other experiments. The differences observed in Experiment 2 were: a) survival of larvae in cultures receiving CA2 bacteria at the two lower bacterial concentrations was reduced (but not

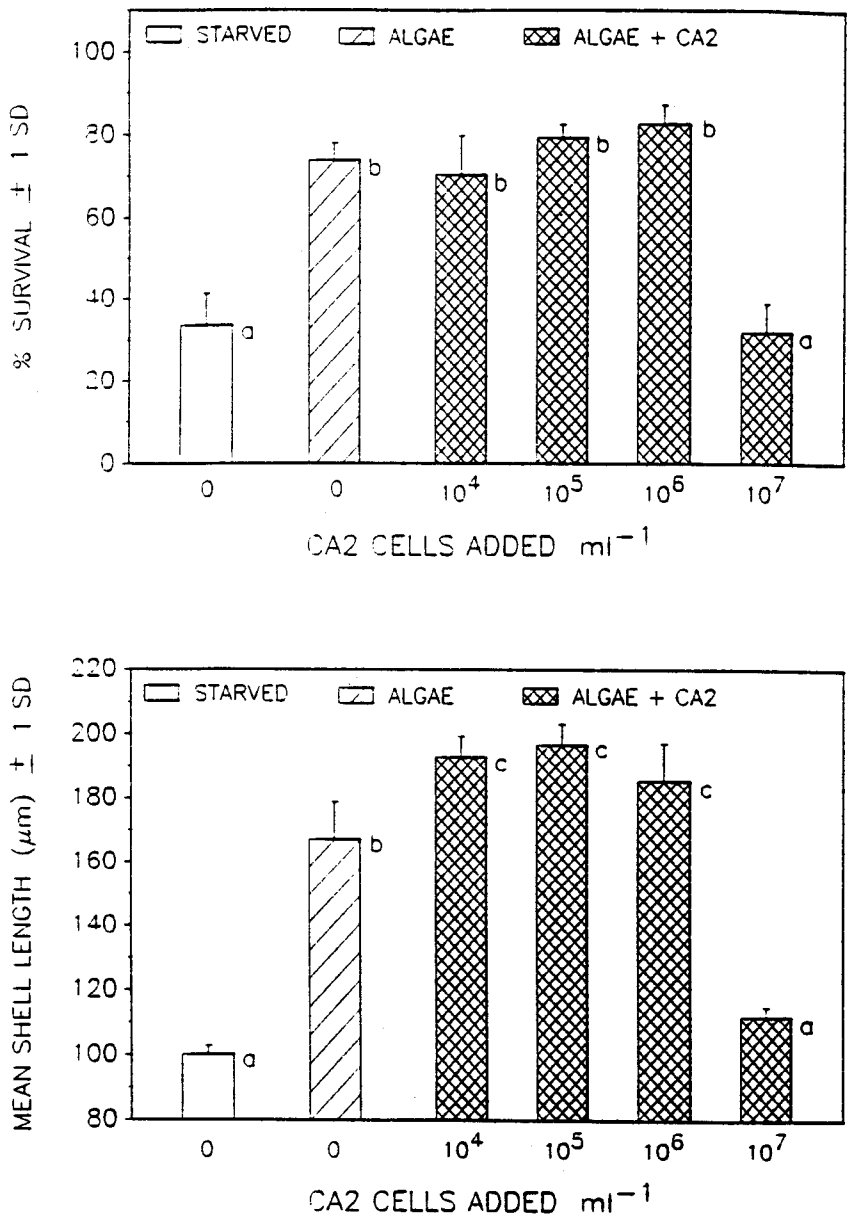

Fig.8. Experiment 1. Survival and growth of oyster larvae at 25° C after 10 days of culture fed on a diet of Isochrysis galbana supplemented with different concentrations of CA2 bacteria. Treatments with common superscripts are not significantly different (Tukey's HSD pairwise comparisons test, $p<0.05$).

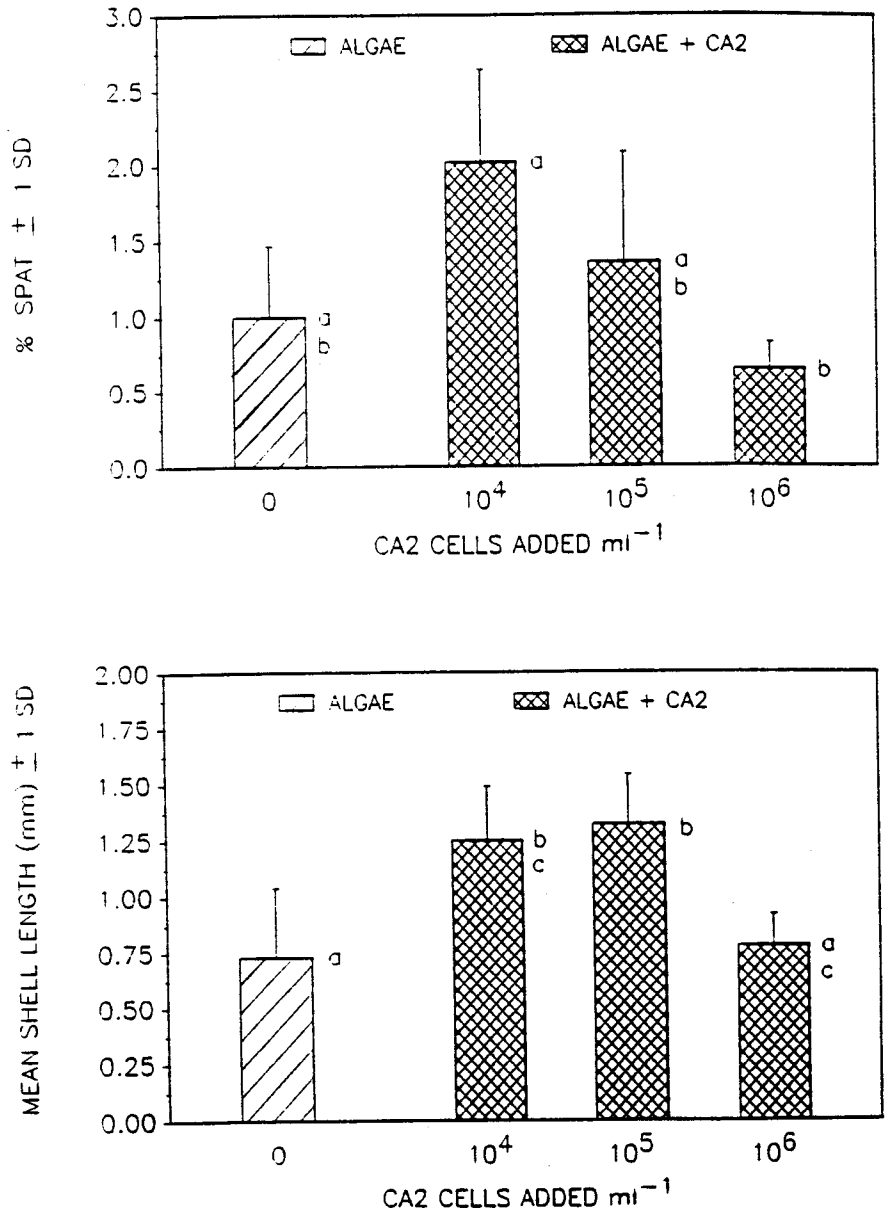

Fig.9. Experiment 1. Percent initial larvae set at 25° C after 30 days of culture and mean shell lengths of spat in cultures fed Isochrysis galbana, supplemented with different concentrations of CA2 bacteria. Treatments with common superscripts are not significantly different (Tukey's HSD pairwise comparison test, $p<0.05$).

significantly, T-HSD $p>0.05$) compared with that of larvae fed algae alone (Fig.10), and b) the enhancement of larval growth in cultures receiving CA2 bacteria at the three lower concentrations (10^4, 10^5 and 10^6 cells ml^{-1}) was not significant. No spat settlement occurred under any of the treatments after 30 days of culture.

No significant differences in larval survival (two-way ANOVA, $p=0.35$; Appendix III) or growth (two-way ANOVA, $p=0.63$) were detected between Experiments 3 and 4. Larval survival was found not to differ (two-way ANOVA, $p=0.06$) among feeding treatments (starved, algae, algae + CA2). In contrast, a significant effect of feeding treatment was determined for larval growth (two-way ANOVA, $p<0.01$). Data from both experiments were combined and analyzed by one-way ANOVA, followed by Tukey's HSD test. CA2 bacteria added at 10^5 cells ml^{-1} significantly enhanced (T-HSD, $p<0.05$) growth of larvae compared to that of larvae fed on algae alone (Fig.11).

In Experiment 5, larval survival differed (two-way ANOVA, $p=0.04$; Appendix III) among the 3 food regimes (no algae, T-ISO, VA-12) but was not influenced by additions of CA2 bacteria (two-way ANOVA, $p=0.2$). Survival of starved larvae and larvae fed CA2 bacteria alone were similar to those of larvae fed on diets containing algae (Fig.12).

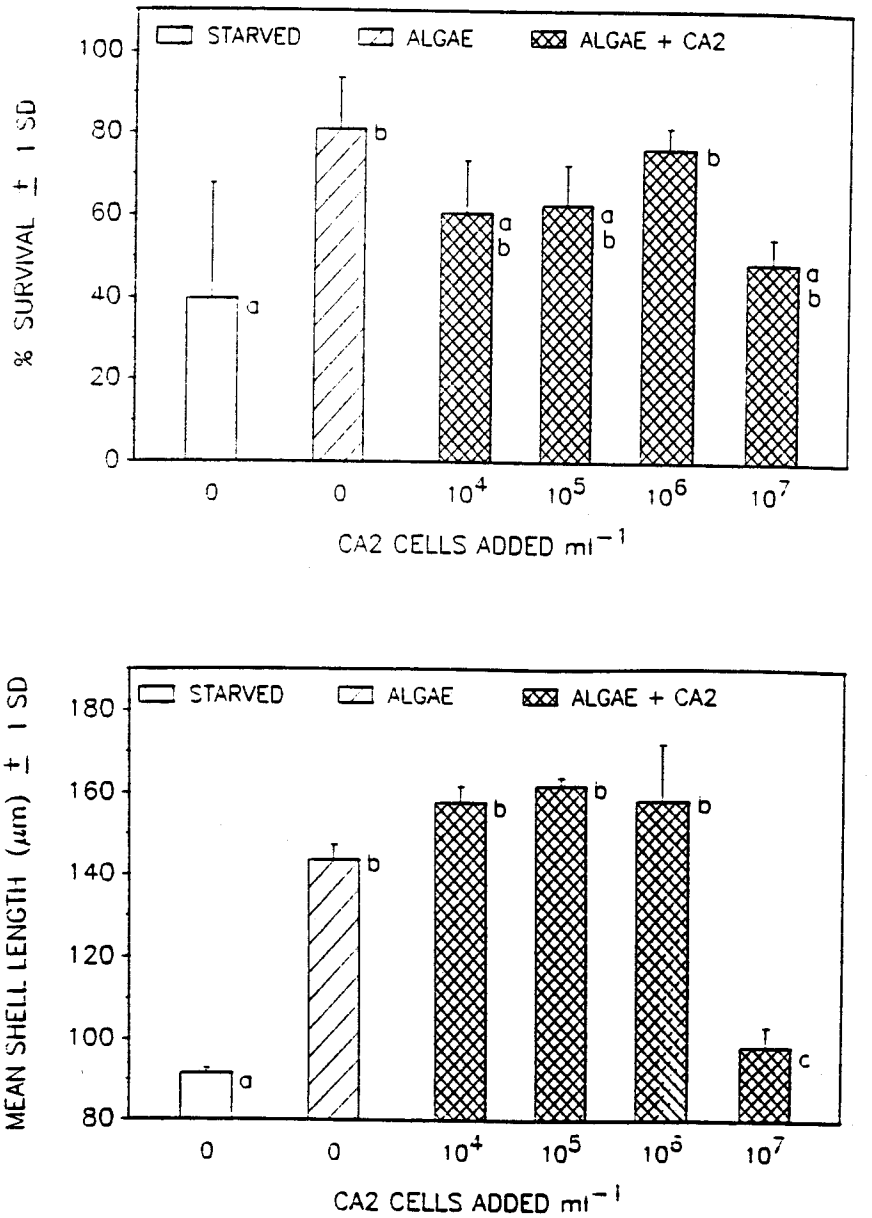

Fig.10. Experiment 2. Survival and growth of oyster larvae at 20° C after 10 days of culture on Isochrysis galbana, supplemented with different concentrations of CA2 bacteria. Survival and growth data analyzed by Games and Howell's and Tukey's pairwise comparisons tests, respectively; both tests at 0.05 % level of significance. Treatments with common superscripts are not significantly different.

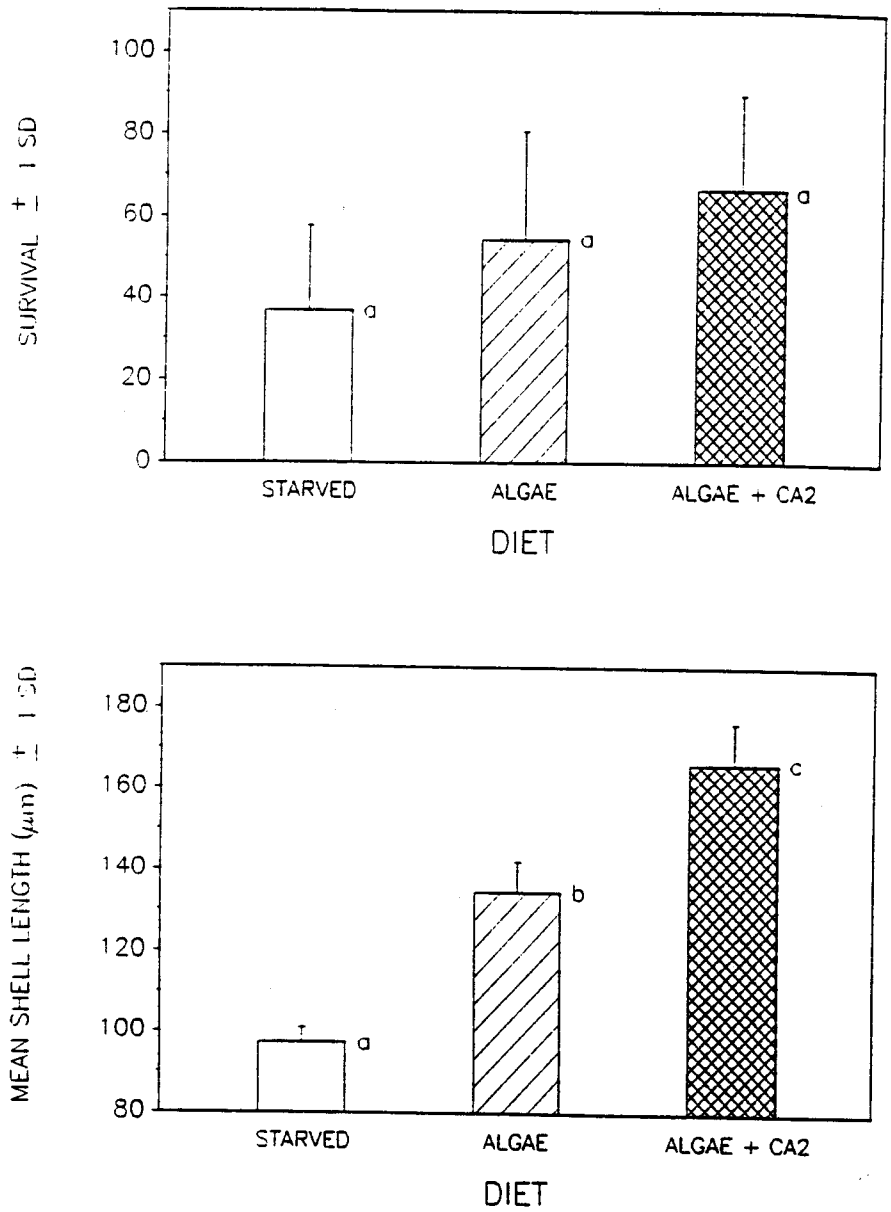

Fig.11. Experiments 3 and 4. Survival and growth of oyster larvae after 10 days of culture on Isochrysis galbana, with or without supplements of CA2 bacteria at 10^5 cells ml^{-1}. Treatments with common superscripts are not significantly different (Tukey's HSD pairwise comparison test, $p<0.05$).

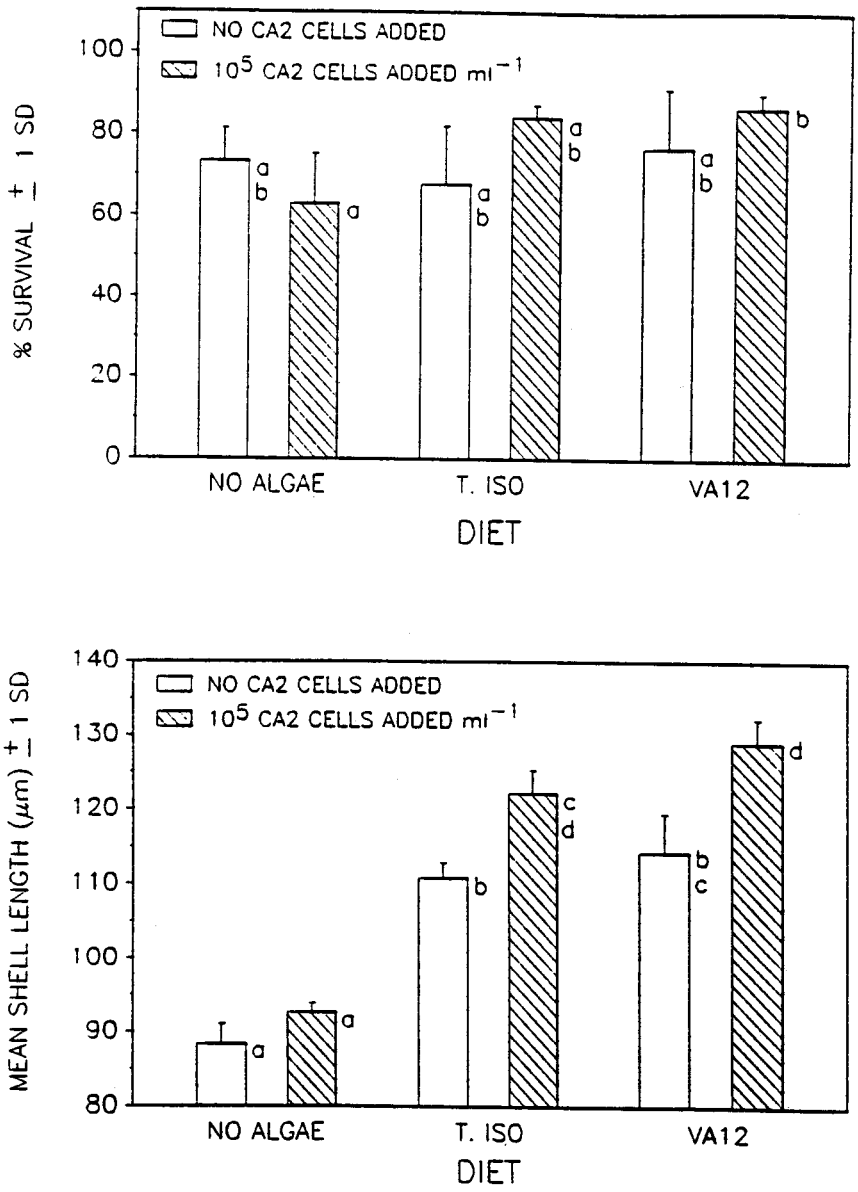

Fig.12. Experiment 5. Survival and growth of oyster larvae after 10 days of culture on either Isochrysis aff. galbana clone T-ISO or Pseudoisochrysis paradoxa clone VA-12, with or without additions of CA2 bacteria at 10^5 cells ml^{-1}. Treatments with common superscripts are not significantly different (Tukey's HSD pairwise comparisons test, $p<0.05$).

Larval growth was found to be significantly (two-way ANOVA, $p<0.01$) affected by different food regimes (no algae, T-ISO, VA-12) as well as by additions of CA2 cells. Bacteria enhanced (T-HSD, $p<0.05$) growth of larvae fed on either of the algal species (T-ISO and VA-12). Shell lengths of larvae fed on CA2 bacteria alone were not significantly different from those of starved larvae.

After 30 days of culture, significant differences (ANOVA, $p<0.01$; Appendix III) in spat numbers were found among treatments (Fig.13). No larvae metamorphosed when fed on T-ISO alone while a few larvae metamorphosed in all cultures receiving T-ISO supplemented with CA2 bacteria. A significantly higher proportion (T-HSD, $p<0.05$) of larvae metamorphosed to produce spat in cultures fed VA-12, and this proportion was further significantly increased (T-HSD, $p<0.05$; Fig.13) when CA2 was added as a supplement. Growth of spat was not found statistically different among treatments in which spat settlement occurred (T-HSD, $p=0.21$).

Average skewness coefficients from size frequency distributions of larvae fed either algae alone or algae and CA2 bacteria ($1x10^5$ cells ml^{-1}) for all experiments are presented in Table 4. Skewness coefficients were found to be significantly (two-way ANOVA, $p< 0.01$; Appendix IV)

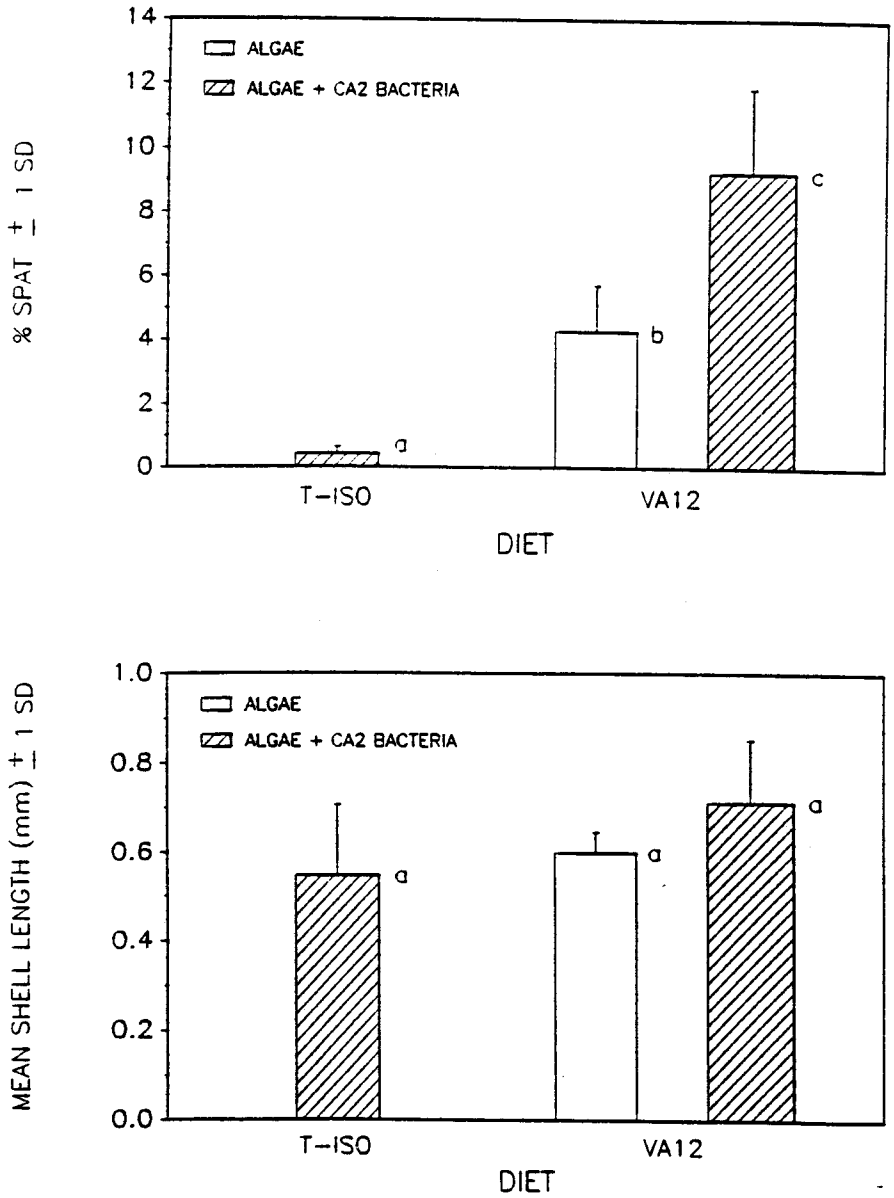

Fig.13. Experiment 5. Percent initial larvae set after 30 days of culture and mean shell length of spat fed on either Isochrysis aff. galbana clone T-ISO or Pseudoisochrysis paradoxa clone VA-12, with or without additions of CA2 bacteria at 10^5 cells ml^{-1}. Treatments with common superscripts are not significantly different (Tukey's HSD pairwise comparisons test, $p<0.05$).

Table 4. Mean skewness coefficients (g1) of size frequency distributions determined in populations of larvae fed on either algae alone or algae with CA2 bacteria added at 10^5 cells ml^{-1}. n=4 per treatment.

		ALGAE ONLY		ALGAE + CA2		ANOVA
EXPERIMENT	ALGAL FOOD	MEAN g1	SD	MEAN g1	SD	Sig. level
1	ISO	-0.602	0.379	-1.056	0.656	0.643
2	ISO	-0.993	0.275	-0.840	0.562	0.276
3	ISO	0.195	0.248	-0.247	0.179	0.028
4	ISO	0.415	0.271	-0.522	0.354	0.006
5	T-ISO	-0.232	0.300	-0.744	0.329	0.061
5	VA-12	0.096	0.212	-0.566	0.219	0.005

different among experiments as well as between feeding treatments (algae and algae + CA2 bacteria). No differences between skewness of larval populations fed algae either alone or with CA2 bacteria were detected in Experiments 1, 2 and 5 (T-ISO), while significant differences were found for Experiments 3, 4 and 5 (VA-12) (Table 4). In the three latter experiments (3, 4 and 5 (VA-12)), the skewness coefficients of populations of larvae fed algae alone were higher (T-HSD, $p<0.05$) than those of populations of larvae fed algae and CA2 bacteria, indicating a lower proportion of small-sized larvae in cultures supplemented with CA2 cells.

DISCUSSION

Additions of strain CA2 to xenic cultures of oyster larvae fed Isochrysis galbana (clone ISO) consistently enhanced growth of oyster larvae regardless of the season of the year. In other words, possible seasonal variation in the bacteria species composition in seawater from Yaquina Bay, Oregon, did not affect enhancement of larval growth due to additions of CA2 bacteria.

Seasonal variation in larval survival and growth was evident in this study. Differences in larval growth, spat yields and spat growth of Ostrea edulis were reported for broods obtained from broodstock spawned over an extended season (Helm et al., 1973). Survival of larval Crassostrea gigas was found to depend largely on the gametogenic condition of the parents (Lannan, 1980). Broodstock conditioning techniques (Loosanoff and Davis, 1963) allow larvae to be obtained throughout most of the year. The duration of the conditioning period has been reported to be a critical factor in determining production of "optimal quality" eggs (Lannan et al., 1980). However, the latter authors reported seasonal differences in spat production regardless of the duration of the conditioning period.

A concentration of 10^5 CA2 cells ml^{-1} was found optimal

for the enhancement of larval cultures. Bacteria concentrations in healthy cultures of bivalve larvae are normally between 10^4 and 10^6 cells ml^{-1} (Jeanthon et al., 1988). The beneficial effects of strain CA2 were not algal-species specific, as indicated by significant growth improvements of larvae fed either Pseudoisochrysis paradoxa (clone VA-12) or Isochrysis aff. galbana (clone T-ISO) when supplemented with CA2 bacteria. The only case where bacterial enhancement of larval growth was not significant was in Experiment 2, which was carried out at 20° C. All other experiments were carried out at 25° C. Seasonal effects were not likely to have been responsible for the absence of CA2 bacterial enhancement of larval growth in Experiment 2, because significant enhancement of larval growth in the presence of supplements of CA2 bacteria was evident in Experiment 5, which was carried out at the same time of the year (winter) as Experiment 2. Culture temperature may have been an important factor in determining the effects of CA2 bacteria, and requires further investigation.

Bacteria have been reported to beneficially interact with animals in various ways. Many organisms remain free of pathogenic bacteria or fungus by maintaining a symbiotic bacterial flora (Zobell, 1946; Marpes, 1969; Fisher, 1983). Nitrogen-fixing bacteria found in guts of marine

invertebrates may contribute to the protein budget of the host (Carpenter and Culliney, 1975; Guerinot and Patriquin, 1981). Microbes can also improve the digestive capabilities of bacterivorous consumers by liberating active enzymes into the digestive system of the consumer (Martin and Kukor, 1984). Prieur (1982) reported a high proportion of bacteria strains able to release extracellular enzymes, such as proteases and lipases in the guts of bivalve larvae. Beneficial bacteria strains with antibiotic properties have been isolated and used to enhance survival of shrimp and growth of crabs in aquaculture (Maeda, 1988; Maeda and Nogami, 1989). The extracellular products of other beneficial bacteria strains act as inducers of setting of bivalve larvae (Alteromonas clone LST; Weiner et al., 1982) and sea urchin larvae (Pseudomonas sp.; Miller, 1989).

The shape of a size frequency distribution can be described by its skewness coefficient (g1; Sokal and Rohlf, 1981). For a normal distribution, the g1 coefficient is 0. Positive values indicate positive skewness (distributions with greater proportions of small-sized individuals), while negative values indicate negative skewness (distributions with greater proportions of large-sized individuals). Populations of axenic larvae fed axenic Isochrysis galbana had g1 coefficients ranging from 0.38 to 0.79. In contrast,

populations of axenic larvae cultured on algae and strain CA2 had g1 coefficients ranging from -0.04 to -0.06 (Chapter 1). Selective mortality of slow growing larvae would affect size frequency distributions; however, larval survival rates were higher in cultures receiving additions of strain CA2 than in cultures not receiving the bacterial supplement. Furthermore, larvae were not limited by the quantity of algae available during the culture period (Breese and Malouf, 1975). Consequently, growth enhancement of small larvae may be attributed to CA2 bacteria supplying: a) essential nutrients not present in algae to some individuals in the population, or b) enzymes which could enhance larval digestive processes. The bacteria gut flora of bivalve larvae has been found to consist of a high proportion of strains that produce extracellular enzymes, such as proteases and lipases (Prieur, 1982)

Skewness coefficients determined from size frequency distributions of populations of larvae fed algae under xenic culture conditions were not as skewed as those from cultures fed algae under bacteria-free conditions (Table 4, this chapter; Table 1, Chapter 1), except for Experiment 4. Naturally-occurring microbes present in culture media possibly reduced the proportion of small individuals in populations of xenic algal-fed larvae, in a similar way to that observed when CA2 bacteria were added to axenic

cultures of algal-fed larvae. Nevertheless, additions of CA2 cells to xenic cultures significantly reduced the proportion of small larvae in populations examined in Experiments 3, 4 and 5 (VA-12).

A nutritional contribution of naturally-occuring bacteria to oyster larvae was evident in experiments where survival of starved axenic larvae (Chapter 1) was significantly lower after 10 days of culture than that of starved xenic larvae (this chapter). Furthermore, in Experiments 3, 4 and 5 in this chapter, survival of starved larvae was not different from that of larvae fed algae (Figs. 11 and 12). Enhancement of larval survival by naturally occuring microflora present in seawater may explain why additions of CA2 cells enhanced survival of axenic algal-fed larvae (Figs. 2 and 3, Chapter 1) but did not enhance survival of xenic larvae fed algae (Figs. 8, 11 and 12, this chapter).

Another reason for the shift in larval size frequency distribution associated with addition of CA2 bacteria may have been that CA2 bacteria removed metabolic substances released by bivalves (Cockroft, 1990) or algae (Hellebust, 1974), which could have adversely affected larval growth. However, seawater was changed every second day in xenic culture experiments and significant accumulation of

metabolic products is unlikely.

Additions of CA2 bacteria significantly improved the proportion of larvae that metamorphosed to produce spat (Experiment 5) and significantly increased the subsequent size of spat after 30 days of culture (Experiment 1) compared with that of algal-fed controls. However, CA2 bacteria could have indirectly enhanced spat growth through beneficial effects on larval growth and development. It is not known whether survival and growth of spat was directly affected by additions of CA2 cells.

In summary, experimental evidence suggests that strain CA2 enhanced cultures of oyster larvae fed on Isochrysis galbana (ISO), I. aff. galbana (T-ISO) and Pseudoisochrysis paradoxa (VA-12) by providing essential nutrients not present in these algal diets or by improving their digestion by supplying digestive enzymes to the larvae. Supplemental feeding of bivalve larvae with CA2 bacteria may be included in current culture practices of bivalve hatcheries to improve production. However, further research to determine conditions that enhance the beneficial effects of strain CA2 should be conducted prior to full-scale commercial application.

CHAPTER 3

Bacterivory in Pacific Oyster Larvae *Crassostrea gigas* (Thunberg)

ABSTRACT

A bacterium (strain CA2) beneficial to the culture of larvae of the oyster Crassostrea gigas was used in a series of experiments to determine the occurrence and potential contribution of bacterivory to the nutrition of bivalve larvae. Size and carbon content of this bacterium was found to be within the range reported for naturally-occurring marine bacteria. Free-swimming, DAPI-stained CA2 cells were readily captured and ingested by oyster larvae and were seen to accumulate in the digestive systems of oyster larvae. Ingestion of ^{14}C-labelled bacteria was significant at bacteria concentrations higher than $5x10^6$ cells ml^{-1}. Experimental methods that reduced label recycling in ^{14}C-feeding trials permitted determination of larval retention efficiencies for bacterial and algal (Isochrysis galbana) carbon when these foods were provided to larvae at equivalent carbon concentrations. Straight-hinged oyster larvae fed CA2 bacteria at $1x10^7$ cells ml^{-1} could meet 140% of their carbon requirements with bacterial carbon retained in "pulse-chase" ^{14}C-feeding experiments. Furthermore, this bacterial carbon represented 46% of the carbon retained by straight-hinged larvae fed on a similar concentration of algal carbon (I. galbana at 54 cells μl^{-1}). The potential contribution of bacterial carbon towards the metabolic carbon requirements of oyster larvae decreased as larvae

grew in size. This is the first reported study of the potential role of bacteria in meeting the carbon requirements of bivalve larvae.

INTRODUCTION

Utilization of bacteria as a food source by marine animals has been the subject of many investigations (e.g. Zobell and Feltham, 1938; Zhukova, 1963; Sorokin, 1968; Boucher and Chamroux, 1976). Zobell and Feltham (1938) have shown that bacteria, as the only particulate food source, could support growth of adult mussels Mytilus californianus. Ingestion and digestion of bacteria have been demonstrated with clams Venus verrucosa (Amouroux, 1986) and Mulinia lateralis (Chalermwat et al., 1991), the oyster Crassostrea virginica (Crosby et al., 1990) and mussels Aulacomya ater (Stuart et al., 1982) and Choromytilus meridionalis (Muir et al., 1986). In contrast, Gillespie et al. (1966) reported no growth of the oyster C. virginica fed on several strains of marine bacteria, and Langdon and Newell (1990) reported that bacterial carbon contributed only 5.5% to the metabolic carbon requirements of adult oysters (C. virginica). Major differences in the activity and distribution of lysozyme-like activity among organs (digestive gland, style, gill, mantle and foot) of adult bivalves could explain why bivalve species differ in their ability to utilize bacteria as a food source (McHenery et al., 1986).

Studies on bacteriolytic activity in bivalve larvae

have not been published and there are conflicting reports on the ability of larvae to utilize bacteria as a source of nutrition. Riisgard et al. (1980) found an apparent decrease in clearance rate of larval Mytilus edulis fed on particles smaller than 2.5 μm and concluded that particles smaller than 1 μm diameter could not be filtered by 13 day-old veligers. Davis (1950; 1953) found that thirteen strains of bacteria did not support growth of larval C. virginica. In contrast, Prieur (1981) using scanning electron microscopy, presented evidence of ingestion of bacteria by mussel larvae. Baldwin and Newell (Horn Point, University of Maryland; unpublished data) found that larval C. virginica accumulated ^{3}H-activity when exposed to 0.2-0.8 μm sized, naturally-occurring particles labelled with ^{3}H-thymidine, and Gallager (Woods Hole Oceanographic Institution, Massachusetts, unpublished data) demonstrated ingestion of Synechococcus sp. (1 x 0.5 μm in size) by larval Mercenaria mercenaria. Accumulation of radioactivity by larval Mytilus galloprovinciallis fed ^{14}C-labelled bacteria has been documented by Mengus (1978); however, the long exposure period (>2 h) of larvae to labelled bacteria may have permitted recycling of the label, resulting in indirect uptake of radioactivity rather than direct uptake through bacterivory. Nutrient fluxes between bacteria, bacterivorous protozoa and algae, as described by Berman et al. (1987), may also occur in a system with bacteria,

bivalve larvae and algae. Recycling of radiolabel should be a cause for concern, considering reported significant uptake of dissolved organic matter by bivalve larvae (Manahan and Richardson, 1983).

In order to determine if oyster larvae exhibited bacterivory, both ingestion of bacteria cells and assimilation of bacterial cellular products should be clearly demonstrated. Difficulties associated with measuring assimilation of food by zooplankton have been discussed by Johannes and Satomi (1967), Conover and Francis (1973) and Lampert (1977). Several methods proposed for determination of assimilation efficiencies cannot be used with bivalve larvae because of methodological considerations or violations of the assumptions on which these methods are based. One of these methods consists of measuring the radioactivity of animals at two different times after the gut is filled with radioactive food (Rigler, 1971). Possible problems in larval-feeding studies with this technique include label recycling while the larvae are filling their guts with radioactive food as well as non-linearity in the accumulation of radioactivity by larvae. The ^{14}C-^{51}Cr dual tracer method (Calow and Fletcher, 1972) has been used with bivalve larvae (Nelson and Siddall, 1988); however, sources of error discussed by these authors included different gut passage times for the

two isotopes, dissolution of feces, and the confounding effects of bacterial and algal respiration.

Another ^{14}C-method for determination of carbon assimilation has been described by Rigler (1971) and consists of feeding animals for a definite period of time with radioactive food, followed by transfer of animals to non-radioactive food to purge their guts of radioactive material, and finally measurement of the remaining radioactivity incorporated into the animals' tissues. Losses of ^{14}C through respiration and excretion should be added to the radioactivity incorporated by the grazers in order to determine assimilated ^{14}C. Respiration of ^{14}C by the grazers should be determined during the feeding period on radioactive food (Lampert, 1977) as well as during the purge period with non-radioactive food. One difficulty in obtaining accurate estimates of assimilated matter is determination of the distribution of ^{14}C among fractions of labelled dissolved organic matter corresponding to larval excretion, leakage of ingested carbon (Pechenik, 1979), disintegration of feces and release of label by bacteria. A second difficulty involves separation of ^{14}C-respiration of labelled-bacteria from that of larvae during the larval grazing period and during the purge period. Estimation of retention efficiencies, defined as the percentage of ingested carbon which is retained by an animal after it has

emptied its gut (Johannes and Satomi, 1967; Pechenik and Fisher, 1979), can be used to avoid these potential sources of error. Carbon retention is equal to carbon assimilation minus metabolic losses of carbon occuring during the experiment. Therefore, carbon retention is a conservative estimate of carbon assimilation.

The objective of this study was to determine if oyster larvae can utilize marine bacteria as a carbon source. Effects of marine bacteria on algal-fed, axenic larval Crassostrea gigas have been found to be strain-specific (Chapter 1). A bacterial strain found beneficial to larval cultures (strain CA2; Chapters 1 and 2) was used for this investigation in order to eliminate the possibility of adverse bacterial effects reducing estimates of the potential contribution of marine bacteria to larval carbon requirements.

METHODS

INGESTION OF CA2 CELLS BY OYSTER LARVAE: DIRECT OBSERVATION

A technique was developed for feeding oyster larvae on live, stained bacteria which permitted direct observation of ingestion of CA2 cells by 24 h old larvae. CA2 cells grown for 4 days at 25° C on Marine Agar 2216 (Difco) were transferred to a 50 ml screw-capped, plastic centrifuge tube containing 35 ml of 0.2 μm-filtered, autoclaved seawater (FSSW) at a salinity of 30 ppt. The cells were suspended in FSSW overnight at 20° C, then washed by centrifugation at 20,000 x g for 10 minutes and re-suspended in 5 ml FSSW. A 0.2 μm-filtered solution of the stain 4′,6-Diamidino-2-phenyl-indole (DAPI)(Sigma) was added at a final concentration of 10 μg ml^{-1}. This concentration did not appear to harm bacteria, considering that no reduction in cell numbers or motility was detected 24 h following staining. After 10 minutes of staining, cells were washed 3 x with FSSW at 20 ppt by repeated centrifugation, in order to remove unbound stain, and then the cells were re-suspended in 30 ppt FSSW. Stained cells were filtered at low vacuum pressure (<2 cm Hg) through 3 μm followed by 1 μm-Nuclepore filters to remove clumped cells. Concentrations of stained bacteria were determined by measuring absorbances of suspensions at 600 nm, using a Beckman DU-6 spectrophotometer. Absorption values were

converted to equivalent cell concentrations by the equation (Chapter 1):

$$\text{CA2 cells ml}^{-1} = (2.034\text{x}10^{9} \times \text{absorbance}) + 8.129\text{x}10^{5} \quad (r = 0.99)$$

Straight-hinged oyster larvae were obtained from the Whiskey Creek Hatchery, Netarts Bay, Oregon, or by spawning adults at the Hatfield Marine Science Center, Newport, Oregon. Larvae suspended at a density of 15 ml^{-1} in FSSW were exposed to DAPI-stained, CA2 cells at a concentration of $1x10^{7}$ cells ml^{-1}. Samples of larvae were withdrawn every five minutes with a Pasteur pipette, mounted on a glass slide with a depression and observed by epifluorescence microscopy using a combination of fluorescent light to detect the stained bacteria, and bright light to determine the location of the larvae. A saturated solution of $MgCl_2$ was slowly added to narcotize larvae with their vela extended. Photographs were taken (Kodak Ektachrome ASA 200 film) with a MC63 photomicrography system mounted on a Zeiss Universal epifluorescence microscope.

INGESTION OF CA2 CELLS BY OYSTER LARVAE: TRACER TECHNIQUES

A second method to confirm ingestion of CA2 cells by larvae consisted of feeding radioactively-labelled bacteria to oyster larvae and measuring accumulation of ^{14}C over time. CA2 bacteria grown on Marine Agar 2216 for 4 days

were transferred to 1/10 recommended concentration Marine Broth 2216 (3.74 g l^{-1}) at a salinity of 30 ppt and enriched with 0.5 g glucose l^{-1}. Microbial cultures were incubated at 25° C and agitated on an orbital shaker. After 4 days of growth, the cells were washed twice in FSSW by repeated centrifugation and re-suspension, and then added to 1/10 Marine Broth 2216. All centrifugations, unless specified otherwise, were carried out at 20,000 x g for 10 minutes and salinity of FSSW was 30 ppt. D-(U-14C)glucose (304.7 mCi $mmol^{-1}$; New England Nuclear, NEC-042X) was added to the broth at a concentration of 125 μCi l^{-1}. CA2 cells were incubated in the labelling medium for 4 days, harvested and washed twice with FSSW by centrifugation and re-suspension, and then transferred to 1/10 Marine Broth 2216 enriched with 0.5 g glucose l^{-1}. Following a chase period of four hours, the cells were washed three times by centrifugation and re-suspension in FSSW. After complete dispersion of cells in FSSW, a 4 ml sample was taken, preserved by addition of 1 ml 10% (w/v) formaldehyde and stored in the dark for up to five days at 5° C, for determination of cell numbers by the acridine orange staining technique of Hobbie et al. (1977). Four samples of 1 ml were each filtered at low vacuum pressure (<2 cm Hg) through 0.45 μm Gelman Metricel GN-6 filters, washed with 5 ml 0.5 M ammonium formate and transferred to scintillation vials which each received 4 ml Aquasol 2 (New England

Nuclear) and 2 ml distilled water. Samples were gelled by vigorous agitation on a Vortex mixer. Radioactivity was measured with a Beckman LS 8000 or LS 6000 TA liquid scintillation counter (LSC). Quench correction was by the external standard ratio method. The specific ^{14}C-activity of bacterial cells was determined by dividing the activity of $PO^{14}C$ in one ml by the number of cells per ml. Rapid determinations of bacteria concentrations were necessary in larval feeding experiments and these determinations were performed spectrophotometrically (see above).

Twelve hours prior to each feeding experiment, larvae were placed at a density of 0.1 to 0.2 ml^{-1} in 200 l Nalgene tanks containing sand-filtered seawater at 25° C. Algal cells of Isochrysis galbana (clone ISO; Center for Culture of Marine Phytoplankton, Maine) were added at an initial concentration of 40 cells μl^{-1}. Algal concentrations greater than 20 cells μl^{-1} were present in culture tanks prior to removal of larvae for use in labelling experiments; therefore, larvae used in the feeding experiments were pre-fed. Larvae were gently washed with FSSW on a 64 μm Nitex screen, placed in a separatory funnel and non-swimming larvae were allowed to settle out of suspension. Swimming larvae were suspended in FSSW at a density of 10 ml^{-1}. A sample of larvae was preserved in 2% (w/v) buffered formaldehyde (pH = 8) and copiously rinsed

with FSSW on a Nitex screen. Formaldehyde-killed larvae were added to another series of beakers at a density of 10 ml^{-1} and were used as controls for passive uptake of label by larvae.

^{14}C-labelled bacteria were added at $1x10^5$, $1x10^6$, $5x10^6$ or $1x10^7$ cells ml^{-1} to beakers containing either live larvae, formalin-killed larvae, or FSSW alone. The volume of FSSW in each of the flasks was 120 ml. All treatments were duplicated.

Water samples for determination of initial concentrations of bacteria and $PO^{14}C$ were withdrawn after agitation of the larval cultures with plastic plungers. Samples were withdrawn using 10 ml pipettes with 37 μm Nitex screens covering their tips, to prevent removal of larvae. Samples were processed as described above and initial ^{14}C-activity per bacterial cell calculated.

To determine background ^{14}C-activities, 4 subsamples of live larvae and formalin-killed larvae were collected on 8 μm (25 mm) Nuclepore filters, washed with 5 ml 0.5 M ammonium formate, dried by vacuum and transferred while held on the filters, to Petri dishes where larvae were counted under a dissecting microscope. Moist Whatman GF/C filters (25 mm) were then carefully placed over the larvae

held on the Nuclepore filters, to prevent loss of larvae during their transfer from the Petri dishes to 20 ml scintillation vials. Protosol (New England Nuclear) tissue solubilizer (0.5 ml) and distilled water (0.2 ml) were added to the scintillation vials and the larvae digested at 50° C for 24 h. After complete digestion of larval tissues, 4 ml of Aquasol 2 and 2 ml distilled water were added, the mixture gelled by vigorous agitation and larval radioactivity determined by LSC. ^{14}C-activity per larva was calculated by dividing measured ^{14}C-activity by the number of larvae in each sample.

Accumulation of radioactivity in bacteria-fed larvae at each of the four tested bacteria concentrations was determined by sampling larvae every 0.5 h during a 2 h feeding period. Passive adsorption of radioactivity by formalin-killed larvae (dpm-ADS $(larva)^{-1}$) was assessed at the same sampling intervals. Volumetric pipettes were used to withdraw 20 ml samples of larval suspension from each culture flask. To facilitate rapid sampling, a battery-operated pipettor was used.

In order to estimate the contribution of $DO^{14}C$ to larval uptake of radioactivity, larvae were exposed to particle-free filtrates (0.2 μm Nuclepore filtered; <2 cm Hg) of the culture medium used in feeding larvae on

bacteria for 2 h. Unlabelled larvae were added at a density of 10 ml^{-1} to the filtrates containing $DO^{14}C$ released by labelled bacteria. Larvae were sampled and processed for accumulated radioactivity after 0.5, 1.0, 1.5 and 2.0 h exposure. These measurements were used to estimate active absorption of $DO^{14}C$ by larvae (dpm-ABS $(larva)^{-1}$).

Radioactivities accumulated by larvae were calculated by subtracting the mean larval background ^{14}C-activity from the ^{14}C-activities of larvae determined in each treatment. Radioactivities accumulated by larvae ingesting ^{14}C-labelled bacteria (dpm ingested $(larva)^{-1}$) were estimated by subtracting the sum of both the mean ^{14}C-activity accumulated by passive uptake of label (dpm-ADS $(larva)^{-1}$) and the mean active uptake of labelled dissolved organic material (dpm-ABS $(larva)^{-1}$) from the mean ^{14}C-activity accumulated by live larvae (dpm $(larva)^{-1}$) for each sampling time and bacterial concentration.

SIZE AND BIOVOLUME OF CA2 CELLS

Cells of the bacterium strain CA2 were cultured and stained with DAPI as described above. Suspensions of stained cells and fluorescent microspheres, 2 μm in diameter (Fluoresbrite; Polysciences) were filtered onto 0.2 μm black Nuclepore filters. DAPI-stained cells and fluoresbrite microspheres fluoresce when excited at the

same wavelength. The filters were mounted on a slide under high viscosity, low fluorescence immersion oil (Resolve; Stephens Scientific) and observed with an epifluorescence microscope (Zeiss Universal). Photographs (Kodak Ektachrome ASA 400) were taken of areas of the filters where bacteria and microspheres were simultaneously in focus, thus reducing halo effects. Photographs were enlarged (3000x) and the length and width of 100 bacteria cells were determined with an image analysis system (Java; Jandel Corporation), which was calibrated by measuring the diameter of 50 microspheres 2 μm in diameter. Error due to measurement was determined by measuring a single microsphere 20 times. Bacterial biovolume was calculated assuming that the volume of the rods corresponded to the volume of a cylinder with hemispherical ends:

$$\text{Biovolume of a rod} = \pi\ r^2\ (L - 2/3\ r)$$

where r is half of the width of the cell and L is the length of the cell.

LARVAL RETENTION OF CA2 CARBON

In order to determine retention of bacterial carbon, larvae had to be completely purged of non-absorbed ^{14}C-material. However, live algal foods used as a purge substrate could take up $DO^{14}C$ released by larvae, and thus

affect estimation of larval ingestion and retention of bacterial ^{14}C. Consequently, dead ^{60}Co-irradiated cells were used to purge the digestive systems of larvae because irradiated cells were ingested at high rates by oyster larvae (Chapter 1), and would not recycle ^{14}C as there would be no active uptake of label. ^{60}Co-irradiated cells were prepared by firstly centrifuging cells from axenic cultures of Isochrysis galbana. The algal paste was stored in a 20 ml scintillation vial under a nitrogen atmosphere. The paste was sterilized by ^{60}Co-irradiation (5 megarads) at the Radiation Center of Oregon State University and stored in the dark at 5°C. Irradiated cells did not grow when incubated under conditions that facilitated growth of living cells. Sterility of the irradiated paste was confirmed by aerobic or anaerobic (BBL Gas Pack Pouch) incubations in 1/10 diluted Marine Broth 2216 (3.74 g l^{-1}) at 25° C.

Larvae were placed in beakers filled with FSSW at a density of 10 ml^{-1}. ^{14}C-labelled CA2 cells were added to the beakers at $1x10^7$ cells ml^{-1}, a bacteria concentration that resulted in highest bacterial ingestion rates for larvae in the preceding experiment. After a period of 2 h, in which larvae were fed on ^{14}C-labelled cells, larvae were washed with FSSW on a 64 μm Nitex screen and transferred to beakers containing 380 ml of a suspension of 30 cells μl^{-1}

of ^{60}Co-irradiated Isochrysis galbana. A 20 ml sample (ca. 200 larvae) was immediately taken from each flask for determination of larval ^{14}C-activity, followed by additional 20 ml samples taken every ten minutes during the first two hours of the purge period and thereafter at 3, 4, 6.3, 24 and 48 h in order to determine rates of gut evacuation.

Control treatments for both passive ^{14}C-uptake and active ^{14}C uptake were set up as described in the previous experiment. After 2 h exposure to either labelled bacteria or to the labelled dissolved fraction, both live and formalin-killed control larvae were washed and transferred to suspensions of irradiated algae to determine the loss of accumulated label over time.

Radioactivities were calculated by subtracting mean larval background ^{14}C-activity from the larval ^{14}C-activity determined for different treatments. Labelled carbon remaining in larvae during or after depuration was determined by subtracting the sum of the mean activities of larvae in controls for passive uptake of label (dpm-ADS $(larva)^{-1}$) and for active uptake of dissolved label (dpm-ABS $(larva)^{-1}$), from the activity remaining in bacteria-fed larvae (dpm $(larva)^{-1}$) at each sampling time. In this way, the rate of gut evacuation was determined.

CARBON RETENTION EFFICIENCIES FOR LARVAE FED ON LIVE OR DEAD BACTERIA

A second experiment was designed to test the hypothesis that ingested CA2 carbon was digested and retained by oyster larvae. Retention efficiencies of axenic larvae fed on either live or heat-killed bacteria were determined and compared. The ability of axenic larvae to retain ^{14}C from ingested ^{14}C-labelled CA2 cells after completely purging them of ^{14}C material would indicate that larvae do not need a gut flora to digest CA2 cells. Furthermore, retention of ^{14}C by axenic larvae, fed on heat-killed CA2 cells, would indicate that ^{14}C-activity accumulated and retained by larvae fed on living CA2 cells was not due to label recycling by CA2 cells, or to bacterial colonization of the digestive systems of larvae, but due to larval ingestion, digestion and absorption of ^{14}C from ^{14}C-labelled CA2 cells.

All glassware was previously acid washed, rinsed seven times with distilled water and baked overnight at 450°C. Filtered (0.2 μm) seawater was added to beakers which were capped with aluminum foil and sterilized by autoclaving. Screened chambers (described below) were sterilized separately in autoclavable bags, and then aseptically placed in the sterile beakers. Manipulations were performed under a laminar-flow hood.

A batch of CA2 cells was labelled with ^{14}C-glucose as described above. Half the CA2 culture was washed by centrifugation and resuspension in 35 ml of FSSW and then the suspension was heated at 95° C for 6 h. This treatment killed the cells (no growth in either solid or liquid Marine 2216 culture media) without destroying their structural integrity. The other half of the culture of ^{14}C-labelled bacteria was washed by centrifugation and resuspension with 35 ml FSSW, and then transferred to 1/10 diluted Marine Broth 2216 (3.74 g l^{-1}) enriched with 0.5 g unlabelled glucose l^{-1} and cultured for a 6 h "chase" period. Both living and heat-killed bacteria were then washed three times by centrifugation and re-suspension in 35 ml FSSW. Bacteria concentrations were estimated by optical density (see above).

Bacteria-free, straight-hinged oyster larvae were obtained and tested for axenicity following methods presented in Chapter 1. Axenic larvae were washed with FSSW on a sterile 64 μm Nitex screen and transferred at a density of 25 ml^{-1} to 4 sterile screened chambers held in 1 liter beakers filled with 400 ml FSSW. Each screened chamber consisted of a PVC tube (radius = 3.85 cm) with a 64 μm Nitex screen covering its base. Either live or heat-killed bacteria were added to larval suspensions at a concentration of 10^7 cells ml^{-1}. Each treatment was

duplicated. Initial bacterial ^{14}C-activities were determined as described in preceding experiments. Control treatments for passive label adsorption, active $DO^{14}C$ uptake and rates of loss of activity during the purge period were determined as described above.

After allowing larvae to feed on ^{14}C-labelled cells for 10 minutes, the screened chambers containing the larvae were removed from the bacteria suspensions, washed with FSSW and transferred to beakers to allow larvae to purge themselves on a diet of axenic ^{60}Co-irradiated algae (30 cells μl^{-1}). Immediately after resuspension of the larvae, two 20 ml samples per chamber were taken for determination of accumulated radioactivity in larvae. Subsequently, screened chambers containing larvae were removed from suspensions of irradiated algae every 3 h, washed with FSSW and transferred to fresh, axenic, irradiated algae suspensions. Frequent water changes reduced the risk of re-ingestion of ^{14}C-labelled fecal material by larvae. Duplicate samples of larvae were taken from each chamber after transfer to fresh, irradiated algae suspensions. The volume of the irradiated algae suspension was reduced at each transfer in order to maintain constant larval densities over the purge period. After a purge period of 9 h, larvae were next transferred to fresh, irradiated algae suspensions at 24 h.

In order to test for microbial contamination, samples of larvae were taken at the end of the experiment from treatments previously fed heat-killed bacteria. Samples were added to 1/10 Marine Broth 2216 and incubated for a month either under aerobic or anaerobic (BBL Gas Pack Pouch) conditions while other samples were tested for axenicity by epifluorescence microscopy using DAPI-staining techniques (Porter and Feig, 1980).

Average ^{14}C-activities per larva were calculated from duplicate samples taken from each replicated beaker at each sampling time. Rates of gut emptying under both treatment conditions (live or heat-killed bacteria) were determined as described in the preceding experiment. Radioactivity determined in larvae after 10 minutes feeding on ^{14}C-labelled bacteria (dpm ingested $(larva)^{-1}$ $(10\ minutes)^{-1}$) permitted estimation of ingestion rates because the feeding period was shorter than the larval gut passage time. Ingestion rates (IR) for larvae fed on CA2 cells over a 10 minute feeding period were calculated as follows:

$$IR = DPM\ ingested\ (larva)^{-1}\ 10(minutes)^{-1}\ /\ DPM\ (cell)^{-1}$$

Clearance rates (μl $(larva)^{-1}$ h^{-1}) defined here as the volume of water swept clear of particles in unit time were estimated by dividing ingestion rates (extrapolated to

hourly rates as cells $(larva)^{-1}$ h^{-1}) by the initial concentration of bacteria in the larvae suspension (cells μl^{-1}).

Retention efficiency of bacterial ^{14}C (RE) was calculated as:

$$RE = (DPM\ retained\ (larva)^{-1} / DPM\ ingested\ (larva)^{-1}) \times 100$$

Larval retention efficiencies determined for each bacterial cell type were arcsine-transformed and compared using a two-sample t-test (Sokal and Rohlf, 1981).

CARBON RETENTION EFFICIENCIES FOR LARVAE OF 2 DIFFERENT SIZES WHEN FED LIVE CA2 CELLS

Axenic experiments were initially necessary to unequivocally demonstrate that oyster larvae could utilize CA2 bacteria as a carbon source without possible beneficial digestive effects due to a microbial gut flora. However, the method used to obtain axenic larvae in the preceding experiment (storage of starved larvae in the same culture medium for five days at 5° C) may have stressed larvae. Having established direct utilization of bacterial carbon by oyster larvae, three independent non-axenic experiments were carried out in which utilization of bacterial carbon by larvae of 2 size groups was compared. Straight-hinged

(≈100 μm) and 10-12 day old larvae (200-300 μm) were obtained from the Whiskey Creek Hatchery in Netarts Bay, Oregon, held at 0.1 to 0.2 larva ml^{-1} in sand-filtered seawater at 25° C and fed on 40 cells of Isochrysis galbana μl^{-1} for 12 h prior to experimentation.

Experimental methods and control treatments (dpm-ABS, dpm-ADS) were identical to those described in the experiment using axenic larvae, except that when sampling larvae, only one 20 ml sample was taken from each beaker. In addition, activity of ^{14}C-labelled CA2 cells was determined immediately after addition of cells to larvae suspensions and also at the end of the 10 minutes feeding period in order to detect any possible reduction in bacterial ^{14}C-activity during the larval grazing period. All treatments were duplicated.

Cell ingestion rates over the 10 minute feeding period (IR), clearance rates (CR) and retention efficiencies for bacterial ^{14}C (RE) were calculated for each replicated treatment. Experimental variation due to both larval size and experiment were analyzed by two-way ANOVA after arcsine-transforming retention efficiencies. Where significant differences among treatments were indicated, Tukey's honestly significant difference test (T-HSD) was used to determine differences among individual treatments,

at the 0.05 level of probability.

CARBON RETENTION EFFICIENCIES FOR STRAIGHT-HINGED LARVAE FED ON ALGAE.

A medium for labelling axenic Isochrysis galbana with ^{14}C was prepared by aseptically acidifying 150 ml FSSW to pH 3 with 1 N HCl to drive off CO_2 and then restoring the pH to 8 by the addition of 1 N NaOH. Sterile nutrients (f/2 medium; Guillard and Ryther, 1962) and axenic I. galbana cells ($3.4x10^5$ cells ml^{-1}, final concentration) were added to the medium. The ^{14}C label was added as $NaH^{14}CO3$ (50 mCi $mmol^{-1}$; New England Nuclear, NEC-086H) at a concentration of 1 μCi ml^{-1}. Algae were cultured at 20° C under cool-white fluorescent light, emitting 2500-3000 lux 24 h per day. Exponentially growing ^{14}C-labelled algal cells were washed three times with FSSW by repeated centrifugation (10 minutes at 3000 x g) and re-suspended in FSSW. The labelled algal stock suspension was filtered through an 8 μm Nuclepore filter to remove clumped cells and algal concentrations were determined by counting 10 samples using a hemocytometer.

Straight-hinged larvae were fed ^{14}C-labelled algae at a concentration of 54 cells μl^{-1} for a period of 10 minutes. The larvae were then purged by transfer to a suspension of 30 cells μl^{-1} of ^{60}Co-irradiated Isochrysis

galbana. Sampling methods were identical to those described in previous experiments, except that 3 samples of larvae (20 ml each) were taken at each sampling time from each treatment flask. Average ^{14}C-activities per larva for each treatment flask were calculated from triplicate samples. All treatments were duplicated.

Algal ingestion rates over the 10 minute feeding period (IR), clearance rates (CR) and retention efficiencies (RE) were calculated in the same way as described in experiments with CA2 cells.

CARBON CONTENT OF CA2 CELLS

Four day-old cultures of CA2 cells grown on Marine Agar 2216 were transferred to 1/10 Marine Broth 2216 enriched with 0.5 g glucose l^{-1}. After a further 4 days of culture, the cells were washed twice in FSSW by repeated centrifugation and resuspension, and were finally re-suspended in 18 l of 1/10 Marine Broth 2216 supplemented with 0.41 μmol glucose l^{-1} and 2.25 ml ethanol l-1. Ethanol was added at this concentration with ^{14}C-glucose to culture media in preceding experiments and, therefore, bacteria sampled for carbon analysis were cultured in similar ethanol concentrations. After a 4 day incubation period on an orbital shaker at 25° C, the cells were concentrated at 20° C by centrifugation at 20,000 x g (Sorval RC-2).

Finally, the cells were washed by centrifugation and re-suspended in 40 ml 0.5 M NaCl in a pre-weighed plastic centrifuge tube. A 0.5 ml sample of the bacteria suspension was taken, diluted with 4.5 ml FSSW and fixed in 2% (w/v, final concentration) buffered formaldehyde (pH=8). Sub-samples of 50 μl from the fixed suspension were diluted in 39.95 ml FSSW and bacteria were counted by the acridine-orange staining technique of Hobbie et al. (1977). All remaining bacteria were concentrated by centrifugation at 30,000 x g, the pellet chilled at -70° C and then freeze-dried. The weight of the centrifuge tube containing the dry bacteria pellet was determined (Sartorius R160P balance), and the weight of the pellet estimated by subtraction of the initial tube weight. Dry weight per CA2 cell was estimated by dividing the dry weight of the bacterial pellet by the number of cells in the pellet. Percent carbon, hydrogen and nitrogen (CHN) content of cells were estimated by analysis of 4 samples, each from separate bacterial cultures by Dr. R. L. Petty, University of California, Santa Barbara. The CHN analyzer (Control Equipment Corporation model 240XA) provided data with a precision of ± 0.3 weight percent. Carbon content per CA2 cell was calculated by multiplying the dry weight of the cell by its percent carbon content. The average carbon content per cell resulting from analysis of bacteria from the four cultures was used for computations of bacterial

carbon utilization by larvae.

CARBON CONTENT OF ISOCHRYSIS GALBANA

Axenic Isochrysis galbana cells were cultured under identical conditions to those employed to culture algae for the algal-feeding experiment, except that no $NaH^{14}CO_3$ was added to the cultures. Exponentially growing cells were harvested after reaching similar cell concentrations to those obtained for algal-feeding experiments. Cell volumes were determined using a Coulter Channelyser 256, in conjunction with a Counter Counter (Model BZ1). Latex beads of diameters 2.17 μm (Interfacial Dynamics Corporation), 3.43 and 4.54 μm (Polysciences) were used for volume calibration of the Channelyser. Ten estimates of cell volume were carried out and an average cell volume was calculated. Two replicate algal cultures were analyzed.

Carbon content of algal cells was determined by using the relationship between volume and carbon content, as determined by Strathmann (1967):

$$\text{Log } C = 0.866 \log V - 0.46$$

where C is the carbon per cell in picograms and V is the cell volume in cubic microns.

CARBON RETENTION BY LARVAE FED BACTERIA OR ALGAE

Assuming that CA2 and Isochrysis cells were homogeneously labelled after culture for several days in ^{14}C-labelled media, then the amount of carbon retained by each larva (C.RET.) after 10 minutes feeding on labelled food, followed by a complete purge of the digestive system with non-labelled food, was estimated as:

$$C.RET. = IR \times \text{cell carbon content} \times RE / 100$$

where IR was ingestion rate expressed in terms of cells $(larva)^{-1}$ $(10\ minutes)^{-1}$ and the carbon content of CA2 or Isochrysis cells was expressed as pg C $(cell)^{-1}$.

The amounts of bacterial carbon retained by larvae fed live or heat-killed CA2 cells were calculated (Table 5). Similarly, the bacterial carbon retained by larvae of 2 different sizes (Experiments 1, 2 and 3), and the algal carbon retained by straight-hinged larvae (Experiment 4) were calculated and presented in Table 6. Variation in larval carbon retention due to larval size and experiment (Experiments 1, 2 and 3) was analyzed by two-way ANOVA. Data for retained carbon were log-transformed to reduce heterogeneity of variances (Cochran's test). Where significant differences among experiments were indicated, Tukey's honestly significant difference test (T-HSD) was

used to determine differences among individual experiments, at the 0.05 level of probability.

Amounts of particulate organic carbon (POC) provided as CA2 cells in Experiments 1, 2 and 3 and as algal cells in Experiment 4 were calculated by multiplying cell concentrations (cells ml^{-1}) by carbon content per cell (pg C $(cell)^{-1}$). Carbon ingestion rates (CIR) for straight-hinged larvae fed on these two diets were determined by multiplying cell ingestions rates (cells $(larva)^{-1}$ $(10\ minutes)^{-1}$) by carbon content per cell (pg C $(cell)^{-1}$). POC (pg C ml^{-1}), CIR (pg C $(larva)^{-1}$ $(10\ minutes)^{-1}$), CR (μl $(larva)^{-1}\ h^{-1}$, arcsin-transformed RE units and log-transformed C.RET. were compared for algae and bacteria-fed, straight-hinged larvae in each of these four experiments by one-way ANOVA, after confirmation of homogeneity of variances (Cochran's test). Where significant differences were indicated, Tukey's (HSD) test was used, at the 0.05 level of probability, to determine differences among individual treatments.

METABOLIC REQUIREMENTS OF LARVAE

Respiration has conventionally been used to estimate metabolism. Gerdes (1983a) presented an allometric equation relating dry tissue weight to oxygen consumption of pre-fed

larval Crassostrea gigas, determined at a salinity of 25 ppt and at 25° C:

$$VO_2 = 0.00282\ W^{0.96}$$

where VO_2 is the rate of oxygen consumption expressed as ml O_2 h^{-1} $(larva)^{-1}$ and W is the dry tissue weight (mg) per larva.

Dry tissue weight was determined for straight-hinged larvae obtained from two different spawnings. Larvae were washed with 0.5 M ammonium formate, collected by vacuum onto pre-washed, pre-weighed (Perkin Elmer AD-2Z) 8 μm Nuclepore filters (25 mm) and counted under a dissecting scope. Filters were folded to avoid loss of larvae, placed on small, pre-weighed aluminum foil trays and dried at 60° C to constant weight. Three samples from each spawning containing approximately 900 larvae each were processed and the weight per larva estimated by dividing the weight of larvae by the number of larvae on each filter. Dry tissue weight was estimated based on reports that 75% of larval dry weight corresponds to shell weight (Walne, 1965; Millar and Scott, 1967). Dry-tissue weights of oyster larvae of different sizes were also obtained using an equation based on data of Gerdes (1983b, Tables 4, 5 and 6 and 1983a, Table 3) relating larval shell length to dry tissue weight.

Similarity between these two estimates of dry tissue weights of straight-hinged larvae supported use of Gerdes' formula in the present study.

Estimates of metabolic energy requirements and equivalent weights of organic carbon respired for differently sized larvae were calculated using the following equation:

$$\text{mg C utilized (time)}^{-1} = \text{ml } O_2 \text{ consumed (time)}^{-1} \times 12 \times 22.4^{-1} \times RQ$$

A respiratory quotient (RQ) of 0.8 (Brody, 1945) was used for all calculations.

The amount of carbon retained by larvae can be compared to estimates of larval metabolic requirements for carbon in order to determine if the diet could potentially provide enough carbon to meet larval requirements. Percent carbon metabolic requirements of larvae potentially supplied by diets were calculated as:

$$(\text{C.RET./ C metabolic requirements (larva)}^{-1} \text{ (10 minutes)}^{-1}) \times 100$$

RESULTS

INGESTION OF CA2 CELLS BY OYSTER LARVAE: DIRECT OBSERVATION

Microscopic observations of straight-hinged larvae fed DAPI-stained CA2 cells revealed that after only 5 minutes of grazing, large accumulations of stained bacteria were present in the digestive systems of larvae (Fig.14). Stained bacteria were concentrated in the stomach, digestive gland and upper intestine of some larvae. A few single bacterial cells were released with feces after only 15 minutes of grazing, but most bacteria were retained in the digestive systems of larvae during this period of initial feeding. DAPI-fluorescence of bacteria in the guts of larvae faded within several hours following exposure to stained cells, while non-ingested stained cells were fluorescent for over 24 h. Patterns of stain accumulation and gut passage times were consistent among repeated observations. Larvae exposed to dilute concentrations of DAPI absorbed the stain through the velum, resulting in a different distribution of stain in the larvae from that occuring in larvae fed on stained bacteria, where fluorescence only appeared in the digestive system.

INGESTION OF CA2 CELLS BY OYSTER LARVAE: TRACER TECHNIQUES

Larvae accumulated radioactivity when exposed to bacteria concentrations between $1x10^5$ and $1x10^7$ cells ml^{-1}.

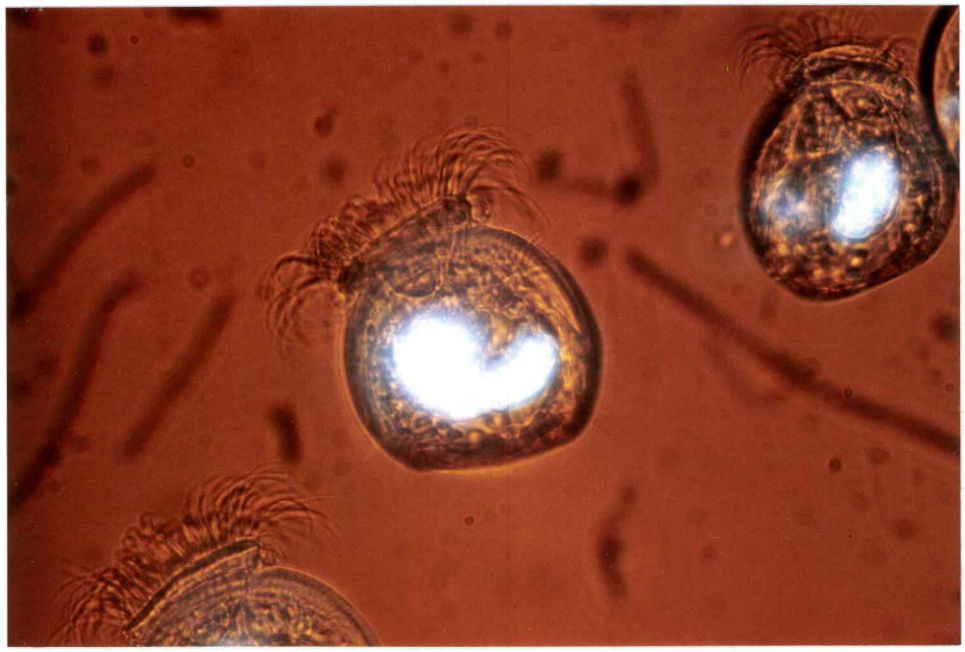

Fig.14. Photograph of straight-hinged oyster larvae (≈100 µm in shell length) after 5 minutes grazing on DAPI-stained CA2 cells. Fluorescence of larval digestive systems attributed to ingestion of stained CA2 cells.

This radioactivity determined in larvae corresponded to net accumulated activity because of losses of ^{14}C due to larval respiration and excretion and, therefore, was an underestimate of assimilated ^{14}C. Uptake of label by active absorption of $DO^{14}C$ (dpm-ABS (larva)$^{-1}$) accounted for 14.6% ± 6.7% ($\bar{x}$ ± SD) of net accumulated larval activity. Passive adsorption of label by formalin killed larvae (dpm-ADS (larva)$^{-1}$) was equal to 9.5% ± 3.4% of net accumulated activity. Larval radioactivity after subtraction of dpm-ABS (larva)$^{-1}$ and dpm-ADS (larva)$^{-1}$, represented net activity accumulated through ingestion of $PO^{14}C$ (Fig.15).

SIZE AND BIOVOLUME OF CA2 CELLS

High viscosity oil allowed both DAPI-stained bacteria and microspheres to be positioned so that both were in focus in a same photograph (Fig.16). The average length of CA2 cells was estimated to be 0.87 μm (SD = 0.16 μm), while the average width was 0.41 μm (SD = 0.047 μm). The average cell biovolume was estimated to be 0.0968 μm^3.

LARVAL RETENTION OF CA2 CARBON

Active absorption (dpm-ABS (larva)$^{-1}$) and passive adsorption (dpm-ADS (larva)$^{-1}$) were found to represent 13.7% ± 5.9% ($\bar{x}$ ± SD) and 7.8% ± 4.3%, respectively, of the ^{14}C-activity accumulated by larvae after a 2 h grazing period. After subtraction of dpm-ABS (larva)$^{-1}$ and dpm-ADS (larva)$^{-1}$

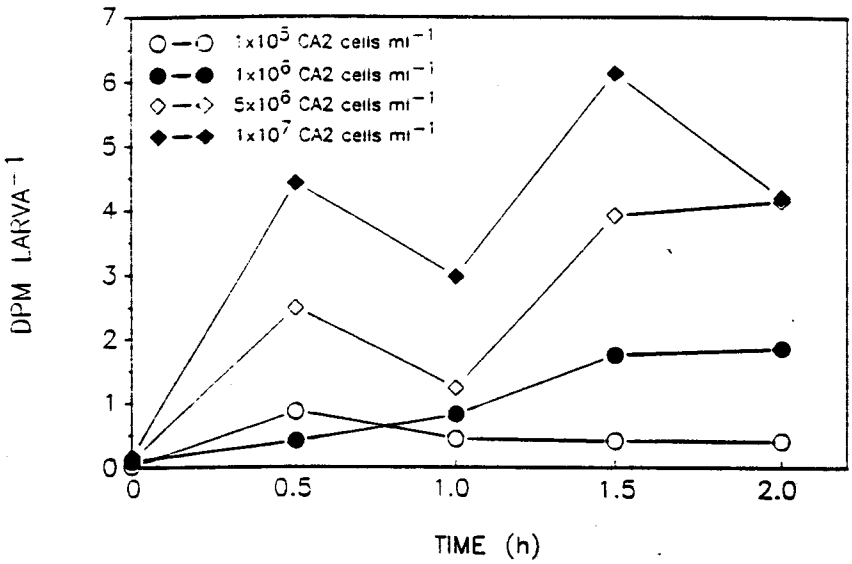

Fig.15. Patterns of ingestion of $PO^{14}C$ in straight-hinged oyster larvae fed on ^{14}C-labelled CA2 bacteria at different cell concentrations. Symbols represent mean values of two replicate samples, standard deviations omitted for clarity.

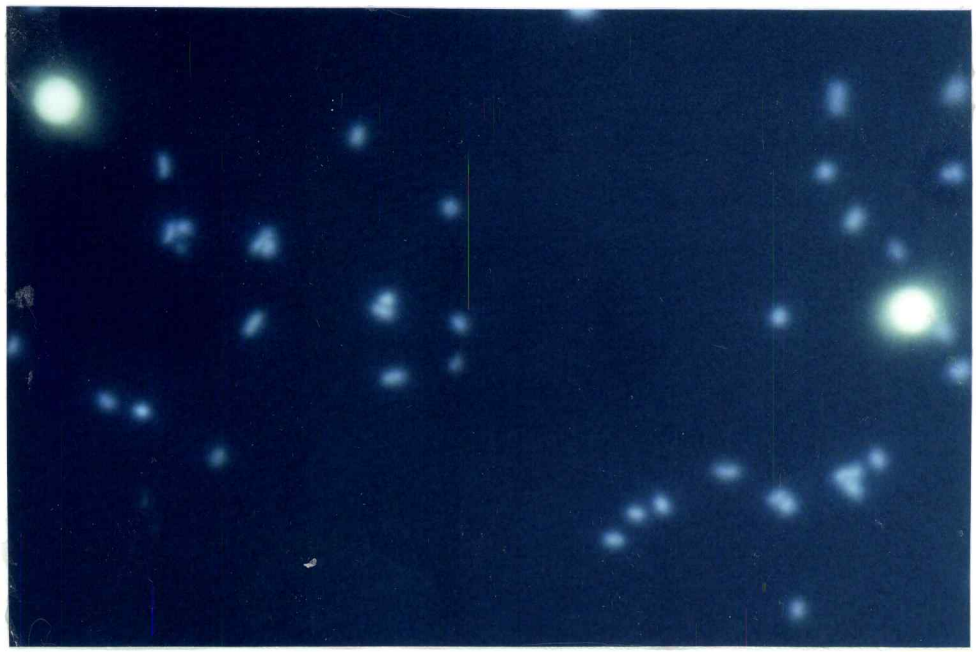

Fig.16. Photograph of DAPI-stained CA2 bacteria and 2 μm size fluorescent microspheres.

from the total ^{14}C-activity of larvae at each sampling time, the rate of gut evacuation was characterized by a rapid decline in ^{14}C-activity during the first 10 minutes and a more gradual decrease in activity during the next 48 h of the purge period (Fig.17). At 48 h, larvae retained 34.15% ± 7.36 ($\bar{x}$ ± SD) of activity accumulated after 2 h exposure to ^{14}C-labelled CA2 cells. A purge period of 4 h was considered sufficient to allow for complete defecation of ingested ^{14}C material.

CARBON RETENTION EFFICIENCIES FOR LARVAE FED ON LIVE OR DEAD CA2 CELLS

^{14}C-activities of live and heat-killed CA2 cells immediately after addition of cells to larvae suspensions were determined to be $1.503 \times 10^{-4} \pm 2.97 \times 10^{-6}$ ($\bar{x}$ ± SD) and $1.077 \times 10^{-4} \pm 3.32 \times 10^{-6}$ dpm $(cell)^{-1}$, respectively. Active uptake of dissolved ^{14}C by larvae exposed to leachates of live or heat-killed CA2 cells accounted for 8.1% ± 1.3% ($\bar{x}$ ± SD) and 13.4% ± 2.7%, respectively, of net accumulated ^{14}C-activity in larvae fed on these cell types. Passive adsorption of ^{14}C (dpm-ADS $(larva)^{-1)}$ was determined to be 6.3% ± 0.6% and 5.9% ± 1.2% of net ^{14}C-activities accumulated in larvae fed live or heat-killed CA2 cells, respectively.

Microscopic examination revealed that even after

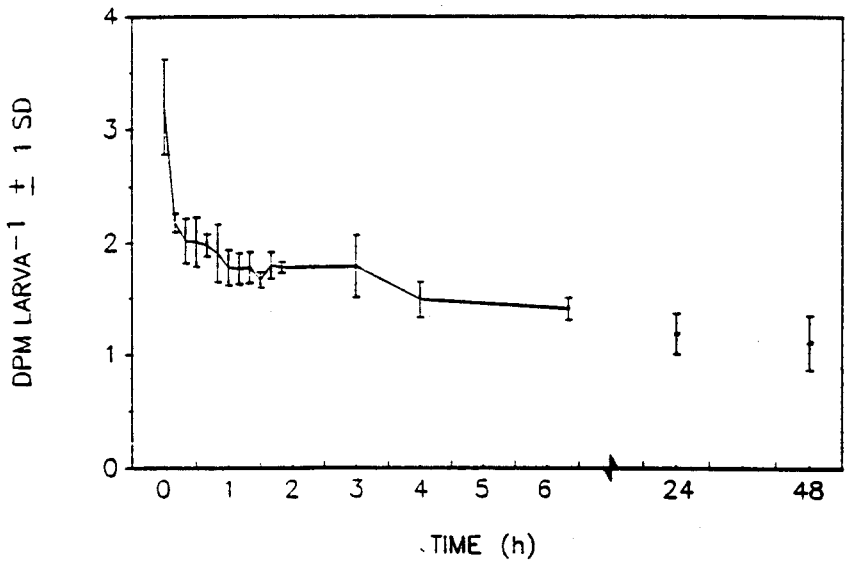

Fig.17. Rate of gut evacuation of straight-hinged oyster larvae fed for 2 h on ^{14}C-labelled CA2 bacteria at $1x10^7$ cells ml^{-1} and then depurated in filtered seawater containing 30 cells μl^{-1} of ^{60}Co-irradiated Isochrysis galbana. Symbols represent values of means and standard deviations for four replicate samples.

filtration through a 1 μm Nuclepore filter, heat-killed CA2 cells tended to clump together when suspended in seawater. This could explain why larvae ingested heat-killed CA2 cells at higher rates than living cells (Table 5). Rates of gut evacuation were faster for heat-killed than for live CA2 cells (Fig.18). Larvae retained carbon from both heat-killed and live CA2 cells after a 24 h purge period. Larvae fed on heat-killed bacteria were still axenic at the end of the 24 h purge period. Consequently, retention of bacterial carbon by larvae was not due to microbial recycling of label or due to the establishment of a resident microbial gut flora consisting of ^{14}C-labelled CA2 cells.

Cell concentrations, cell ingestion rates over the 10 minute feeding period, clearance rates, and carbon retention efficiencies of straight-hinged larvae grazing on live or heat-killed CA2 cells are given in Table 5. Cell concentrations and the number of cells ingested by larvae differed between treatments (t-test, $p=0.01$ and $p=0.03$, respectively). Although clearance rates, retention efficiencies and the amount of carbon retained by larvae fed on heat-killed cells appeared higher than for larvae fed on live CA2 cells, the differences were not found to be statistically significative (t-test; $p=0.09$, $p=0.12$ and $p=0.06$, respectively).

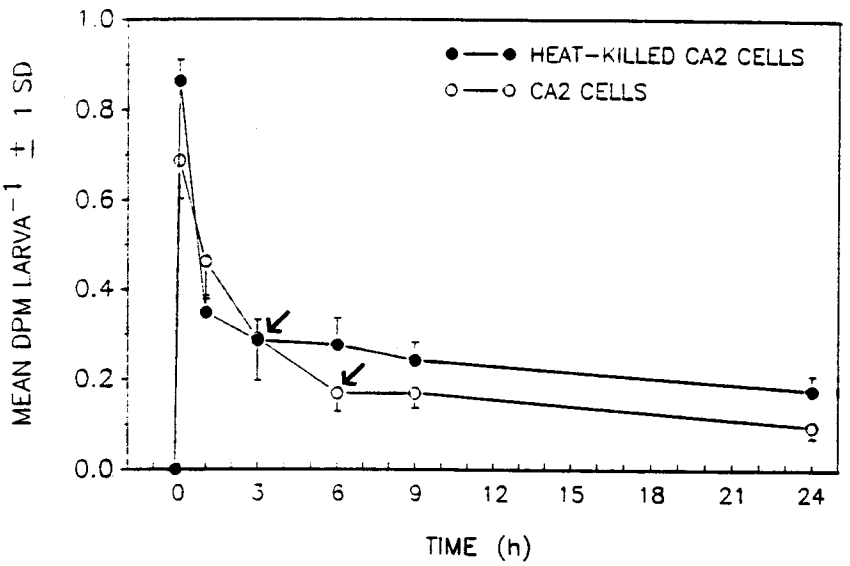

Fig.18. Rate of ingestion and gut evacuation of bacteria-free, straight-hinged oyster larvae fed for 10 minutes on either live or heat-killed ^{14}C-labelled CA2 bacteria and then depurated in filtered seawater containing 30 cells μl^{-1} of ^{60}Co-irradiated Isochrysis galbana. Symbols represent values of means and standard deviations of two replicates. Arrows indicate points after which larvae were considered purged of ingested ^{14}C-material.

Table 5. Summary of results of ^{14}C-retention experiment conducted with bacteria free, straight-hinged oyster larvae fed for 10 minutes on either live or heat-killed ^{14}C-labelled CA2 cells and then purged for 24 h in seawater containing 30 cells μl^{-1} of ^{60}Co-irradiated Isochrysis galbana. Values are the means of two replicates ± 1 standard deviation.

	CA2 cell type		2 sample t-test
	live	heat-killed	p values
CA2 concentration [cells ml^{-1}]	$2.19 \times 10^7 \pm 1.13 \times 10^6$	$3.28 \times 10^7 \pm 1.16 \times 10^6$	0.010
Clearance rate [μl (larva)$^{-1}$ h^{-1}]	1.251 ± 0.065	1.469 ± 0.073	0.087
Ingestion rate [cells (larva)$^{-1}$ (10 minutes)$^{-1}$]	4573 ± 474	8037 ± 682	0.027
Percent carbon retention efficiency	24.40 ± 2.74	32.89 ± 3.63	0.117
CA2 carbon retained [pg C (larva)$^{-1}$ (10 minutes)$^{-1}$]	28.95 ± 6.23	68.51 ± 13.30	0.062

CARBON RETENTION EFFICIENCIES FOR LARVAE OF 2 SIZES WHEN FED LIVE CA2 CELLS

Decreases in the ^{14}C-activity of bacteria cells during 10 minute grazing periods were negligible (<2.4%) in all three experiments. Nevertheless, average cell activities were calculated for each experiment, based on the assumption of a linear decrease in ^{14}C-activity from initial to final cell activities over the 10 minute grazing period. Accordingly, CA2 cell activities in Experiments 1, 2 and 3 were estimated to be $2.77 \times 10^{-4} \pm 7.78 \times 10^{-6}$ ($\bar{x} \pm$ SD), $2.08 \times 10^{-4} \pm 6.36 \times 10^{-6}$, and $1.81 \times 10^{-4} \pm 5.66 \times 10^{-6}$ dpm $(cell)^{-1}$, respectively. Active uptake of label in dissolved form (dpm-ABS $larva^{-1}$) represented 6.8% ± 1.5% ($\bar{x}$ ± SD) and 10.5% ± 2.1% of net accumulated activity in large and small larvae, respectively. Passive adsorption of label by formalin-killed larvae accounted for 4.7% ± 0.8% and 4.3% ± 0.6% of net accumulated activity in large and small larvae, respectively.

Results from the three comparative feeding experiments are presented in Figure 19 and Table 6. Significant differences occurred among experiments in bacteria cell densities ($p<0.01$), ingestion ($p<0.01$) and clearance rates ($p=0.028$)(Appendix IV). Bacteria cell densities were significantly different in all three experiments (T-HSD, $p<0.05$). No significant differences were found among values

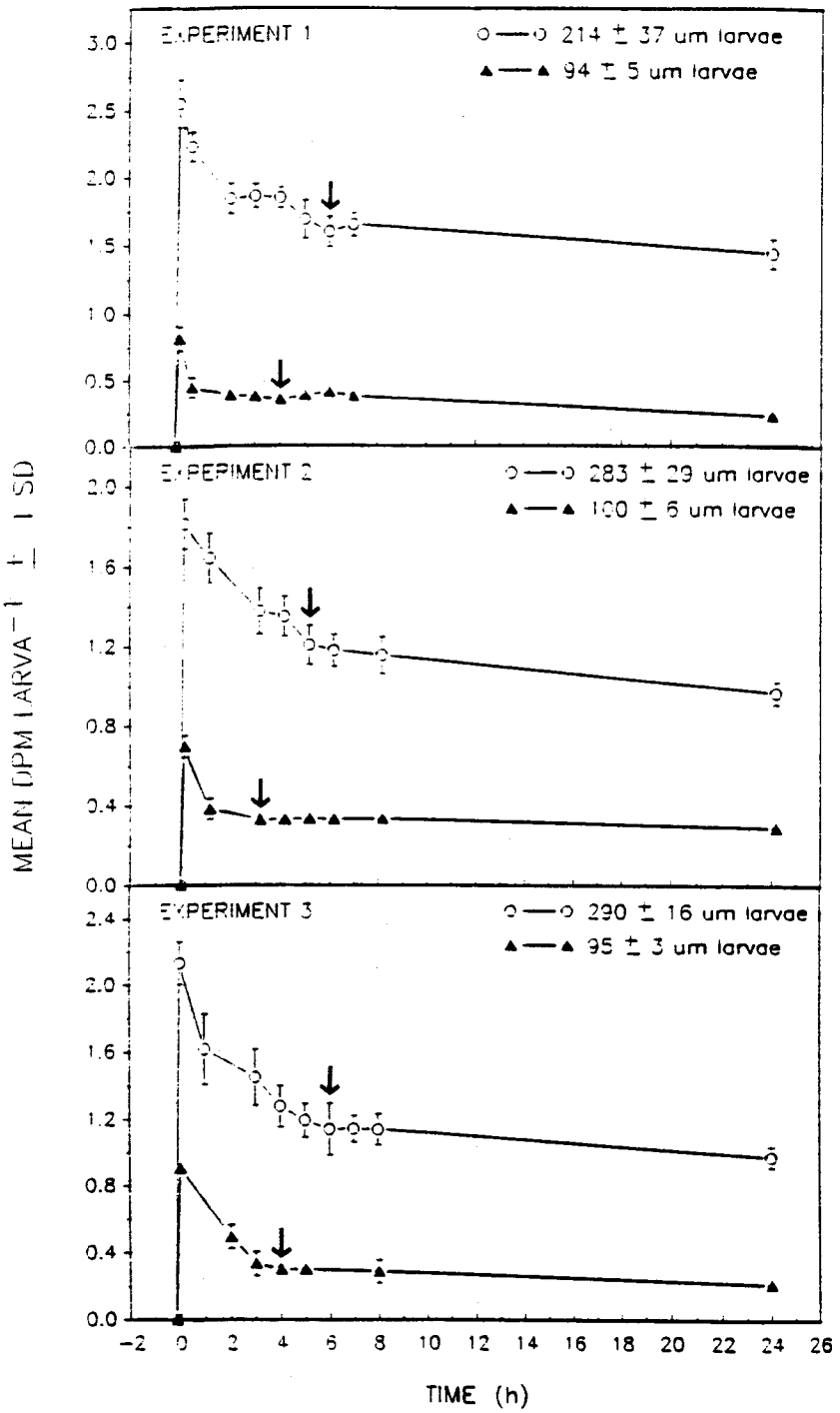

Fig.19. Rates of ingestion and gut evacuation for large (214-290 µm shell length) and small (94-100 µm shell length) oyster larvae fed for 10 minutes on ^{14}C-labelled CA2 bacteria and then depurated in filtered seawater containing 30 cells μl^{-1} of ^{60}Co-irradiated Isochrysis galbana. Symbols represent means and standard deviations of two replicates. Arrows indicate points after which larvae were considered purged of ingested ^{14}C-material.

Table 6. Summary of results of ^{14}C-retention experiments conducted with oyster larvae of different sizes. Larvae grazed for 10 minutes on either ^{14}C-labelled CA2 bacteria or ^{14}C-labelled Isochrysis galbana and were then depurated in filtered seawater containing 30 cells μl^{-1} of ^{60}Co-irradiated I. galbana. Values are the means of two replicates except for larval size measurements where n=20. Values in parentheses are standard deviations.

EXPERIMENT	SIZE OF LARVAE (μm)	DIET	CELL CONCENTRATION		INGESTION RATE		CLEARANCE RATE	RETENTION EFFICIENCY	CARBON RETAINED	% LARVAL METABOLIC REQUIREMENTS
			(cells ml^{-1})	(ng C ml^{-1})	(cells (larva)$^{-1}$ 10 minutes^{-1})	(pg C (larva)$^{-1}$ (10 minutes)$^{-1}$)	(μl (larva)$^{-1}$ h^{-1})		(pg C (larva)$^{-1}$ (10 minutes)$^{-1}$)	
1	94 (5)	CA2	1.60×10^7 (5.37×10^5)	412.80 (13.86)	2,982 (387)	76.94 (9.98)	1.116 (0.108)	42.74 (1.72)	32.80 (2.94)	137.92 (12.37)
	214 (37)		1.62×10^7 (4.24×10^5)	417.96 (10.95)	9,244 (897)	238.50 (23.14)	3.421 (0.243)	62.77 (8.62)	148.70 (6.04)	110.60 (4.50)
2	100 (6)	CA2	1.53×10^7 (2.55×10^5)	394.74 (6.57)	3,319 (373)	85.63 (9.61)	1.301 (0.124)	44.43 (1.55)	38.12 (5.60)	146.90 (21.58)
	283 (29)		1.53×10^7 (2.83×10^5)	394.74 (7.30)	8,753 (865)	225.83 (22.31)	3.430 (0.276)	66.92 (9.91)	150.02 (7.44)	41.23 (2.05)
3	95 (3)	CA2	2.65×10^7 (3.18×10^5)	683.83 (8.21)	4,925 (348)	127.07 (8.98)	1.115 (0.092)	33.38 (5.81)	42.15 (4.38)	174.70 (18.16)
	290 (16)		2.64×10^7 (2.90×10^5)	680.99 (7.48)	11,782 (1083)	303.98 (27.95)	2.677 (0.217)	53.41 (3.93)	162.92 (26.86)	40.48 (6.67)
4	98 (3)	ISO	54,546 (4,554)	401.09 (40.17)	16.97 (1.06)	149.70 (9.33)	1.869 (0.040)	52.46 (2.26)	78.64 (8.28)	312.06 (32.84)

for cell ingestion rates in Experiments 1 and 2, while overall significantly higher cell ingestion rates occurred in Experiment 3 than in Experiments 1 and 2 (T-HSD, $p<0.05$). Larval clearance rates were found to differ between Experiments 2 and 3, but neither were found to differ from clearance rates obtained in Experiment 1 (T-HSD, $p<0.05$). No differences among experiments were detected for retention efficiencies (two-way ANOVA, $p=0.07$). Cell densities were not significantly different between flasks holding large and small larvae in each experiment (two-way ANOVA, $p=0.89$), but cell ingestion and clearance rates were significantly higher for large compared with small larvae in all three experiments (two-way ANOVA, $p<0.01$). Large larvae required a longer period of time to clear their guts of ^{14}C-labelled material, compared with straight-hinged larvae (Fig.19). Overall, large sized larvae showed significantly higher carbon retention efficiencies than small sized larvae (two-way ANOVA, $p<0.01$).

CARBON RETENTION EFFICIENCIES FOR STRAIGHT-HINGED LARVAE FED ON ALGAE.

Algal ^{14}C-activity did not decrease significantly (<3 %) during the 10 minute feeding period. The average cell activity was 0.29 ± 0.009 ($\bar{x} \pm SD$) dpm $cell^{-1}$. Active uptake of dissolved ^{14}C from labelled algal leachates (dpm-

ABS $(larva)^{-1}$) and passive uptake from labelled algal suspensions (dpm-ADS $(larva)^{-1}$) accounted for 11.7% ± 2% and 3.3% ± 0.7%, respectively, of net accumulated activity in larvae fed algae.

Patterns of gut depuration were similar for larvae fed on either algae or bacteria. Rapid larval loss of ^{14}C was detected during the first 1.5 h of depuration (Fig.20). ^{14}C-activity of larvae decreased at a more gradual rate over the following 6 h. After 7.5 h depuration, the decrease in larval activity followed a constant slope, indicating that the gut was purged of labelled organic matter.

CARBON CONTENT OF STRAIN CA2 AND ISOCHRYSIS

The average weight of freeze-dried CA2 cells was 6.17×10^{-14} g $cell^{-1}$ ± 0.53×10^{-14} ($\bar{x}$ ± SD). Average values for percent carbon, hydrogen and nitrogen for CA2 cells were 41.875% ± 1.94%, 6.45% ± 0.65%, 12.15% ± 1.42% and the carbon to nitrogen ratio was 3.47% ± 0.31%. The average carbon content per CA2 cell was thus estimated to be 25.8 ± 2.6 fg $(cell)^{-1}$.

The mean volume of cells of Isochrysis galbana was determined to be 41.99 ± 2.22 μm^3 ($\bar{x}$ ± SD). The carbon content of an algal cell with this volume was calculated,

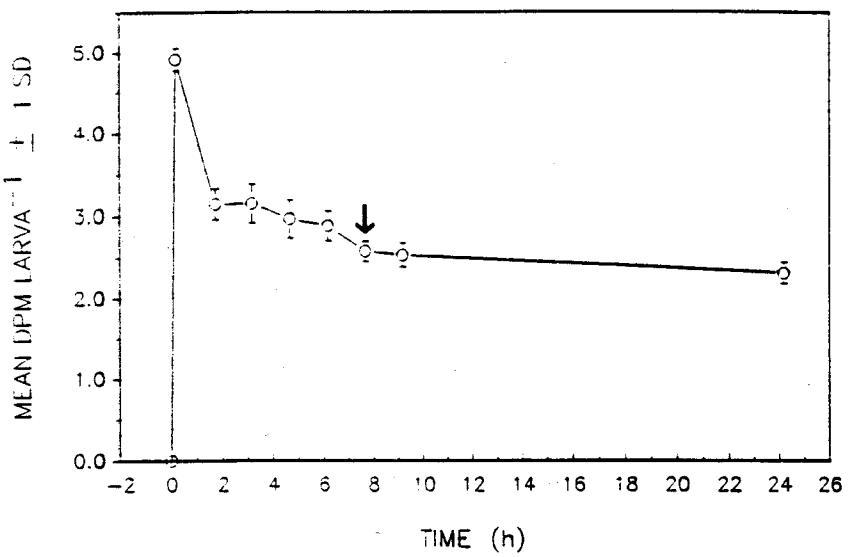

Fig.20. Rates of ingestion and gut evacuation for straight-hinged oyster larvae fed for 10 minutes on ^{14}C-labelled Isochrysis galbana and then depurated in filtered seawater containing 30 cells μl^{-1} of ^{60}Co-irradiated I. galbana. Symbols represent means and standard deviations of two replicates. Arrow indicate point after which larvae were considered purged of ingested ^{14}C-material.

using Strathmann's equation (1967), as 8.82 pg $(cell)^{-1}$. This estimate of algal carbon content allowed calculation of the potential contribution of algae to the metabolic carbon requirements of larvae.

CARBON RETENTION OF LARVAE FED BACTERIA OR ALGAE

No differences among experiments 1, 2 and 3 were detected for the amounts of carbon retained by large and small larvae (two-way ANOVA, $p=0.18$). Overall, large sized larvae retained significantly greater amounts of carbon than small sized larvae (two-way ANOVA, $p<0.01$).

The amount of POC added to cultures in the form of CA2 cells in Experiments 1, 2 and algal cells in Experiment 4 did not differ significantly among experiments, while a significantly higher amount of POC was provided in Experiment 3 (T-HSD, $p<0.05$) (Appendix V). No significant differences in carbon ingestion rates were detected between Experiments 1 and 2 and between Experiments 3 and 4 (T-HSD, $p<0.05$). Carbon ingestion rates were higher in Experiments 3 and 4 than in Experiments 1 and 2 (T-HSD, $p<0.05$). Clearance rates and amounts of carbon retained by larvae did not differ among experiments with CA2 bacteria (Experiments 1, 2 and 3), while both parameters were significantly higher (T-HSD, $p<0.05$) for larvae fed on algae (Experiment 4). Larval retention efficiencies for

ingested carbon were not significantly different among Experiments 1, 2 and 4 and among Experiments 1, 2 and 3, but a significant difference was found between Experiments 3 and 4 (T-HSD, $p < 0.05$).

METABOLIC REQUIREMENTS OF LARVAE

The dry tissue weight of straight-hinged larvae was determined to be 84.93 ± 19.39 ng $(larva)^{-1}$ ($\bar{x}$ ± SD). Because of close agreement between this value and the dry tissue weight calculated for straight-hinged larvae using the formula derived from Gerdes (1983a), it was considered satisfactory to employ Gerdes' method to determine weights of variously sized larvae which, in turn, would allow estimation of larval metabolic requirements. Oxygen consumed by variously sized larvae and the equivalent amount of carbon utilized per larva are given in Table 7. Straight-hinged oyster larvae fed CA2 bacteria at 1.5×10^7 cells ml^{-1} could meet 146% of their carbon metabolic requirements with the bacterial carbon retained after complete purge of their digestive systems. This bacterial carbon represented 46% of the carbon retained by larvae fed on a similar concentration of carbon in the form of algae. The bacterial carbon contribution towards larval metabolic requirements decreased as larvae increased in size.

Table 7. Metabolic carbon requirements of various sized oyster larvae. Methods used for calculations presented in text.

Size of larva [μm]	94	95	98	100	214	283	290
Weight of larva [ng]	81.02	82.24	86.04	88.67	492.01	1388.11	1542.10
Oxygen consumption [pl O_2 (larva)$^{-1}$ (10 minutes)$^{-1}$]	55.50	56.30	58.80	60.50	314.00	849.00	939.00
Metabolic carbon requirements [pg C (larva)$^{-1}$ (10 minutes)$^{-1}$]	23.78	24.13	25.20	25.95	134.45	363.83	402.50

DISCUSSION

Straight-hinged oyster larvae were able to capture and ingest free-swimming cells of strain CA2 as demonstrated by: a) direct observation, using DAPI-stained bacteria and, b) quantifying the accumulation of radioactivity, corrected for absorption and adsorption processes, in larvae exposed to ^{14}C-labelled cells. Use of live DAPI-stained bacteria instead of heat-killed stained bacteria, as used by Sherr et al. (1987) to feed protozoa, had the advantage of avoiding possible changes in chemical composition and physical structure of bacterial cells due to heat treatment. Discrimination against heat-killed compared to living bacteria has been demonstrated in feeding experiments with marine protozoa (Landry et al., 1987). Cultured bacteria are sometimes larger in size than bacteria from the natural environment (Peterson et al., 1978). The size of strain CA2 (0.87 x 0.41 μm) was within the size range reported for bacteria in estuarine and coastal waters (Wright et al., 1982; Palumbo et al., 1984). Therefore, ingestion rates for larvae fed on CA2 bacteria reported in this study, could be representative of ingestion rates for larvae feeding on natural bacterioplankton, assuming that larvae do not discriminate among different bacterial strains on the basis of other factors apart from size.

Considerable larval ingestion of bacteria cells occurred at concentrations above $5x10^6$ cells ml^{-1}. Concentrations of bacteria in the marine environment usually range within 10^5 and 10^6 cells ml^{-1} (Sieburth et al., 1978; Furhman, 1981; Chretiennot-Dinet, 1982; Moriarty et al., 1985; Andersen and Sorensen, 1986). However, higher concentrations of bacteria have been reported in eutrophic coastal waters ($1.5x10^7$ cells ml^{-1}; Andersen and Sorensen, 1986), coastal waters following algal blooms ($1.2x10^7$; Tracey et al., 1988); estuaries ($1.5-1.8x10^7$ cells ml^{-1}; Wright et al., 1982; Palumbo and Ferguson, 1978); salt marshes ($1.9x10^7$ cells ml^{-1}; Wilson and Stevenson, 1980); oyster ponds ($2x10^7$ cells ml^{-1}, Frikha and Linley, 1988/1989) and in larval culture tanks in hatcheries ($3.8x10^6$ cells ml^{-1}, Jeanthon et al., 1988). The cyclical pattern of accumulation rates of radioactivity by larvae fed at the two highest bacterial concentrations ($5x10^6$ and $1x10^7$ cells ml^{-1}) is similar to the pattern observed by Gallager et al. (1989) with scallop larvae (Argopecten irradians) fed ^{14}C-labelled algae. The latter authors suggested that this pattern was caused by cycles of gut filling and emptying of the digestive systems of larvae. The pattern of gut depuration of oyster larvae after feeding on ^{14}C-labelled bacteria in this study was similar to patterns observed with mud snail Nassarius obsoletus larvae (Pechenik and Fisher, 1979) and scallop A. irradians

larvae (Gallager et al., 1989) when both were fed on ^{14}C-labelled algae.

Several potential problems in feeding experiments with suspension-feeding marine bivalves and bacteria should be considered. 1) Larval re-ingestion of feces during the purge period may affect carbon retention determinations, and water changes during the purge period should be as frequent as practical. 2) Certain bacteria strains do not seem to be digested and may even multiply while passing through the guts of bivalve larvae (Prieur, 1981). Therefore, ingested bacteria may resist digestion and colonize the guts of larvae so that ^{14}C-activity present after the purge period would not be due to absorbed carbon but due to the presence of a gut microflora of ^{14}C-labelled bacteria. The use of heat-killed bacteria control for this latter possibility. 3) The presence of bacteriolytic activity associated with bacteria isolated from the crystaline styles of Mytilus edulis (Seiderer et al., 1987) suggests that gut bacteria may affect the results of assimilation studies in which bacteria are fed to bivalve larvae by enhancing the digestion of ingested bacterial cells. The use of axenic larvae without a gut microflora allows determination of the ability of the larvae alone to utilize ingested bacteria as a food source.

Retention efficiencies for ingested bacterial ^{14}C were

used to quantify utilization of bacterial carbon by oyster larvae. This estimate required an accurate quantification of ingestion of labelled cells. Observations of larvae fed on DAPI-stained CA2 cells revealed that some bacteria would pass unharmed through the guts of larvae after 15 minutes exposure of larvae to the microbial suspension. Estimates of a gut passage time of 10 minutes have been reported for larvae of the oyster Crassostrea virginica (Baldwin and Newell, Horn Point, University of Maryland; unpublished data) and 30 minutes for scallop larvae fed fluorescent paint particles (Nelson and Siddall, 1988). A short term (10 min) pulse-feed with ^{14}C-labelled bacteria allowed estimation of ingestion rates, reduced both the probability of label recycling among bacteria, larvae and seawater, as well as the probability of larval ingestion of defecated bacteria. The use of ^{60}Co-irradiated algae for purging larvae eliminated the possibility of label recycling through active uptake of dissolved ^{14}C-label by algae. Clumping of bacteria cells due to heat treatment probably increased the efficiency with which cells were captured by larvae and, consequently, resulted in the significantly higher ingestion rates for larvae fed heat-killed bacteria. Possible changes in the chemical structure of bacteria cell walls due to heat treatment may have facilitated digestion; however, carbon retention efficiencies were not significantly different between larvae fed heat-killed or

live bacteria.

Larvae fed on bacteria at different concentrations in Experiments 1, 2 and 3 showed significantly different clearance and ingestion rates. Retention efficiencies decreased, although not significantly, in Experiment 3 where ingestion rates were highest. The resulting effect was that larvae retained similar amounts of carbon during each experiment despite variation in bacteria cell concentrations from 1.5 to 2.6×10^7 cells ml^{-1}. Further experiments are required to determine if larvae regulate carbon retention rates when exposed to a wide range of bacteria concentrations.

Differently sized larvae were exposed to similar bacteria concentrations in each one of three experiments (Experiments 1, 2 and 3); however, in all cases, large larvae (214-290 μm shell length) showed significantly higher clearance rates, ingestion rates, retention efficiencies and consequently, retained larger amounts of carbon, than small larvae (94-100 μm shell length). Because of the dramatic increase in metabolic requirements as larvae grow in size (Table 7), the potential contribution of retained bacterial carbon to the metabolic requirements of larvae is more significant for small than for large larvae.

Clearance rates for straight-hinged larvae fed Isochrysis galbana at $5x10^4$ cells ml^{-1} equalled 1.869 μl $(larva)^{-1}$ h^{-1}. A clearance rate of 1.2 μl $(larva)^{-1}$ h^{-1} was estimated by Gallager et al., (1989) for 4 day-old scallop larvae fed I. galbana at $5x10^4$ cells ml^{-1}. Gerdes (1983b) reported clearance rates varying from 0.92 to 3.62 μl $(larva)^{-1}$ h^{-1} for 95-105 μm sized larval Crassostrea gigas fed $1x10^5$ cells ml^{-1}, while larvae 91-99 μm in size had clearance rates ranging from 1.9 to 12.3 μl $(larva)^{-1}$ h^{-1} when fed I. galbana at $2.5x10^4$ cells ml^{-1}.

Average ingestion of Isochrysis galbana by straight-hinged larval Crassostrea gigas during the 10 minute feeding period equalled 16.97 cells $(larva)^{-1}$. Most estimates of algal ingestion rates have been carried out with large sized bivalve larvae (e.g. Walne, 1965; Bayne, 1965). Ingestion rates have been related to shell length of larval Mytilus edulis according to equations developed by Sprung (1982) and Jespersen and Olsen (1982), which predict ingestion rates of 15.1 and 53.2 cells $(larva)^{-1}$ in 10 minutes, respectively, for mussel larvae 98 μm in shell length when fed on algae at 54 cells μl^{-1}. An ingestion rate of 10.3 cells $larva^{-1}$ was calculated for scallop larvae fed for 10 minutes on I. galbana based on data of Gallager et al. (1989). Similarly, ingestion rates calculated from data of Gerdes (1983b) for larval C. gigas

(91 to 105 μm shell length) ranged between 7.7 and 52 cells $(larva)^{-1}$ over a 10 minute feeding period. Experimental factors that could have affected measurements of ingestion rates in this study include the gut-fullness of larvae at the beginning of the labelling period. Larvae were pre-fed algae at optimal concentrations for 24 h, but were starved for 20 minutes while being manipulated at the beginning of each experiment. The feeding rate in short-term experiments is partly dependent on the nutritional adaptation of the organism (McMahon and Rigler, 1965). Feeding rates of bivalve larvae have been found to be 25 times higher in larvae with empty guts than in larvae with full guts (Gallager, 1988). However, algal ingestion rates determined in this study were not higher than published values, suggesting that the methods used in this study did not result in enhanced grazing activity. Similar methods were used to determine larval ingestion rates for CA2 cells; therefore, no enhanced grazing activities for larvae fed bacteria would be expected to result from experimental manipulations.

Assimilation efficiencies have been found to be affected by factors such as temperature, animal size, food concentration and diet (Lampert, 1977; Lampert and Egg, 1977). Assimilation efficiencies of mussel larvae have been reported to decrease from 45 to 31% as algal concentrations

increase (Jespersen and Olsen, 1982). Similarly, a decrease in assimilation efficiencies from 80 to 30% as a result of higher algal concentrations was reported by Crisp et al. (1985). Assimilation efficiencies of 71% were reported for larval Mytilus edulis (Bayne, 1976); 12.5-40% (Walne, 1965) and 40-77% (Gabbott and Holland, 1973) for Ostrea edulis; and 39-60% for scallop (Argopecten irradians) larvae (Gallager et al., 1989). A carbon retention efficiency of 52% determined in this study, corresponds to the upper reported estimates of assimilation efficiencies for bivalve larvae. The amount of algal carbon retained by straight-hinged larvae (98 μm shell length) was equivalent to more than 3 times the estimated larval metabolic requirements.

In order to compare utilization of bacteria and algae by oyster larvae, computations were carried out based on the carbon content of each food type. The method used to determine the amount of carbon per bacteria cell may have overestimated the weight of the cells because these were not washed-free of 0.5 M sodium chloride solution prior to freeze-drying. The carbon-dry weight ratio determined for CA2 cells was 0.4187. Lower (0.344; Ferguson and Rublee, 1976) and higher (0.5; Luria, 1960) carbon-dry weight ratios have been reported for bacteria. The amount of carbon in CA2 bacteria was determined to be 25.8 fg $(cell)^{-1}$. Lee and Furhman (1987) found that marine bacterioplankton had a carbon content of 20 fg $(cell)^{-1}$,

while Bratbak (1985) determined the amount of carbon in Pseudomonas putida to be between 129 and 312 fg $(cell)^{-1}$. Variation in the size of marine bacteria combined with the finding that small bacteria tend to have higher carbon to volume ratios (Lee and Furhman, 1987) could explain the range of values reported for the carbon content per cell for marine bacteria. Consequently, bacterial carbon-to-biovolume ratio (g C cm^{-3}) is a more useful estimate for comparisons. Any errors in the estimate of bacterial cell biovolume will accordingly affect the carbon-to-biovolume conversion ratio. Biovolume of CA2 cells was determined to equal 0.0968 μm^3. This estimate of bacteria biovolume is higher than the range for naturally occuring bacterioplankton (0.035-0.075 μm^3) determined by Lee and Fuhrman (1987) and Frikha and Linley (1988/1989), but within the range (0.02-0.17 μm^3) determined by Fergusson and Rublee (1976); Fuhrman (1981); Palumbo et al. (1984) and Riemann et al. (1984). Reported carbon-to-biovolume ratios for bacteria in g C cm^{-3} vary from 0.106 (Nagata, 1986) to 0.56 (Bratbak, 1985). Therefore, the carbon- to-biovolume ratio estimated for strain CA2 (0.266 g C cm^3) is within the reported range for marine bacteria.

A wide range of biovolume estimates for cells of Isochrysis galbana has been reported, extending from 23 μm^3 (Crisp et al., 1985) to 80 μm^3 (Fabregas et al., 1986;

Omori and Ikeda, 1984). An intermediary biovolume of 41.99 μm^3 was determined for I. galbana in this study. Not surprisingly, the calculated carbon content per cell (8.82 pg C $(cell)^{-1}$) represents an intermediate value for published estimates, which range from 5.1 pg C $(cell)^{-1}$ (Omori and Ikeda, 1984) to 20 pg C $(cell)^{-1}$ (Falkowski et al., 1985).

When cells of Isochrysis galbana and CA2 bacteria were added to larval cultures at similar particulate organic carbon concentrations (POC), highest ingestion rates, clearance rates and amounts of carbon retained were evident in larvae fed algae. However, carbon retention efficiencies were not significantly different for larvae fed on either food type. In contrast, when higher concentrations of bacterial POC were added to larval cultures than algal POC, then similar carbon ingestion rates were evident in larvae fed on either food type, but clearance rates, amounts of carbon retained and carbon retention efficiencies were significantly higher for larvae fed algae than for larvae fed bacteria. Consequently, under all conditions tested, larvae retained larger amounts of carbon from algae than from bacteria.

Carbon assimilation rate does not necessarily reflect the nutritional value of the assimilated diet (Lampert,

1977); for example similar assimilation efficiencies were determined for Daphnia fed either blue-green algae of poor food value or green algae of high food quality (Arnold, 1971; Lampert, 1977). Bacteria alone were found to be an inadequate diet for oyster larvae (Chapters 1 and 2), probably due to lack of long-chain polyunsaturated fatty acids (Kates, 1964; Perry et al. 1979) and sterols (Lehninger, 1975), both probably required by bivalves (Trider and Castell, 1980; Langdon and Waldock, 1981). However, bacteria may be a valuable food item because of its high nitrogen content, as demonstrated by the low C/N ratio (3.47) of CA2 bacteria. Furthermore, valuable nutrients for bivalve larvae such as vitamins (Kutsky, 1981) are produced by bacteria. Bacterial supply of essential nutrients was proposed as a mechanism by which additions of CA2 bacteria significantly enhanced the growth of larvae fed algal diets (Chapters 1 and 2).

Preferential ingestion of bacteria over phytoplankton has been demonstrated in asteroid larvae in the Antarctic (Rivkin et al. 1986) whereas phytoplankton is the principal food source for some temperate echinoderm species (Strathmann, 1971). Some crustaceans have been found to switch between various food sources over time, adapting to whatever food resource becomes available or abundant (Lee et al. 1976). Food selection may be an adaptation to

differences in the natural availability of food (Rivkin et al., 1986), or to reduce interspecific competition (Hicks and Coull, 1983). Provasoli et al. (1959) suggested that supplemental feeding (i.e. feeding on more than one food source) was a trophic strategy used by consumers in nature; for example, some copepods have been found to use detritus to supply the majority of their energy needs while requiring algae for egg production (Heinle et al., 1977). Ingestion and retention of bacterial carbon was demonstrated in differently sized oyster larvae, therefore, bacteria can be used as a food source throughout all larval development. However, the bacterial contribution to larval carbon metabolic requirements may be more significant for small sized larvae than for larger larvae.

The energy requirements for survival and growth of bivalve larvae have been reported to be greater than that which could be met by algae alone, at algal concentrations commonly reported in coastal waters (Walne, 1956b; Crisp et al., 1985). Up to 90% of the biomass (measured as chlorophyll a) of marine phytoplankton has been attributable to picoplankton (size range 0.2-2.0 μm) (Li et al., 1983); however, their contribution to the nutrition of bivalve larvae is not clear. Clam larvae were able to ingest and digest the cyanobacterium Synechococcus (Gallager, S. M., Woods Hole Oceanographic Institution,

Massachusetts; unpublished data). This diet did not support growth of larvae when provided alone, but growth of larvae fed algae of good nutritional quality was enhanced when Synechococcus was added as a dietary supplement. Scallop larvae efficiently digested cells of Aureococcus anophagefferens; however the contribution of this picoplankton species to the nutrition of bivalve larvae may be very limited due to inefficient cell capture by larvae, poor growth of larvae fed only on this diet, adverse effects on larval growth when fed in combination with Isochrysis, and deleterious effects on survival and growth when fed to larvae at near bloom concentrations (Gallager et al., 1989).

Crisp et al. (1985) suggested that non-algal food sources make a significant contribution to bivalve larvae nutrition in the marine habitat. Imai and Hatanaka (1949) cultured larval Crassostrea gigas on diets of naked heterotrophic flagellates. Straight-hinged oyster larvae have a mouth opening of less than 10 μm (Ukeles, 1969); therefore this should limit the size of protozooplankton that could be ingested by small larvae. Heterotrophic nanoplankton concentrations in coastal and open ocean environments range from 10^2 to 10^3 cells ml^{-1}, increasing to 10^4 cells ml^{-1} in eutrophic waters (Fenchel, 1982; Caron, 1983; Sherr and Sherr, 1984). These

concentrations are not different from those of phytoplankton determined in the same environments (Caron, 1983).

Direct utilization of dissolved organic matter as a food source of invertebrates was advanced early this century by Pütter (1909), and other researchers examined the role of different dissolved compounds in the nutrition of bivalve larvae (see review by Ukeles, 1969; Manahan, 1990). Manahan (1989) demonstrated that axenic Crassostrea gigas larvae can take up dissolved amino acids from seawater. Larvae exposed to high amino acid concentrations (10 μM) could potentially meet 100% of their energy requirements for oxidative metabolism by amino acid uptake. As recognized by Manahan (1989), these concentrations are unrealistically high for the marine environment (amino acid concentrations in marine habitats ca. <0.1 μM: Heinrichs and Williams, 1985; Williams 1986) and may only be found in "microzones" of high nutrient concentration (Nissen et al., 1984). The ability of larvae to take advantage of the high nutrient concentrations of microzones has yet to be demonstrated. Utilization of detritus prepared from plant and animal material was demonstrated to occur in adult oysters (Gavard, 1927) and larvae (DeBoer, 1975); however, the chemical composition of these organic aggregates may have differed significantly from that of natural detritus.

No growth improvement of larvae resulted from additions of marine detritus from different sources to larval cultures (Davis, 1950), and low absorption (3%) of cellulosic material was reported by adult Crassostrea virginica (Langdon and Newell, 1990).

Bacteria are ubiquitous in the marine environment, and concentrations up to 10^7 cells ml^{-1} have been reported in environments where bivalve larvae are likely to be found (see below). Vertical heterogeneity in bacterial abundance, with concentrations varying by up to 3.5 fold over distances of 10 cm, was reported in coastal waters by Mitchell and Furhman (1989). Further research is required to determine concentrations of bacteria that bivalve larvae may encounter while swimming through the water column.

In summary, bacterivory was shown to be important in the nutrition of oyster larvae in this study, in which CA2 bacteria were fed to larvae in experiments designed to reduce major sources of error (e.g. label recycling) in estimating utilization of bacteria as a food source. The contribution that bacteria may have to the carbon requirements of small (94-100 μm) oyster larvae was shown to exceed larval carbon metabolic requirements. Cell size and carbon content of bacteria strain CA2 were within reported ranges for bacteria found in natural environments;

however, not all bacteria strains have similar effects on bivalve larvae (Chapter 1), and extrapolation of results obtained with strain CA2 to describe interactions between populations of bacteria and bivalve larvae in natural conditions should be undertaken with caution.

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APPENDICES

APPENDICES

APPENDIX I

COMPARISON OF INGESTION RATES OF OYSTER LARVAE FED EITHER LIVE OR ^{60}Co-IRRADIATED ALGAE (ISOCHRYSIS GALBANA).

Ingestion rates of larvae fed on either live or ^{60}Co-irradiated Isochrysis galbana were determined as follows: both types of algae cells were initially suspended separately in FSSW and subsequently each suspension was equally subdivided into eight 250 ml beakers. Suspensions of 5 day old oyster larvae were added at a final density of 20 ml^{-1} to four beakers containing each algal type, while equal volumes of FSSW were added to the other 4 culture flasks so as to maintain identical algal concentrations in flasks with or without larvae. The final seawater volume in each flask was 70 ml. Initial algal concentrations were determined by removing 4 ml sub-samples from each flask, after evenly dispersing algae and larvae by agitation with a perforated plastic plunger. Samples were diluted with 16 ml of FSSW and the concentrations of algae determined using a Coulter Counter. After a 105 minute grazing period, a second set of samples were taken from all flasks and algal concentrations determined. Ingestion rates (I_p) were

determined as described in Checkley (1980):

$$a = (1 / T) \ln (P_{T'} / P_{0'})$$

$$m = (1 / T) \ln (P_T / P_0)$$

$$g_p = a - m$$

$$P = (1 / T) \int_0^T P_0 e^{mT} dt = (P_0(e^{mT} - 1) / (mT)$$

$$C = Vg_p / N_F$$

$$I_p = PC$$

where:

a	Instantaneous rate of change of phytoplankton cell concentration in the control flask	(h^{-1})
m	Instantaneous rate of change of phytoplankton cell concentration in the treatment flask	(h^{-1})
g_p	Instantaneous rate of change of phytoplankton cell concentration attributed to grazing by oyster larvae	(h^{-1})
T	Duration of the experiment	(h^{-1})
$P_{0'}$	Initial food concentration in control flask	(cells ml^{-1})
$P_{T'}$	Final food concentration in control flask	(cells ml^{-1})
P_0	Initial food concentration in treatment flask	(cells ml^{-1})
P_T	Final food concentration in treatment flask	(cells ml^{-1})
P	Average food concentration in treatment flask	(cells ml^{-1})
C	Clearance rate	(ml $(larva)^{-1}$ h^{-1})
V	Volume of flask	(ml)

N_F Number of live larvae in treatment flask (larvae)

I_p Rate of ingestion of phytoplankton (cells (larva)$^{-1}$ h^{-1})

Table I. Clearance and ingestion rates of 5 days-old oyster larvae fed live or ^{60}Co-irradiated Isochrysis galbana for 105 minutes. Four replicate larval cultures were used per treatment.

	CLEARANCE RATE (ml larva^{-1} h^{-1})	INGESTION RATE (cells larva^{-1} h^{-1})
ALGAE	0.0041	214
	0.0050	259
	0.0048	248
	0.0026	137
mean ± s.d.	0.0041 ± 0.0011	214 ± 55.10
IRRADIATED	0.0104	511
ALGAE	0.0123	587
	0.0151	691
	0.0151	690
mean ± s.d.	0.0132 ± 0.0023	619.75 ± 87.39

Two-sample t-tests for ingestion of either live or ^{60}Co-irradiated Isochrysis galbana by oyster larvae in a 105 minutes grazing period.

		SAMPLE		
ALGAE TYPE	MEAN	SIZE	S.D.	S.E.
Live	214.4	4	54.88	27.44
^{60}Co-irradiated	619.6	4	87.55	43.77

	t	DF	P
Equal variances	-7.84	6	0.0002
Unequal variances	-7.84	5	0.0005

	F	NUM DF	DEN DF	P
Test for equality of variances	2.54	3	3	0.2317

APPENDIX II

STATISTICAL ANALYSES OF RESULTS OF LARVAL SURVIVAL AND GROWTH IN CULTURE EXPERIMENTS IN CHAPTER 1.

Results of one-way analyses of variance of transformed data of larval survival [arcsin (square root (percent survival x 100^{-1})] and of Kruskal-Wallis analyses of data of larval growth in Experiments 1 to 5 in Chapter 1.

Experiment 1

Survival Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	13	5.7613	0.4432	11.14	0.0000
Within	41	1.6210	0.0386		
Total	55	7.3823			

Growth

Kruskal-Wallis statistic	50.8839
P value, using chi-squared approximation	0.0000

Experiment 2

Survival Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	13	3.9816	0.3063	21.73	0.0000
Within	42	0.5919	0.0141		
Total	55	4.5736			

Growth

Kruskal-Wallis statistic	39.1422
P value, using chi-squared approximation	0.0002

Experiment 3

Survival Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	4	2.2969	0.5742	31.53	0.0000
Within	35	0.6374	0.0182		
Total	39	2.9343			

Growth

Kruskal-Wallis statistic	35.1271
P value, using chi-squared approximation	0.000

Experiment 4

Survival Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	6	1.9297	0.3216	19.59	0.0000
Within	21	0.3447	0.0164		
Total	27	2.2744			

Growth

Kruskal-Wallis statistic	24.4741
P value, using chi-squared approximation	0.0004

Experiment 5

Survival Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	4	1.9415	0.4854	37.14	0.0000
Within	35	0.4574	0.0131		
Total	39	2.3988			

Growth

Kruskal-Wallis statistic	25.7828
P value, using chi-squared approximation	0.0000

APPENDIX III

STATISTICAL ANALYSES OF RESULTS OF LARVAL SURVIVAL AND GROWTH IN CULTURE EXPERIMENTS IN CHAPTER 2.

One-way analyses of variance of larval survival and growth under different feeding treatments in Experiment 1, Chapter 2.

Survival		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	5	10,611	2,122	51.40	0.0000
Within	18	743	41		
Total	23	11,354			

Growth		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	5	36,079	7,215.8	115.16	0.0000
Within	18	1,123	62.4		
Total	23	37,203			

One-way analyses of variance of number and size of spat produced under different treatments in Experiment 1, Chapter 2.

Number of spat Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	3	10,334	3,444.6	4.8	0.0202
Within	12	8,616	718.0		
Total	15	18,950			

Size of spat Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	3	1.1183	0.3727	6.75	0.0064
Within	12	0.6627	0.0552		
Total	15	1.7811			

One-way analysis of variance of transformed data of larval growth [arcsin (square root ((ln L_t - ln L_0) t^{-1})), where L_t = final mean shell length (μm), L_0 = initial mean shell length (μm) and t = culture period (10 days)] and summary of Kruskal-Wallis test of data of larval survival in Experiment 2, Chapter 2.

Growth Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	5	0.09797	0.01959	149.87	0.0000
Within	18	0.00235	0.00013		
Total	23	0.10032			

Survival

Kruskal-Wallis statistic	15.0086
p value, using Chi-squared approximation	0.0103

Two-way analyses of variance of transformed data of larval survival [arcsin (square root (percent survival x 100^{-1}))] and growth [arcsin (square root ((ln L_t - ln L_0) t^{-1})), where L_t = final mean shell length (μm), L_0 = initial mean shell length (μm) and t = culture period (10 days)] in different treatments in Experiments 3 and 4, Chapter 2.

Survival Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	1	0.07385	0.07385	0.89	0.3571
Treatment	(B)	2	0.52471	0.26236	3.17	0.0660
Interactions	(A*B)	2	0.22429	0.11215	1.36	0.2827
Replicates	(C)					
Error	(A*B*C)	18	1.4885	0.08269		
Total		23	2.3113			

Growth Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	1	0.00006	0.00006	0.4	0.5259
Treatment	(B)	2	0.0688	0.0344	243.9	0.0000
Interactions	(A*B)	2	0.0004	0.0002	1.4	0.2642
Replicates	(C)					
Error	(A*B*C)	18	0.00254	0.00014		
Total		23	0.07184			

Two-way analyses of variance of transformed data of larval survival [arcsin (square root (percent survival x 100^{-1}))] and growth [arcsin (square root ((ln L_t - ln L_0) t^{-1})), where L_t = final mean shell length (μm), L_0 = initial mean shell length (μm) and t = culture period (10 days)] in different feeding treatments in Experiments 5, Chapter 2.

Survival			Sum of	Mean		Sig.
Source of variation		d.f.	squares	squares	F-ratio	level
Diet	(A)	2	0.1081	0.05409	3.76	0.0432
CA2 addition	(B)	1	0.0244	0.02440	1.70	0.2092
Interactions	(A*B)	2	0.0954	0.04774	3.32	0.0593
Replicate	(C)					
Error	(A*B*C)	18	0.2589	0.0143		
Total		23	0.4870			

Growth			Sum of	Mean		Sig.
Source of variation		d.f.	squares	squares	F-ratio	level
Diet	(A)	2	0.03342	0.01667	274.06	0.0000
CA2 addition	(B)	1	0.00320	0.00320	52.77	0.0000
Interactions	(A*B)	2	0.00012	0.00006	1.03	0.3761
Replicates	(C)					
Error	(A*B*C)	18	0.00109	0.00006		
Total		23	0.03777			

One-way analyses of variance of number and size of spat produced under different treatments in Experiment 5, Chapter 2.

Number of spat Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	2	414,800	207,400	28.31	0.0001
Within	9	65,930	7,320		
Total	11	480,700			

Size of spat Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	2	0.0568	0.0284	1.80	0.2193
Within	9	0.1417	0.0157		
Total	11	0.1986			

APPENDIX IV

STATISTICAL ANALYSES OF SKEWNESS COEFFICIENTS (g1) OF SIZE FREQUENCY DISTRIBUTIONS OF POPULATIONS OF XENIC LARVAE FED EITHER ALGAE ALONE OR ALGAE WITH CA2 BACTERIA.

Two-way analysis of variance of skewness coefficients (g1) of size frequency distributions determined in populations of xenic larvae fed either algae alone or algae with CA2 bacteria added at 10^5 cells ml^{-1}. Data include results of Experiments 1, 2, 3 and 4 carried out with Isochrysis galbana (clone ISO). Data of Experiment 5 were split in two groups depending on the algal species used (Isochrysis aff. galbana clone T-ISO, Pseudoisochrysis paradoxa clone VA-12) and analyzed as two independent experiments. Chapter 2.

g1 coefficients			Sum of	Mean		Sig.
Source of variation		d.f.	squares	squares	F-ratio	level
Experiment	(A)	5	5.944	1.188	9.20	0.0000
CA2 addition	(B)	1	2.719	2.719	21.04	0.0001
Interactions	(A*B)	5	1.293	0.258	2.00	0.1021
Replicates	(C)					
Error	(A*B*C)	36	4.654	0.129		
Total		47	14.611			

Analyses of variance of skewness coefficients (g1) of size frequency distributions of populations of larvae fed either algae alone or algae and CA2 bacteria added at 10^5 cells ml^{-1}. Data of Experiment 5 were analyzed as 2 independent experiments, depending on the algal species used (Isochrysis aff. galbana clone T-ISO, Pseudoisochrysis paradoxa clone VA-12). Chapter 2.

Experiment 1		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	1	0.047	0.047	0.24	0.6429
Within	6	1.177	0.196		
Total	7	1.224			

Experiment 2		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	1	0.4116	0.4116	1.43	0.2763
Within	6	1.7221	0.2870		
Total	7	2.1337			

Experiment 3		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	1	0.3915	0.3915	8.36	0.0277
Within	6	0.2811	0.0468		
Total	7	0.6727			

Experiment 4		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	1	1.7586	1.7586	17.65	0.0057
Within	6	0.5978	0.0996		
Total	7	2.3565			

Experiment 5 (T-ISO)		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	1	0.5250	0.5250	5.28	0.0613
Within	6	0.5970	0.0995		
Total	7	1.1220			

Experiment 5 (VA-12)		Sum of	Mean		Sig.
Source of variation	d.f.	squares	squares	F-ratio	level
Between	1	0.8794	0.8794	18.90	0.0048
Within	6	0.2792	0.0465		
Total	7	1.1586			

APPENDIX V

STATISTICAL ANALYSES OF RESULTS OF THREE FEEDING EXPERIMENTS IN WHICH BACTERIA WERE FED TO EITHER LARGE (214-290 μm SHELL LENGTH) OR SMALL (94-100 μm SHELL LENGTH) OYSTER LARVAE. CHAPTER 3.

Two-way analysis of variance table for concentration of CA2 cells in cells ml^{-1}.

Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	2	3.0x1014	$1.5x10^{14}$	1162.02	0.000
Size	(B)	1	$2.7x10^{9}$	$2.7x10^{9}$	0.02	0.891
Interactions	(A*B)	2	$4.9x10^{10}$	$2.4x10^{10}$	0.19	0.835
Replicates	(C)					
Residual	(A*B*C)	6	$7.9x10^{11}$	$1.3x10^{11}$		
Total		11	$3.1x10^{14}$			

Tukey (HSD) pairwise comparisons of concentration of CA2 cells (in cells ml^{-1}) by experiment, at a 0.05 probability level.

Experiment	Mean	groups
3	$2.6x10^{7}$	I
1	$1.6x10^{7}$	..I
2	$1.5x10^{7}$	I

Two-way analysis of variance table for clearance rates of CA2 bacteria in μl (larva)$^{-1}$ h^{-1}.

Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	2	0.490	0.245	6.77	0.0289
Size	(B)	1	11.978	11.978	330.48	0.0000
Interactions	(A*B)	2	0.301	0.150	4.16	0.0737
Replicates	(C)					
Residuals	(A*B*C)	6	0.217	0.036		
Total		11	12.988			

Tukey (HSD) pairwise comparisons clearance rates (in μl (larva)$^{-1}$ h^{-1}) of CA2 cell by experiment, at a 0.05 probability level.

Experiment	Mean	Groups
2	2.365	I
1	2.268	I I
3	1.896	..I

Two-way analysis of variance table for ingestion rates of CA2 cells in cells $(larva)^{-1}$ $(10\ minutes)^{-1}$.

Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	2	$1.3x10^{7}$	$6.9x10^{6}$	13.26	0.0063
Size	(B)	1	$1.1x10^{8}$	$1.1x10^{8}$	219.57	0.0000
Interactions	(A*B)	2	$1.0x10^{6}$	$5.1x10^{5}$	0.98	0.4291
Replicates	(C)					
Residual	(A*B*C)	6	$3.1x10^{6}$	$5.2x10^{5}$		
Total		11	$1.3x10^{8}$			

Tukey (HSD) pairwise comparisons of ingestion rates of CA2 cells (in cells $(larva)^{-1}$ $(10\ minutes)^{-1}$) by experiment, at a 0.05 probability level.

Experiment	Mean	Groups
3	$8.3x10^{3}$	I
1	$6.1x10^{3}$	..I
2	$6.0x10^{3}$	..I

Two-way analysis of variance table for arcsin-transformed carbon retention efficiencies originally expressed as percentages.

Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	2	0.03534	0.01767	4.22	0.0716
Size	(B)	1	0.13565	0.13565	32.43	0.0013
Interactions	(A*B)	2	0.00048	0.00024	0.06	0.9447
Replicates	(C)					
Residuals	(A*B*C)	6	0.02510	0.00418		
Total		11	0.19657			

Two-way analysis of variance table for log-transformed carbon retention in pg C $(larva)^{-1}$ $(10\ minutes\ feeding)^{-1}$.

Source of variation		d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Experiment	(A)	2	0.0106	0.0053	2.34	0.1775
Size	(B)	1	1.1280	1.1280	497.11	0.0000
Interactions	(A*B)	2	0.0029	0.0014	0.65	0.5536
Replicates	(C)					
Residuals	(A*B*C)	6	0.0136	0.0022		
Total		11	1.1551			

APPENDIX VI

STATISTICAL ANALYSES OF RESULTS OF FEEDING EXPERIMENTS IN WHICH STRAIGHT-HINGED OYSTER LARVAE WERE FED ON EITHER BACTERIA (STRAIN CA2) OR ALGAE (ISOCHRYSIS GALBANA).

One-way analysis of variance table for POC (in pg C ml^{-1}) added as diet.

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between treatments	3	105,300	35,100	73.26	0.0006
Within treatments	4	1,916	479.1		
Total	7	107,200			

Tukey (HSD) pairwise comparisons of POC (in pg C ml^{-1}) added as diet by experiment.

Experiment	Mean	Groups
3	683.8	I
4	481.1	..I
1	412.8	..I
2	394.7	..I

One-way analysis of variance table for clearance rates in μl (larva)$^{-1}$ h^{-1}.

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between treatments	3	0.7619	0.2540	27.20	0.0040
Within treatments	4	0.0373	0.0093		
Total	7	0.7993			

Tukey (HSD) pairwise comparisons of clearance rates (in μl (larva)$^{-1}$ h^{-1}) by experiment.

Experiment	Mean	Groups
4	1.869	I
2	1.301	..I
1	1.116	..I
3	1.115	..I

One-way analysis of variance table for carbon ingestion in pg C $(larva)^{-1}$ $(10\ minutes)^{-1}$

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between treatments	3	7,110	2,370	26.36	0.0043
Within treatments	4	359.7	89.9		
Total	7	7,470			

Tukey (HSD) pairwise comparisons of carbon ingestion (in pg C $(larva)^{-1}$ $(10\ minutes)^{-1}$) by experiment.

Experiment	Mean	Groups
4	149.7	I
3	127.1	I
2	85.63	..I
1	76.93	..I

One-way analysis of variance table for arcsin-transformed carbon retention efficiencies originally expressed as percentages.

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	3	0.0383	0.0127	10.52	0.0228
Within	4	0.0048	0.0012		
Total	7	0.0432			

Tukey (HSD) pairwise comparisons of arcsin-transformed carbon retention efficiencies (originally expressed as percentages) by experiment.

Experiment	Mean	Groups
4	0.810	I
2	0.729	I I
1	0.712	I I
3	0.615	..I

One-way analysis of variance table for carbon retention expressed in pg C $(larva)^{-1}$ $(10\ minutes\ feeding)^{-1}$.

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio	Sig. level
Between	3	2,603	867.7	27.20	0.0040
Within	4	127.6	31.9		
Total	7	2,731			

Tukey (HSD) pairwise comparisons of carbon retention (in pg C $(larva)^{-1}$ $(10\ minutes)^{-1}$) by experiment.

Experiment	Mean	Groups
4	78.64	I
3	42.15	..I
2	38.12	..I
1	32.80	..I