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Nutrient Limitation in the Symbiotic Association between Zooxanthellae and Reef-building Corals: A United States–Israel Workshop

Edited by Paul L. Jokiel

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"Nutrient Limitation in the Symbiotic Association between Zooxanthellae and Reef-building Corals"

A workshop held at the Hawaii Instititue of Marine Biology, Coconut Island, Hawaii, 25 to 30 August 1991.

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Results of the 1991 United States–Israel Workshop, "Nutrient Limitation in the Symbiotic Association between Zooxanthellae and Reef-building Corals"[1]

PAUL L. JOKIEL,[2] ZVY DUBINSKY,[3] AND NOGA STAMBLER[3,4]

ABSTRACT: An intensive research effort was organized as a 1-week workshop with the specific goal of resolving difficult questions concerning whether or not zooxanthellae are nutrient limited within host corals. Over 30 scientists participated. Participants applied various techniques to the same set of corals, which had been preincubated under different nutrient regimes for various time periods. Interdisciplinary research projects of this scale and intensity have rarely been attempted by coral reef biologists, yet the evidence developed during the workshop demonstrates the usefulness of this approach. This was a true "workshop" rather than a "talk-shop" and produced important research results while allowing participants to compare research methods and discuss various theories and research philosophies. Important new data were developed. Apparent contradictions were resolved through development of models that consider the dynamics of carbon fixation relative to nutrient availability.

REEF CORALS ARE mutualistic associations between marine invertebrate hosts and microalgal symbionts known as zooxanthellae. Reef corals typically flourish in nutrient-poor tropical and subtropical waters, creating massive biogenic structures. It has been argued that the plant and animal partners in such symbioses exchange vital nutrients and thus have a natural advantage in nutrient-poor tropical waters (Muscatine and Porter 1977). Carbon fixed by algal photosynthesis is translocated to the coral host and inorganic plant nutrients from the animal are taken up by the algae.

Corals are capable of taking up, retaining, and recycling both inorganic and organic dissolved nutrients (Muscatine and Porter 1977, Rahav et al. 1989). In addition, corals obtain nutrients derived from digestion of prey (Erez 1990). The coral *Stylophora pistillata* Esper from the Red Sea, Israel, responds to enrichment with ammonium, or ammonium + phosphate, mostly by increasing the algal density (Muscatine et al. 1989). The same was found for the coral *Pocillopora damicornis* (Linnaeus) from Kaneohe Bay, Hawaii (Stambler et al. 1991). The photosynthetic rate of the nitrogen-enriched colonies increases in comparison with that of controls, although the photosynthetic rate per algal cell decreases (Dubinsky et al. 1990).

There is some question as to whether or not the zooxanthellae are limited by nitrogen or phosphorous while in hospice. Results of previous studies are contradictory. On one hand, the areal density of zooxanthellae in hospice is reported to increase in response to nutrient enrichment (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Dubinsky et al. 1990, Stambler et al. 1991). Such increased algal density in response to increased nutrient is the classical response of nutrient-limited populations. On the other hand, flux of nitrogen from host metabolism appears to exceed requirements of zooxanthellae for

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growth, so the algae should not be nutrient limited in hospice (Rahav et al. 1989). The conclusion that zooxanthellae are nutrient limited as deduced from the increase in zooxanthellae densities in response to nutrient loading is not consistent with conclusions reached by Miller and Yellowlees (1989), based on biochemical indicators. Cook and D'Elia (1987) investigated nitrogen uptake using the nonmetabolizable ammonium analog ^{14}C methylammonium and concluded that zooxanthellae are, in fact, nutrient limited.

Such conflicting results were presented and discussed during the session on nutrient limitation during the Sixth International Coral Reef Symposium in Townsville, Australia, in 1988. These discrepancies may well be due to differences in geographic location, season, cnidarian and algal species involved, or experimental technique. It was obvious that this issue could not be resolved without further investigation, because existing results were based on experiments done in various seas and seasons, under different nutrient levels, and with different organisms. It was agreed that a "hands-on" workshop would be undertaken where participants from different disciplines could work together on the same organisms and samples. The ideal location for such an experiment was the Hawaii Institute of Marine Biology (HIMB) at Coconut Island with its excellent laboratories, access to reefs, and living accommodations.

Participants of the symposium asked P. L. Jokiel and Z. Dubinsky to organize the project. The workshop was sponsored by HIMB in collaboration with the United States–Israel Binational Science Foundation and the University of Hawaii Sea Grant Program and took place at HIMB on Coconut Island, in Kaneohe Bay, Oahu. Over 30 coral reef specialists from the United States, Israel, and four additional countries, among them coral reef biologists, geochemists, physiologists, biochemists, biophysicists, and molecular biologists, joined forces to further our understanding of the nutrient status and fluxes in corals and coral reefs. They brought their various techniques and specialized instrumentation to Coconut Island, and all worked simultaneously on samples from coral colonies that were preincubated for periods of time extending up to 8 weeks under four different ammonium concentrations—nutrient-stripped water ($\ll 1$ μM ammonium), seawater at ambient nutrient concentration (< 1 μM ammonium), and two levels of ammonium enrichment (20 μM and 50 μM). The experiment is described in detail in the following article by Stambler et al. (1994*b*).

The true "experiment" here was not that of nutrient limitation, but rather whether or not such a massive undertaking could be completed within 1 week. This was a true workshop and not a "talk-shop" in that all scientists participated directly in the experiment and worked around the clock. The only organized oral presentations were made during an introductory organizational meeting on Sunday and a summary discussion at the end of the session on the following Friday morning.

RESULTS AND DISCUSSION

Detailed biochemical, physiological, and morphometric data were gathered during the experiment. Results of many of the studies conducted during the workshop are contained in this issue of *Pacific Science*, and other results are forthcoming. A brief summary of detailed findings is as follows:

The carbohydrate, lipid, and protein composition of the zooxanthellae and animal fractions of corals grown under different nutrient regimes were measured (Achituv et al. 1994). Total amino-N and glutamine (gln) to glutamate (glu) ratios were also measured (McAuley 1994). The gln : glu ratios appear to be a sensitive indicator of zooxanthellae response to exogenous nitrogen and suggest that the added ammonium was directly utilized by the symbiotic zooxanthellae. Most of the nitrogen from the elevated seawater ammonium was retained by the zooxanthellae rather than the animal fraction (Muller-Parker et al. 1994*a*), although both plant and animal biomass increased in the 20-μM treatment (Muller-Parker et al. 1994*b*). Analysis of enzymes in the plant and animal fractions

revealed that the animal fraction may play an important role in nitrogen uptake (Yellowlees et al. 1994).

The 50-μM ammonium treatment may have been slightly toxic to the corals. Biomass parameters did not increase with time in that treatment (Muller-Parker et al. 1994*b*). Zooxanthellae in corals exposed to the 20-μM ammonium treatment had mitotic indices (percentage of cells dividing) that were two to three times higher than those of the controls (Høegh-Guldberg 1994). Cells in both ammonium-enriched treatments divided at a higher rate than in the controls. Division of zooxanthellae was still phased in the 20-μM treatment, but there were more cells dividing out of phase compared with the control treatment. Cells in the 50-μM ammonium treatment were out of phase, suggesting destabilization of the symbiosis. Micromorphometric technique (Berner and Izhaki 1994) as well as elemental analysis (Muller-Parker et al. 1994*a*) showed decreasing availability of surplus carbon, giving further evidence of an impaired symbiotic relationship in ammonium-enriched treatments.

The work of Atkinson et al. (1994) emphasizes that delivery rate of ammonium to the coral is controlled by water motion as well as by ammonium concentration. To a degree, "nutrient limitation" must consider "water motion limitation" that involves physical barriers to mass transfer. The results of Muscatine and Kaplan (1994) remind us that corals may become "nutrient-limited" only under high light conditions that allow extremely high photosynthetic rates. Further, different species of corals show different responses to the same alterations in nutrient regime (Stambler et al. 1994*a*).

By the end of the workshop, several of the participants had formulated a model that resolved many previous contradictions (Falkowski et al. 1993). In essence, the argument is made that under normal conditions in nutrient-poor tropical seas, zooxanthellate corals are successful because they are closed systems with respect to nitrogen. Growth of zooxanthellae under these conditions is not balanced with respect to fixed carbon because of the low rate of nitrogen supply. As a result, the excess carbon is translocated to the animal host. Increasing the nitrogen supply leads to rapid growth of the zooxanthellae, with consequent reduction of translocated carbon to the host. Eutrophic conditions allow the zooxanthellae to outgrow their hosts and the host loses control over the population of its symbiotic algae. Thus, maintenance of a balanced coral symbiotic association appears to require low ambient nutrient concentrations. Other aspects of the dynamic carbon-nitrogen model can be clarified by examining the ratio of energy and nutrient fluxes in the regulation of the symbiosis (Dubinsky and Jokiel 1994).

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Nutrient Limitation in the Symbiotic Association between Zooxanthellae and Reef-building Corals: The Experimental Design[1]

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ABSTRACT: The question of nutrient limitation and of its regulatory effect on population densities of zooxanthellae in hospice was studied by an international team of researchers during an intensive 5-day workshop. Participants studied colonies of two coral species that were preincubated over different time periods ranging from 0 to 8 weeks under four different nutrient concentrations. A broad spectrum of parameters was measured simultaneously at the molecular, cellular, and colony levels of organization using a variety of techniques. This paper describes the overall experimental design.

THE WORKSHOP "Nutrient Limitation in the Symbiotic Association between Zooxanthellae and Reef-building Corals" was organized in response to scientific needs described in the preceding paper (Jokiel et al. 1994). The experimental approach chosen was to provide researchers with preincubated corals grown under different nutrient regimes for various lengths of time. These corals would be analyzed simultaneously by several teams using different techniques. All important parameters would be measured on the same corals. The result would be an extensive data set that might provide new insights into the dynamics of nutrient metabolism and resolve apparent conflicts between previous studies. Nitrogen was selected as the nutrient species to be investigated. Previous studies showed that the response of reef corals to nitrogen enrichment is more pronounced than that to phosphorus enrichment (Stambler et al. 1991). Further, it was important to keep the experimental design simple. Inorganic nitrogen was supplied in the form of ammonium, which has been shown to be rapidly taken up by reef corals (Kawaguti 1953, Muscatine and D'Elia 1978, Burris 1983, Muscatine et al. 1984).

MATERIALS AND METHODS

Two common species of Hawaiian reef corals were selected for this study. The highly branched imperforate species *Pocillopora damicornis* (Linnaeus) has been widely used for physiological studies throughout the Indo-Pacific (e.g., Richmond 1985). The perforate coral *Montipora verrucosa* Vaughan was selected as a second species for comparison. Corals were preincubated over different periods ranging from 0 to 8 weeks, under four different nutrient concentrations.

Colonies of *P. damicornis* about 10 cm in diameter and colonies of *M. verrucosa* were collected from Kaneohe Bay (Oahu, Hawaii). All of the colonies were initially collected from the same reef over a period of a few days and maintained thereafter in holding tanks under the same conditions as the control (ambient) treatment. At various time intervals, groups of corals were moved from the holding tanks into the experimental treatments.

Experiments were carried out in eight white fiberglass tanks with a water volume of ca. 400 liters (1.15 by 1.15 by 0.27 m). Each tank was supplied with unfiltered running seawater, at a rate of 4 liters min^{-1}. All tanks were aerated. Tanks were located in sunlight and covered with neutral-density shade cloth

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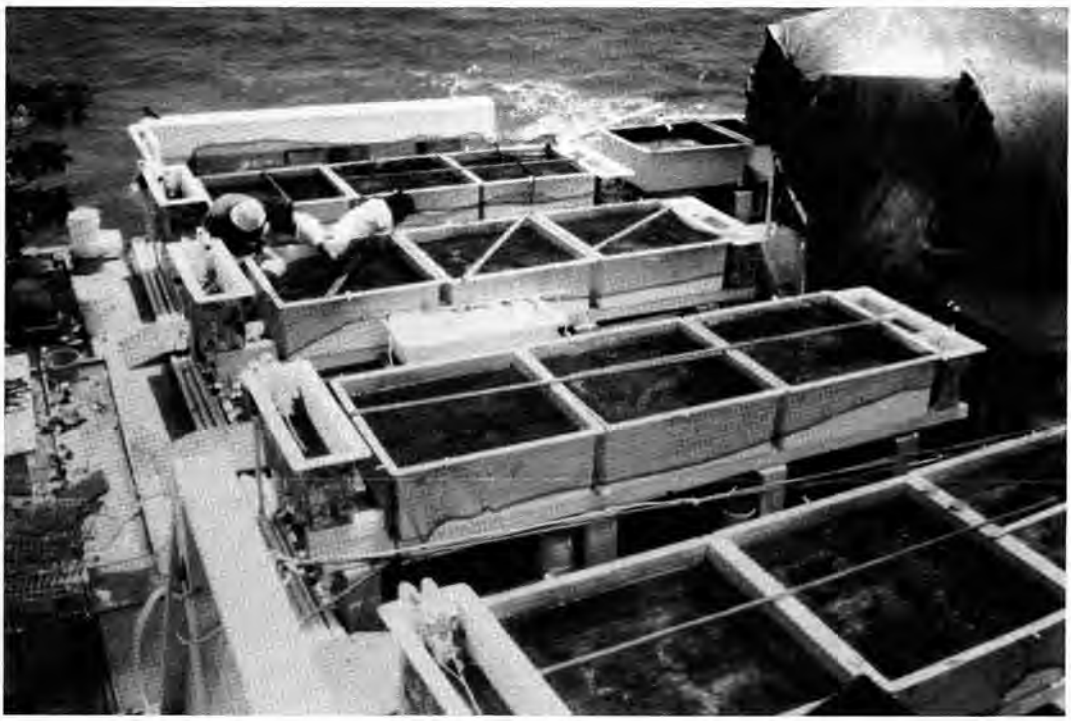

FIGURE 1. View of the experimental tank facility.

so as to expose the corals to 80% of full solar radiation (Figure 1).

Six to eight colonies of *P. damicornis* from the holding tanks were transferred to the experimental tanks every 2 weeks. Thus, colonies preincubated for 0, 2, 4, 6, and 8 weeks were available to the investigators for simultaneous analysis during the 5-day workshop.

Ammonium $[(NH_4)_2SO_4]$ solution was pumped into the intake flow of the tanks with a peristaltic pump at a rate sufficient to raise the nutrient level to either 20 μM or 50 μM. Water entering the "ambient" control tanks was not altered, so values in those tanks remained the same as nutrient concentrations on reefs in Kaneohe Bay (<1 μM). Water supplied to the "nutrient stripped" treatment was first passed through a flume (4 m long, 40 cm wide, 40 cm deep) filled with the macroalga *Gracillaria salicornia* (C. Agardh) Dawson (Figure 2). Water leaving the flume had undetectable ammonium concentration. Water within the "stripped" tanks, however, showed ammonium concentrations approaching that found in the ambient tanks. Possible sources of this nitrogen include nitrogen excreted and lost from the experimental corals added to the "stripped tanks" and nitrogen fixed by algae growing within the tanks.

From an ecological point of view, levels of nitrogen in the 20-μM and the 50-μM treatments are clearly above values encountered in nature. However, to investigate the dynamics of the symbiosis, it is useful to load the symbiotic relationship above the normal limits and observe the outcome.

In the course of the workshop the following parameters were determined: (1) coral growth rate; (2) density of the zooxanthellae within their host; (3) photosynthesis rate of the zooxanthellae, in hospice and after isolation; (4) dark respiration rates of the intact colony and of freshly isolated zooxanthellae; (5) division rate of the zooxanthellae; (6) levels and activity of the key enzymes involved

FIGURE 2. Flume containing macroalgae used to remove nutrients from the "stripped" treatment.

in the uptake and assimilation of nitrate and ammonium by the zooxanthellae; (7) levels and activity of carbonic anhydrase, postulated to facilitate CO_2 uptake under limiting conditions; (8) the chemical composition of both the zooxanthellae and animal fractions of the symbiosis; (9) release rate of zooxanthellae; (10) ultrastructure of zooxanthellae.

RESULTS AND DISCUSSION

During preliminary experiments, concentrations of 100 μM ammonium were shown to be toxic to the two species of Hawaiian corals, although this concentration was previously used successfully with the coral *Stylophora pistillata* Esper (Muscatine et al. 1989). Treatment levels of 50 μM ammonium, 20 μM ammonium, ambient (<1 μM ammonium), and "stripped" ($\ll 1$ μM) treatments were selected for the experiment. The colonies in all four treatments remained alive throughout the course of the experiment. Colonies grown in the highest ammonium concentration appeared to be stressed. Often the polyps were contracted, and many of the colonies lost tissues from the lower portion of the branches.

The most apparent effect of the ammonium concentration on the colonies was the color change. Colonies at the high ammonium concentration were darker than the control colonies. The colonies in the stripped treatment were lighter in color than the control corals. The color changes developed gradually during the first 2 to 3 weeks of incubation. After that time the color remained unchanged.

The workshop participants succeeded in applying the various measurement techniques to the same experimental corals in a coordinated and systematic manner. The critical factor in the success of this venture was the sequential flow of the same sample from one "work station" to the following one (Figure 3). We began with the nondestructive procedures, proceeded through the cellular procedures, and concluded with biochemical studies and analyses of preserved samples. These, in turn, ultimately led to data reduction and analysis. This approach also allowed each participant to observe and participate in an unusually diverse array of methods. Ongoing discussions centered on the merits and pitfalls of the various methodologies and research philosophies. The application of all these techniques to the very same samples eliminated possible differences in results and conclusions stemming from differences among different species, regions, and seasons. Results of research conducted by the various research teams of this workshop are presented in papers contained in this volume. Other papers are still in preparation, so additional results are forthcoming. Collaboration and follow-up discussions were an important result of this workshop. For example, a session involving the participants of the workshop was held at the Seventh International Coral Reef Symposium in Guam in 1992.

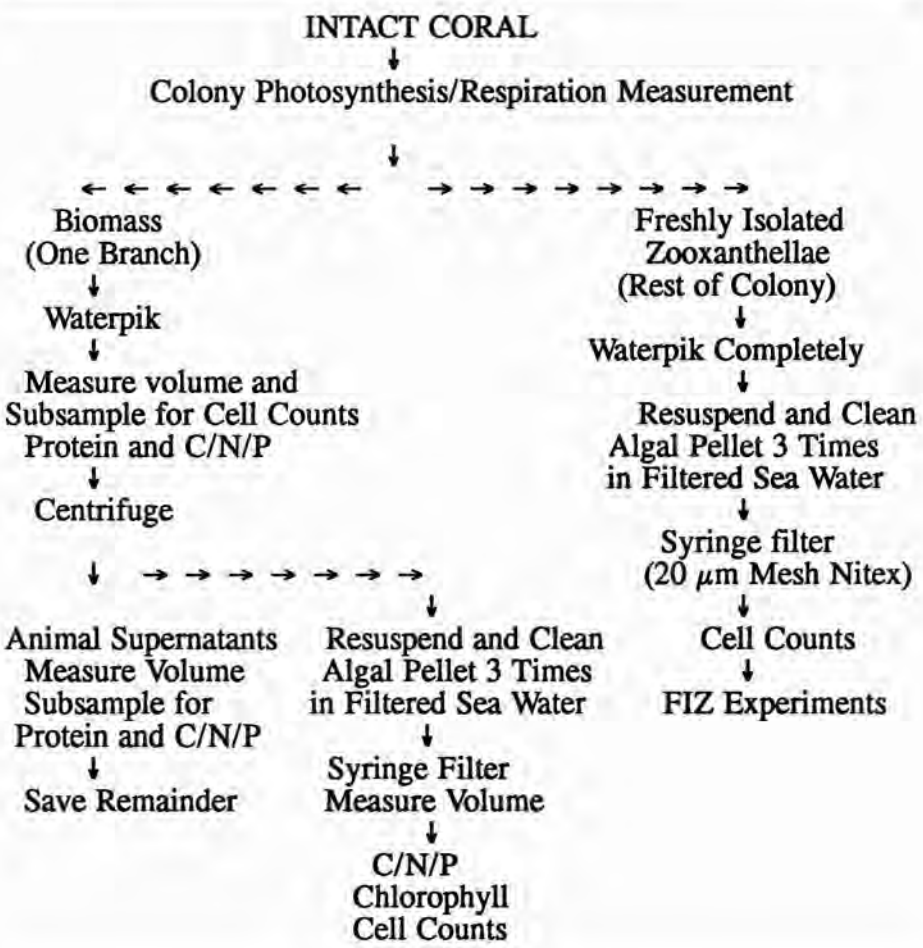

FIGURE 3. Procedures and routing of samples during final analysis of the corals.

Further, other workshops based on this successful approach are now in the planning stage.

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Carbohydrate, Lipid, and Protein Composition of Zooxanthellae and Animal Fractions of the Coral *Pocillopora damicornis* Exposed to Ammonium Enrichment[1]

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ABSTRACT: The carbohydrate, lipid, and protein composition of coral tissue and zooxanthellae were compared in Hawaiian *Pocillopora damicornis* (Linnaeus) colonies kept at different ammonium levels. Corals were maintained at two levels of ammonium enrichment (20 μM and 50 μM), in locally drawn seawater with <1 μM ammonium, and in water stripped of ammonium by running over a flume with macroalgae. No significant differences due to the treatment were found in the biochemical composition of the coral tissue. The values from control corals were 900, 275, and 170 $\mu g/cm^2$ for protein, carbohydrates, and lipids, respectively. Under all treatments the carbohydrate levels of zooxanthellae were inconsistent, but did not differ much from the control value of about 650 pg per cell. Lipid content in the control of nonenriched algae remained at ca. 140 pg per cell. However, in the 20-μM treatment algal lipid content increased to about 200 pg per cell during the second and fourth weeks, decreased slightly at 6 weeks, and remained at 164 pg per cell after 8 weeks. In the 50-μM ammonium treatment, there was a decrease to levels of about 40 pg lipids per cell for the entire period. Protein content increased from a control value of 590 pg per cell to ca. 950 pg per cell after 2 and 4 weeks of 20-μM ammonium enrichment and then after 6 weeks dropped back to the control level. At 50-μM ammonium the algal protein content increased after 2 weeks and remained at about 900 pg per cell after 6 and 8 weeks. The preliminary nature of this study is emphasized.

DATA PRESENTED IN this paper are from a workshop on nutrient limitation in the symbiosis between zooxanthellae and reef-building corals, which took place at the Hawaii Institute of Marine Biology during August 1991. The chemical oceanographic context of the workshop was covered by Atkinson (1988), and the overall structure of the various experiments and their relation to the biological aspects of nutrient limitation in zooxanthellate corals are introduced by Stambler et al. (1994).

Literature on phytoplankton indicates that a change in nitrogen supply influences the relative composition of their carbohydrates, lipids, and protein. Planktonic algae have received scientific attention mainly in relation to their nutritional value to other organisms. Parsons et al. (1961) analyzed the chemical composition of 11 phytoplankton species grown in culture under similar chemical and physical conditions. Their results showed that, under uniform conditions, marine phytoplankton have very similar organic composition regardless of their size or the taxon to which they belong. Strickland et al. (1969) compared the composition of phytoplankton cells grown in large tanks where conditions were closer to natural and showed that biochemical composition is not greatly affected by differences in the nutrient concentration of the environment. They did

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find, however, that differences occur depending on the source of the nitrogen. In the peridinian dinoflagellate *Cachonina niei* (Loeblich) Morrill & Loeblich, a deficiency in nutrient salts leads to an increase in carbohydrate concentration. *Ditylum brightwellii* synthesizes more lipids when nitrogen is supplied in the form of nitrate rather than as ammonium. On the other hand, there are results indicating major changes in the carbohydrate, lipid, and protein content of phytoplankton, grown in large enclosed experimental bags, associated with nutrient limitation (Morris et al. 1983).

Changes in the biomass production and protein and lipid content of two green algae, *Chlorella vulgaris* Beij. (K. & H.) and *Scenedesmus obliquus* (Turp.) Kütz, grown under different nitrogen regimes were studied by Piorreck et al. (1984). They found that high nitrogen levels led to an increase in biomass and protein content. At low nitrogen levels the algae contained a high percentage of total lipids. Harrison et al. (1990), working with three species of marine phytoplankton, *Isochrysis galbana* Parke, *Chaetoceros calcitrans* Ehrenberg, and *Thalassiosira pseudonana* Cleve, grown in batch or semicontinuous culture, found changes in protein, carbohydrate, and lipid concentrations under different nutritional conditions. Under N starvation, the percentage of lipids remained relatively constant, while carbohydrate percentage increased and protein levels decreased. On the other hand, cultures of *Euglena* in nitrogen-deficient conditions accumulated carbohydrates and lipids (Coleman et al. 1988). Shifrin and Chisholm (1981) measured the lipid content of about 30 species of phytoplankton. They found that nitrogen deprivation for 4 to 9 days resulted in a two- to three-fold increase in the lipid content of green algae, whereas both increase and decrease were noted in diatoms, depending on the species.

The effect of salinity and high nutrient concentrations on the marine microalga *Isochrysis galbana* using $NaNO_3$ as a nitrogen source was studied by Fabregas et al. (1986*b*). When nutrient concentration was increased up to 8 mM, the total protein content of the cells increased. At higher nutrient concentrations the total protein content of the cells diminished drastically, probably as a result of toxic effects. The same authors (Fabregas et al. 1986*a*) demonstrated great variability in chemical composition in cultures of *Dunaliella tertiolecta* Butcher. During logarithmic growth, protein concentration was not related to nutrient concentrations, but in the stationary phase, protein cell content reached its maximum value at 16 mM nitrate. Maximum quantities of carbohydrates were also obtained at that nitrate concentration.

In contrast, there is limited information available on the biochemical composition of symbiotic algae. Muscatine et al. (1989) measured the effect of ammonium and phosphate enrichment on the carbohydrate, lipid, and protein content of both zooxanthellae and coral tissue of *Stylophora pistillata* Esper. Analysis of coral tissue revealed no trends with treatment; however, in the zooxanthellae carbohydrate content decreased under ammonium enrichment.

An essential difference exists between free-living algae and symbiotic zooxanthellae in terms of fate of their metabolites. In free-living phytoplankters, most of the excess metabolites are directed toward cell reproduction. In symbiotic zooxanthellae, most of the metabolites are translocated and used by the host (Davis 1984, Muscatine et al. 1984, Achituv and Dubinsky 1990). Muscatine (1990) reviewed the role of zooxanthellae in energy flux in reef corals and concluded that, in general, the in situ growth rate of zooxanthellae in corals appears to be relatively slow and represents a relatively small sink for photosynthetically fixed material. Because of translocation, zooxanthellae concentration in the host's cells remains essentially constant and can be regarded as being in a stationary phase. However, because there is evidence that some zooxanthellae are continually released by the coral, we have to assume that the increase in zooxanthellae numbers has to exceed that required to maintain a constant density in a growing coral, because it also has to account for losses via expulsion. Expulsion of zooxanthellae by *Stylophora pistillata* did not exceed 0.1% of the standing stock of

algae, which in terms of carbon represents 0.01% of the total daily fixed carbon (Høegh-Guldberg et al. 1987). Because most of the metabolites are translocated, it can be assumed that changes in the nutritional conditions of zooxanthellae are also likely to affect the nutritional condition of the coral animal host. Nitrogen content is higher in zooxanthellae from *Porites furcata* Lamarck colonies with resident grunt fish schools than in zooxanthellae from colonies without fish (Meyer and Schultz 1985). Colonies of *Porites furcata* and *Acropora palmata* (Lamarck) with fish schools also showed an increase in coral tissue nitrogen. Nitrogen enrichment by the grunt excretory and fecal material might be responsible for the increase in nitrogen in the zooxanthellae and coral tissue.

A nutrient is limiting when an increase in the flux of that nutrient elicits a metabolic response (Parsons et al. 1984). It has been hypothesized that coral reefs are nutrient-limited and that nutrient enrichment will increase their metabolic response and change the gross productivity (Atkinson 1988, Muscatine et al. 1989). This was one of the hypotheses examined during the Hawaii workshop. Carbohydrate, lipid, and protein content were measured in animal tissue and in zooxanthellae from corals grown in different levels of ammonium enrichment.

MATERIALS AND METHODS

The experimental procedure and treatment of the *Pocillopora damicornis* (Linnaeus) colonies are described by Stambler et al. (1994). After collection, the colonies were incubated in seawater containing 20 μM or 50 μM ammonium, in ambient seawater with <1 μM ammonium, and in nutrient-deprived water, which was run over the macroalga *Gracilaria salicornia* Greville for nutrient stripping. Separation and processing of animal tissue and zooxanthellae are described by Muller-Parker et al. (1994). The number of colonies analyzed for each experimental treatment is given in Table 1. The coral tissue was removed from the skeleton using the Water Pik technique (Johannes and Wiebe 1970) in filtered seawater (glass filter/C), and subsamples from a known volume of the homogenate were taken for cell counts. A subsample of the total coral homogenate was centrifuged, and the algal pellet was resuspended two times in filtered seawater. A subsample of the combined supernatant was used for the determination of carbohydrates, lipids, and proteins of the animal tissue. The algal pellet was resuspended in a known volume of filtered seawater, and the algal cell concentration of this suspension was determined (Muller-Parker et al. 1994). Known volumes (ca. 10 ml) of the algal suspension and of the animal fraction were freeze-dried and used for the analysis of biochemical constituents of the zooxanthellae and coral tissue at a later date at Bar-Ilan University in Israel. Surface area of the corals and algal concentrations were obtained from calculations made by G. Muller-Parker (pers. comm.).

The freeze-dried material was resuspended in distilled water and made up to the original volume. The zooxanthellae samples were sonicated for 5 min, then analyzed for protein concentration using the method of Lowry et al. (1951) with BSA as a protein standard. Lipids were extracted from the samples using chloroform : methanol (2 : 1); then subsamples were transferred to test tubes and evaporated to dryness. Total lipids were analyzed by the microanalytical method of Marsh and Winstein (1966) with palmatic acid as standard. Carbohydrates were analyzed using the anthrone method (Roe 1955) and glucose as standard.

To eliminate the effect of increased algal density in corals exposed to elevated nitrogen concentrations (Muller-Parker et al. 1994), the biochemical constituents of the zooxanthellae are expressed as concentration per cell in contrast to the coral tissue, which is expressed as concentration per surface area of animal tissue.

RESULTS

The biochemical composition (total protein, lipids, and carbohydrates) of isolated

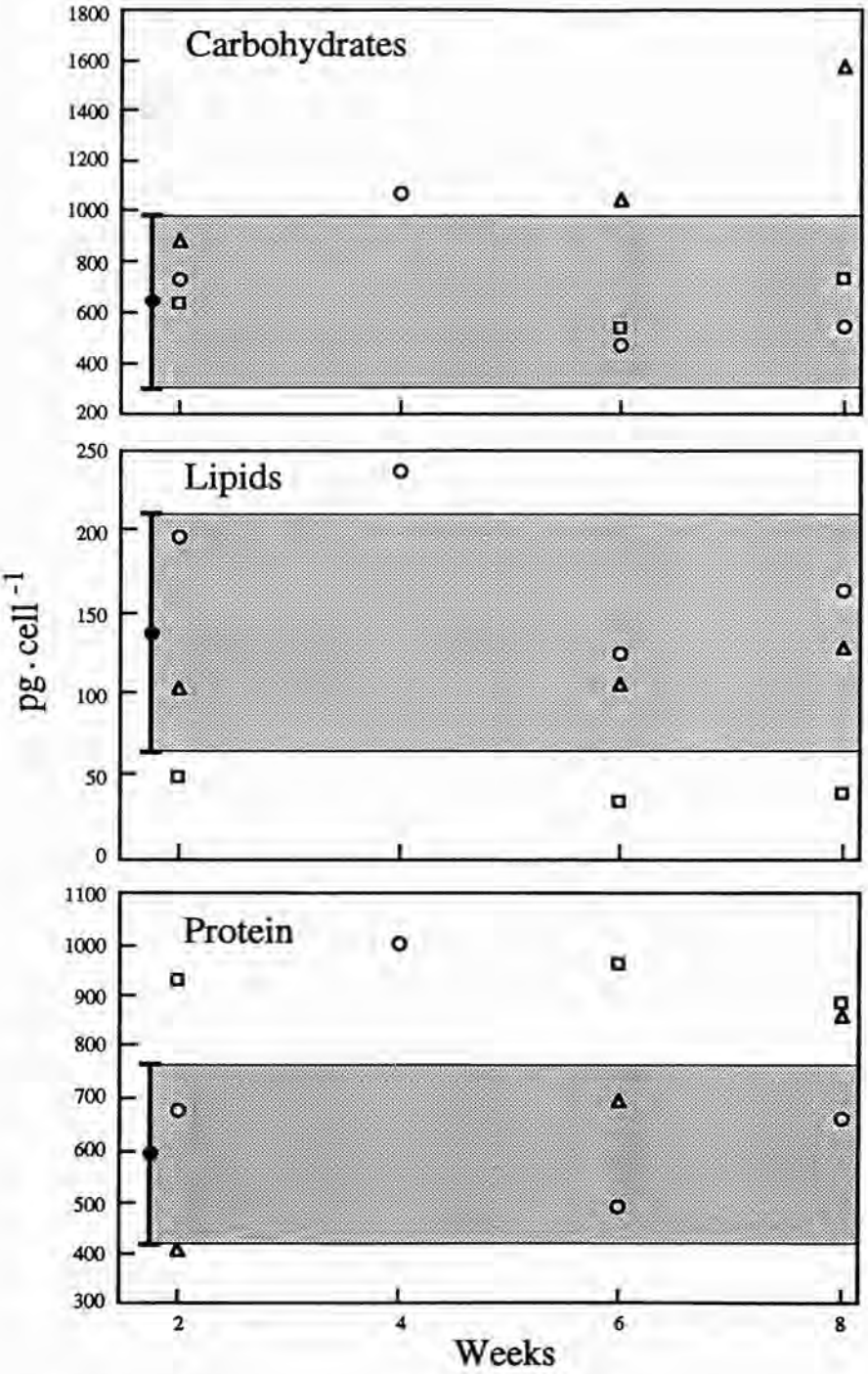

FIGURE 1. Carbohydrate, lipid, and protein content of zooxanthellae from the coral *Pocillophora damicornis*. Filled circle, average control value; its 95% confidence interval is presented as a shaded area; open circle, nitrogen-enriched colonies (20-μM ammonium treatment); open square, nitrogen-enriched colonies (50-μM ammonium treatment); open triangle, nitrogen-stripped colonies. For number of samples see Table 1.

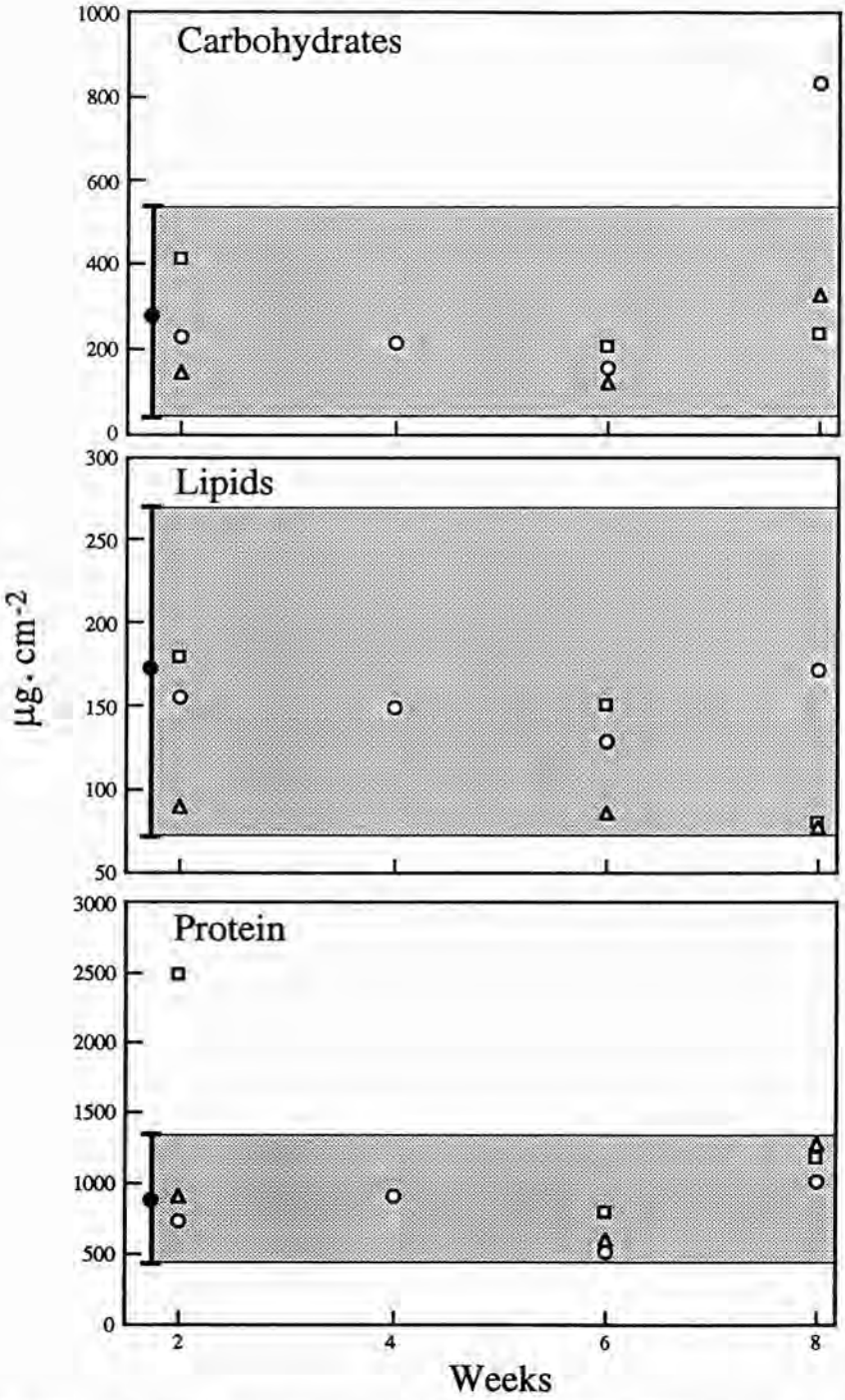

FIGURE 2. Carbohydrate, lipid, and protein content of animal tissue of *Pocillophora damicornis*. For explanation of symbols, see Figure 1.

algae and coral animal tissue at control (ambient seawater) and at three ammonium treatment levels are presented in Figures 1 and 2, respectively, and in Table 1. Because the control corals exposed to ambient seawater were expected to remain unchanged throughout the entire experimental period with respect to biochemical composition, a single, pooled average value was calculated from all time points for each biochemical fraction. This average with its 95% confidence interval is presented as the shaded area in Figures 1 and 2. An examination of the data shows no significant effect of nitrogen enrichment on the biochemical composition of the coral tissue (Table 1, Figure 2).

In the algal cells there was a decrease in lipid content for the 50-μM ammonium enrichment samples (Figure 1, Table 1). At that ammonium concentration, the values remained fairly constant throughout the experimental period. With respect to carbohydrate content, the 50-μM enrichment samples clearly did not differ from the control and again were not so variable. Protein level at that ammonium concentration was constant and always above that found in the control samples. However, the lipid content of zooxanthellae in corals exposed to the 20-μM ammonium treatment was higher than the mean control value, but still within the 95% confidence interval of control corals. At that concentration the changes in lipids, protein, and carbohydrates did not show a constant trend: during the first half of the experiment (i.e., 4 weeks), there was an increase in all three constituents, but during the second 4 weeks there was a decrease in lipid, carbohy-

TABLE 1

PROTEIN, CARBOHYDRATE, AND LIPID COMPOSITION OF ZOOXANTHELLAE AND ANIMAL TISSUE FROM COLONIES OF *Pocillopora damicornis* INCUBATED AT DIFFERENT AMMONIUM LEVELS (VALUES ARE MEANS ± SEM ON *n* DETERMINATIONS)

	ZOOXANTHELLAE (pg CELL^{-1})			ANIMAL TISSUE (μg cm^{-2})		
TREATMENT	PROTEIN	CARBOHYDRATES	LIPIDS	PROTEIN	CARBOHYDRATES	LIPIDS
Stripped						
2 weeks	404 ± 195	881 ± 280	102 ± 82.7	904 ± 249	136 ± 0.64	87 ± 21.3
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
6 weeks	694	1,039	106	580	123	87
	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
8 weeks	863 ± 216	1,547 ± 164	128 ± 52.2	1,265 ± 177	322 ± 126	77 ± 22.6
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
Control	594 ± 209	646 ± 399	138 ± 89	902 ± 498	276 ± 267	172 ± 106
	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 8$
20 μM						
2 weeks	936 ± 435	736 ± 147	197 ± 116	755 ± 257	225 ± 6.7	155 ± 104
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
4 weeks	999 ± 597	1,059 ± 196	236 ± 220	923 ± 221	213 ± 10.3	150 ± 13.8
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
6 weeks	495 ± 36	473 ± 464	125 ± 58.4	523 ± 196	155 ± 17.1	130 ± 7.7
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
8 weeks	669 ± 127	548 ± 322	165 ± 63.1	1,020	831	172
	$n = 2$	$n = 2$	$n = 2$	$n = 1$	$n = 1$	$n = 1$
50 μM						
2 weeks	680 ± 39.4	634 ± 331	47.7 ± 22.6	2,472 ± 2,110	411 ± 341	180 ± 198
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$
6 weeks	963	544	34.2	804	208	151
	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$	$n = 1$
8 weeks	890 ± 98.2	729 ± 180	41.1 ± 7.5	1,175 ± 739	236 ± 91.7	80.4 ± 14.0
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$

drate, and protein content of zooxanthellae, but the values did not differ from those of zooxanthellae from the control.

In the nitrogen-stripped samples there was a slight and gradual increase in cellular carbohydrates and protein toward the end of the experiment; lipid content remained more or less constant and within the 95% confidence interval of control corals.

DISCUSSION

The results of this investigation show that exposure of the hermatypic coral *Pocillopora damicornis* to nitrogen enrichment did not alter significantly ($P = 0.05$) the biochemical composition of the coral animal tissue. Our coral tissue analyses agree with those of Muscatine et al. (1989), whose values also did not show any clear-cut effect of the various enrichment treatments, including nitrogen enrichment. Muller-Parker et al. (1992) found that the C : N : P ratio of the animal tissue in samples from the very same colonies of *P. damicornis* we studied, and the same experimental treatments, did not change with ammonium enrichment. Although Muller-Parker et al. (1994), also as part of the same experiment, observed an increase in areal animal protein concentration after 8 weeks of exposure to the 20-μM ammonium treatment, we did not detect such an increase.

Our results show that in the zooxanthellae, the amount of protein per algal cell increased in both ammonium concentrations. This change was already noticeable after 2 weeks of ammonium enrichment, and most of the changes occurred within the first 2 weeks of exposure. This agrees with Muller-Parker et al. (1994), who also found that beyond the first 2 weeks continuous ammonium enrichment had little further effect on the algal biomass parameters. The changes in the biochemical composition of zooxanthellae (Table 1) expressed per cell were not caused by differences in cell size. Berner and Izhaki (1994) show that cell diameters of all zooxanthellae isolated from the corals were equal, regardless of nitrogen treatment.

The carbohydrate-to-protein and lipid-to-protein ratios in zooxanthellae and in animal tissue are presented in Table 2. The carbohydrate-to-protein ratio in the zooxanthellae at 20 μM and 50 μM ammonium enrichment did not deviate much from the control value, which was around unity. In the ammonium-stripped corals, this ratio was

TABLE 2

RATIO OF LIPIDS TO PROTEIN AND CARBOHYDRATES TO PROTEIN IN ZOOXANTHELLAE AND ANIMAL TISSUE IN COLONIES OF *Pocillopora damicornis* INCUBATED AT DIFFERENT AMMONIUM LEVELS

	ZOOXANTHELLAE		ANIMAL TISSUE	
TREATMENT	LIPIDS/PROTEIN	CARBOHYDRATES/PROTEIN	LIPIDS/PROTEIN	CARBOHYDRATES/PROTEIN
Stripped				
2 weeks	0.229	2.276	0.097	0.181
6 weeks	0.153	1.498	0.149	0.212
8 weeks	0.161	1.876	0.063	0.246
Control				
	0.242	1.165	0.234	0.317
20 μM				
2 weeks	0.204	0.841	0.193	0.269
4 weeks	0.207	1.363	0.165	0.283
6 weeks	0.248	0.924	0.264	0.299
8 weeks	0.241	0.881	0.169	0.815
50 μM				
2 weeks	0.069	0.919	0.061	0.492
6 weeks	0.036	0.565	0.188	0.258
8 weeks	0.046	0.813	0.090	0.233

higher because of the decrease in carbohydrate content of the algal cells. The ratio between lipids and protein in zooxanthellae from the control samples, and in those from corals exposed to the 20-μM ammonium treatment, was between 0.20 and 0.25. In algae from corals kept in 50 μM ammonium, this ratio dropped from 0.04 to 0.07, which reflects the decrease in lipid content and concomitant increase in protein.

Muller-Parker et al. (1992) showed that zooxanthellae from N-enriched *P. damicornis* had less C and more N per cell than those from the seawater control corals. Analysis of the relative abundance of carbohydrates, lipids, and protein in zooxanthellae from *Stylophora pistillata* (Muscatine et al. 1989) revealed that carbohydrates per cell were significantly lower for N-treated corals than in control colonies. However, we could not demonstrate the same effect in zooxanthellae from *P. damicornis*.

In phytoplankton there is a general predominance of protein over other constituents. However, in the Dinophyceae, to which the zooxanthellae belong, the ratio of protein to carbohydrates is lower than in other algae and also approaches unity (Parsons et al. 1984). In the coral tissue, carbohydrate levels are lower, and therefore the ratio between carbohydrates and protein is rather low. Parsons et al. (1961) emphasized that their results, showing similarity in the chemical composition of various species of free-living phytoplankton cells, were obtained with no nutrient limitation, and the results would have been different had the cells been grown under conditions of nutrient deficiency. Fabregas et al. (1986*a*,*b*) found that under high nitrogen concentrations there was an increase in protein in *Isochrysis galbana* and in both protein and carbohydrates in *Dunaliella tertiolecta*. However, those authors used nitrogen concentrations that were more than two orders of magnitude higher than those used in our experiments. It is not unreasonable to expect that to detect notable changes in the biochemical composition of algae, the colonies should be exposed to much higher concentrations than those used in our experiments.

Berner and Izhaki (1994) show, by analyzing the ultrastructure of zooxanthellae, that in the control corals and in corals grown in nitrogen-stripped seawater the percentage of cell volume of starch and lipid inclusions is higher than in the zooxanthellae from corals grown at elevated nitrogen levels. The storage materials occupied only 15% of the algal cell volume. These findings are not clearly reflected in our results. However, it should be emphasized that our determinations include the total amounts of the biochemical components, of which the storage material comprises only a small portion. Furthermore, the storage bodies found by Berner and Izhaki (1994) may also contain nitrogenous compounds. Nevertheless, as mentioned previously, in the zooxanthellae of nitrogen-stripped and seawater control samples there was an increase in carbohydrate content toward the end of the experimental period. Lipid content of zooxanthellae from the 50-μM ammonium enrichment was higher than that of the control and the nitrogen-stripped zooxanthellae; this reflects the trend found by Berner and Izhaki (1994).

In the N-enriched corals the density of the zooxanthellae increases (Muscatine et al. 1989, Muller-Parker et al. 1994), and this is associated with a relative decrease in lipids in the zooxanthellae. It can be assumed that under these conditions either lipids were not synthesized to the same extent as in the low-nitrogen zooxanthellae or lipids were used for algal proliferation, and thus nitrogen might regulate zooxanthellae density within the coral. Høegh-Guldberg (1994) found that the mitotic index of zooxanthellae from the same corals exposed to ammonium enrichment was two to three times higher than in the control corals. Coleman et al. (1988) suggested that nitrogen deficiency inhibits cell division so that the carbohydrates and lipids produced are divided among fewer cells, increasing the quantity of storage products per cell.

Some of the differences and inconsistencies in our results might be due to the high heterogeneity of the samples. G. Muller-Parker (pers. comm.) reported a high variability in the density of algal cells in the coral

tissue of our control samples, commonly ranging over an order of magnitude.

In conclusion, this initial study on the biochemical composition of coral tissue and zooxanthellae shows that we could not demonstrate major consistent changes in the biochemical composition of coral tissue. In some cases, a general similarity between zooxanthellae and free-living microalgae does exist; nevertheless, differences can be found as well. In free microalgae, considerable variations as a function of species and ecological conditions were shown to exist. It is possible that in different host corals and at different initial nutritional conditions the zooxanthellae will exhibite different responses. This species-specific response might also be reflected in the deviation from results presented by other investigators. Furthermore, because most of the changes, especially under high ammonium concentrations, probably occurred within the first 2 weeks of exposure, the time course and dynamics of change should be studied. Any assumptions drawn from our preliminary measurements must be confirmed by further studies on a larger scale.

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Elemental Composition of the Coral *Pocillopora damicornis* Exposed to Elevated Seawater Ammonium[1]

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ABSTRACT: The elemental composition (C, N, and P) of zooxanthellae and host tissue from the coral *Pocillopora damicornis* (Linnaeus) was determined after maintenance in flowing seawater with 20-μM and 50-μM ammonium enrichments for periods of 2 to 8 weeks. Compared with ambient seawater controls, total zooxanthellar nitrogen (μg N cm^{-2} colony surface) increased four-fold during exposure to 20 μM ammonium. This resulted from increases in N content of zooxanthellae and in zooxanthellae population densities. C : N ratios of zooxanthellae decreased from 19.7 ($\pm$4.0) to 10.3 ($\pm$3.0), and N : P ratios increased from 21.4 ($\pm$3.1) to 30.4 ($\pm$2.2) after 8 weeks in 20 μM ammonium. Zooxanthellae from the 8-week 50-μM ammonium corals had values of 8.9 ($\pm$0.6) for C : N and 40.4 ($\pm$2.3) for N : P. Coral animal C, N, and P content were not affected by ammonium-enriched seawater. The C : N ratio of coral animal tissue was 5.2 ($\pm$0.0), and the N : P ratio was 20.1 ($\pm$0.2) after 8 weeks in 20-μM ammonium seawater. There were no changes in host C : N, N : P, or C : P with ammonium enrichment. Thus, most of the N from the elevated seawater ammonium is retained by the zooxanthellae of *P. damicornis*, rather than by the animal tissue. Accordingly, sustained high concentrations of ammonium are likely to result in increased N storage by zooxanthellae and to affect the relative size of zooxanthellar to animal N pools.

ADDITION OF A LIMITING nutrient increases the size of the resident population of zooxanthellae living within animal hosts, including corals and sea anemones (Cook et al. 1988, Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Stambler et al. 1991, Stimson and Kinzie 1991, Muller-Parker et al. 1994). Increased densities of zooxanthellae within corals subjected to ammonium [$(NH_4)_2SO_4$] enrichment clearly increase the productivity of the symbiotic association (Høegh-Guldberg and Smith 1989, Dubinsky et al. 1990). However, the effects of added nutrients on the balance between algal growth and animal growth, and the nutrient fluxes and pools of reef corals are not yet well understood. These effects are likely to depend on the nutrient status of the coral zooxanthellae before nutrient enrichment.

There is some evidence that C : N ratios are related to the nitrogen status of symbiotic zooxanthellae. The C : N of zooxanthellae increased with starvation of the host anemone *Aiptasia pallida* (Verrill) in low-nutrient seawater (Cook et al. 1988) and decreased in zooxanthellae in the coral *Stylophora pistillata* Esper with ammonium enrichment of the seawater (Muscatine et al. 1989). Schools of fish that defecate on coral heads also increase the N and P content of coral tissue (Meyer and Schultz 1985). If the ratio of C : N : P reflects the availability of ambient nutrients, as originally proposed by Redfield et al. (1963) and confirmed by studies with

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cultured phytoplankton (e.g., Rhee 1974, 1978) and macroalgae from high-nutrient and low-nutrient environments (Atkinson and Smith 1983, Lapointe et al. 1992), comparisons of the elemental ratios of zooxanthellae isolated from nutrient-enriched and unenriched corals are a rapid way to assess the nutrient status of symbiotic zooxanthellae in corals from different environments.

Knowledge of the C, N, and P content of zooxanthellae is important for relating carbon productivity rates to N and P production and turnover. Current interest in the effect of nutrients on corals relates to the effect of nutrient additions on the nutritional and energetic exchanges between the host animal and its zooxanthellae and to the effect of potential increases in coral reef anthropogenic nutrients on coral growth. Also, the effect of long-term additions of inorganic nutrients on the elemental composition and C : N : P of the separate zooxanthellae and coral fractions is unknown. Continuous additions of nutrient-enriched seawater (N provided as 20 and 50 μM ammonium) to the Hawaiian coral *Pocillopora damicornis* (Linnaeus) resulted in increased densities of zooxanthellae (Muller-Parker et al. 1994). These corals provided the opportunity to explore the effect of ammonium enrichment on the C, N, and P content of corals. Furthermore, we were able to evaluate the utility of the C : N : P ratio as a potential indicator of nutrient status of zooxanthellae in corals.

MATERIALS AND METHODS

The experimental design and details of the maintenance of *P. damicornis* under the different ammonium treatments are described by Stambler et al. (1994). Colonies were maintained in seawater enriched with 20 and 50 μM ammonium, or in ambient seawater (≤ 2 μM NH_4^+), for periods ranging from 2 to 8 weeks. Control corals kept in ambient flowing seawater for 8 weeks served as the zero time point. Corals from the different treatments were separated into animal and zooxanthellae (Muller-Parker et al. 1994), and these fractions were used to prepare samples for elemental analysis. The surface area corresponding to the amount of tissue removed from each coral specimen was obtained by a leaf area measuring device (Li-Cor Model 3100), as described by Muller-Parker et al. (1994).

Freshly isolated zooxanthellae were passed sequentially through 73-μm and 20-μm Nitex screening to remove animal debris, and known volumes were collected by filtration onto glass fiber filters (Whatman GF/F) under vacuum (<250 mm Hg). Precombusted (6 to 8 hr at 500°C) 25-mm GF/F filters were used for the samples for C and N analysis, and noncombusted 25-mm GF/F filters were used for the P samples. Cell counts were taken with a hemacytometer before filtration of samples. One million to 3 million zooxanthellae were collected on filters, which were then rinsed with 3–5 ml of filtered seawater followed by about 200 μl of distilled water to remove salts. Preliminary tests showed that this treatment did not affect the amount of C and N per zooxanthella. All sample sets included appropriate filter blanks and controls (equivalent volumes of seawater filtered). The final filter samples were placed in individual aluminum foil packets and frozen before analysis.

Samples for elemental analysis of the animal fraction were prepared by absorbing a measured volume of each sample into pairs of filters (for separate C and N, and P analyses). Samples of frozen coral animal fraction stored in microfuge tubes were thawed and mixed thoroughly on a vortexer before applying them to the filters. Filters were arranged on a ridged clean piece of aluminum foil, and a total of 400 μl (100 μl at a time) of well-mixed animal sample was placed on each filter using a calibrated micropipette. Filters were dried under a heat lamp between each 100-μl addition so that no sample leaked from the filter. Control filter blanks were prepared by spotting filters with equivalent volumes of filtered seawater. Contamination of filters by handling was avoided by wearing gloves throughout the procedure and using forceps to transfer all filters. Dried filters were stored in individual aluminum foil packets.

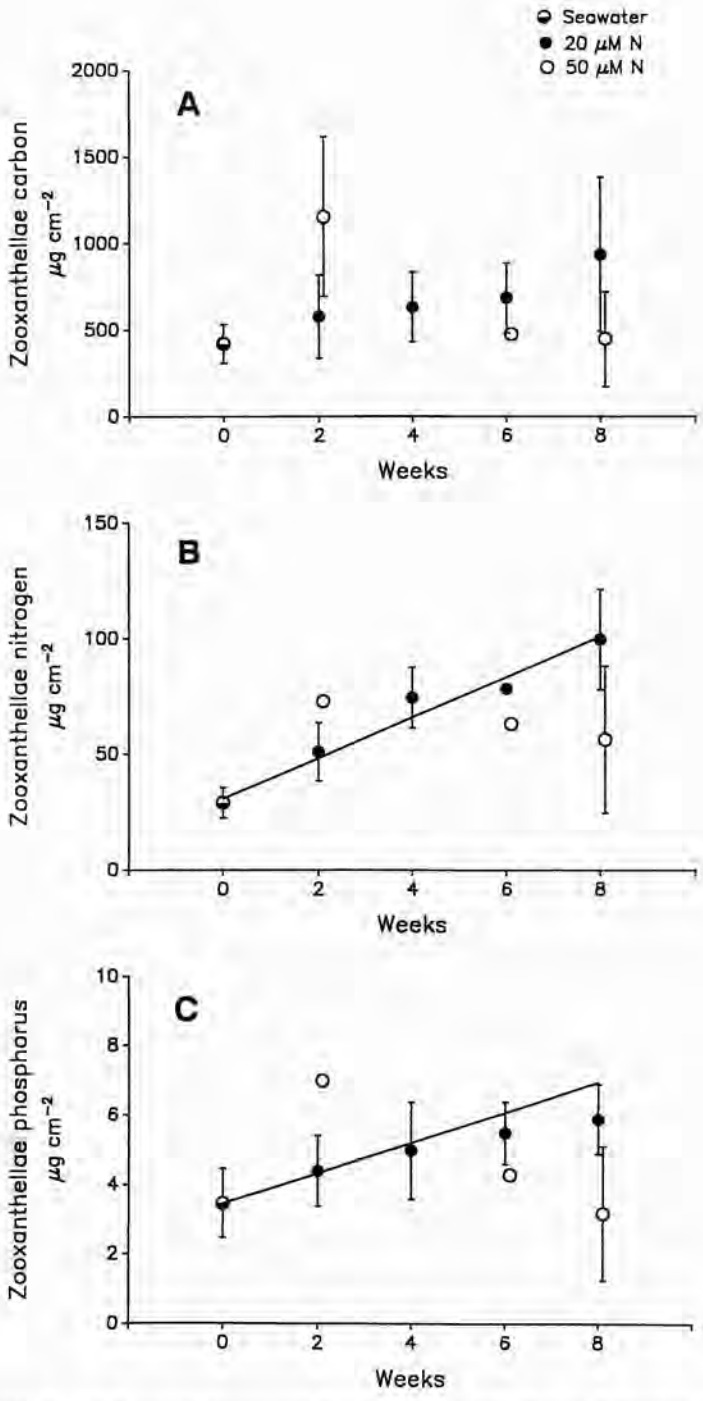

FIGURE 1. Areal C, N, and P content of zooxanthellae in colonies of *Pocillopora damicornis* as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater. Ambient seawater control colonies maintained under the same light and water flow conditions are used for the zero time point. Data are expressed as the total amount of carbon (*A*), nitrogen (*B*), and phosphorus (*C*) in zooxanthellae per unit colony surface area. $n = 6$ colonies for zero time point, and $n = 2$ for all others except $n = 1$ for the 6-week 50-μM ammonium time point. Error bars are ± 1 SE. The x axis for the 50-μM ammonium data is slightly shifted to the right for clarity. Regression lines are provided for significant effects observed with 20 μM ammonium. *B*: $r = 0.869$, $P < 0.01$; *C*: $r = 0.625$, $P < 0.05$.

Weights of C and N for each filter were determined using an elemental analyzer (Control Equipment Corp. Model 240X). Filters were dried to constant weight before analysis (45°C). Weight of P per filter was measured according to the procedure of Aspila et al. (1976). Each set of analyses included appropriate filter and control seawater blanks that were subtracted from sample results before calculating the C, N, and P content of coral animal and zooxanthellae fractions from volume equivalents.

For statistical purposes, corals maintained in ambient flowing seawater for 8 weeks were presumed to represent corals before the addition of ammonium and were used as the zero time point. The effects of ammonium addition on elemental content and elemental ratios of zooxanthellae and host animal tissue were examined by linear regression over time, using the correlation coefficient (r) to indicate significance of treatments. Elemental ratios were not transformed before analysis.

RESULTS

Figure 1 shows the areal C, N, and P content of zooxanthellae during the 8-week exposure to 20 μM and 50 μM ammonium-enriched seawater. There was a marginally significant ($0.06 > P > 0.05$) trend of increasing C content of zooxanthellae per square centimeter at 20 μM ammonium during this period (Figure 1*A*). Total zooxanthellar N increased four-fold (Figure 1*B*; $P < 0.001$) and total P of zooxanthellae doubled (Figure 1*C*; $P < 0.05$) over the 8-week exposure period to 20 μM ammonium. There were no significant time-related changes in the areal C, N, and P content of zooxanthellae from corals maintained in 50 μM ammonium during the 8-week experiment (Figure 1A–C).

The data in Figure 1 show the changes in total C, N, and P content of zooxanthellae, reflecting both the elemental content of each cell and the areal population density of zooxanthellae in each coral. Figure 2 shows the amount of C, N, and P per zooxanthella isolated from *P. damicornis* after 2 to 8 weeks exposure of the corals to ammonium enrichment. The C and P content of zooxanthellae from corals held in 20 μM ammonium-enriched seawater did not change with time (Figure 2*A*,*C*), but nitrogen per cell increased significantly ($P < 0.05$) over the 8-week period (Figure 2*B*). Different results were obtained for colonies maintained at 50 μM ammonium. There was a significant decline ($P < 0.05$) in C and P content of zooxanthellae during the 8 weeks in 50 μM ammonium (Figure 2*A*,*C*). After 8 weeks, the P content of these cells was half that of zooxanthellae from control corals. The nitrogen content of zooxanthellae from corals exposed to 50 μM ammonium remained constant with time (Figure 2*B*).

The relative changes in C, N, and P of *P. damicornis* zooxanthellae with exposure to added ammonium are shown by the ratios of these elements. The C : N of zooxanthellae from corals kept in 20 μM ammonium-enriched seawater was reduced by half during the first 4 weeks and then remained constant for the next 4 weeks (Figure 3*A*); however, the overall change in C : N with time was not significant ($0.07 > P > 0.06$). The N : P of zooxanthellae in these corals increased by 42% during the 8-week period (Figure 3*B*; $P < 0.05$), with most of the increase resulting from increased N content (cf. Figure 1*B*,*C*). There was no significant change in C : P (Figure 3*C*). N : P also increased in zooxanthellae from corals in the 50-μM ammonium treatment (Figure 3*B*), due in part to the decrease in P content of these algae (Figure 2*C*). Coral zooxanthellae from the 8-week samples had an extremely high N : P of 40. Neither C : N nor C : P of zooxanthellae was affected by exposure of the corals to 50 μM ammonium (Figure 3*A*,*C*).

The C : N and N : P ratios of all zooxanthellae samples from *P. damicornis* were compared with zooxanthellae population densities (zooxanthellae cm^{-2}) to determine if there was a density-dependent effect on elemental ratios. There was no significant correlation of C : N and of N : P with cell density of corals maintained in ambient and in nutrient-stripped seawater.

Figure 4 shows the areal C, N, and P con-

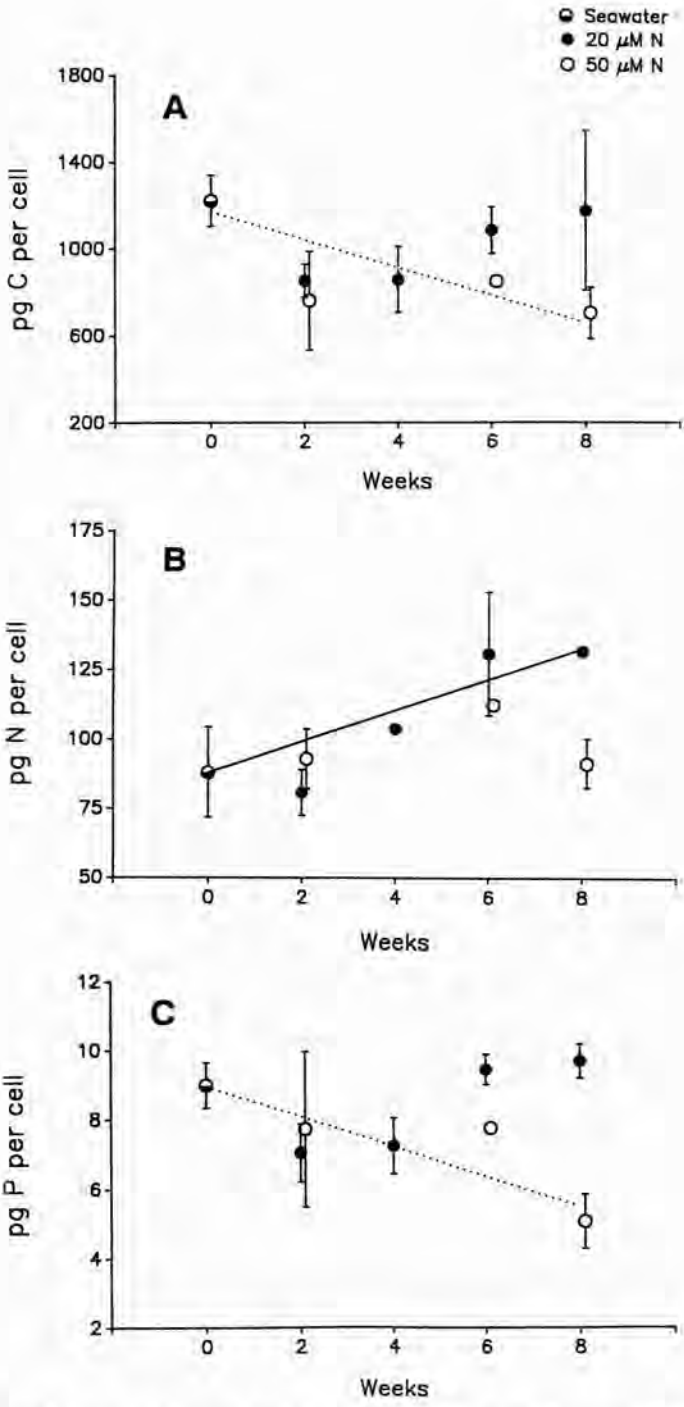

FIGURE 2. C, N, and P content of zooxanthellae in colonies of *Pocillopora damicornis* as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater. Ambient seawater control colonies maintained under the same light and water flow conditions are used for the zero time point. Data are expressed as the weights of carbon (*A*), nitrogen (*B*), and phosphorus (*C*) per zooxanthella. $n = 8$ colonies for zero time point, and $n = 2$ for all others except $n = 1$ for the 6-week 50-μM ammonium time point. Error bars are ± 1 SE. The x axis for the 50-μM ammonium data is slightly shifted to the right for clarity. Regression lines are provided for significant effects (solid lines: corals in 20-μM ammonium treatment; dotted lines: corals in 50-μM ammonium treatment). *A*: $r = 0.573$, $P < 0.05$; *B*: $r = 0.478$, $P < 0.05$; *C*: $r = 0.612$, $P < 0.05$.

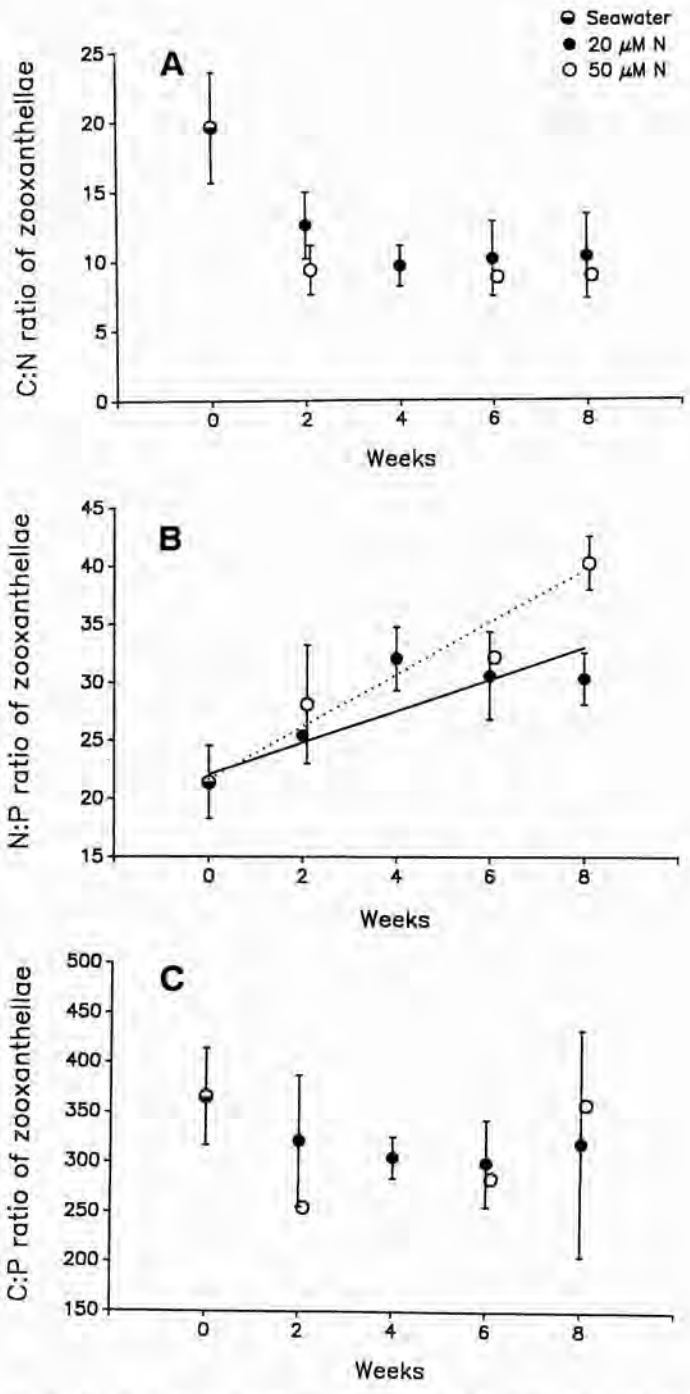

FIGURE 3. C : N, N : P, and C : P ratios (by atoms) of zooxanthellae from individual colonies of *Pocillopora damicornis* as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater. Ambient seawater control colonies maintained under the same light and water flow conditions are used for the zero time point. C : N (*A*), N : P (*B*), and C : P ratios (*C*) are derived from data in Figure 2 (same conventions). *B*: 20 μM ammonium comparison: $r = 0.532$, $P < 0.05$; 50 μM ammonium comparison: $r = 0.703$, $P < 0.01$.

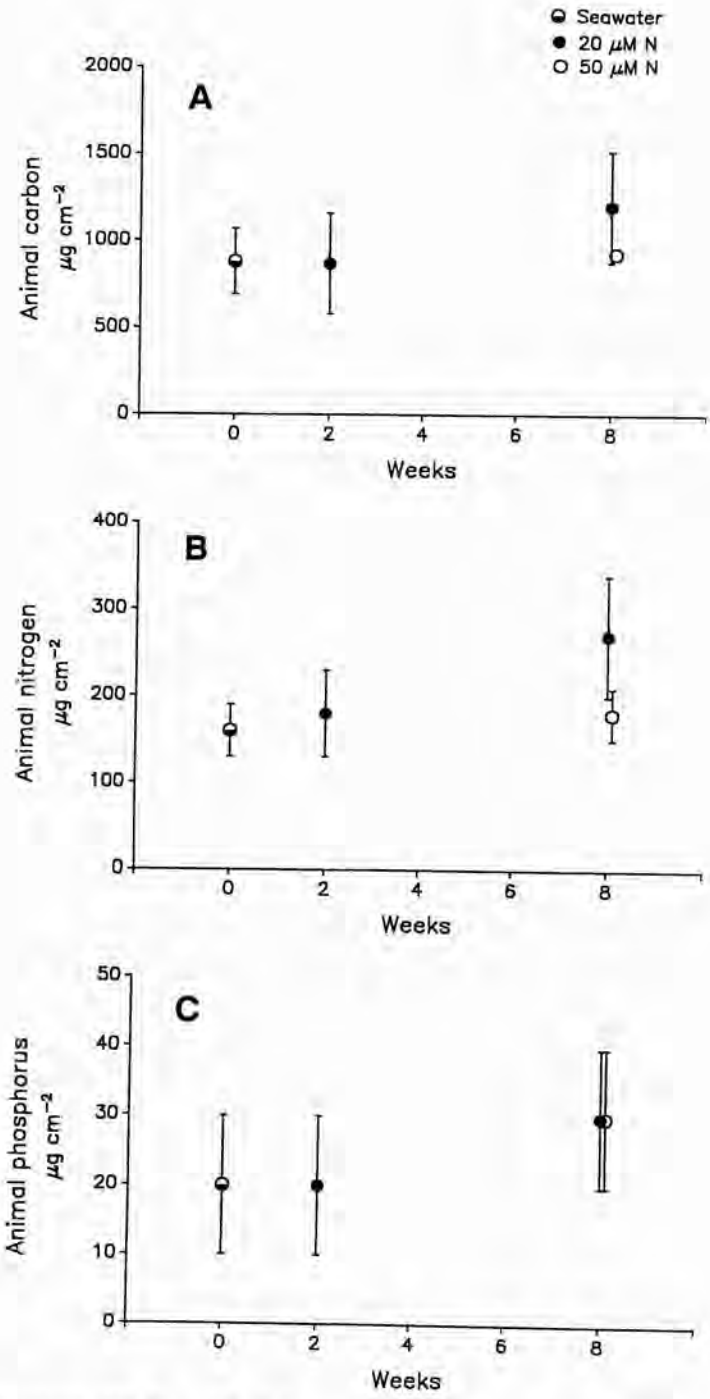

FIGURE 4. Areal content of C, N, and P of the coral animal fraction of *Pocillopora damicornis* colonies as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater. Ambient seawater control colonies maintained under the same light and water flow conditions are used for the zero time point. $n = 4$ colonies for zero time point, and $n = 2$ for all other time points. Error bars are ± 1 SE. The x axis for the 50-μM ammonium data is slightly shifted to the right for clarity. Data are expressed as the weight of animal carbon per square centimeter (*A*), animal nitrogen per square centimeter (*B*), and animal phosphorus per square centimeter (*C*).

tent of host coral tissue during exposure to ammonium. There were no changes with time in any of these parameters in corals maintained in 20 μM ammonium, in contrast to the increases in N and P of zooxanthellae isolated from these corals. Ammonium-N appears to have been retained by the zooxanthellae, rather than by the animal tissue. The lack of effect on host C, N, and P is reflected in the elemental ratios; there were no changes in host C : N, N : P, or C : P with ammonium addition with time (Table 1). Only two samples of animal tissue were taken from corals exposed to 50 μM ammonium (at 8 weeks), precluding statistical analysis.

Table 1 contains the C : N and N : P ratios of animal tissue and zooxanthellae of *P. damicornis* and shows the amount of C, N, and P in whole coral (sum of animal and algal fractions) and the percentage of each element that is present in the zooxanthellae. A comparison of the zooxanthellae and animal elemental ratios shows that the N : P ratios are similar for both fractions, but C : N ratios are consistently lower in animal tissue and were not changed by ammonium enrichment. Zooxanthellae comprised from 26 to 42% of the total C content of *P. damicornis*, from 13 to 27% of the total N, and from 11 to 28% of total P (Table 1).

DISCUSSION

Effect of Ammonium Enrichment on C, N, and P of Zooxanthellae in Pocillopora damicornis

The addition of 20 μM ammonium to seawater surrounding the colonies of *P. damicornis* resulted in significant increases in the areal N and P content of zooxanthellae (Figure 1). Total areal N content of the zooxanthellae increased four-fold over the 8-week period (Figure 1*B*). This increase was caused by an increase in the density of zooxanthellae (Muller-Parker et al. 1994) and in N per cell (Figure 2*B*). The increase in areal P of zooxanthellae was due solely to an increase in zooxanthellae population density, because P per cell did not change as a function of time of ammonium addition (Figure 2*C*). The C content of zooxanthellae was not affected by the length of exposure to 20 μM ammonium (Figures 1*A*, 2*A*). Because the population density of zooxanthellae increased over this period, the lack of change in C indicates that these cells lost C and gained N during the experiment. In the 50-μM ammonium treatment, the C and P content of zooxanthellae was significantly reduced with time (Figure 2*A*,*C*). This decline in C per cell with ammonium addition is consistent with a decline in starch and lipid observed in morphometric analyses of micrographs of zooxanthellae (Berner and Izhaki 1994). However, areal C and P were not affected (Figure 1*A*,*C*), presumably because of an increase in the numbers of zooxanthellae with time.

The increase in N content of zooxanthellae from *P. damicornis* colonies maintained at 20 μM ammonium could result from an increase in either one or more of the following cellular constituents: amino acid pools, protein, or chlorophyll. Results of biochemical analyses performed on zooxanthellae from the same specimens eliminated free amino acids and protein as N pools. McAuley (1994) found no differences in the size of the amino acid–N pools of zooxanthellae from ammonium-enriched *P. damicornis* and corals maintained in ambient seawater. Although Achituv et al. (1994) observed an initial increase in zooxanthellae protein during the first month of enrichment with 20 μM ammonium, protein content declined during the second month. The significant increases in chlorophyll content of zooxanthellae exposed to 20 μM ammonium (Muller-Parker et al. 1994) may account for the higher N in these cells. There is also the possibility that ammonium is directly stored by zooxanthellae. It is interesting that maintenance of the host in ammonium did affect the amino acid pools of zooxanthellae in a symbiotic anemone; Ferrier (1992) found that zooxanthellae isolated from *Aiptasia pallida* maintained for 4 weeks in 20 μM ammonium exhibited higher concentrations of basic amino acids.

The C and P content of zooxanthellae from corals in the 50-μM ammonium treatment declined during the 8-week period (Fig-

TABLE 1

Elemental ratios and amount of C, N, and P in the coral *Pocillopora damicornis* exposed to 20 μM ammonium and 50 μM ammonium-enriched seawater for 8 weeks

	ANIMAL			ZOOXANTHELLAE						ZOOXANTHELLAE		
P. damicornis	C : N	N : P	C : P	C : N	N : P	C : P	TOTAL C (μg/cm^2)	TOTAL N (μg/cm^2)	TOTAL P (μg/cm^2)	% OF TOTAL C	% OF TOTAL N	% OF TOTAL P
Ambient seawater												
Avg.	6.37	26.57	172	19.66	21.41	365	1,236	182	18	26	13	15
SE	0.40	4.36	35	3.99	3.12	49	310	36	6	4	1	2
n	4	4	4	8	8	8	4	4	4	4	4	4
20 μM ammonium 2 weeks												
Avg.	5.64	17.10	96	12.59	25.45	321	1,443	227	27.3	39	23	16
SE	0.34	0.02	6	2.40	0.38	66	534	63	8	2	1	1
20 μM ammonium 8 weeks												
Avg.	5.21	20.12	105	10.31	30.38	320	2,138	368	36.7	42	27	20
SE	0.03	0.22	2	3.00	2.24	114	765	91	8	6	1	2
50 μM ammonium 8 weeks												
Avg.	6.25	14.92	88	8.92	40.41	359	1,381	235	32.2	29	22	11
SE	0.84	5.80	24	0.65	2.31	6	322	65	4	13	8	7

The 20-μM ammonium treatment corals also were sampled 2 weeks after enrichment. $n = 2$ except where indicated (ambient seawater corals).

ure 2*A*,*C*). Achituv et al. (1994) found that the lipid content of zooxanthellae isolated from 50 μM ammonium-enriched corals was extremely low. Whether the low C, N, P, and lipid content of these zooxanthellae resulted from shrinkage of algal cells in response to the high ammonium or from changes in the biochemical composition of the zooxanthellae cannot be determined without appropriate cell size measurements. It is possible that the high ammonium concentration was toxic to the zooxanthellae and that the anomalous trends observed reflect a response to a stressful nutrient environment.

C : N : P ratios reflect the nutritional history of organisms. The elemental ratios of the zooxanthellae were clearly affected by the ambient ammonium concentration to which the coral was exposed, although only the N : P ratio of zooxanthellae showed a significant increase with time of exposure to both 20 and 50 μM ammonium (Figure 3). However, the C : N of zooxanthellae dropped immediately from 20 to <10 after 2 weeks of ammonium treatment (Figure 3*A*).

Elemental ratios are often associated with changes in the growth rate of the algae. The near-Redfield ratios of oceanic phytoplankton were used to suggest nutrient-saturated growth rates for these algae (Goldman et al. 1979), although others have reasoned that Redfield ratios can occur when growth is limited by other factors (Tett et al. 1985). Because the accumulation of nitrogen in zooxanthellae with 20 μM ammonium enrichment increased after 1 month of exposure to N (Figure 2*B*), it is likely that the increased growth rate of zooxanthellae in response to additions of both 20 and 50 μM ammonium (Høegh-Guldberg 1994) accounted for the steady N content and for the reduction of C and P during this period. After 6 weeks, the C and P levels of zooxanthellae exposed to 20 μM ammonium were similar to the levels in zooxanthellae in control corals kept in unenriched seawater (Figure 2*A*,*C*), indicating that zooxanthellae stored nitrogen primarily during the second month of ammonium enrichment.

Our data show the importance of a baseline (or zero time point) for comparison of elemental ratios of algae subjected to different nutrient regimes. For example, the C : N of N-enriched *P. damicornis* zooxanthellae (Figure 3*A*, Table 1) was higher than C : N ratios of cultured zooxanthellae maintained in nutrient-enriched media (Domotor and D'Elia 1984; unpublished data) and of zooxanthellae isolated from well-fed sea anemones (*Aiptasia pallida* [Cook et al. 1988]). Based on the Redfield ratio for phytoplankton growing in seawater with unlimited nutrient supply of C : N : P ratio of 106 : 16 : 1, we might infer that *P. damicornis* zooxanthellae from N-enriched corals had high C : N ratios. However, the C : N of zooxanthellae from the ambient seawater control corals was twice as high (Figure 3*A*).

It is therefore likely that the elemental composition of zooxanthellae is species-specific and quite variable. Comparison with an extensive data set for Bermuda corals (G.M.P., C.B.C., and Porter, unpublished data) shows that the C : N of *P. damicornis* zooxanthellae from corals maintained in ambient seawater is about twice that of the zooxanthellae in the Atlantic corals *Montastrea annularis* (Ellis & Solander) and *Madracis mirabilis* (Duch. & Mich.) from Bermuda, but the N : P of *P. damicornis* zooxanthellae is lower. The C : N and N : P of N-enriched *P. damicornis* zooxanthellae are similar to those of zooxanthellae from the field populations of the Atlantic corals *M. annularis* and *Madracis mirabilis* (G.M.P., C.B.C., and Porter, unpublished data).

Effect of Ammonium Enrichment on C, N, and P of Animal Tissue of Pocillopora damicornis

It is clear that most of the effects of seawater ammonium enrichment are observed in changes in the areal N content of zooxanthellae (Figure 1) and not in the animal tissue (Figure 4). Unlike zooxanthellae, the N content of the coral animal tissue did not increase significantly with time during exposure to 20 μM and to 50 μM ammonium (Figure 4*B*). The highest N content of animal tissue, obtained in corals maintained in 20-μM ammonium-enriched seawater for 8 weeks

(Figure 4*B*), is consistent with an increase in protein content obtained by Muller-Parker et al. (1994), but was a small and nonsignificant increase compared with that observed in zooxanthellae from the same corals (Figure 1*B*). However, the number of animal tissue samples analyzed was limited and may have contributed to the lack of effect observed with time. If animal assimilation of ammonium occurred, the amount assimilated is relatively small in comparison with assimilation of N by the zooxanthellae. Furthermore, Achituv et al. (1994) concluded that the biochemical composition of the coral animal tissue, including protein, remained constant under all treatments. Høegh-Guldberg and Smith (1989) observed a slight (also nonsignificant) increase in animal tissue protein content with ammonium addition to two species of corals. Muscatine et al. (1989) obtained no differences in protein, lipid, and carbohydrate content of animal tissue from *Stylophora pistillata* exposed to added nutrients. Using $^{15}NH_4^+$, Lipschultz and C.B.C. (unpublished data) found that the zooxanthellae of *P. damicornis* had a higher rate of ammonium assimilation over 24 hr than did the animal tissue. However, they did find appreciable incorporation of ammonium-N into host macromolecules at the end of the study.

This is the first report of the C, N, and P composition of the separated animal and algal fractions of a coral. Other investigators have reported on the elemental ratios of whole coral tissue. The C : N of coral tissue in *Stylophora pistillata* ranged from 6.26 to 4.86 (Muscatine et al. 1989), and the N : P of two Caribbean corals ranged from 21 to 26 (Meyer and Schultz 1985). The N : P ratios of animal tissue and zooxanthellae in *P. damicornis* are similar, but C : N ratios are consistently lower in animal tissue (Table 1). Ammonium enrichment did not alter animal C : N ratios (Table 1). However, C : N and N : P ratios may provide information about the relative importance of inorganic nutrients dissolved in seawater and nutrients obtained via capture and assimilation of zooplankton prey. By varying the relative input of dissolved inorganic nutrients and food, it may be possible to assess the relative contribution of these sources to the elemental composition of an organism. Nutrient fluxes between the seawater environment and the host are affected by the nutritional status of the zooxanthellae (Muller-Parker et al. 1990, Szmant et al. 1990). A comparison of the relative elemental ratios, in conjunction with knowledge of uptake rates of inorganic nutrients and prey capture rates, may indicate which source is more important.

Ecological Implications

Changes in inorganic nitrogen input affect the nitrogen balance of reef corals, because the relative proportion of zooxanthellar to animal nutrient pools changes with enrichment. These changes are also likely to affect net coral production and the stoichiometry of C, N, and P metabolism on reefs (Atkinson 1988). Our results suggest that corals living in high-nutrient environments will have more N stored in the autotrophic zooxanthellae. The mass balance of C, N, and P will change as the proportion of zooxanthellae to animal biomass changes under different conditions, ranging from conditions that result in loss of zooxanthellae (bleaching) to eutrophic conditions that promote high population densities of zooxanthellae.

Because the nutrient status of zooxanthellae in field corals is difficult to determine without experimental manipulations of the ambient nutrient regime, the use of "indices" of algal nutrient status such as the C : N : P ratios, the ammonium enhancement of dark carbon fixation (Cook et al. 1992, 1994), and amino acid ratios (Flynn 1990) to test the nutrient status of zooxanthellae in field populations of symbiotic anemones and corals is a promising approach. More work is needed to determine the pathways of nutrient acquisition and storage products of nitrogen.

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Amino Acid Content of Zooxanthellae Freshly Isolated from *Pocillopora damicornis*[1]

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ABSTRACT: Total amino-N content and glutamine to glutamate ratios (gln : glu) were determined in zooxanthellae freshly isolated from colonies of the coral *Pocillopora damicornis* (Linnaeus) incubated in ambient seawater or in seawater supplemented with ammonium to give a final concentration of 20 or 50 μM. Addition of ammonium did not change total amino-N content but did increase gln : glu from 0.25 to 0.47–0.48, suggesting that ammonium was directly utilized by the symbiotic zooxanthellae. Gln : glu in zooxanthellae from corals maintained in seawater "stripped" of ammonium fell to 0.18. Sizes of pools of most free amino acids in zooxanthellae from *P. damicornis* were roughly two to five times those of zooxanthellae from the temperate sea anenome *Anemonia viridis*, but the latter, which is not believed to be N-limited, exhibited higher gln : glu ratios. These data indicate that gln : glu is a sensitive measure of the response of symbiotic zooxanthellae to exogenous dissolved nitrogen, but despite an increase in gln : glu when seawater is supplemented with ammonium, it cannot be concluded that individual zooxanthellae are normally N-limited.

SEVERAL STUDIES have shown that addition of ammonium causes an increase in population density of coral symbionts (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Dubinsky et al. 1990, Stambler et al. 1991, Muller-Parker et al. 1994*b*). However, although increase in population density as a response to elevation of supply of dissolved inorganic nitrogen (DIN) is taken as an indication that the growth rate of populations of symbiotic zooxanthellae is nitrogen-limited (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Szmant et al. 1990), so far little attention has been paid to physiological and biochemical parameters that are indicative of nutrient status in microalgae. Cook and D'Elia (1987) have shown that uptake of the ammonium analog ^{14}C-methylammonium by freshly isolated zooxanthellae was indicative of nitrogen-limited metabolism, but other parameters remain to be investigated. The Hawaii Institute of Marine Biology workshop provided the opportunity to measure both elemental composition of zooxanthellae (Muller-Parker et al. 1994*a*) and size and composition of zooxanthellar amino acid pools.

Size of amino acid pools has been used to determine nitrogen status in a variety of microalgae, including *Chlorella* symbiotic with green hydra (Ohmori et al. 1984, Dortch et al. 1985, McAuley 1987, 1992, Flynn et al. 1989). In particular, it has been pointed out that the ratio of glutamine to glutamate content (gln : glu) is very sensitive to nitrogen status, because ammonium is assimilated into glutamine via glutamine synthetase (GS) (Flynn et al. 1989, Flynn 1990, 1991). In nitrogen-limiting conditions, glutamine levels fall, but glutamate pools appear to be conserved, perhaps because the latter play a central role in aminating reactions. Davidson et al. (1992), studying batch culture growth of the marine phytoplankter species *Isochrysis galbana* Parke, found a drop in gln : glu upon

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exhaustion of the extracellular N source. A similar drop in gln : glu was recorded in cells of a strain of symbiotic *Chlorella* transferred to N-free medium (McAuley 1992). Changes in zooxanthellar glutamine and glutamate pools in response to addition of DIN to the intact symbiosis may also indicate whether or not zooxanthellae directly assimilate DIN.

Here, amino acid levels were measured in zooxanthellae from the coral *Pocillopora damicornis* (Linnaeus) to determine whether change in ammonium concentration caused perturbation of zooxanthellar amino acid pools. The effect of ammonium addition on total pool size and on glutamine : glutamate was determined, and composition of amino acid pools was compared with that of zooxanthellae isolated from the temperate-water symbiosis *Anemonia viridis*.

MATERIALS AND METHODS

The collection and maintenance of colonies of *Pocillopora damicornis* in seawater supplemented with different levels of ammonium or "stripped" by passage through a flume containing macroalgae are described by Stambler et al. (1994). Zooxanthellae were isolated from colonies of *P. damicornis* using a Water Pik (Johannes and Wiebe 1970) and cleaned by centrifugation as previously described (Muller-Parker et al. 1994*b*).

Total amino acid content was estimated from extracts of duplicate aliquots of zooxanthellae. Pellets were extracted with 5% TCA for 1 hr at room temperature, neutralized with NaOH, and amino acid-N content was estimated by a modification of the ninhydrin method of Wylie and Johnson (1962). Absorbance of samples was measured at 570 nm using a spectrophotometer (Hewlett Packard HP 8452A) and compared with that of known amounts of glycine.

Contents of amino acid pools were measured by high performance liquid chromatography (HPLC). Then 100 μl of zooxanthellae suspension (containing between 5×10^4 and 2×10^5 cells) were extracted by adding 400 μl absolute ethanol. Extracts were dried by evaporation and resuspended in 12.5 μM αamino butyric acid (AABA, used as internal standard). Aliquots were prederivatized with *o*-phthaldialdehyde and separated on a Waters Resolve 5 μm C18 column using a methanol : sodium-acetate stepped gradient (Jones et al. 1981). Sample injections were performed using a Rheodyne injection valve equipped with a 20-μl sample loop; the gradient was generated by an HPLC pump (LKB model 2150) and LC controller (model 2152) with a flow rate of 0.8 ml min^{-1}. Derivatized amino acids were detected using a Milton Roy Fluoromonitor III equipped with a 418-nm cut-off filter, and peak areas were integrated with a Spectra Physics Chromjet integrator. Amounts of amino acids were calculated from the fluorescence of samples of 12.5-μM standards run on the same day, corrected for differences in detector sensitivity by reference to the fluorescence of AABA internal standards.

Anemonia viridis was maintained in filtered seawater from St. Andrews Bay, Fife, Scotland, at 15°C in constant light (60 μmol photons m^{-1} sec^{-1}) and were fed to repletion on chopped whitefish meat once per week. Ammonium content of the seawater varied between 2 and 5 μM, higher than that of surface seawater from Kaneohe Bay in which control corals were maintained (Stambler et al. 1994), but lower than levels in supplemented seawater treatments. Zooxanthellae were isolated by homogenization of clipped tentacles, washing three times by centrifugation, and filtration through two layers of 60-μm mesh plankton netting followed by a final washing by centrifugation. Microscopic examination revealed that the resulting pellet was essentially free of animal contamination, particularly nematocysts, but preliminary experiments showed that the animal fraction of the homogenate contained high levels of glycine and taurine, and even small amounts of contamination could give falsely high values for pools of those amino acids in isolated zooxanthellae. To ensure that only amino acids on zooxanthellar internal pools were measured, samples were filtered onto Whatman GF/C filter papers using low vacuum pressure, washed with 20 ml seawater, and extracted in 80% ethanol for amino acid analysis. This

procedure removed contaminating animal material but did not harm the zooxanthellae.

Statistical analysis was performed by one-way analysis of variance using Minitab (Minitab Inc., State College, PA) statistical software.

RESULTS

Total Amino Acids

Measurement of total amino acid content (as amino acid-N) showed no difference between control coral heads (incubated in untreated seawater from Kaneohe Bay) and those incubated in seawater supplemented with 20 or 50 μM ammonium (Table 1). Ammonium treatment did not increase amino acid pools of zooxanthellae in terms of total N content. Indeed, two measurements of total amino acid content of zooxanthellae from colonies incubated in "stripped" seawater showed that this value was higher than in control corals.

Glutamine : glutamate Ratios

Gln : glu ratios were compared in zooxanthellae isolated from colonies in "stripped" or ambient seawater with those from colonies treated with ammonium (Table 1). Ratios in zooxanthellae from untreated corals were between 0.14 and 0.34, but ratios in zooxanthellae from ammonium-treated corals were considerably higher, between 0.30 and 0.58 in zooxanthellae from corals treated with 20 μM ammonium and between 0.34 and 0.57 from corals treated with 50 μM ammonium. Conversely, zooxanthellae isolated from corals incubated in "stripped" seawater exhibited reduced gln : glu ratios (0.14–0.24, mean = 0.18) compared with controls.

Zooxanthellae isolated from corals incubated with either 20 or 50 μM ammonium showed elevated gln : glu ratios within 2 weeks. This is consistent with observations that supply of ammonium caused changes in algal biomass parameters within the first 2 to 4 weeks of addition of ammonium (Muller-Parker et al. 1994*b*). In the case of zooxanthellae from *P. damicornis* treated with seawater containing 20 μM ammonium, elevation in gln : glu was a result of increase in glutamine rather than decrease in glutamate. Glutamine levels increased steadily over the 6 weeks of treatment for which these measurements were made. Comparison of summed means of all 20-μM treatments with controls showed that glutamine levels were significantly increased by ammonium treatment, and there was also a less significant increase in glutamate levels (glutamate, $F = 3.67$, $df = 10$, $P < 0.10$; glutamine, $F = 15.62$, $df = 10$, $P < 0.005$). If it is assumed that ammonium assimilation proceeded via GS, increased amounts of glutamine would be predicated from an increased supply of ammonium, and increased gln : glu in zooxanthellae suggests that they were able to take up ammonium directly and assimilate it into amino acids.

Zooxanthellae from *P. damicornis* incubated in the 50-μM ammonium treatment exhibited increased glutamine levels but decreased glutamate levels compared with controls, although because of high variation in treated samples, in neither case was the difference significant (glutamate, $F = 1.26$, $df = 9$, $P < 0.294$; glutamine, $F = 1.75$, $df = 9$, $P < 0.222$). Although the resulting gln : glu was similar to that of zooxanthellae from *P. damicornis* incubated in 20 μM ammonium, other workers have noted that *P. damicornis* treated at the higher ammonium level exhibited signs of stress (Høegh-Guldberg 1994, Muller-Parker et al. 1994*b*).

Amino Acid Pool Composition

To my knowledge, no analyses of amino acid pools of coral zooxanthellae have been published. Table 2 shows the size of intracellular pools of 16 amino acids from five replicate extracts of zooxanthellae from *P. damicornis* colonies incubated in ambient seawater. Histidine could not be measured because its peak co-resolved with an unknown nonprotein amino acid that has been found in a number of marine phytoplankton species (Flynn and Flynn 1992). As might be expected from its role in primary amino

TABLE 1

TOTAL AMINO ACID-N CONTENT AND GLUTAMINE TO GLUTAMATE RATIOS OF ZOOXANTHELLAE FRESHLY ISOLATED FROM *Pocillopora damicornis*

TREATMENT	TOTAL AMINO ACID-N (PG PER CELL)	GLUTAMATE (FMOL PER CELL)	GLUTAMINE (FMOL PER CELL)	GLN:GLU
Ambient seawater control				
Avg.	0.265	11.12	2.80	0.25
SE	0.017	1.13	0.5	
n	5	5	5	5
20 μM ammonium, 2 weeks				
Avg.	0.277	11.32	5.46	0.48
SE	0.003	1.15	0.79	
n	2	2	2	2
20 μM ammonium, 4 weeks				
Avg.	0.257	15.94	6.89	0.43
SE	0.017	0.53	2.23	
n	2	2	2	2
20 μM ammonium, 6 weeks				
Avg.	0.283	14.70	7.96	0.55
SE	—	0.39	0.29	
n	1	2	2	2
20 μM ammonium, 8 weeks				
Avg.	0.269	—	—	—
SE	0.105	—	—	—
n	2			
50 μM ammonium, 2 weeks				
Avg.	0.263	8.68	4.04	0.45
SE	—	1.30	1.61	
n	1	2	2	2
50 μM ammonium, 6 weeks				
Avg.	0.334	9.65	3.30	0.34
SE	—	—	—	
n	1	1	1	1
50 μM ammonium, 8 weeks				
Avg.	0.224	9.08	4.98	0.56
SE	0.035	4.43	2.39	
n	2	2	2	2
"Stripped," 2 weeks				
Avg.	—	18.29	3.43	0.20
SE	—	2.53	0.30	
n		2	2	2
"Stripped," 6 weeks				
Avg.	—	12.13	2.03	0.17
SE	—	—	—	
n		1	1	1
"Stripped," 8 weeks				
Avg.	0.420	12.91	2.20	0.18
SE	0.026	1.52	0.16	
n	2	2	2	2

acid metabolism, glutamate was the largest component of the total amino acid pool. Amounts of amino acids in free pools were similar to those in zooxanthellae isolated from two species of reef-forming coral in the Caribbean (P.J.M. and V. J. Smith, unpubl. data), but were generally higher than those of zooxanthellae freshly isolated from 1-day starved *Anemonia viridis* (a temperate-water symbiosis). Only glycine, methionine, and taurine levels were similar in the two types of zooxanthellae; *Anemonia* symbionts con-

TABLE 2

Free Amino Acid Content of Zooxanthellae Freshly Isolated from *Pocillopora damicornis* and from *Anemonia viridis*

	Pocillopora ($n = 5$)		*Anemonia* ($n = 4$)	
FREE AMINO ACID	FMOL PER CELL	% TOTAL	FMOL PER CELL	% TOTAL
asp	3.72 ± 0.60	6.93	0.77 ± 0.06	3.30
glu	10.45 ± 0.99	19.45	3.02 ± 0.39	12.87
asn	1.03 ± 0.14	1.92	0.20 ± 0.01	0.85
ser	5.53 ± 1.50	10.29	1.55 ± 0.10	6.60
gln	2.74 ± 0.48	5.10	4.36 ± 0.29	18.53
gly	4.12 ± 0.58	7.66	4.79 ± 1.67	20.39
thr	1.20 ± 0.08	2.23	0.64 ± 0.19	2.73
arg	3.56 ± 0.47	6.62	0.30 ± 0.06	1.29
tau	3.92 ± 0.98	7.29	4.05 ± 1.16	17.23
ala	5.15 ± 0.82	9.59	1.25 ± 0.15	5.33
tyr	1.77 ± 0.20	3.29	0.45 ± 0.04	1.90
met	0.36 ± 0.19	0.68	0.37 ± 0.02	1.56
val	3.15 ± 0.71	5.86	0.76 ± 0.13	3.22
phe	1.69 ± 0.28	3.15	0.46 ± 0.07	1.96
iso	1.23 ± 0.35	2.30	0.23 ± 0.01	0.99
leu	3.10 ± 0.58	5.77	0.29 ± 0.01	1.24
Total	52.72 ± 7.00	100.00	23.50 ± 3.72	100.00

Note: Aliquots of cells were extracted in 80% ethanol, resuspended in distilled water containing 12.5 μM AABA as internal standard, and stored at −40°C for HPLC. Figures are means ± SE; number of determinations are given in parentheses.

tained higher levels of glutamine. The gln : glu ratio in *Anemonia* symbionts was also high: 1.44 compared with 0.26 in zooxanthellae isolated from coral heads incubated in untreated seawater and 0.48 in zooxanthellae from coral heads incubated in 20 μM ammonium. This did not appear to be a result of recent host feeding, because levels remained unchanged during 2 weeks starvation of hosts (unpubl. data).

DISCUSSION

Incubation of colonies of the coral *Pocillopora damicornis* in seawater containing elevated levels of ammonium did not alter total amino acid content of the symbiotic zooxanthellae. This is consistent with the findings of Achituv et al. (1994), who found that protein content of zooxanthellae showed no consistent trend related to ammonium supplementation. However, ammonium supplementation of the seawater in which colonies were incubated did increase the glutamine : glutamate ratio of zooxanthellae. This suggests that the nitrogen metabolism of the zooxanthellae was directly affected by increased levels of ammonium in the seawater, because if it is assumed that assimilation of ammonium proceeds via GS, then elevated levels of ammonium would lead to elevated levels of glutamine. This conclusion is supported by the findings of Muller-Parker et al. (1994*a*), who measured an increase in elemental N content and C : N ratios of zooxanthellae, but not animal tissue of *P. damicornis* incubated in 20 μM ammonium. Zooxanthellae also responded to ammonium supplementation by increased chlorophyll contents (Muller-Parker et al. 1994*a*) and mitotic indices (Høegh-Guldberg 1994).

Although zooxanthellae respond directly to ammonium supplementation in a number of ways, it is not yet clear if they are normally nitrogen limited in the symbiosis. Flynn

(1990, 1991) suggested that gln : glu ratios of <0.2 may be indicative of nitrogen-limited growth in microalgae. Here, mean gln : glu ratios of zooxanthellae isolated from *P. damicornis* ranged from 0.18 ("stripped"), 0.25 (controls), and up to 0.56 (ammonium supplemented). Only zooxanthellae from colonies maintained in "stripped" seawater, which were exposed only to ammonium produced by host catabolism, could be considered N-limited by Flynn's (1990, 1991) criteria. Zooxanthellae isolated from *Anemonia viridis*, which has a symbiont population density similar to that measured in *P. damicornis* (unpubl. observations; Muller-Parker et al. 1994*b*), possessed higher gln : glu than zooxanthellae isolated from *P. damicornis*, even after coral colonies had been incubated in 20 or 50 μM ammonium for 6–8 weeks. Growth of populations of *A. viridis* symbionts does not appear to be N-limited, because expulsion of zooxanthellae by the host is continuous in normal conditions, and both mitotic index and levels of free amino acids do not fall during 2 weeks host starvation in the light (unpubl. observations).

Although availability of ammonium may limit population density of zooxanthellae, as shown by increase in numbers of zooxanthellae per unit area in colonies treated with supplemented seawater (Muller-Parker et al. 1994*b*), tight recycling of N within the symbiosis may mean that although overall population size may be N-limited, individual zooxanthellae may not be physiologically N-limited. Nitrogen status of individual symbionts depends not only upon flux of DIN through the symbiosis, but also upon the population density of symbionts, rates of flux through symbiont amino acid pools (dependent upon rates of protein synthesis and amino acid catabolism), and ability of symbionts to utilize stored N. More observations are required before it can be determined whether or not individual cells of zooxanthellae symbiotic with *P. damicornis* are physiologically N-limited. In particular, it would be of interest to measure changes in amino acid pools of cultured zooxanthellae grown under varying conditions of N-limitation to provide baseline data for comparison with freshly isolated symbionts. However, these preliminary results show that HPLC analysis of amino acid pools of zooxanthellae freshly isolated from corals is a useful and sensitive method to determine response of zooxanthellae to changes in supply of DIN to the symbiosis.

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Effect of Exogenous Nitrogen Levels on Ultrastructure of Zooxanthellae from the Hermatypic Coral *Pocillopora damicornis*[1]

TAMAR BERNER[2] AND IDO IZHAKI[3]

ABSTRACT: Branches of the hermatypic coral *Pocillopora damicornis* (Linnaeus) were exposed for 2, 4, 6, and 8 weeks to ammonium concentrations of <1 μM (nutrient-stripped), 2 μM (seawater as a control), 20 μM, and 50 μM (enriched), after which their symbiotic zooxanthellae were examined for changes in their ultrastructure. No significant differences among treatments were detected in cell diameter or in relative volume of any of the cellular organelles of zooxanthellae subjected to the various nitrogen levels. The surface density of thylakoids was higher in cells from the elevated-nitrogen treatments. However, there was a significant increase in accumulation of starch grains and lipid droplets in zooxanthellae in corals maintained in unenriched and nutrient-stripped seawater, occupying about 15% of the cell volume. Storage of these N-free compounds showed that under N-limited conditions photosynthate cannot be used as carbon skeletons in synthesis of amino and nucleic acids, both required for cell doubling. We believe that our results further demonstrate the uncoupling of photosynthesis from population growth under C : N ratios deviating from those needed to support balanced growth.

INCREASING EUTROPHICATION of the Earth's oceans has brought about increasing public awareness of the possible effects of this process on coral reefs. Among the major sources of marine pollution are a range of inorganic nitrogen compounds. It is known that growth and chemical composition of microalgae are affected by the availability of nitrogen, as they are with most other limiting nutrients (Glibert 1988). Symbiotic dinoflagellates in marine invertebrates are no exception. The coral host, as a habitat for the algae, is a source of nitrogen for algal growth, in addition to the dissolved inorganic sources available directly to the algae, albeit in low concentrations. Muscatine et al. (1989), working with the common Red Sea hermatypic coral *Stylophora pistillata* Esper, showed that nitrogen enrichment resulted in a decrease in the C : N ratio of the zooxanthellae, as well as an increase in zooxanthellae numbers, that was proportional to the increase in cellular protein within the algae. This suggests that availability of inorganic nitrogen leads to increased protein synthesis in zooxanthellae and growth in their areal concentrations.

On the other hand, under N limitation, algal cells, even with adequate CO_2 supply and optimal irradiance, cease to multiply and start to accumulate different carbohydrates (Criscuolo et al. 1981, Coleman et al. 1988). Lipids are also common storage substances in nitrogen-starved microalgae (Shifrin and Chisholm 1981, Aaronson et al. 1983). Nutrient limitation may cause excretion of carbohydrates, instead of their accumulation, by phytoplankton (Zlotnik 1986) or, in the case of armored dinoflagellates, production and shedding of polyglucane shells (Criscuolo et al. 1981). Both the storage and elimination of such nitrogen-deficient compounds suggest an uncoupling between photosynthesis and protein synthesis, leading to uncoupling between photosynthesis and cell multiplication.

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There is, however, at least for some time, ongoing production of carbon skeletons. This process, as a result of nitrogen shortage, shifts toward synthesis of storage lipids and carbohydrates rather than of amino acids and nucleotides, both prerequisites for cell doubling. Høegh-Guldberg (1994) conveys another reason for the increasing growth rates of symbiotic algae under N-enriched conditions: the toxic effect of ammonium on the host with concomitant lower translocation and higher availability of nutrients to the zooxanthellae.

The addition of ammonium to the seawater growth medium of corals has been reported to cause a small increase in chlorophyll per zooxanthella cell in some studies (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Dubinsky et al. 1990) or to have no effect in other studies (Høegh-Guldberg and Smith 1989, Stambler et al. 1991).

Studies on ultrastructure of zooxanthellae and of free-living microalgae under various light regimes showed that the volume fraction of chloroplasts and the surface density of thylakoid lamellae increase with cellular chlorophyll content (Berner et al. 1987, Lesser and Shick 1990).

However, very little data exist on the influence of nitrogen concentrations on the ultrastructure of algal cells of both phytoplankton and symbiotic algae. Morphometric analysis has been conducted on *Isochrysis galbana* Parke (Haptophyceae) cultured under high and low nitrogen concentrations (A. Sukenik and T.B., unpubl. data) and on the zooxanthellae from *S. pistillata* (Stambler 1992), comparing the effects of ambient and elevated nitrogen levels on cell ultratructure. The relative volume of chloroplast and thylakoid surface density increased slightly with nitrogen rise in *I. galbana*. Zooxanthellae did not show any change in dimensions of chloroplast and thylakoids as a result of elevated nitrogen concentrations in the seawater surrounding their coral host. However, neither study examined the possible effects of nitrogen levels on the volume fraction of accumulation bodies. Ultrastructural information can contribute to our understanding of the ways by which N limitation regulates the populations of symbiotic algae. Chloroplast volume and surface density of thylakoids, as well as the different amounts of storage products, can serve as indicators of photosynthetic potential and the fate of photosynthate.

The aim of this study was to determine if there were any specific changes in the characteristics of the ultrastructure of the zooxanthellae of the hermatypic coral *Pocillopora damicornis* (Linnaeus), resulting from exposure of the host to four nitrogen levels, for various lengths of time.

MATERIALS AND METHODS

Colonies of *P. damicornis* were exposed for 2, 4, 6, and 8 weeks to the following nitrogen levels in flowing seawater: <1 μM, (nutrient-stripped), about 2 μM (ambient) as a control, and enriched to 20 μM or 50 μM (Stambler et al. 1994). Freshly isolated zooxanthellae from branches taken from four single corals, each of which was exposed to a treatment, were mixed together and fixed for transmission electron microscopy (TEM) in 2.5% glutaraldehyde. Samples were then concentrated using the bovine serum albumin (BSA) technique (Oliveira et al. 1989). After postfixation in OsO_4, samples were dehydrated by serial transfers through progressive aqueous-ethanol series and finally embedded in Spurr's resin (Spurr 1969). Sections were cut and subsequently stained with uranyl acetate (Stempak and Ward 1964), followed by lead citrate (Reynolds 1963), and were observed with a TEM (JEOL 1200x) operating at 80 kV.

From each treatment the diameter of 40 cells was measured under a light microscope. Morphometric analysis of the relative volume of chloroplasts, nuclei, pyrenoids, mitochondria, and starch and lipid storage bodies to cell volume, and the surface density of thylakoids was calculated by the superimposition of an array of short lines on the TEM photographs (Weibel et al. 1966, Freere and Weibel 1967).

Because the main effect studied was N concentration, a one-way analysis of variance

(ANOVA) procedure was used to assess the cell diameter, the relative volume of various organelles, and the relative surface density of the thylakoids as a function of exposure for 2, 4, 6, and 8 weeks to the four experimental nitrogen levels. All one-way ANOVAs were followed by posteriori comparisons using a Duncan multiple range test ($P < 0.05$) (SAS Institute 1982).

RESULTS AND DISCUSSION

Because our morphometric analysis determined the fraction of the cell volume occupied by the various organelles, it was important to check whether there was any change in the cell size under the different treatments. Figure 1 shows that the difference in cell diameter, although significant, does not show any consistent trend in relation to nitrogen level. Cell diameter under any nitrogen concentration did not deviate by more than 10% from its mean value for all treatments. Our conclusion that nitrogen availability does not affect the cell volume of zooxanthellae is in agreement with the results reported by Høegh-Guldberg and Smith (1989). It seems that intrinsic genetic disposition, rather than environmental factors, determines the cell size of zooxanthellae. Likewise, the relative volumes of the nuclei, mitochondria, pyrenoids, and vacuoles were not significantly affected by nitrogen levels. Similar results were reported by Stambler (1992) and by Sukenik and T.B. (unpubl. data).

The relative volume of the chloroplast, although showing significant differences among treatments, did not show any particular trend with respect to external nitrogen concentrations (Figure 2). Again, our results are in agreement with the studies mentioned above (Stambler 1992; Sukenik and T.B., unpubl. data). The values for the relative chloroplast volume resembled those measured in other algae grown under high to moderate light intensities (500–800 μmole quanta m^{-2} min^{-1}) (Berner et al. 1987, Sukenik et al. 1989). It is likely that the high irradiance to which the corals in the treatment tanks were exposed (Stambler et al. 1994) cancelled the effect of increased algal density and mutual shading caused by high nitrogen concentration. The combination of these opposite effects resulted in no change in chloroplast to cell volume ratios. Nevertheless, the ratios of surface

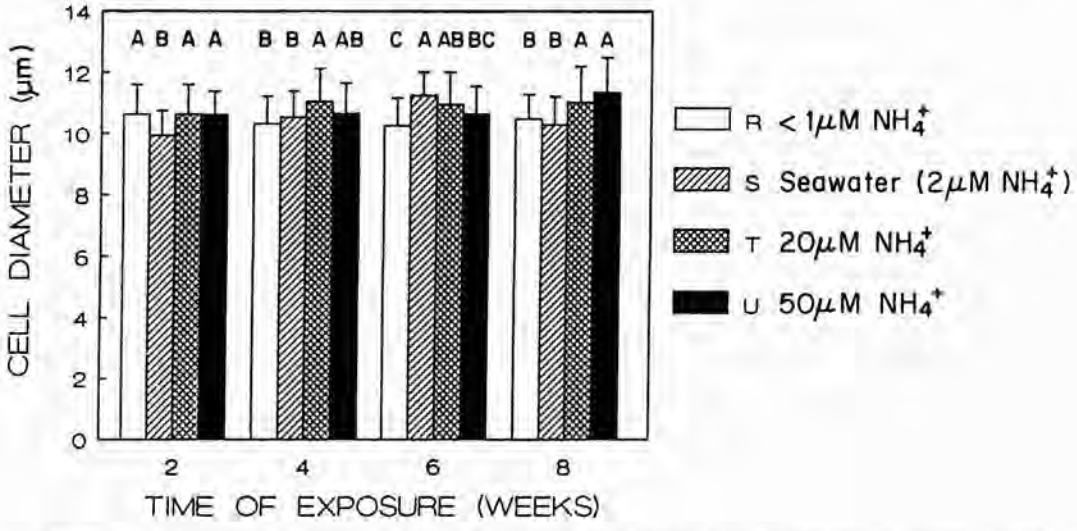

FIGURE 1. Cell diameter as a function of time of exposure to different levels of nitrogen. Bars represent standard errors. Different letters above columns show that means are significantly different according to the Duncan multiple range test; $n = 40$.

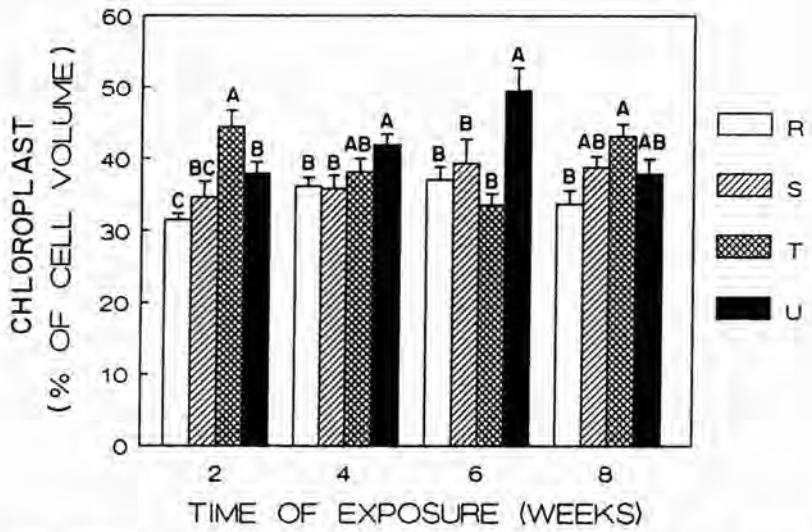

FIGURE 2. Volume ratio of chloroplast to cell as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

densities of thylakoids to cell volume in N-enriched cells (Figure 3) yielded values similar to those of microalgae under moderate light intensities (Sukenik et al. 1989; Sukenik and T.B., unpubl. data). Surface density is significantly higher in cells grown at elevated nitrogen concentration compared with the control. This increase in surface density of thylakoids to cell volume mirrors the concomitant rise in chlorophyll per cell at elevated nitrogen levels (Dubinsky et al. 1990, Muller-Parker et al. 1994).

The increase in surface density of the thylakoids under increased nitrogen is of special interest because all corals were incubated under the same irradiance. Therefore, one should look for additional factors affecting thylakoid lamellae, other than light level. We interpreted the similarity between nitrogen-enriched low or moderate light–adapted cells as the combined result of two factors. Nitrogen enrichment leads to increased population densities of the zooxanthellae, which, in turn, causes mutual shading requiring photoacclimation (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Dubinsky et al. 1990, Høegh-Guldberg 1994). The second factor is the light-driven change in the C : N ratio in the ingoing nutrient flux under different irradiance levels. Under the high photosynthetic rates typical of high-light corals, ambient nitrogen levels are far too low to satisfy cell growth, but the converse is true under light-limited conditions, where the same nitrogen levels are sufficient for the low photosynthetic rates. Therefore, for the limited carbon flux into the cells, under low light and low photosynthetic rates, there may well be sufficient nitrogen. The very same nitrogen concentration in the water, but under high light and photosynthesis, will be experienced by the zooxanthellae as severe nitrogen limitation. The increase in cell density and mutual shading and the relative nitrogen sufficiency of light-limited cells explain the similarities we see among corals grown under these treatments in the study reported here (Dubinsky and Jokiel 1994).

A striking difference occured in ultrastructure of zooxanthellae from the four treatments. Lipid and starch storage bodies accumulated in cells under low nitrogen or were scarce in cells incubated in the nitrogen-enriched treatments. The stored photosynthates of the zooxanthellae from *P. damicornis*, as seen in the electron micrographs, were starch and lipids. The starch was mainly deposited around the pyrenoid, but was occa-

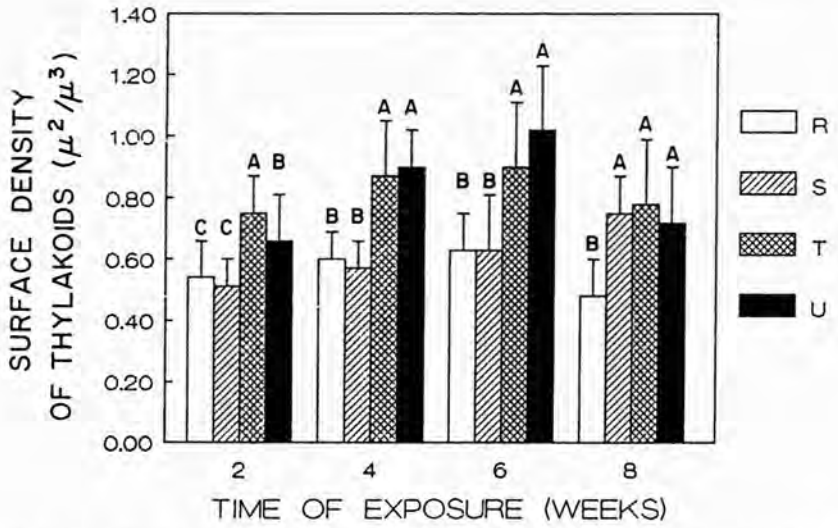

FIGURE 3. Surface density of thylakoids in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

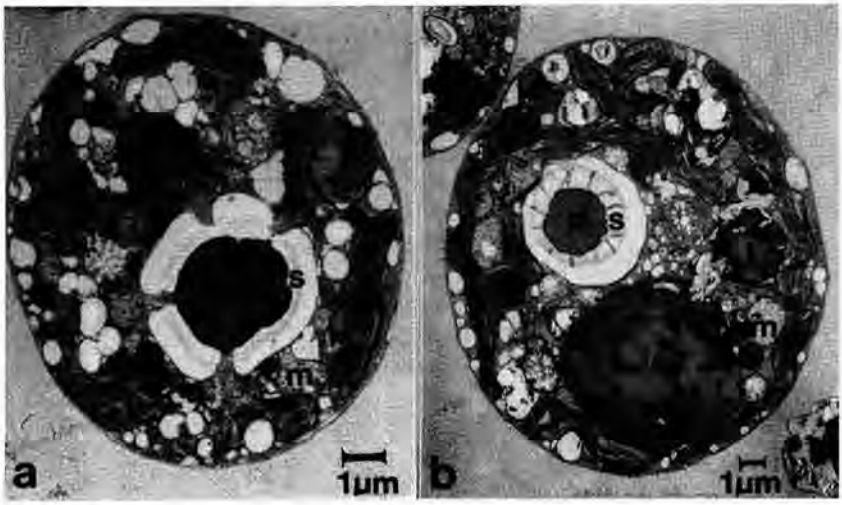

FIGURE 4. Electron micrographs of low (*a*) and high (*b*) nitrogen concentrations. c, chloroplast; l, lipids; m, mitochondria; n, nucleus; p, pyrenoid; s, starch.

sionally seen as starch granules in the cytoplasm. The lipids were stored as droplets, scattered throughout the cytoplasm (Figure 4). It should be emphasized that the starch and lipids seen in the electron micrographs represent only the free fraction of their share in the cell constituent. Most of the carbohydrates and lipids exist in the cell solution and as structural components of the cell organelles.

The most pronounced accumulation of starch was seen in cells from corals incubated under reduced nitrogen concentration. The presence of starch granules remained high throughout the experiment (Figure 5). There was an accumulation of starch in the

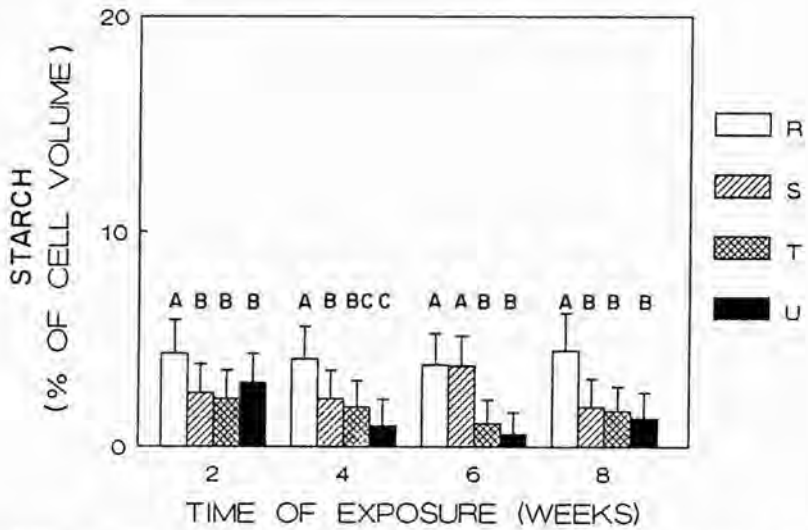

FIGURE 5. Volume ratio of starch to cell in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

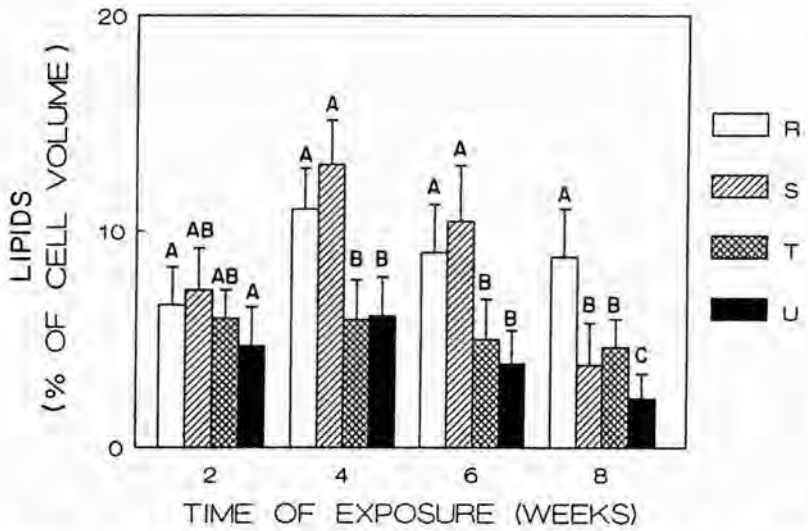

FIGURE 6. Volume ratio of lipids to cell in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

control until 6 weeks into the experiment, when it reached the same level of the lowest (nutrient-stripped) concentration. It is possible that the control reached cellular N limitation, which acted as a trigger for switching cellular biosynthesis from protein toward starch production, later than cells exposed to reduced N concentration. This is conceivable, because the cells under ambient N conditions may have depleted their nutrient cell quota later then those kept in nutrient-depleted seawater. The amount of starch present at higher nitrogen levels was lower than that of the control.

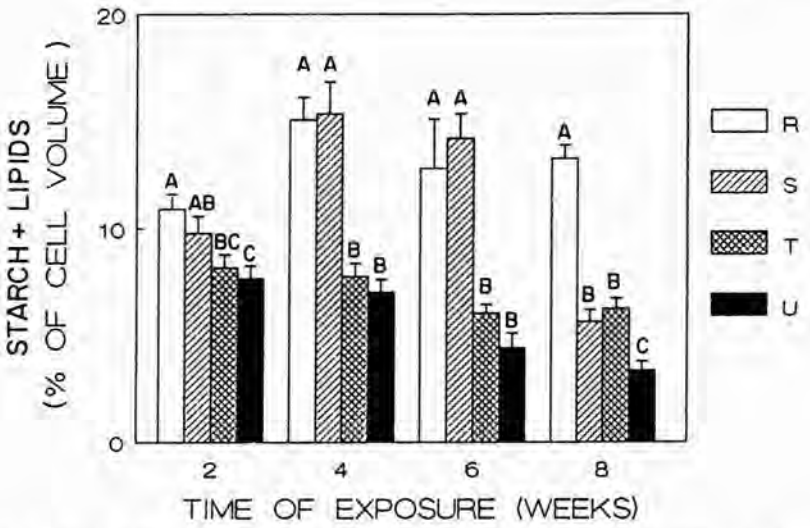

FIGURE 7. Combined volume ratio of starch and lipids to cell in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

The accumulation of lipids in the control and in the reduced nitrogen concentration became evident after 2 weeks, but it became significantly different from algae under N-enriched conditions after 4 and 6 weeks (Figure 6). In the zooxanthellae from *P. damicornis*, lipids were more abundant than starch as storage compounds, and they occupied twice its volume.

If the volumes of starch and lipids are combined (Figure 7), it can be clearly seen that after 4 and 6 weeks they significantly exceeded the amount of these substances under N-enriched conditions. At times, these compounds, as visible accumulation structures, may occupy up to 15% of the cell volume.

Muller-Parker et al. (1994) show that zooxanthellae from N-enriched corals had less C and more N per cell than those from unenriched controls. Under a high C : N ratio there is an excess of photosynthetic products over protein synthesis, resulting in the accumulation of N-deficient compounds such as starch and lipids. It is likely that by their exclusion from the osmotic system, they facilitate the maintenance of stable Redfield ratios in the cell solutes.

Stimson and Kinzie (1991) showed that under N-enriched conditions the lipid level in coral tissues was reduced, possibly as a result of the decrease in the rate of carbon translocation from the algae to the coral. Their conclusion is consistent with our results that the lipid content in the algae under such conditions is low. Lipid droplets may be transferred to the animal, as in the symbiotic anemone *Condylactis gigantea* (Kellogg and Patton 1983). Whether the end result is that under N-enriched conditions less lipids are translocated to the animal, resulting in its starvation, or that it causes the shift of its diet to the consumption of other external organic compounds by predation on zooplankton remains an open question.

We conclude that morphometric analysis of cellular ultrastructure is a valuable method complementing biochemical analysis. On the whole, we show that under N limitation there is visible storage of photosynthate as starch and lipids, results that allow insights into the cellular consequences of nutrient limitation and enrichment, which cannot be derived from chemical analyses alone.

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Population Dynamics of Symbiotic Zooxanthellae in the Coral *Pocillopora damicornis* Exposed to Elevated Ammonium [$(NH_4)_2SO_4$] Concentrations[1]

OVE HØEGH-GULDBERG[2]

ABSTRACT: Division synchrony and growth rate of symbiotic zooxanthellae was investigated for populations living in colonies of the reef-building coral *Pocillopora damicornis* (Linnaeus) exposed to different concentrations of ammonium [$(NH_4)_2SO_4$] in seawater. Presence of low concentrations of ammonium (0.2 μM) did not affect (compared with corals growing in ammonium-stripped seawater) either division synchrony or growth rate. Exposure to higher concentrations of ammonium (20 or 50 μM), however, affected the population dynamics of the zooxanthellae residing in *P. damicornis*. Zooxanthellae in corals exposed to 20 μM ammonium had mitotic indices (percentage of total cells dividing) that were two to three times higher than mitotic indices of zooxanthellae in control (0.2 μM) corals. Although division of zooxanthellae was still phased in corals exposed to 20 μM ammonium, there were many more cells dividing out of phase compared with control corals. Division of zooxanthellae in corals exposed to 50 μM was not phased. Calculated growth rates of zooxanthellae exposed to 20 or 50 μM ammonium were higher than those representative of zooxanthellae living in control corals, although growth rate of both carbon and nitrogen pools was lower in 50 μM as compared with 20 μM ammonium. These data support the conclusion that the population dynamics of symbiotic zooxanthellae within *P. damicornis* are affected by concentrations of ammonium in seawater that are equal to or higher than 20 μM and that 50 μM ammonium concentrations may be toxic to some extent. These data taken in isolation, however, do not constitute an effective test of the hypothesis that zooxanthellae are limited by the supply of ammonium under ambient conditions and further emphasize the importance of enrichment studies concentrating on growth and nitrogen incorporation rates measured for the entire symbiotic association.

POPULATIONS OF SYMBIOTIC zooxanthellae are characterized by low growth rates relative to populations of cultured zooxanthellae (Wilkerson et al. 1983, Cook and D'Elia 1987). The low growth rates exhibited by symbiotic zooxanthellae have been cited as evidence of the host influence over the metabolism of symbiotic zooxanthellae, either passively (via restricted access to space and nutrients [Muscatine and Pool 1979, Cook and D'Elia 1987]) or actively (via host-specific mitogenic or cytogenic factors [Muscatine and Pool 1979]). A key experiment in identifying the importance of passive "control" mechanisms is to supply an excess of a particular nutrient and examine the response of the growth rate of the zooxanthellae. If an increase in the growth rate occurs after the addition of a nutrient (all else being equal), then the passive supply of the nutrient is a significant factor in explaining the low growth rate of zooxanthellae in hospice. The relative importance of the availability of a particular nutrient can then be determined by examining how closely the measured growth rate under surplus matches the maximum growth rate attained by symbiotic zooxanthellae under optimal growth conditions (e.g., in culture).

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Ammonium [$(NH_4)_2SO_4$] was supplied to the coral *Pocillopora damicornis* (Linnaeus) during the Hawaii Institute of Marine Biology (HIMB) experiment (see Stambler et al. [1994] and Muller-Parker et al. [1994]). Elevated ammonium has been shown to affect several characteristics of symbiotic zooxanthellae in reef-building corals, including their population density, photosynthetic rate, and chlorophyll *a* concentration (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Stambler et al. 1991, Stimson and Kinzie 1991). It is interesting that the specific growth rate of zooxanthellae (μ_z) in these studies did not differ between treatments. Cook et al. (1988) demonstrated a stimulatory effect of adding NH_4Cl (together with phosphate) on the division rate of populations of symbiotic zooxanthellae in previously starved individuals of the sea anemone *Aiptasia pallida* (Verrill), but the effect of NH_4Cl on division was seen in only five of 23 anemones examined. The lack of a demonstrable effect of elevated nutrients on the division rates of zooxanthellae despite substantial increases in the population density of symbionts (seen at the end of the experiments reported by Cook et al. [1988]) suggests that the effect of elevated NH_4Cl on the division of zooxanthellae may be transitory and short-lived (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989).

This paper reports the changes that occurred in the population dynamics of zooxanthellae in *P. damicornis* when exposed to elevated concentrations of $(NH_4)_2SO_4$ (as part of the U.S.–Israel 1991 HIMB workshop). To explore the possible transitory effect of increased ammonium availability, observations of the population dynamics of symbiotic zooxanthellae were made at both short (days) and long (weeks) time scales.

MATERIALS AND METHODS

Diel Patterns of Division by Symbiotic Zooxanthellae in Pocillopora damicornis

The diel patterns of cell division of symbiotic zooxanthellae in *P. damicornis* were investigated by sampling coral colonies incubated for 8 weeks in four different concentrations of $(NH_4)_2SO_4$ in seawater (three coral colonies per treatment [see Stambler et al. (1994) for details of maintenance of corals during the experiment]) every 3 hr for 48 hr. Small branch tips (≤ 1.5 cm) were broken off the coral colonies during each sampling time and were placed in 10% formalin in seawater. After fixing the tissues in this solution for at least 5 hr, the branch tips were decalcified in 4% nitric acid (in seawater) as described by Stimson (1990). The decalcified tissue was then rinsed in seawater and homogenized using a glass-glass homogenizer in a small volume of seawater (0.5 ml). The number of dividing cells in 1000 cells was counted using a hemacytometer (Bright-line, American Optical Corp.). Mitotic index was calculated by expressing the number of dividing cells as a percentage of the total number of cells inspected.

The mitotic index of symbiotic zooxanthellae in *P. damicornis* was also examined as a function of exposure time to elevated concentrations of ammonium. Three colonies of *P. damicornis* that had been exposed to ammonium for 0 (collected within 24 hr [=freshly collected]), 2, 4, 6, and 8 weeks were sampled at 0230 hr (time of peak division), and the mitotic index was measured, as described above. In a separate set of experiments, three colonies were moved between treatments to investigate the short-term influences of changes in concentrations of ammonium. Three different treatments were used as follows: corals were moved from the same treatment and back again, or they were moved from high to low concentrations or from low to high concentrations.

Calculation of Specific Growth Rates (μ_z), Duration of Division (t_d), and Changes in the Carbon and Nitrogen Pools of Symbiotic Zooxanthellae in Pocillopora damicornis

The specific growth rate of the zooxanthellae (μ_z) in *P. damicornis* was calculated using formulas for phased and unphased division of populations of zooxanthellae as described by Wilkerson et al. (1983). The duration of

division (t_d) was also calculated using the method outlined by Wilkerson et al. (1983) for those populations of zooxanthellae exhibiting phased cell division. Changes in the standing stock of carbon and nitrogen within the symbiotic zooxanthellae during nutrient enrichment were calculated by multiplying μ_z by the population density and the carbon (C) or nitrogen (N) content of the zooxanthellae (Muller-Parker et al. 1994).

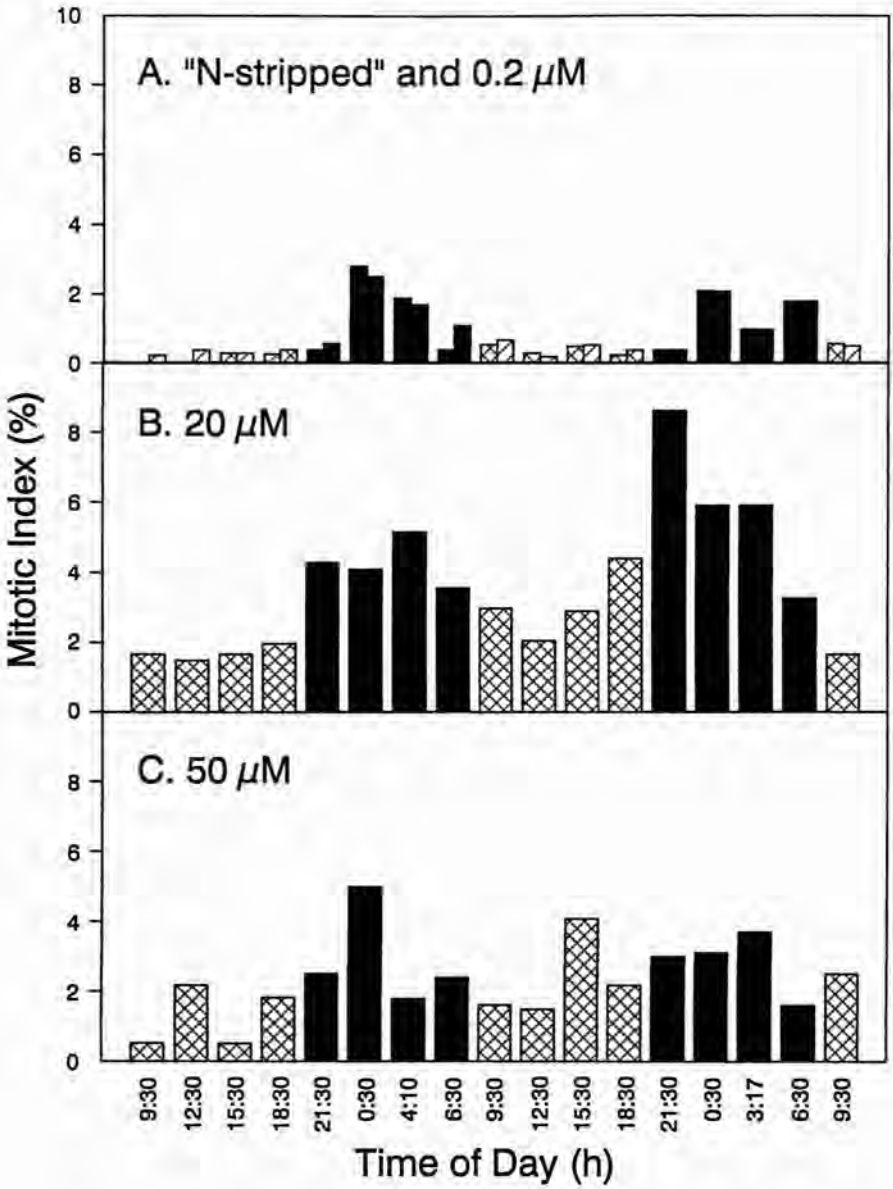

FIGURE 1. Mitotic index (percentage total zooxanthellae dividing) as a function of time of day for colonies of the coral *Pocillopora damicornis* exposed to: *A*, N-stripped (control) and ambient (0.2 μM); *B*, 20 μM; and *C*, 50 μM ammonium. Black bars indicate hours of darkness (night). No difference was detected between sampling times ($n = 3$). The pooled standard error of the mean averaged 8.1% of the mean.

RESULTS

Diel Pattern and the Duration of Division (t_d) of Symbiotic Zooxanthellae

Symbiotic zooxanthellae in *P. damicornis* given low levels of dissolved ammonium ("N-stripped" and 0.2 μM, ambient [measured concentrations given in Stambler et al. (1994)]) showed a diel pattern of division that was phased and had maxima that occurred between 0000 hr (midnight) and 0600 hr (Figure 1*A*). Maximum mitotic indices (f_{max}) were 2.81 and 2.50% on day 1 (first 24 hr of measurement) and were 2.10% on day 2 (second 24 hr) in both cases (Table 1). Mitotic indices at times outside the period of peak division activity were low ($<0.5\%$). Division was phased in *P. damicornis* incubated in 20 μM ammonium, although the number of dividing cells found between peak periods (Figure 1*B*) was approximately five-fold greater than that seen among zooxanthellae in N-stripped or 0.2-μM treatments. Maximum division rates were also two to three times greater (Figure 1*A* and *B*, Table 1). When corals were treated with 50 μM ammonium, division rates were not phased, although the highest value for mitotic index was recorded at 0030 hr (Figure 1*C*), which is the beginning of the period of maximal division for zooxanthellae in *P. damicornis* when division was phased in other treatment conditions.

The mitotic index of symbiotic zooxanthellae in *P. damicornis* was also examined as a function of exposure to surplus ammonium (Figure 2). Zooxanthellae in *P. damicornis* receiving 0.2, 20, and 50 μM ammonium for 2 weeks had higher mitotic indices than those of freshly collected *P. damicornis*. Although the mitotic index of zooxanthellae in the 0.2-μM treatments did not vary with exposure ranging from 2 to 8 weeks, the mitotic index of zooxanthellae in *P. damicornis* receiving 20 and 50 μM ammonium increased as the length of their exposure to ammonium increased (Figure 2).

The duration of division (t_d) was calculated for those populations of zooxanthellae exhibiting phased cell division (Table 2). The time of division was comparable between treatments, although it was higher in treatments receiving 20-μM concentrations of ammonium (0.34, 0.39, and 0.66 day, for N-stripped, 0.2- and 20-μM treatments, respectively). The overall mean (for all treatments pooled on each day) was 0.47 and 0.46 days for the first and second day, respectively.

The Specific Growth Rate (μ_z) and the Growth Rate of Carbon and Nitrogen Pools in Symbiotic Zooxanthellae

The μ_z of zooxanthellae within the tissues of *P. damicornis* incubated in N-stripped or 0.2-μM ammonium seawater ranged between 0.021 and 0.028 day^{-1} (Table 1). Zooxanthellae within *P. damicornis* incubated in 20 and 50 μM ammonium had mitotic indices that ranged between 0.042 and 0.072 day^{-1}. When

TABLE 1

SPECIFIC GROWTH RATES OF ZOOXANTHELLAE (μ_z) IN *Pocillopora damicornis* 8 WEEKS AFTER THE BEGINNING OF NUTRIENT ENRICHMENT WITH AMMONIUM

AMMONIUM (μM)	MITOTIC INDEX (%)	μ_z (day^{-1})	C (μmol cm^{-2} day^{-1})	N (μmol cm^{-2} day^{-1})
0	2.81, 2.10	0.028, 0.021	1.32, 0.99	0.10, 0.08
0.2	2.50, 2.10	0.025, 0.021	0.89, 0.74	0.05, 0.04
20	4.29, 4.17	0.042, 0.057	3.09, 4.19	0.30, 0.40
50	2.09, 2.58	0.045, 0.055	1.55, 1.89	0.17, 0.21

NOTE: Rates were calculated using the equations described by Wilkerson et al. (1983) for phased (0, 0.2-, and 20-μM treatments; $t_d = 0.46$ days) and unphased cell division (50 μM). Values for mitotic index were taken from data set shown in Figure 1. The mean of two highest mitotic indices were averaged for each day for the treatments 0, 0.2, and 20 μM, whereas the overall mean mitotic index of each day was used for calculating μ_z in the 50-μM treatment. The first value of each pair represents data collected during the first 24 hr; the second value represents data collected in the second 24-hr period.

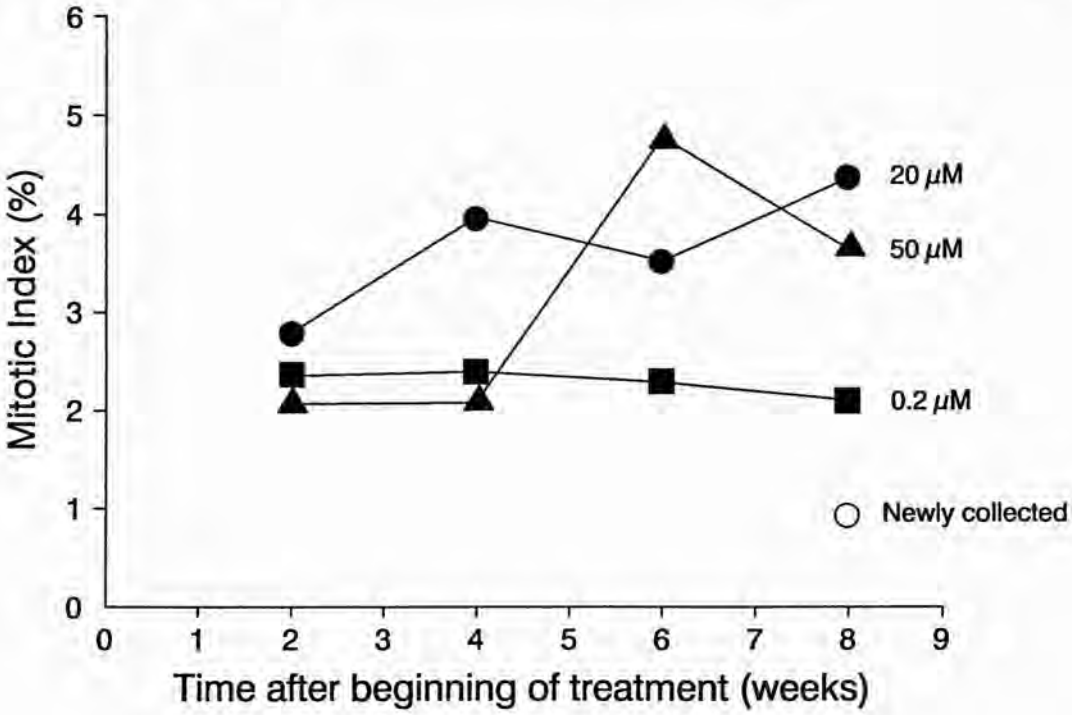

FIGURE 2. Mitotic index (percentage total zooxanthellae dividing) as a function of length of time that host corals (*Pocillopora damicornis*) were exposed to three different concentrations of ammonium in seawater. Concentrations are indicated at the end of each trajectory. Each point represents the mean of two measurements from different corals.

TABLE 2

DURATION OF DIVISION (t_d) OF SYMBIOTIC ZOOXANTHELLAE IN *Pocillopora damicornis* EXPOSED TO ELEVATED CONCENTRATIONS OF AMMONIUM

	CONCENTRATION OF AMMONIUM (μM)			
SPECIFIC GROWTH RATES (day^{-1})	0	0.2	20	50
Using equation 1:				
Day 1 (using f_{max})	0.028	0.025	0.042	N/A
Day 2 (using f_{max})	0.021	0.021	0.057	N/A
Using equation 2:	0.017	0.019	0.072	0.050
Duration of division (t_d):				
Day 1	0.29	0.35	0.78	N/A
Day 2	0.38	0.42	0.57	N/A

NOTE: Calculation is based on method outlined by Wilkerson et al. (1993, equations 1 and 2 indicated below). Refer to Table 1 for explanation of day 1 and day 2.

corals were transferred from one treatment to another, 24 hr before sampling, μ_z changed only when corals incubated in 20 μM were transferred to 0.2 μM (Table 3, simple t test, $P \leq 0.05$). These data indicate that the response time of μ_z to increased availability of ammonium is generally greater than 24 hr. The data also suggest that responses in μ_z

TABLE 3

SHORT-TERM EFFECT OF ELEVATED AMMONIUM

AMMONIUM (μM)	μ_z (day^{-1}) MEAN	SEM	95% confidence interval Min.	Max.
0.2 ⇒ 20	0.025	0.004	0.007	0.043
0.2 ⇒ 50	0.022	0.001	0.016	0.028
0.2 ⇒ 0.2	0.021	0.003	0.010	0.032
20 ⇒ 0.2	0.035*	0.003	0.023	0.046
20 ⇒ 20	0.061	0.002	0.054	0.069
50 ⇒ 0.2	0.023	0.001	0.020	0.026
50 ⇒ 50	0.030	0.004	0.013	0.046

NOTE: Specific growth rates (μ_z) calculated from mitotic indices of zooxanthellae populations in colonies of *Pocillopora damicornis* ($n = 3$) transferred to other treatment conditions. Samples were taken from the colonies at 0230 hr, 24 hr after transfer. Arrows indicate direction of transfer. Shown are means, standard errors of the mean (SEM), and 95% confidence intervals. Asterisk (*) indicates treatments where transfer resulted in a significant change ($P < 0.05$) relative to controls within each group.

may show hysteresis with respect to sudden increases versus decreases in the availability of ammonium.

The specific growth rate of zooxanthellae (μ_z) in *P. damicornis* was converted into a measure of the rate of growth of the organic carbon and nitrogen pools within the zooxanthellae, using other data collected during the study (Muller-Parker et al. 1994). Zooxanthellae isolated from corals incubated in relatively low concentrations of ammonium (≤ 0.2 μM) had organic carbon and nitrogen pools that grew at a rate of 0.99 μmol-C cm^{-2} day^{-1} (± 0.213 SD, $n = 4$, N-stripped and 0.2-μM treatments pooled) and 0.07 μmol-N cm^{-2} day^{-1} (± 0.024 SD, $n = 4$, Table 1), respectively, whereas zooxanthellae from corals incubated in 20 μM ammonium had organic carbon and nitrogen pools that grew at a rate of 3.64 μmol-C cm^{-2} day^{-1} (mean, $n = 2$) and 0.35 μmol-N cm^{-2} day^{-1} (mean, $n = 2$, Table 1), respectively. Growth of the carbon and nitrogen pools of zooxanthellae isolated from corals incubated in 50 μM ammonium were lower than those in corals incubated with 20 μM ammonium and were 1.72 μmol-C cm^{-2} day^{-1} (mean, $n = 2$) and 0.19 μmol-N cm^{-2} day^{-1} (mean, $n = 2$, Table 1), respectively.

DISCUSSION

Ammonium ions [$(NH_4)_2SO_4$], when supplied at concentrations of 20 μM and above, have a marked effect on the population dynamics of symbiotic zooxanthellae growing within the tissues of *P. damicornis*. The stimulatory effect of ammonium on the growth rate of zooxanthellae in *P. damicornis* is reflected ultimately in an increased density of zooxanthellae within the tissues of *P. damicornis* (Muller-Parker et al. 1994). One possible interpretation of these data is that the low growth rates of zooxanthellae in symbioses involving reef-building corals are a function of the restricted availability of ammonium (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989). The validity of this idea is at the heart of the aims of the joint U.S.–Israel 1991 HIMB workshop.

Is Phased Cell Division and Low μ_z Characteristic of Populations of Symbiotic Zooxanthellae Found within Reef-building Corals?

The cell division of symbiotic zooxanthellae within the tissues of *P. damicornis* is phased, with a peak in the percentage of dividing cells (mitotic index) occurring between 0000 and 0600 hr. The cell division of zooxanthellae in the reef-building corals *Seriatopora hystrix* Dana (Høegh-Guldberg and Smith 1989), *Stylophora pistillata* Esper, *Fungia repanda*, and *P. damicornis* (Smith and Høegh-Guldberg 1987) from Lizard Island (northern sector of the Great Barrier Reef) was also phased. It is interesting that zooxanthellae in nine species of reef-building corals investigated by Wilkerson et al. (1988) in Discovery Bay, Jamaica, did not have phased cell division. Similar data were reported for *S. pistillata* from the Red Sea (Wilkerson et al. 1983). The exact reason for this difference between these studies is not clear. However, if elevated ammonium (50 μM) can lead to the

disappearance of phased division from populations of zooxanthellae in *P. damicornis* (as suggested here), then differences in how specimens are handled (e.g., aquarium ammonium concentration) may have important influences on the diel pattern of cell division measured for populations of symbiotic zooxanthellae. Until this peculiar difference is explored further, it is not possible to generalize that the division of symbiotic zooxanthellae is phased under normal conditions.

The specific growth rates (μ_z) of zooxanthellae found in reef-building corals are low relative to the μ_z exhibited by zooxanthellae under optimal growth conditions. Values of μ_z calculated from the mitotic indices measured in the study reported here ranged between 0.021 and 0.057 day^{-1} (calculated using equation 1 of Wilkerson et al. [1983]). Zooxanthellae from two other pocilloporid corals also had similar specific growth rates (*Seriatopora hystrix*: 0.040 to 0.082 day^{-1}; *Stylophora pistillata*: 0.028 to 0.032 day^{-1} [Høegh-Guldberg and Smith 1989]). These values are also similar to those reported for *S. pistillata* growing in the Red Sea (range: 0.013 to 0.094 day^{-1} [Wilkerson et al. 1983, Muscatine et al. 1985]). To date, the majority of specific growth rates determined for symbiotic zooxanthellae in reef-building corals fall into this range (Wilkerson et al. 1988). Given the potential for zooxanthellae to grow faster under more optimal conditions (e.g., at low population densities: up to 0.40 day^{-1} [Høegh-Guldberg and Hinde 1986]) or when cultured (up to 0.43 day^{-1} [Chang et al. 1983, Fitt and Trench 1983]), the μ_z characteristic of symbiotic zooxanthellae in reef-building corals is still relatively low under normal or low concentrations of ammonium.

Estimates of the rate of growth of organic carbon (C) and nitrogen (N) pools within the symbiotic populations of zooxanthellae were calculated using μ_z, the population density, and the amount of C and N per zooxanthella. Rates of growth of organic C (0.99 ± 0.213 μmol-C cm^{-2} day^{-1}) and N (0.07 ± 0.024 μmol-N cm^{-2} day^{-1}) were comparable with rates reported for zooxanthellae residing in the coral *Stylophora pistillata* (1.36 ± 0.686 μmol-C cm^{-2} day^{-1} and 0.17 ± 0.027 μmol-N cm^{-2} day^{-1} [data calculated using Tables 1 and 2 of Muscatine et al. (1989)]).

Are the Population Dynamics of Symbiotic Zooxanthellae in Pocillopora damicornis *Influenced by Elevated Concentrations of Ammonium* [$(NH_4)_2SO_4$]?

Ambient levels of ammonium (0.2 μM) had no detectable effect (relative to N-stripped seawater) on the growth dynamics of zooxanthellae growing within *P. damicornis*. At concentrations of 20 μM or greater, the zooxanthellae within *P. damicornis* showed increased maximum rates of division as well as a greater number of zooxanthellae dividing out of phase. When corals were exposed to 50 μM, the response of the zooxanthellae was complicated by a loss of division synchrony (phase) as well as a reduction (relative to the 20-μM treatment) in the number of zooxanthellae entering division at any one time. In both cases, however, the calculated specific growth rates were two to three times higher when compared with those calculated for the treatments receiving ammonium at concentrations of 0.2 μM or less. These data suggest that elevated ammonium can influence the population dynamics of symbiotic zooxanthellae in reef-building corals.

The relative importance of the availability of ammonium in determining the growth rate of zooxanthellae in *P. damicornis* can be assessed by comparing the maximal specific growth rates of zooxanthellae achieved in this study with the maximum growth rate attained by symbiotic zooxanthellae under more optimal growth conditions. Despite elevated concentrations of ammonium (20 μM) in the water surrounding the corals in this study, the maximum μ_z in this study was $<20\%$ of that when zooxanthellae are growing at very low population densities (0.40 day^{-1} [Høegh-Guldberg and Hinde 1986]) or when cultured (0.43 day^{-1} [Chang et al. 1983, Fitt and Trench 1983]). At a higher concentration (50 μM), the growth rate in this study was reduced relative to growth rates in 20 μM, and ammonium ions apparently have

a toxic effect. These data strongly suggest that ammonium is not the sole factor limiting the growth of zooxanthellae in hospice.

The direct influence of elevated ammonium on the specific growth rate of zooxanthellae (μ_z) within reef-building corals has not been reported before, although two studies (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989) specifically measured μ_z of symbiotic zooxanthellae in the presence of elevated NH_4Cl. Those authors explained the unanticipated result of "no effect" as a function of the transient and short-lived effect of NH_4Cl on symbiotic zooxanthellae (as demonstrated by Fitt [1988] and Cook et al. [1988] for zooxanthellae in the hydrozoan *Myrionema amboinense* and the sea anemone *Aiptasia pallida* [Verrill]). This possibility does not appear to be supported here. The effect of ammonium on the mitotic index of zooxanthellae in *P. damicornis* was long-lived and was still present in populations that had been receiving ammonium for up to 8 weeks. A possible explanation of this discrepancy is that previous studies took single time-point samples based on the assumption that symbiotic zooxanthellae in low and high NH_4Cl treatments had similar diel patterns of cell division. Changes in the number of cells dividing out of phase (as seen in the study reported here), however, would lead to both under- and overestimated values of μ_z and a generally obscured picture of how μ_z responds to the increased availability of ammonium.

Stimulation of the specific growth rate of the zooxanthellae in corals exposed to 20 μM ammonium also translated into increased rates of growth of the N and C pools within the zooxanthellae. Specifically, rates of N and C pool growth were three to five times higher than those seen in corals exposed to ≤ 0.2 μM ammonium. Rates of N and C growth were lower (by a factor of 2) for zooxanthellae exposed to 50 μM ammonium (when compared with the 20-μM treatment), which corroborates other data (loss of division synchrony, and reduced N and C per cell in the 50-μM treatment [Muller-Parker et al. 1994]) that suggest that corals exposed to 50 μM are affected negatively by exposure to concentrations of ammonium in the vicinity of 50 μM.

Are Zooxanthellae in Reef-building Corals Limited by the Availability of Ammonium?

The general response of the population dynamics of zooxanthellae in *P. damicornis* reported here (increases in μ_z and the appearance of cells dividing out of phase) does not itself constitute proof that symbiotic zooxanthellae are limited by the supply of inorganic nitrogen. Most of the organic nitrogen synthesized by symbiotic zooxanthellae is probably translocated to the host (Muscatine and Cernichiari 1969, Trench 1979) and, therefore, by having been removed from the zooxanthella pools, is not actually accounted for by the observed changes in the growth rate of the zooxanthellae. Translocation also represents a confounding factor in the use of population dynamics to assess whether or not symbiotic populations are nitrogen-limited. For example, an equally tenable explanation for the observed increase in μ_z under elevated ammonium conditions is that translocation decreased in response to the toxic effects of ammonium on host metabolism, thereby leading to a greater retention of organic carbon for the growth of the zooxanthellae.

The resolution of the question as to whether symbiotic zooxanthellae are nitrogen-limited lies in the careful measurement of changes to the total size of the inorganic nitrogen pool of zooxanthellae in reef-building corals when exposed to elevated ammonium. The demonstration, for example, that ammonium uptake by reef-building corals is not saturated under ambient conditions would constitute confirmation of the proposal that the zooxanthellae are limited by the availability of ammonium under ambient conditions. This proposal is in fact supported by the observed increases in the total protein of corals in this study when exposed to elevated inorganic nitrogen (Muller-Parker et al. [1994]). This latter observation suggests that the growth of zooxanthellae is limited by the availability of inorganic nitrogen and that the observed increases in the frequency of dividing cells were due to bona fide increases in the

growth rate of zooxanthellae in response to the addition of inorganic nitrogen. This final point further highlights the importance of studies focusing on growth rates and nitrogen incorporation rates measured as a function of the entire symbiotic association for resolving the questions as to whether or not symbiotic zooxanthellae are limited by the availability of inorganic nitrogen under normal field conditions.

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Effect of Ammonium Enrichment on Animal and Algal Biomass of the Coral *Pocillopora damicornis*[1]

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ABSTRACT: Algal and animal biomass parameters of colonies of the Pacific coral *Pocillopora damicornis* (Linnaeus) were measured as a function of time of exposure to elevated concentrations of seawater ammonium (20 and 50 μM $[(NH_4)_2SO_4]$) ranging from 2 to 8 weeks. Areal concentrations of zooxanthellae, chlorophyll, and protein increased with 20 μM ammonium addition. During the 8-week period of exposure to 20 μM ammonium, the population density of zooxanthellae increased from 3.5 to 7.5 $\times$ 10^5 cells cm^{-2}, chlorophyll *a* content of zooxanthellae increased from 5.7 to 8.6 pg, and animal protein concentration doubled (from 0.74 to 1.38 mg cm^{-2}). These data indicate that both the coral animal and the zooxanthellae respond to the addition of exogenous dissolved inorganic nitrogen provided as 20 μM ammonium. Growth of the symbiotic association in response to the addition of 20 μM ammonium adds further evidence to support the argument that growth of tropical symbioses is limited by the availability of nitrogen. However, the coral response is likely to depend on the concentration of ammonium provided, because the biomass parameters of corals held at 50 μM ammonium did not change significantly with time of exposure to the added nutrient.

SYMBIOTIC DINOFLAGELLATES (zooxanthellae) are found at high population densities in reef-building corals and other cnidarians living in the low nutrient concentrations characteristic of tropical seawater (Muscatine 1980). The abundance of these algal symbionts in their hosts, despite the external oligotrophic conditions, is attributed to conservation of nutrients. Nutrients acquired from ambient seawater and from metabolites resulting from host digestion are conserved via exchanges between the animal and zooxanthellae (Muscatine and Porter 1977, Rahav et al. 1989, Szmant et al. 1990). In spite of the inferred advantage of an abundant supply of nutrients for zooxanthellae in animal hosts, the actual nutrient status of the zooxanthellae is unknown. One approach to resolving the question of whether or not zooxanthellae are limited by the supply of nutrients is to experimentally manipulate the two sources of nutrients and to observe the response of the symbiotic partners. Nutrients are withheld from the symbiosis by maintaining the host in low-nutrient seawater for long periods of time without feeding (e.g., Cook et al. 1988). Nutrients are added to the symbiosis by intensive feeding or maintenance of the host in seawater enriched with dissolved inorganic nutrients. A positive growth response of the zooxanthellae to an added nutrient supports the hypothesis that growth of zooxanthellae may be limited by the supply of that nutrient under ambient seawater conditions (Cook

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and D'Elia 1987; but see Høegh-Guldberg [1994]).

Several studies have demonstrated that zooxanthellae population densities in corals increase with the addition of dissolved inorganic nitrogen supplied as ammonium to seawater flowing over the corals. Muscatine et al. (1989) found that 20 μM ammonium caused zooxanthellae population densities in *Stylophora pistillata* Esper to double after 2 weeks of exposure, but phosphate and particulate food (brine shrimp) additions had no effect on algal populations. Similar results were obtained with ammonium in *S. pistillata* and *Seriatopora hystrix* Dana in Australia by Høegh-Guldberg and Smith (1989) and in the coral *Pocillopora damicornis* (Linnaeus) in Hawaii (Stambler et al. 1991, Stimson and Kinzie 1991). Natural additions of nutrients, from excretion and defecation by haemulid fishes over coral colonies, also resulted in increased numbers of zooxanthellae within the tissues of Jamaican corals (Meyer and Schultz 1985).

Although an increase in zooxanthellae population density with the addition of ammonium has been well documented for *S. pistillata* and *P. damicornis*, the time course of the response and the long-term effects of sustained high inorganic nitrogen levels on the coral are unknown. This study describes the biomass parameters (animal and zooxanthellae) of *P. damicornis* exposed to 20 and 50 μM ammonium for periods ranging from 2 to 8 weeks. Corals maintained in ambient seawater were used as a basis for comparison (see Stambler et al. 1994). We measured the areal concentrations of zooxanthellae, chlorophyll, and animal protein as a function of length of exposure to ammonium-enriched seawater. Companion papers describe the changes in the elemental (C, N, and P) composition of *P. damicornis* under the various treatments (Muller-Parker et al. 1994) and in amino acid levels of the zooxanthellae (McAuley 1994).

MATERIALS AND METHODS

The collection and maintenance of *Pocillopora damicornis* under the different ammonium and seawater treatments are described by Stambler et al. (1994). Colonies from the various treatments were processed immediately for animal and zooxanthellae biomass parameters, and for the isolation of zooxanthellae for use by other investigators (see other papers in this issue). The number of colonies from each treatment that were processed ranged from eight for the ambient seawater controls to two for all ammonium treatments except the 6-week 50-μM ammonium treatment and the field sample, where only one colony of each was processed. The field colony was collected from the windward reef flat of Coconut Island, Kaneohe Bay, on 27 August 1991 and processed that same day.

Preparation of Animal and Zooxanthellae Fractions

Each coral colony was processed as shown in Figure 1. One branch of each colony was used for determination of biomass parameters; the rest of the colony was used to obtain zooxanthellae. Tissue was removed from coral skeletons using filtered (0.45-μm) seawater (FSW) and a Water Pik (Johannes and Wiebe 1970). The volume of the final homogenate solution was measured and sampled for hemacytometer counts of zooxanthellae. Homogenate samples were also frozen for biochemical and elemental analyses. The remaining homogenate was then separated into supernatant (soluble, or "animal fraction") and pellet (zooxanthellae) fractions by centrifugation at ca. 7000 rpm for 3–4 min. Zooxanthellae pellets were rinsed several times with FSW to remove any remaining animal tissue. The supernatants of pellet rinses were combined with the animal fraction. To remove animal particulate debris and skeletal $CaCO_3$ fragments, the zooxanthellae were then resuspended in FSW and passed sequentially through 73-μm and 20-μm Nitex screens held in a syringe filter apparatus (Gelman). Zooxanthellae numbers in the final suspensions were determined by hemacytometer counts. Samples were prepared from animal and zooxanthellae fractions and kept frozen until analyzed. The volume of each fraction, and that of the homogenate,

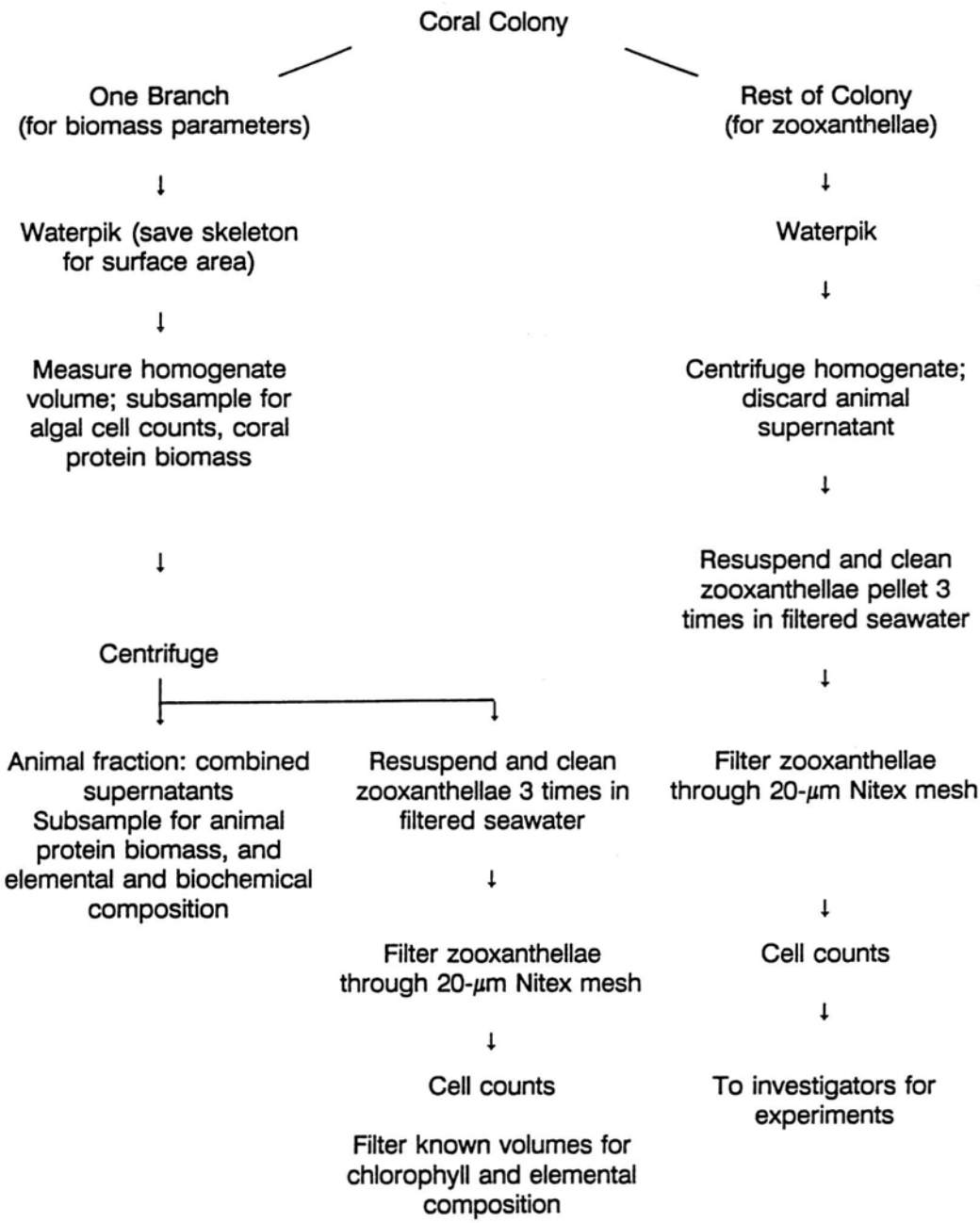

FIGURE 1. Summary of analytical procedures used to obtain animal and zooxanthellae biomass parameters of *Pocillopora damicornis*.

was related to the surface area of individual colonies.

Protein, Chlorophyll, and Coral Surface Area

Protein content of the homogenate and the animal fraction of each coral colony was determined by the method of Lowry (Lowry et al. 1951), using bovine serum albumin (BSA) as a standard. Samples were solubilized in 0.05 N NaOH at 40°C for 0.5 hr before proceeding with the protein analysis. After color development, samples were centrifuged to remove precipitate formed by the interaction of reagents with seawater, and the absorbance of the samples was read at 660 nm in a spectrophotometer (P-E Lambda).

For chlorophyll determinations, known numbers of zooxanthellae were filtered under vacuum (<250 mm Hg) onto 25-mm GF/C filters and stored frozen until analysis within 1 week of sampling. Filters were ground in ice-cold 100% acetone using a motorized tissue grinder. Chlorophyll was extracted for 18 hr at 4°C. The absorbance of acetone extracts was measured at 630, 663, and 750 nm on a diode-array spectrophotometer (Hewlett-Packard) after centrifugation to pellet filter material. The absorbance at 750 nm was used to correct for any turbidity at 630 and 663 nm. Chlorophylls *a* and c_2 of zooxanthellae were determined using the spectrophotometric equations of Jeffrey and Humphrey (1975).

The surface area corresponding to the amount of tissue removed from each coral specimen was obtained with a leaf area measuring device (Li-Cor Model 3100). Areas were calibrated by using branches of corals whose surface area was determined by the aluminum foil method (Marsh 1970). The branches of the coral skeletons were broken into lengths of relatively straight segments small enough to pass through the conveyor belt of the surface area meter. Multiple trial runs on the same collection of fragments from one coral produced results that varied <4%.

For statistical purposes, corals maintained in ambient flowing seawater for 8 weeks were considered "controls" and were presumed to represent corals at the start of the ammonium experiment. The effects of ammonium addition on the biomass parameters of corals maintained under the two ammonium enrichments were examined by linear regression over time, using the correlation coefficient (r) to indicate significance of treatments.

RESULTS

There was a significant increase in the areal population density of zooxanthellae in colonies of *P. damicornis* exposed to 20 μM ammonium ($P < 0.01$; Figure 2*A*). The number of zooxanthellae per square centimeter doubled over the 8-week period; zooxanthellae increased from 3.5 to 6×10^5 cells cm^{-2} within the first 2 weeks of ammonium enrichment (Figure 2*A*). Zooxanthellae densities in *P. damicornis* maintained in "N-stripped" seawater averaged 4.5×10^5 cells cm^{-2} and were not significantly different from those of the ambient seawater control corals. Although the increased zooxanthellae density of ammonium-enriched corals relative to seawater controls is apparent, the zooxanthellae density of a freshly collected colony from the reef was close to that of the N-enriched corals (6.79×10^5 cells cm^{-2}).

Zooxanthellae density expressed on the basis of animal protein biomass did not increase with time of exposure to 20 μM ammonium (Figure 2*B*). Although increases in zooxanthellae numbers were observed for corals maintained at 50 μM ammonium at the 2-week time point, there was no significant effect of time on population density of zooxanthellae for the 50 μM ammonium corals (Figure 2*A*,*B*).

The areal animal protein content of the corals increased significantly ($P = 0.05$) with time of exposure to 20 μM ammonium (Figure 2*C*). Protein content of these corals almost doubled over the 8-week period, paralleling the increase in zooxanthellae numbers in these corals (Figure 2*A*) and accounting for the lack of change in zooxanthellae density normalized to animal protein (Figure 2*B*). The animal protein content of corals exposed to 20 μM ammonium was 0.96 mg

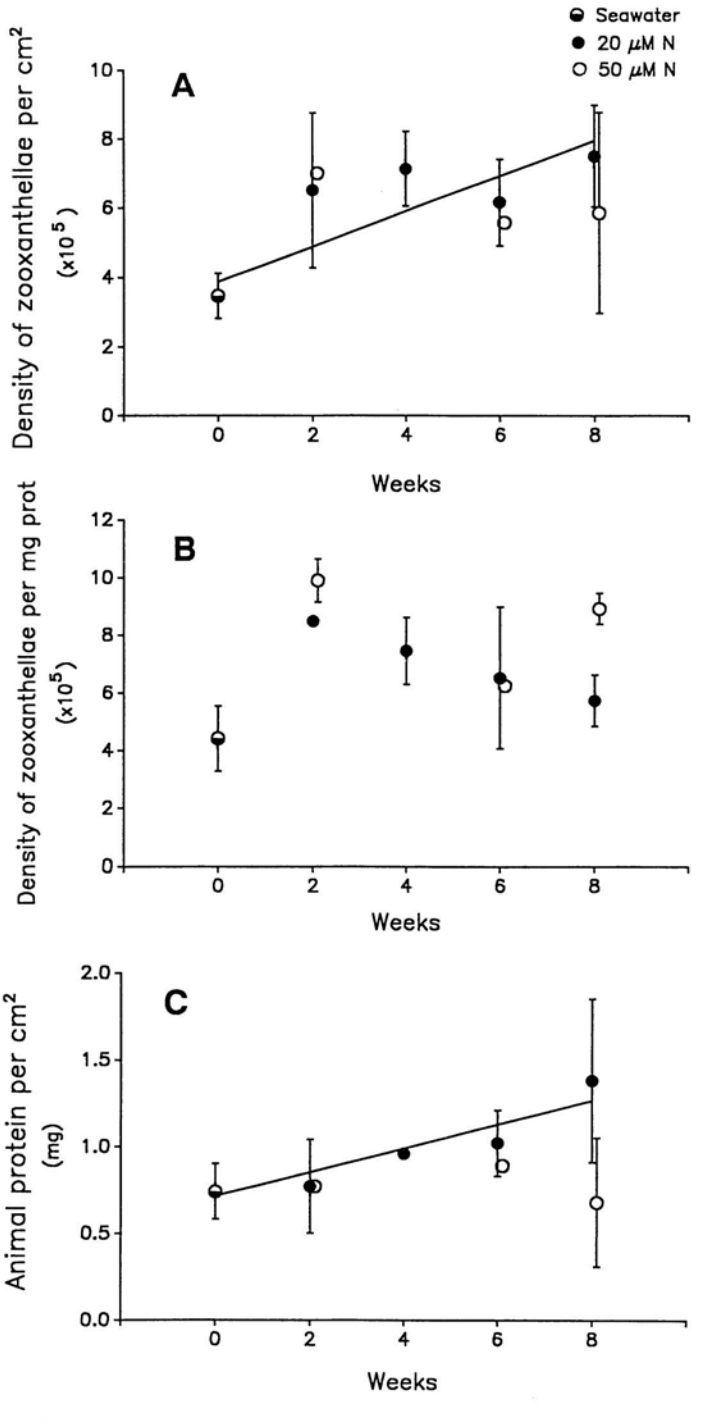

FIGURE 2. Density of zooxanthellae and animal protein in different colonies of *Pocillopora damicornis* as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater. Ambient seawater control colonies maintained under the same light and water flow conditions were used for the zero time point. Data are expressed as numbers of zooxanthellae normalized to coral surface area (*A*) and to protein biomass (*B*). Animal protein biomass per unit surface area is included in *C*. Error bars are ± 1 SE. The *x* axis for the 50 μM ammonium data is shifted slightly to the right for clarity. Regression lines are provided for significant effects observed with 20 μM ammonium (*A*: $r = 0.613$, $P < 0.01$; *C*: $r = 0.511$, $P = 0.05$).

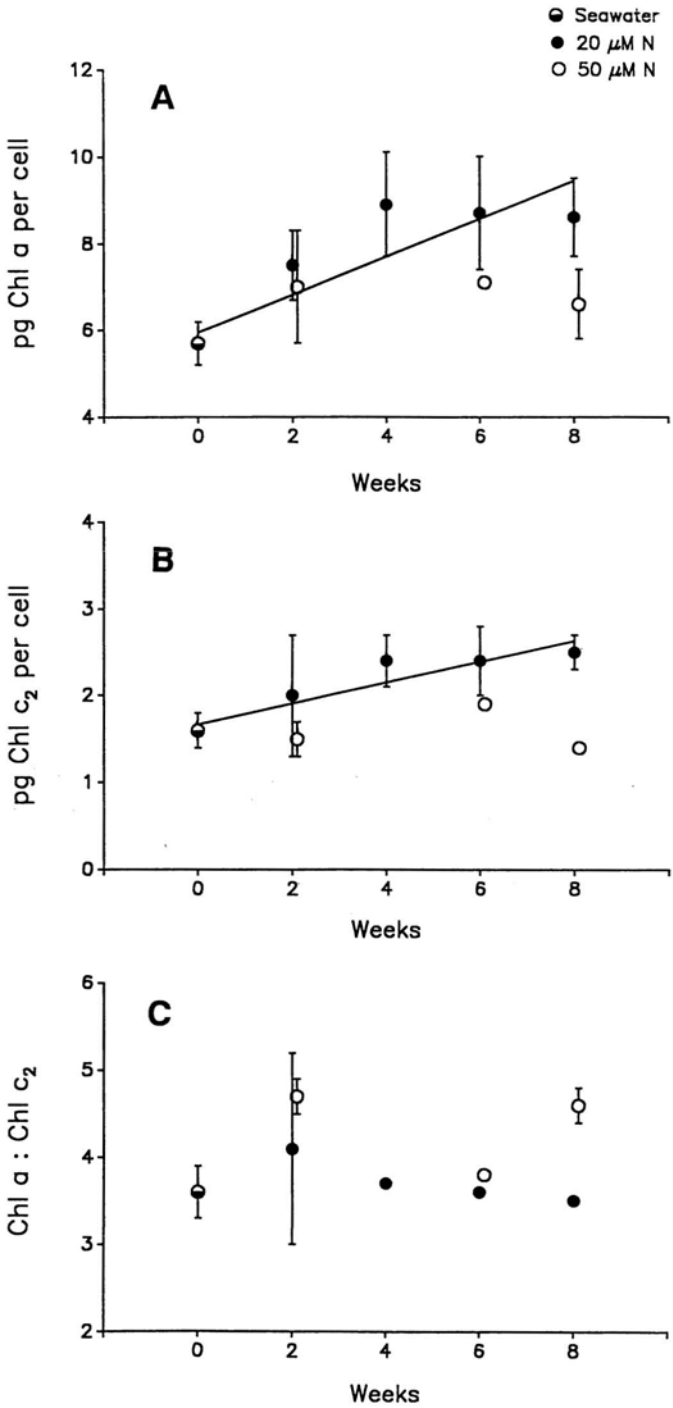

FIGURE 3. Chlorophyll of zooxanthellae isolated from different colonies of *Pocillopora damicornis* as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater: chlorophyll *a* cell^{-1} (*A*), chlorophyll c_2 cell^{-1} (*B*), and the ratio of chlorophyll *a*: c_2 (*C*). Ambient seawater control colonies maintained under the same light and water flow conditions were used for the zero time point. Error bars are ± 1 SE. The x axis for the 50-μM ammonium data is shifted slightly to the right for clarity. Regression lines are provided for significant effects observed with 20 μM ammonium (*A*: $r = 0.663$, $P < 0.01$; *B*: $r = 0.572$, $P < 0.05$).

cm^{-2} after 4 weeks and increased to 1.38 mg cm^{-2} by week 8; the animal protein content of corals exposed to 50 μM ammonium declined from 1.8 at 2 weeks to 0.68 mg cm^{-2} at 8 weeks (Figure 2*C*).

The relationship between density of zooxanthellae expressed on the basis of surface area and animal protein content was explored further by regressing these two parameters for the pooled coral colonies, including colonies maintained in ambient seawater, 20 μM ammonium, 50 μM ammonium, "N-stripped" seawater, and a field colony. The correlation between these two measures of algal density was significant ($r = 0.391$; $P = 0.048$), showing that there is generally good agreement between the two measures of algal population density.

The increase in numbers of zooxanthellae with 20 μM ammonium enrichment was accompanied by a significant increase in chlorophyll *a* and chlorophyll c_2 of zooxanthellae (Figure 3*A*,*B*). The chlorophyll *a* of zooxanthellae from corals maintained at 50 μM ammonium was consistently lower than that of zooxanthellae from the corals maintained at 20 μM ammonium (Figure 3*A*,*B*) and was not significantly different from that of the zooxanthellae in the seawater controls (Figure 3*A*). The chlorophyll *a* : c_2 ratio remained unchanged with exposure to 20 and 50 μM ammonium (Figure 3*C*) and averaged 3.7 for the corals maintained at 20 μM ammonium. The chlorophyll content of zooxanthellae isolated from the field colony (6.0 pg chlorophyll *a*, 1.6 pg chlorophyll c_2, 3.8 chlorophyll *a* : chlorophyll c_2) was identical to the mean values obtained for zooxanthellae from the corals in ambient seawater.

The combined effect of increased chlorophyll per cell and numbers of zooxanthellae on changes in the areal chlorophyll content of 20 μM ammonium-enriched corals is shown in Figure 4. The average amount of chlorophyll ($a + c_2$) per square centimeter was three times higher at 8 weeks (Figure 4*C*). There was no consistent increase in the areal distribution of chlorophyll with length of exposure to 50 μM ammonium (Figure 4). Because chlorophyll *a* per cell showed a significant positive correlation with algal density ($P = 0.038$; Figure 5), it was not possible to separate the effect of ammonium addition from self-shading due to increased zooxanthellae density.

DISCUSSION

The increase in the density of zooxanthellae in *P. damicornis* with ammonium enrichment was evident within the first 2 weeks after the initial addition of ammonium. The first few days after the start of ammonium addition seem not to have received adequate attention in coral studies; they may reveal significant information about nutrient-induced zooxanthellae population dynamics. Most studies have determined the effect of ammonium on the population density of zooxanthellae in corals after periods ranging from 13 days (Stambler et al. 1991) and 14 days (Muscatine et al. 1989) to 19 days (Høegh-Guldberg and Smith 1989). All of those studies reported increased densities of zooxanthellae with ammonium addition during periods that coincide with our first measurement of increased algal density in *P. damicornis* (Figure 2*A*).

This is the first study to examine the effect of time on the response of a coral to sustained elevated ammonium. Although the surface area–based density of zooxanthellae in corals maintained in 20 μM ammonium increased with time, it is important to distinguish between long-term responses (months to years) and short-term responses (days to weeks) to addition of ammonium. *P. damicornis* exposed to 17 μM ammonium for 2–4 months had algal densities in the branch tips that were three times those of branch tips in controls (Stimson and Kinzie 1991). Because that study was conducted during the winter season and algal densities are provided for tips (not whole colonies) and for one time point only, it is not possible to compare algal densities directly and infer that the differences in densities are related to the duration of exposure to high concentrations of ammonium. Future studies should concentrate on changes in biomass of corals during the first few days of exposure to nutrients, as well as

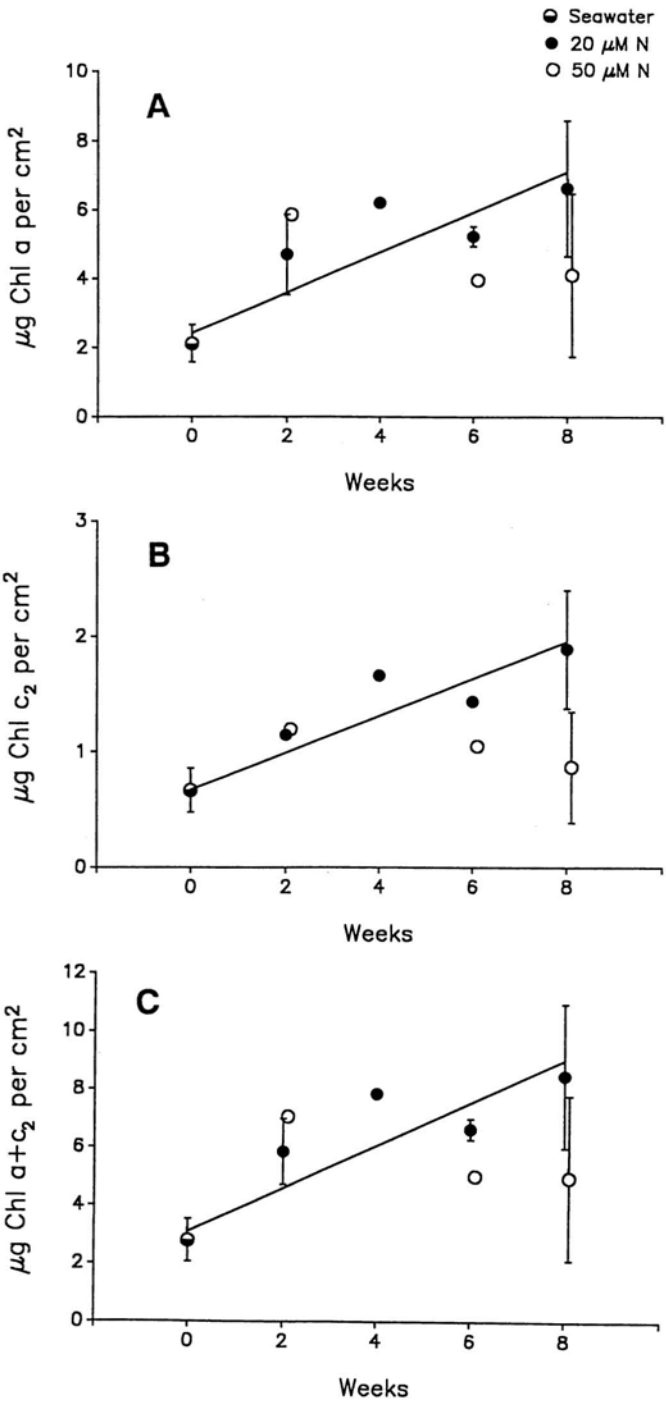

FIGURE 4. Areal chlorophyll content of *Pocillopora damicornis* colonies as a function of time of exposure to 20 μM and 50 μM ammonium additions in seawater: chlorophyll a/cm^2 (*A*), chlorophyll c_2/cm^2 (*B*), and total chlorophyll (a and c_2)/cm^2 (*C*). Ambient seawater control colonies maintained under the same light and water flow conditions were used for the zero time point. Error bars are ± 1 SE. The x axis for the 50-μM ammonium data is shifted slightly to the right for clarity. Regression lines are provided for significant effects observed with 20 μM ammonium (*A*: $r = 0.756$, $P < 0.001$; *B*: $r = 0.762$, $P < 0.001$; *C*: $r = 0.761$, $P < 0.001$).

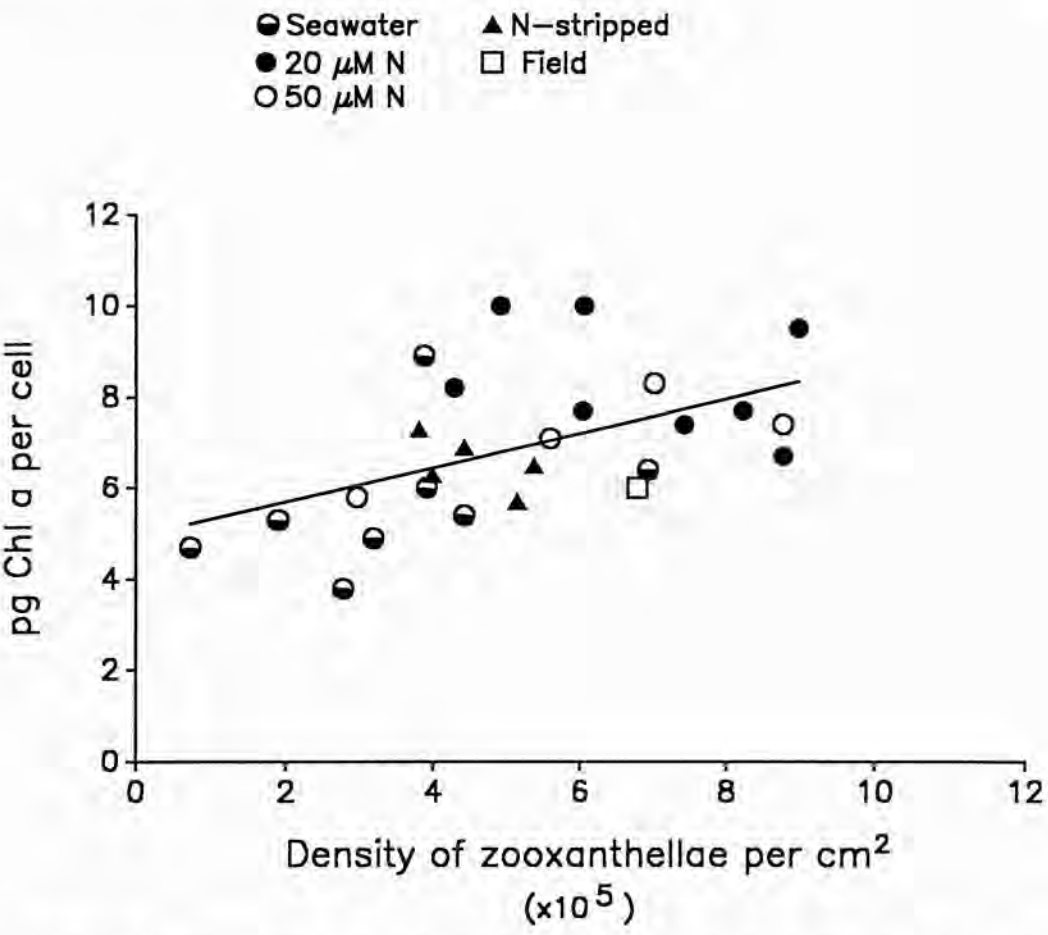

FIGURE 5. Chlorophyll *a* per zooxanthella versus population density of zooxanthellae in individual colonies of *Pocillopora damicornis* exposed to three experimental treatments: 20 μM ammonium, 50 μM ammonium, and "N-stripped" seawater, or maintained in ambient seawater. Data for all time points and a field-collected colony are included. The correlation between these two parameters was significant ($r = 0.40$; $P < 0.05$).

those obtained with corals subjected to high nutrient levels for periods exceeding 2 months (e.g., Stimson and Kinzie 1991).

Zooxanthellae density in the single freshly collected coral field colony was relatively high in comparison with densities in the ambient seawater control colonies. This difference may be related to the prolonged maintenance of the ambient seawater corals in shallow seawater tanks. However, the chlorophyll contents of zooxanthellae from the field colony were the same as those of zooxanthellae from the ambient seawater corals, indicating similar exposures to light and nutrients (see also Figure 5).

The increase in chlorophyll of zooxanthellae in *P. damicornis* exposed to 20 μM ammonium (Figure 3) may result from a combined response to ammonium and a photoadaptive response to increased self-shading of zooxanthellae at high population densities (Figure 2*A*), because there is a significant correlation between chlorophyll *a* per zooxanthella and density of zooxanthellae (Figure 5). Muscatine et al. (1989) did not obtain a significant increase in chlorophyll *a* per cell in *Stylo-*

phora pistillata with ammonium, in spite of increased density of zooxanthellae. Our results suggest that a significant increase in chlorophyll with ammonium enrichment of *S. pistillata* might have occurred in Muscatine et al.'s (1989) study if they had extended their experiment beyond 2 weeks. However, chlorophyll content of zooxanthellae may be independent of algal density under conditions where N supply limits photoadaptation (Dubinsky et al. 1990).

Addition of 20 μM ammonium caused a significant increase in animal protein biomass (Figure 2*C*). This suggests that either ammonium is directly assimilated into protein by the host animal or zooxanthellae are translocating a greater quantity of N-rich compounds. Support for the lack of direct incorporation of ammonium into animal protein is provided by Ferrier (1992), who found that intracellular free amino acid pools in the host tissue of both symbiotic and aposymbiotic anemones (*Aiptasia pallida* [Verrill]) maintained in 20 μM ammonium were not significantly different from those of anemones maintained in low-nutrient seawater. It would be worthwhile to determine the effect of ammonium enrichment on the quality and quantity of products translocated from the zooxanthellae to the animal. Our results clearly show that the addition of 20 μM ammonium caused a significant increase in animal protein with time, but that 50 μM ammonium had no effect. Single time point comparisons by Muscatine et al. (1989) and Achituv et al. (1994) showed that the protein of coral animal tissue does not vary significantly with ammonium addition.

Algal density based on animal protein biomass (Figure 2*B*) showed no significant effect of time, but density based on surface area increased significantly for the corals held in 20 μM ammonium-enriched seawater. This suggests that there are differences in the rate of animal tissue growth and skeletal extension in these corals. However, the significant correlation between zooxanthellae density based on animal protein and surface area for the pooled coral colonies shows that there is generally good agreement between the two methods of measuring population density of zooxanthellae.

No significant trends with time were observed for any biomass parameter of corals maintained exposed to 50 μM ammonium. In some cases, this may be attributed to the smaller sample size for this group of corals. However, it is clear that animal protein (Figure 2*C*), cell-specific chlorophyll (Figure 3), and areal distributions of chlorophyll (Figure 4) were reduced for the colonies in the 50-μM ammonium treatment. This suggests that 50 μM ammonium may be less stimulating than 20 μM ammonium to the growth of zooxanthellae or perhaps even stressful to the coral. The latter suggestion is supported by a decrease in the specific growth rate of zooxanthellae in corals exposed to 50 μM as compared with 20 μM ammonium (Høegh-Guldberg 1994).

The data show generally that the addition of 20 μM ammonium results in a time-course change in zooxanthellae and animal biomass of *P. damicornis*. The response of *P. damicornis* to the addition of inorganic nitrogen adds further evidence to the argument that growth of tropical symbioses, such as that between zooxanthellae and their coral hosts, is limited by the availability of inorganic nitrogen.

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We thank N. Stambler for maintaining the corals, and all of the workshop participants who helped with the biomass analyses. R. Bidwell and L. Faure were particularly instrumental in coordinating the coral biomass procedures; we could not have completed the project without their help. S. Coles helped with data collection, A. Verde with chlorophyll analyses, and M. D. Ferrier with protein analyses.

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Effect of Ammonium Enrichment on Respiration, Zooxanthellar Densities, and Pigment Concentrations in Two Species of Hawaiian Corals[1]

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ABSTRACT: Small branch tips or "nubbins" of two species of Hawaiian corals, *Pocillopora damicornis* (Linnaeus) and *Montipora verrucosa* Vaughan, were exposed to four ammonium concentrations, ammonium-stripped (<2 μM), ambient ($\approx$2 μM), and two enriched (20 μM and 50 μM) in microcosm tanks. Nubbins represent replicates of a single coral colony. We examined the effect of ammonium enrichment on zooxanthellar densities, pigment concentrations, and respiration rates of the nubbins. Nubbins of both *P. damicornis* and *M. verrucosa* showed a trend of increased pigment concentration with elevated ammonium concentration. *Pocillopora damicornis* increased from 9.3 μg chlorophyll *a* cm^{-2} in the ammonium-stripped treatment to 24.8 μg cm^{-2} in the 50-μM ammonium treatment. Similarly, *M. verrucosa* increased from 1.9 to 19.4 μg chlorophyll *a* cm^{-2}. There were no significant differences in algal densities, pigment concentrations per cell, pigment ratios, or respiration rates.

CORAL REEFS DEVELOP in oligotrophic waters where the mutualistic association between coral animal host and endocellular microalgae (the zooxanthellae) enables corals to thrive in the ambient low nutrient concentrations (Muscatine and Porter 1977, Falkowski et al. 1993). During the last decades, increasing areas of coral reefs have been exposed to anthropogenic eutrophication (Davies 1990, Scott 1990). Nutrient concentrations are known to have impacts on coral physiology (Høegh-Guldberg and Smith 1989, Muscatine et al. 1989, Rahav et al. 1989, Dubinsky et al. 1990, Stambler et al. 1991). The response of a coral to any environmental perturbation may be related to physiological differences among species and colony morphologies, including differences between perforate and imperforate colonies.

The family Pocilloporidae has been the focus of many studies on the effects of light and nutrients on coral physiology. The Hawaiian pocilloporid *Pocillopora damicornis* (Linnaeus) is a finely branched coral with an imperforate skeleton. *Montipora verrucosa* Vaughan (Family Acroporidae) occurs in two morphologies, one platelike and one branched (used in this study), and has a perforate skeleton (Jokiel 1978).

These corals have been demonstrated to show species-specific responses to water motion (Jokiel 1978), with *P. damicornis* growing best in moderate water motion and *M. verrucosa* showing the best growth in low water motion. These corals also show different responses to changes in light regime. The perforate species, *M. verrucosa*, responded to changes in light levels with changes in the density of algal cells; the imperforate species, *P. damicornis*, responded with a change in pigment concentration per algal cell (Kinzie et al. 1984).

Small branch tips or "nubbins" of corals can be used to investigate the intra- and intercolony variation in physiological parameters (Davies 1989, 1991). The use of nubbins

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allows replication while optimizing use of available tank space. The results of this study can be compared with values for whole-colony performance. The aim of this study was to examine the effect of ammonium enrichment on nubbins of *P. damicornis* and *M. verrucosa*.

MATERIALS AND METHODS

Nubbin Preparation

Colonies of *P. damicornis* and *M. verrucosa* were collected at 1–3 m depth from several locations within Kaneohe Bay, Hawaii. Large colonies (10–20 cm diameter) were brought to the laboratory in buckets of seawater and held temporarily in tanks with running seawater. Each colony was broken into several nubbins containing one to three branches from the parent colony. Nubbins were ca. 2–3 cm in length. Only healthy nubbins completely covered with live tissue were used in these experiments. To hold nubbins in a vertical orientation, nubbins were glued into 4-cm-long sections of PVC tubing (1.5 cm diameter) using underwater epoxy. Nubbins acclimated for ca. 2 weeks in tanks with running seawater. The sections of PVC tubing fit into holes drilled in wooden racks for placement in the experimental tanks.

Nutrient Experiment

The nutrient experiment was carried out in eight white fiberglass tanks with a water volume of ca. 400 liters (1.15 by 1.15 by 0.27 m). Tanks were supplied with unfiltered running seawater, at a rate of 4 liters min^{-1}. All tanks were aerated and exposed to 80% solar radiation.

Ammonium [$(NH_4)_2SO_4$] was added to four of these tanks at a rate of 1 ml min^{-1} (Stambler et al. 1994). Final ammonium concentrations in these tanks were 20 μM and 50 μM. In the ambient treatment tanks ammonium concentrations were the same as in Kaneohe Bay surface waters, ca. 2 μM.

In the last two tanks, seawater entered after running through a tank filled with the macroalga *Gracilaria salicornia* (C. Agardh) Dawson. These tanks represented the "stripped" nutrients treatment, with <2 μM ammonium in the incoming water.

Nine nubbins of *P. damicornis*, representing three different colonies, were placed in each of the four nutrient treatments. Nine nubbins of *M. verrucosa* were placed in each of the four nutrient treatments, but were kept in different tanks than the nubbins of *P. damicornis*. All nubbins remained in the tanks 21 days. From these nubbins, six from each treatment were used at the end of the experiment for pigment and tissue analyses.

Analytical Methods

At the end of the experiments, the following parameters were determined:

(1) Respiration rates of the nubbins. For each trial, three nubbins from a single parent colony were placed in a 2.5-liter respiration chamber at night. PVC holders had been cleaned of algal films before respiration measurements. Respiration chambers were placed in a tank 0.5 m deep with running seawater, maintaining the temperature in the chambers at $\approx$26°C. Water within the chambers was continually stirred with a magnetic spin bar. Oxygen concentrations were measured with an oxygen probe (Nestor). Incubations lasted 20–30 min, and the rate of oxygen depletion per unit of surface area was calculated for four to seven sets of nubbins.

(2) Density of the zooxanthellae within

TABLE I

EFFECT OF AMMONIUM CONCENTRATION ON RESPIRATION RATES (μmols O_2 min^{-1} cm^{-2}) OF NUBBINS (MEAN $\pm$ SD)

AMMONIUM TREATMENT	*Pocillopora damicornis*	*Montipora verrucosa*
Stripped	-0.034 ± 0.005 $n = 4$	-0.054 ± 0.008 $n = 5$
Ambient	-0.035 ± 0.007 $n = 4$	-0.024 ± 0.004 $n = 4$
20 μM	-0.037 ± 0.007 $n = 7$	-0.042 ± 0.011 $n = 4$
50 μM	-0.036 ± 0.010 $n = 7$	-0.027 ± 0.004 $n = 4$

TABLE 2

EFFECT OF AMMONIUM CONCENTRATION ON ALGAL DENSITIES (number of cells cm^{-2}) OF NUBBINS (MEAN ± SD)

AMMONIUM TREATMENT	*Pocillopora damicornis*	*Montipora verrucosa*
Stripped	$1.06 \times 10^6 \pm 2.75 \times 10^5$ $n = 3$	$1.23 \times 10^6 \pm 2.56 \times 10^5$ $n = 3$
Ambient	$1.90 \times 10^6 \pm 5.94 \times 10^5$ $n = 3$	$8.27 \times 10^5 \pm 3.88 \times 10^5$ $n = 3$
20 μM	$1.33 \times 10^6 \pm 2.13 \times 10^5$ $n = 3$	$6.69 \times 10^5 \pm 3.48 \times 10^5$ $n = 2$
50 μM	$2.08 \times 10^6 \pm 3.72 \times 10^5$ $n = 3$	$7.59 \times 10^5 \pm 1.23 \times 10^5$ $n = 2$

their host. Tissue of the nubbins was removed with a jet of water from a high-pressure unit (WaterPik) (Johannes and Wiebe 1970). The volume of the homogenate was determined, and zooxanthellae were counted using a hemacytometer.

(3) Colony surface area. Cleaned skeletons were dipped in warm paraffin wax (Stimson and Kinzie 1991), and the weight of the wax adhering to the skeleton was compared with a series of samples from blocks or plates of cleaned skeletons of known surface area.

(4) Concentration of pigments in the algae and the nubbins. Two methods of extraction of pigments were used. To determine pigments per algal cell, tissue was removed from nubbins, the homogenate was filtered on GFC filters, and the algal pigments were extracted in 90% acetone. To determine pigments per square centimeter, nubbins were placed in a closed beaker with 90% acetone overnight at 4°C. Chlorophyll *a* (chl *a*) and chlorophyll *c* (chl *c*) were measured using the spectrophotometric equations of Jeffrey and Humphrey (1975). Carotenoids were estimated using the method described by Parsons et al. (1984). Concentrations were normalized per zooxanthella and per unit surface area. The Waller-Duncan test (SAS Institute 1987) was then used to compare treatment means.

RESULTS

Nubbins of both species in the stripped, ambient, and 50-μM treatments seemed normal, but nubbins of *M. verrucosa* in the 20-μM treatment appeared to be bleaching. *Pocillopora damicornis* nubbins in the 20-μM treatment appeared normal.

Respiration rates (Table 1) for *M. verrucosa* nubbins in the four ammonium concentrations were highly variable and not statistically distinguishable. There was less

TABLE 3

EFFECT OF AMMONIUM CONCENTRATION ON PIGMENTS (pg) PER CELL (MEAN ± SD)

PIGMENTS PER CELL (pg)	AMMONIUM TREATMENT	*Pocillopora damicornis*	*Montipora verrucosa*
Chl *a*	Stripped	3.12 ± 0.80 $n = 3$	4.51 ± 0.38 $n = 3$
	Ambient	3.05 ± 0.79 $n = 3$	3.61 ± 0.88 $n = 3$
	20 μM	5.94 ± 3.50 $n = 3$	3.36 ± 0.97 $n = 2$
	50 μM	4.67 ± 1.16 $n = 3$	5.65 ± 1.38 $n = 2$
Chl *c*	Stripped	1.08 ± 0.20 $n = 3$	1.88 ± 1.06 $n = 2$
	Ambient	1.37 ± 0.06 $n = 3$	1.53 ± 0.55 $n = 3$
	20 μM	1.70 ± 0.85 $n = 3$	1.50 ± 0.12 $n = 2$
	50 μM	1.40 ± 0.32 $n = 3$	4.00 $n = 1$
Carotenoids	Stripped	0.51 ± 0.27 $n = 3$	0.93 $n = 1$
	Ambient	0.50 ± 0.07 $n = 3$	0.94 ± 0.07 $n = 3$
	20 μM	2.89 ± 1.69 $n = 3$	1.08 $n = 1$
	50 μM	0.67 ± 0.15 $n = 3$	0.74 ± 0.55 $n = 2$

variability in respiration rates in *P. damicornis*, but no significant differences among respiration rates for the four ammonium treatments.

Zooxanthellar densities were not significantly different for *M. verrucosa* in the four treatments (Table 2). Nubbins of *P. damicornis* in the 50-μM treatment had significantly higher zooxanthellar densities than the nubbins in the stripped treatment. For both species, cell densities were about twice the cell densities recorded for nubbins kept on the reef flat in a natural seawater environment (unpubl. data).

Chl *a* and chl *c* per algal cell in *P. damicornis* and *M. verrucosa* did not differ significantly in any of the four ammonium treatments (Table 3). In both species, chlorophyll levels were three to five times lower than those in nubbins kept on the reef flat in a natural seawater environment (unpubl. data). Carotenoids per cell were not significantly higher in the ammonium treatments. The ratio of chl *a* to chl *c* was around 3 in all treatments for *P. damicornis*, but ca. 1 or less for *M. verrucosa*.

Chl *a* concentrations normalized to surface area in *P. damicornis* showed an increase with increasing concentrations of ammonium although this trend was not significant (Figure 1). For *M. verrucosa*, chl *a* cm^{-2} was significantly higher in the 50-μM treatment.

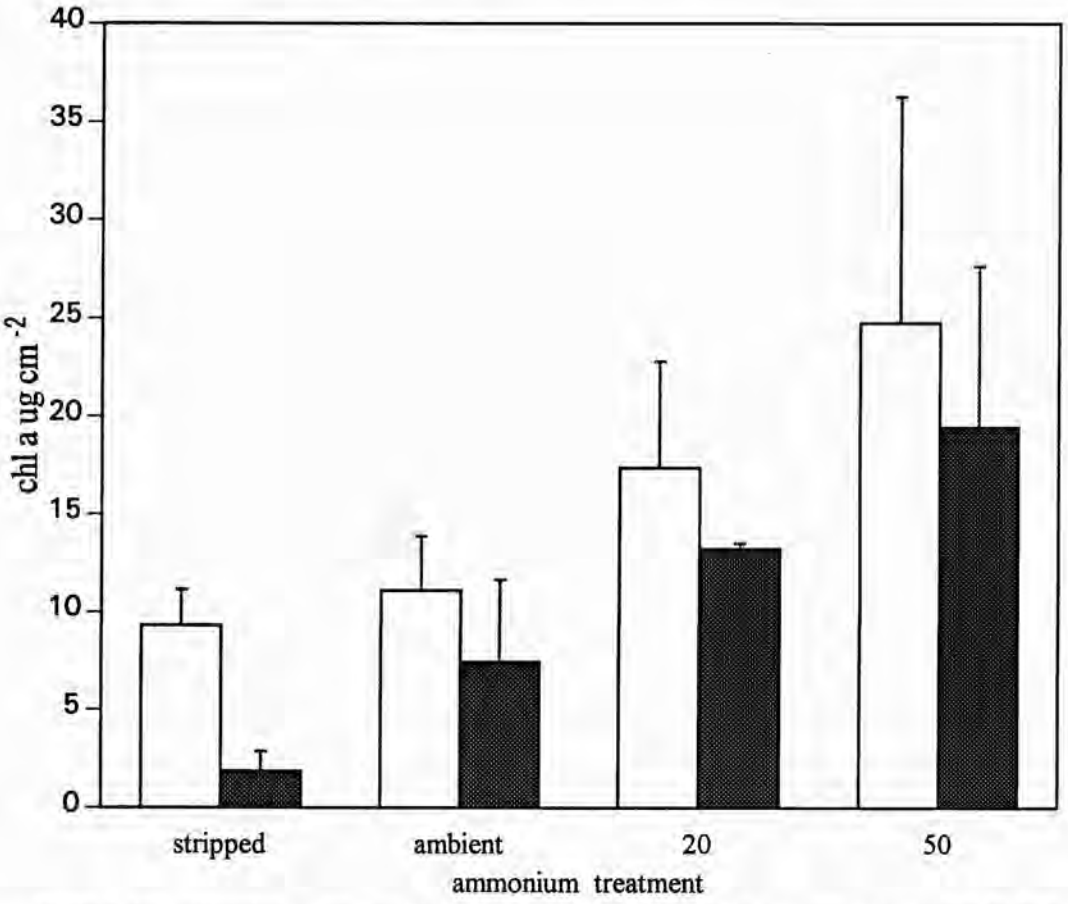

FIGURE 1. Chl *a* concentration (μg cm^{-2}) for *P. damicornis* (open bars) and *M. verrucosa* (solid bars) in the four ammonium treatments (stripped, ambient, 20 μM, and 50 μM).

DISCUSSION

Nitrogen is a limiting factor in oligotrophic seawater, so we expected to find an increase in algal density with increased ammonium concentrations. *Pocillopora damicornis*, but not *Montipora verrucosa*, showed this trend, with highest zooxanthellar densities at the highest ammonium concentration (Table 2). These algal densities for nubbins of *P. damicornis* overlapped values previously reported for intact colonies (Stambler et al. 1991).

In whole colonies of *P. damicornis* and *Stylophora pistillata* Esper, the number of cells per unit surface area increased in response to ammonium enrichment (Muscatine et al. 1989, Dubinsky et al. 1990, Stambler et al. 1991). The response of these experimental nubbins was not as strong as the response of a whole colony. Nubbins may not be representative of the physiology of the whole colony because parts of the colony may respond differently as a result of exposure to different environmental regimes within the colony (Jokiel and Morrissey 1986) or nubbin response may reflect differences in branch age (Titlyanov 1991). Other work with nubbins exposed to elevated ammonium (Høegh-Guldberg and Smith 1989) has demonstrated an increase in algal density in *S. pistillata* but a decrease in algal density in *Seriatopora histrix* Dana.

Ammonium enrichment caused an increase in chl *a* per unit of surface area in both *P. damicornis* and *M. verrucosa* (Table 4, Figure 1). This was also found for *S. pistillata* (Dubinsky et al. 1990), where it represented a combined effect, being the product of increasing algal density and chlorophyll concentration per algal cell. Higher chlorophyll concentrations at high ammonium treatments for *P. damicornis* may have resulted from the trend toward higher zooxanthellar densities and increased chl *a* per cell with enriched ammonium. *Montipora verrucosa* did not increase zooxanthellar densities, but chl *a* per cell showed a trend toward increased pigment at the highest ammonium levels.

Respiration rates of *P. damicornis* and *M. verrucosa* were not affected by the ammonium concentration. Similarly, no significant effect of ammonium concentration was found on respiration rates of entire colonies (Stambler 1992) or nubbins (Høegh-Guldberg and Smith 1989) of *S. pistillata*. Because we cannot separate in vivo the respiration rates of the algae and the animal, and we know that the growth rate of the coral was decreased with ammonium enrichment (Stambler et al. 1991), the lack of differences in respiration rates could be related to an increase in algal respiration, associated with increased algal growth (Falkowski et al. 1985), offset by a decrease in the animal respiration.

The coral-algal association is complex, and disfunction of the symbiosis results from eutrophication (Falkowski et al. 1993). There are demonstrated species-specific responses to light (Kinzie et al. 1984) and nutrient enrichment (Høegh-Guldberg and Smith 1989). In our experiment, although *P. damicornis* re-

TABLE 4

EFFECT OF AMMONIUM CONCENTRATION ON PIGMENTS (μg) PER SQUARE CENTIMETER (MEAN $\pm$ SD)

PIGMENTS PER cm^2 (μg)	AMMONIUM TREATMENT	*Pocillopora damicornis*	*Montipora verrucosa*
Chl *a*	Stripped	9.30 ± 1.83 $n = 3$	1.87 ± 0.99 $n = 3$
	Ambient	11.07 ± 2.80 $n = 3$	7.45 ± 4.19 $n = 3$
	20 μM	17.36 ± 5.44 $n = 3$	13.22 ± 0.28 $n = 2$
	50 μM	24.78 ± 11.48 $n = 3$	19.45 ± 8.23 $n = 3$
Chl *c*	Stripped	2.81 ± 0.58 $n = 3$	5.85 ± 0.89 $n = 3$
	Ambient	3.50 ± 0.83 $n = 3$	13.30 ± 5.35 $n = 3$
	20 μM	4.73 ± 1.59 $n = 3$	11.26 ± 2.20 $n = 2$
	50 μM	6.62 ± 2.69 $n = 3$	11.75 ± 2.10 $n = 3$
Carotenoids	Stripped	5.02 ± 0.63 $n = 3$	5.08 ± 1.50 $n = 3$
	Ambient	6.07 ± 1.46 $n = 3$	6.07 ± 0.57 $n = 3$
	20 μM	8.95 ± 2.63 $n = 3$	13.99 ± 1.83 $n = 2$
	50 μM	12.02 ± 4.47 $n = 3$	16.11 ± 2.74 $n = 3$

sponded to nutrient enrichment with an increase in zooxanthellar density, *M. verrucosa* appeared to show changes in algal pigments rather than zooxanthellar density.

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Effect of Ammonium-supplemented Seawater on Glutamine Synthetase and Glutamate Dehydrogenase Activities in Host Tissue and Zooxanthellae of *Pocillopora damicornis* and on Ammonium Uptake Rates of the Zooxanthellae[1]

D. YELLOWLEES,[2] T. A. V. REES,[3] AND W. K. FITT[4]

ABSTRACT: Host glutamine synthetase activity decreases in *Pocillopora damicornis* (Linnaeus) following exposure of the coral to seawater containing elevated ammonium (20 μM). Zooxanthellae isolated from these corals exhibited lower ammonium uptake capacity and glutamine synthetase activity compared with those from the control corals. Ammonium concentration of the surrounding seawater had no effect on the NADPH-dependent glutamate dehydrogenase activity in the host.

INTRACELLULAR AMMONIUM in corals can be derived both from normal catabolic processes within coral tissue and by uptake of ammonium from seawater. The latter was first observed by Kawaguti (1953). Subsequently, Muscatine and D'Elia (1978) demonstrated that only corals symbiotic with zooxanthellae can take up ammonium and also retain it. They assumed that ammonium can be assimilated within the symbiotic association and that zooxanthellae are essential to this process.

Few enzymes directly utilize ammonium as a substrate. Apart from carbamoyl phosphate synthetase I, which is present in organisms that possess the urea cycle, glutamine synthetase (GS) is the principal enzyme used in ammonium utilization, although NADPH-dependent glutamate dehydrogenase (GDH) has also been implicated in ammonium assimilation despite its low affinity for ammonium. Both GS and NADPH-GDH have been detected in corals and other zooxanthellate symbioses. GS in *Acropora formosa* (Dana) (D.Y., unpubl. data) could not be detected in host tissue, although its presence is assumed because GS is the sole enzyme capable of synthesizing glutamine. Its unlikely absence would indicate either that zooxanthellae must act as the source of glutamine or that it is acquired from heterotrophic feeding. High NADPH-GDH activity has been detected in host tissue of *A. formosa* (Catmull et al. 1987) and *Stylophora pistillata* Esper (Rahav et al. 1989).

GS has been found in zooxanthellae (Wilkerson and Muscatine 1984, Anderson and Burris 1987), although no activity measurements were presented. The presence of GS and GOGAT (glutamate synthetase) in freshly isolated zooxanthellae from corals can also be concluded from the data presented by Summons et al. (1986). GOGAT catalyzes the reductive transfer of the amide-amino group of glutamine to α-ketoglutarate to produce two glutamates. NADPH-GDH is present in freshly isolated zooxanthellae from corals, although its specific activity is an order of magnitude higher after induction with ammonium (Dudler and Miller 1988).

This report examines two of the central aspects of ammonium assimilation in marine alga-invertebrate associations: the relation-

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ship of GS and GDH from both *Pocillopora damicornis* (Linnaeus) and its freshly isolated zooxanthellae to the seawater ammonium concentration; and whether the uptake of ammonium by the zooxanthellae is influenced by the environmental ammonium concentration.

MATERIALS AND METHODS

Corals

Coral branches of *P. damicornis* were obtained as previously described in this issue (Stambler et al. 1994). Samples of control (ambient) corals and corals in seawater supplemented with 20-μM and 50-μM concentrations of ammonium incubated for 2 weeks and 8 weeks were used. Animal tissue was removed from small coral branches with a jet of compressed air from a scuba tank. The tank was connected to a plastic tube and the air delivered through a wide-bore plastic pipette tip inserted in the end of the plastic tube. During this procedure (air piking), the coral branches were wetted with extraction buffer (50 mM Tris-HCl buffer, pH 8.0, containing 1 mM reduced glutathione, 1 mM DTT, 20 mM mercaptoethanol, 1 mM EDTA, and 10 mM $MgSO_4$) as required. Zooxanthellae were removed from air-piked samples by centrifugation for 5 min at room temperature in a microfuge (Beckman). Animal tissue was used for assay of both GDH and GS.

Zooxanthellae

Zooxanthellae were prepared by removing the coral tissue from the skeleton of *P. damicornis* colonies with filtered seawater using the Water Pik technique (Johannes and Wiebe 1970). The zooxanthellae were washed three times with filtered seawater and then resuspended in extraction buffer (containing 5 mM mercaptoethanol) before lysing the cells in a precooled (4°C) Yeda Press at 9000 kPa (1200 psi). The lysate was centrifuged in a microfuge for 5 min to remove unbroken cells and cell debris. The supernatant was used for assay of GDH and GS.

Enzyme Assays

GDH was assayed in 50 mM HEPES-KOH buffer (pH 7.4) containing 100 mM $(NH_4)_2SO_4$, 0.2 mM NAD(P)H, 10 mM α-ketoglutarate, and enzyme extract (total volume = 1.05 ml). The reaction was monitored at 340 nm in a diode array spectrophotometer (Hewlett Packard 8452) at 30°C. Any NAD(P)H oxidase activity was measured for 2 min before initiating the GDH reaction with α-ketoglutarate. The GDH activity was adjusted for any NAD(P)H oxidase activity present. The oxidase activity was always $<20\%$ of the GDH activity.

The transferase and synthetase activities of GS were determined as described by Rhodes et al. (1975) and Guiz et al. (1979), respectively. Protein was measured by the Bradford (1976) method using reagents supplied by BioRad. Bovine serum albumin was used as a calibration standard.

Ammonium Uptake

Zooxanthellae ($0.5–1 \times 10^6$ cells ml^{-1}), prepared by the Water Pik technique and washing three times in filtered seawater, were incubated in seawater containing 20 μM NH_4Cl. The samples were incubated at 30°C under a fluorescent light source (200 $\mu E\ m^{-2}\ sec^{-1}$) and mixed regularly. Aliquots (5 ml) were removed at regular intervals, vacuum-filtered on GF/C filters (Whatman) and the ammonium content of the sample measured (Liddicoat et al. 1975).

RESULTS AND DISCUSSION

Zooxanthellae, freshly isolated from corals exposed to either 20- or 50-μM concentrations of ammonium, incubated in an elevated ammonium concentration took up ammonium ions at a slower rate than zooxanthellae isolated from control corals (Figure 1). The uptake rate for zooxanthellae from control corals (0.042 pmol $NH_4\ hr^{-1}\ cell^{-1}$) was roughly equivalent to that for freshly isolated zooxanthellae from *Acropora formosa* (Gunnersen et al. 1988). However, a five-fold

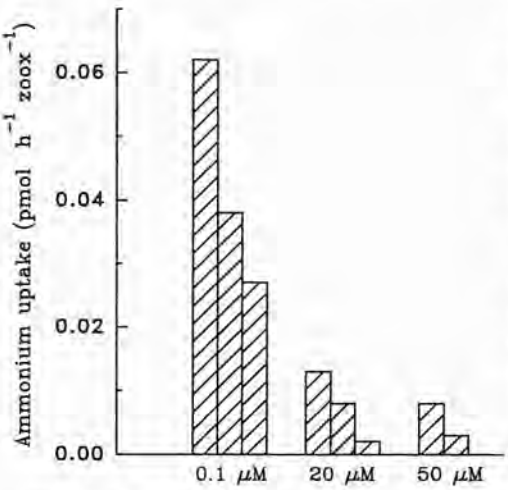

FIGURE 1. The depletion of ammonium from seawater by freshly isolated zooxanthellae from *Pocillopora damicornis*. The corals were maintained in seawater containing either 20-μM ($n = 3$) or 50-μM ($n = 2$) concentrations of ammonium. The ambient ammonium concentration of seawater used for the control corals ($n = 3$) was 0.1 μM.

decrease in the mean uptake rate was observed in zooxanthellae isolated from *P. damicornis* grown in 20-μM-supplemented seawater (8 fmol NH_4 hr^{-1} cell^{-1}). The uptake rate observed with zooxanthellae isolated from corals grown in 50-μM ammonium-supplemented seawater was not significantly different from that of corals grown in 20-μM-supplemented seawater. This indicates that the N status of the zooxanthellae was influenced by the seawater concentration; the ammonium uptake system was repressed with increased concentrations of ammonium. This response has not been previously reported in zooxanthellate symbioses, but Rees (1990) observed a similar result in symbiotic *Chlorella* and used this to argue for a low ambient concentration of ammonium in the perialgal space.

GS activity in host tissue was measured using both the transferase and synthetase assay; however, the measurement of synthetase activity proved unreliable and only the results for transferase are reported in Table 1. The transferase/synthetase ratios for GS in plants (15–100) and giant clam (*Tridacna gigas*) animal tissue (36 [Rees et al. 1994]) indicate that at the transferase levels observed, synthetase activity measurements would have been low. A decrease (>50%) in GS was observed after incubation of corals in seawater supplemented with 20 μM ammonium ions for 2 weeks. Only one sample was measured after 8 weeks exposure, and the GS level in that was further reduced. Although the presence of GS has been assumed, this paper represents the first report of GS activity in coral tissue.

In contrast, no evidence was obtained for changes in GDH in host tissue, indicating that any intracellular change in ammonium concentration resulting from an increase in seawater concentration did not influence the level of GDH. In all instances, >95% of GDH activity measured was NADP-dependent, and there was no evidence for any change in the coenzyme ratio

TABLE 1

GLUTAMINE SYNTHETASE AND GLUTAMATE DEHYDROGENASE ACTIVITIES IN THE CORAL *Pocillopora damicornis* AND ITS ZOOXANTHELLAE

	GLUTAMINE SYNTHETASE		GLUTAMATE DEHYDROGENASE	
TREATMENT	HOST	FRESHLY ISOLATED ZOOXANTHELLAE	HOST	FRESHLY ISOLATED ZOOXANTHELLAE
Control	186 (±23)	15	34 (±3)	ND
2 weeks	77 (±5)	7	31 (±4)	ND
8 weeks	55	—	29	—

NOTE: Units are expressed as nmoles min^{-1} (mg protein)$^{-1}$. Each assay was repeated and the mean taken. The results presented are the means of separate experiments ($n = 3$) with their standard errors. ND denotes not detected.

of dependence. Deaminating activity of GDH was not measured. Aminating activity was, however, 12-fold greater than that reported for *Stylophora pistillata* (Rahav et al. 1989), but six-fold less than that of *Acropora formosa* (Catmull et al. 1987).

Assuming that the concentration of ammonium in seawater influences the intracellular concentration, the results indicate that GS activity must be regulated either directly or indirectly by cytoplasmic ammonium ion concentration. However, without knowing the intracellular concentration of ammonium ions or metabolites derived from its assimilation, it is not possible to speculate further on the control of GS levels in host tissues.

The specific activity of both GS and GDH in zooxanthellae was always $< 10\%$ of that in the host tissue. Indeed, in the case of GDH it was impossible to reliably detect any oxidation of either NADPH or NADH above background, indicating that GDH levels were very low. However, GS was detected in zooxanthellae using the transferase assay, indicating that it must be the major means of ammonium assimilation in zooxanthellae.

From these results, we propose that the host has a role in the assimilation of ammonium ions in the symbiosis. It has been argued that the zooxanthellae are the driving force behind the acquisition and retention of ammonium in the symbiosis (Muscatine and D'Elia 1978). However, uptake of ammonium by zooxanthellae relies on diffusion of ammonium through the host cell and into the perialgal space. With relatively high concentrations of ammonium-assimilating enzymes in the cytoplasm of the control hosts, there is a likelihood that much of the ammonium when it is present at natural seawater concentrations will be assimilated before it can diffuse to the perialgal membrane. The repression of the ammonium uptake rate of zooxanthellae in corals exposed to elevated ammonium ions supports this conclusion.

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Effects of Water Velocity on Respiration, Calcification, and Ammonium Uptake of a *Porites compressa* Community[1]

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ABSTRACT: Colonies of *Porites compressa* Dana were placed in a 10-m-long flume to form a community of coral. Ammonium uptake (N uptake) rate, respiration rate, and calcification rate were measured at different water velocities, ranging from 1 to 57 cm sec^{-1}. N uptake was proportional to concentration from 20 to 0.15 μM N. The first-order rate constant for N uptake varied from 6.8 to 15.6 day^{-1}, only an average of 2.1 times over a 10-fold change in water velocity. First-order rate constants for respiration were less than those for N uptake and ranged from 4.8 to 6.6 day^{-1}. Respiration rate and calcification rate were not correlated with water velocity. The relative turnover of N compared with oxygen (O_2) indicates that 94–98% of N flux must be retained within this coral community.

Uptake rates of phosphate (P) into algal reef-flat communities are positively correlated with water velocity and show a consistent enhancement above that predicted by correlations of mass-transfer from the engineering literature (Atkinson and Bilger 1992). The explanation for the correlation between uptake rate and water velocity is that increased water velocity thins diffusive boundary layers adjacent to organisms. Diffusion of P through these boundary layers can be the rate-limiting step for uptake into communities of coral reef benthos; that is, P can be "mass-transfer limited."

Several preliminary uptake experiments with nitrate and ammonium also showed uptake rates consistent with mass-transfer limitation. A U.S.–Israel Workshop was organized to understand more fully the responses of hermatypic corals to elevated nitrate-ammonium concentrations. Workshop participants studied effects of N enrichment on the composition of zooxanthellae and host (such as carbon : nitrogen : phosphorus [CNP] ratios, amino acids, protein, and enzymes) and growth parameters of zooxanthellae and coral (such as zooxanthellae growth, coral calcification, and photosynthesis). In view of our previous results on the effects of water velocity on P uptake rates (Atkinson and Bilger 1992), we wanted to measure uptake rates of ammonium into a community of hermatypic coral over a range of water velocities and determine whether the uptake rate is mass-transfer limited. (We use the term ammonium uptake even though actual uptake of ammonium by coral can be as ammonia [NH_3].) We also wanted to determine the maximum N-uptake rate, so as to calculate N turnover by the coral community. In addition to N uptake, we also measured two other metabolic rates that have been suggested to be affected by water velocity, calcification and respiration. Respiration rates were useful to calculate the relative turnover of oxygen (O_2) and carbon (C) with that of N. This analysis would indicate the relative amount of N retained by symbiosis between zooxanthellae and host tissue, and how this amount of "retained-N" would change with water velocity.

To be mass-transfer limited, the rate of uptake must have first-order kinetics (rate of uptake is directly proportional to concentration), and the first-order rate coefficient must

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be directly proportional to velocity by a root of 0.5 to 0.8 (Bilger and Atkinson 1992) (that is, $k \sim U_b$ exp [0.5 to 0.8], where k is the first-order rate coefficient and U_b is the velocity in the bulk of the flow, or bulk velocity). Exponents <0.5 are possible, but do not strictly adhere to the algebraic definition of mass-transfer limitation (Bilger and Atkinson 1992). The algebraic definition of mass-transfer limitation requires that the surface concentration of nutrient is negligible compared with the concentration of the bulk water.

MATERIALS AND METHODS

Specimens of *Porites compressa* Dana were collected from the Coconut Island reef flat, Hawaii Institute of Marine Biology, brought to the island, and then placed in an experimental flume, 10 m long, 0.35 m high, and 0.35 m wide (see Atkinson and Bilger [1992] for a discussion of the design of the flume and the formation of side-wall and bottom momentum boundary layers). The experimental community covered 2.1 m^2 (6 by 0.35 m) of the 8-m-long test area of the flume. Water was recirculated through the flume throughout the experiments. Water velocities past the experimental community were controlled from 1 cm sec^{-1} to about 57 cm sec^{-1}. The ratio of water volume to planar surface area of the test area of the flume for these experiments was 0.62 m.

Colonies of *P. compressa* were cleaned of epiphytes and incubated for 4 days in the flume at water velocites of 10 cm sec^{-1} before respiration rates were measured. Daytime respiration rates were measured by placing 4-mil black plastic over the entire flume and measuring changes in O_2 concentration over the next 4–8 hr. O_2 concentration was measured with an O_2 meter (YSI model 58), calibrated to Winkler titrations ($n = 22$, $r^2 = 0.96$). The O_2 probe was mounted in the bulk flow of water over the upstream end of the test benthos. Respiration rates were measured over 10 days from 26 July to 9 August 1991.

N-uptake rates and calcification rates were measured over 7 days during the workshop, 22 to 29 August 1991. Each experiment began in the morning and ended by about 1700 hr. This ensured that both N uptake and calcification were measured through a daylight period. Each morning before the experiment began, coral colonies, side walls, and bottom of the flume were picked clean of epiphytes. This procedure ensured that there were few epiphytes or bacterial films on the walls that could have contributed to N uptake or calcification. After this daily cleaning, the flume was filled with fresh seawater and an ammonium sulphate spike was added over a 1-min period at maximum water velocity to bring the initial concentrations to near 20 μM N. This technique ensured rapid mixing of the spike into the recirculating seawater. Initial water samples were taken after 20 min. Approximately 7–10 water samples were taken throughout the day. During each sampling period, about 10 1-liter subsamples of flume water were siphoned into a bucket. This procedure reduces noise in the data, apparently produced by patches of nutrients in the flume water. The duration of subsampling was the time period for three transits of the water around the flume, which varied from 3 to 24 min depending on the speed of the water. A subsample of the water in the bucket was taken with a 150-ml syringe, and this water was filtered through a GF/C in-line filter into a Nalgene bottle for storage. The subsample of water for nutrients was frozen within 15 min of collection. The water sample for measurement of total alkalinity was left at room temperature. PO_4^{3-}, NO_3^-, NH_4^+, and SiO_4^{4-} were measured on a Technicon II Autoanalyzer, with standard Technicon industrial methods as modified by Walsh (1989). Total alkalinity was determined using the intercept of the linear regression between titrated acid and pH change between pH of 4 and 3 (Edmond 1970). At least 20 points were used in the regression. Our precision using this technique, based on triplicate sampling, was 0.003 meq $liter^{-1}$. Calcification rate is the rate of change of total alkalinity divided by two (Smith and Kinsey 1978, Atkinson and Grigg 1984). Water velocities in the flume were measured by placing a neutrally bouyant

scintillation vial upstream of the community and timing its movement through 6 m of the 8-m test section. Control rates were measured with no organisms in the flume on 29 August. These rates included gas exchange for O_2 and NH_3 and any uptake by bacterial or algal films.

RESULTS

Over the range of O_2 concentrations (3–425 μM O_2), respiration rates were proportional to concentration ($r^2 = 0.996$–1.000, $n = 5$; Table 1). However, the first-order rate coefficient for O_2 uptake, k_O, was not significantly correlated to water velocity, indicating that O_2 uptake is probably not controlled by diffusive boundary layers. The mean k_O was 5.4 day^{-1} $\pm$ 0.7, $n = 8$; the percentage standard deviation was only 13% over a range in water velocities from 1.0 to 36.1 cm sec^{-1} and throughout 14 days. The rate constant for the control, which was measured at the end of the experiments, was 0.46 day^{-1}, only 9% of the mean value. A respiration rate for this community can be calculated by multiplying the mean k_O, 5.4 day^{-1}, times the saturation concentration of 200 μM O_2: 5.4 day^{-1} $\times$ 200 μM O_2 = 1080 mM day^{-1}. Taking the volume to surface area ratio of 0.62 m, the per planar area respiration rate is 670 mmol O_2 m^{-2} day^{-1}.

TABLE 1

SUMMARY OF RESPIRATION EXPERIMENTS ON THE *Porites compressa* COMMUNITY IN THE FLUME

DATE	TIME	VELOCITY (cm sec^{-1})	O_{2i} (μM)	O_{2f} (μM)	k_O (day^{-1})
26 July	9.67–15.72	4.2 $\pm$ 0.26	209	31	6.6
29 July	10.00–15.67	36.1 $\pm$ 1.2	203	44	5.6
31 July	9.77–15.65	5.1 $\pm$ 0.25	200	38	6.2
1 Aug.	9.43–16.47	5.0 $\pm$ 0.20	200	21	5.0
2 Aug.	11.25–15.63	9.3 $\pm$ 0.10	209	78	4.9
5–6 Aug.	15.07–10.97	4.8 $\pm$ 0.09	425	3	5.5
6–7 Aug.	16.65–09.03	1.0 $\pm$ 0.05	325	3	4.8
8–9 Aug.	17.83–09.62	1.0 $\pm$ 0.06	281	3	4.8
Control					
9–11 Aug.	16.34–10.50	4.0 $\pm$ 0.18	188	125	0.46

NOTE: The control experiment without coral, 9–11 August, lasted 2 days. "Time" is the start-end decimal time of day; O_{2i} is the initial O_2 concentration and O_{2f} is final; k_O is the first-order rate coefficient (slope of ln O_2 versus day); r^2s are all above 0.996.

TABLE 2

SUMMARY OF AMMONIUM UPTAKE EXPERIMENTS ON THE *Porites compressa* COMMUNITY IN THE FLUME

DATE	TIME	VELOCITY (cm sec^{-1})	$NH_4^+{}_i$ (μM)	$NH_4^+{}_f$ (μM)	k_N (day^{-1})	r^2 $n = 8$	$CaCO_3$ (mmol m^{-2} day^{-1})	r^2 $n = 10$
22 Aug.	10.78–15.50	47.6 $\pm$ 2.1	18.30	1.70	12.7	0.99	314	0.99
23 Aug.	9.42–17.07	5.6 $\pm$ 0.8	18.41	1.26	7.34	0.95	361	1.00
24 Aug.	9.50–16.00	5.6 $\pm$ 0.6	20.94	3.33	6.84	1.00	333	1.00
25 Aug.	9.00–16.00	28.5 $\pm$ 1.6	18.90	0.79	12.7	0.97	430	1.00
26 Aug.	11.75–15.50	50.6 $\pm$ 2.0	1.11	0.15	13.5	0.99	—	—
27 Aug.	9.50–16.00	56.9 $\pm$ 3.2	21.91	0.41	15.6	0.99	314	0.99
Control								
29 Aug.	9.75–16.47	36.9 $\pm$ 1.0	20.03	17.46	0.47	0.92	13	0.06

NOTE: The control experiment without coral was on 29 August. "Time" is the start-end decimal time of day. $NH_4^+{}_i$ is the initial ammonium concentration and $NH_4^+{}_f$ is final; k_N is the first-order rate coefficient (slope of ln NH_4^+ versus day). Calcification rate is a linear rate and summarized under $CaCO_3$; r^2s for NH_4^+ uptake and $CaCO_3$ are listed after the rate.

Calcification rates were constant throughout the day ($r^2 = 0.993$–0.998; Table 2) and were also not a function of water velocity. The mean calcification rate was 350 mmol $CaCO_3$ m^{-2} day^{-1} $\pm$ 0.048, $n = 5$. The control rate was 13 mmol $CaCO_3$ m^{-2} day^{-1} ($r^2 = 0.063$), only 3.8% of the mean rate. Both respiration and calcification rates are close to metabolic standards for coral reef flats (Kinsey 1985), indicating that the flume community represents a healthy coral assemblage.

Rates of N uptake were proportional to ammonium concentration from 20 to 0.15 μM N ($r^2 = 0.972$ to 0.998); an example of an N uptake experiment is shown in Figure 1. The first-order rate coefficient for N uptake, k_N, was positively correlated to water velocity (Table 2, Figure 2): k_N was 7.3 and 6.8 day^{-1} at 5.6 cm sec^{-1} and 12.7 day^{-1} and 15.6 day^{-1} at 47.6 and 56.9 cm sec^{-1}, respectively. The k_N value for the control measurement was 0.47 day^{-1}, only 6.9% of the minimum observed rate and 3% of the maximum rate. A 10-fold increase in water velocity only increased N uptake 2.1-fold. At 0.15 μM ammonium, there was no apparent net uptake of ammonium (experiment of 26 August, Table 2), indicating that uptake rate of N equaled the release rate of N.

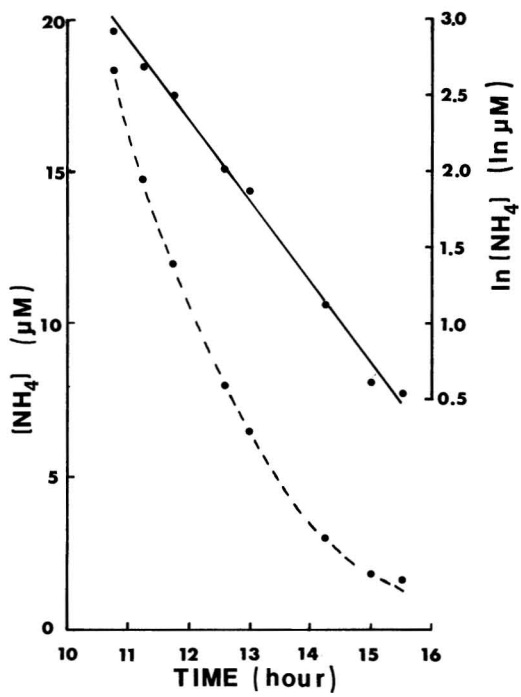

FIGURE 1. Dashed line: ammonium concentration versus time of day for experiment on 22 August (Table 2). Experiments began in the morning with initial concentrations near 20 μM N and continued until 1600 hr. Solid line: ln [NH_4^+] for the same experiment. The slope of the straight line is the first-order rate constant, k_N, for this experiment. The first-order rate constants are reported in Table 1 for O_2 and Table 2 for ammonium.

P concentration in the flume experiments decreased only slightly from initial concentrations of 0.15–0.21 to 0.09–0.20 μM P (Table 3). Similarly, Si concentrations began at 7.65–9.62 and changed to 2.99–9.25 μM Si. In all but one instance, Si decreased. In contrast, $NO_3 + NO_2$ significantly increased from initial concentrations of 0.29–0.63 to final concentrations of 0.63–1.44 μM N. During the experiment where no ammonium was injected into the flume (experiment of 26 August), NO_3 significantly decreased from 0.50 to 0.17 μM N. These results indicate that NO_3 is produced during high ammonium concentrations, but is removed from the water when ammonium concentrations are low.

DISCUSSION

It is well known that hermatypic corals retain or recycle N relative to C (Rahav et al. 1989). The relative turnover of N and C can be estimated for the corals in these experiments. A release rate of ammonium from the coral community can be calculated by assuming that uptake of ammonium equals release of ammonium at 0.15 μM N (experiment of 26 August, Table 2). Uptake rate (and release rate) is therefore equal to k_N x (0.15 μM N). Taking the largest rate coefficient in Table 2, 15.6 day^{-1} at 56.9 cm sec^{-1}, the maximum release rate of ammonium from the experimental coral community in the flume was 1.4 mmol N m^{-2} day^{-1}. The calculated respiration rate of these corals at 200 μM O_2 was 670 mmol m^{-2} day^{-1}. Assuming a respiratory quotient (C/O_2) of 1.0, the C : N ratio

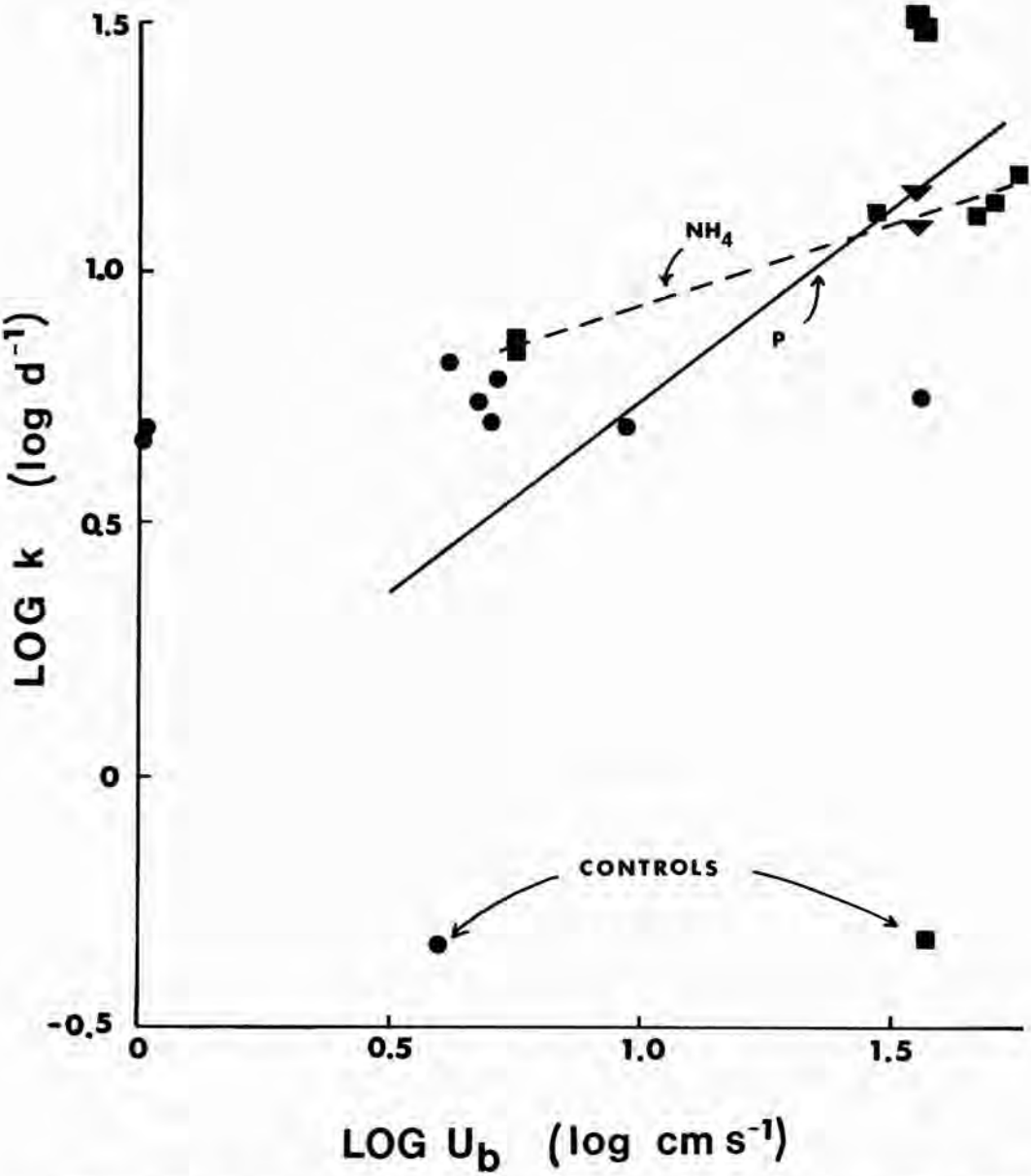

FIGURE 2. First-order rate constants versus water velocity. The solid line is the relationship between P uptake (k_P) into a mixed algal community and water velocity (Atkinson and Bilger 1992). The dashed line connecting the solid squares shows the relationship between ammonium uptake (k_N) and water velocity for our experiments with *Porites compressa*. The solid circles show O_2 uptake, k_O, for these experiments. The large solid squares show ammonium uptake, k_N, for a mixed algal community in the flume. The solid triangles show NO_3 uptake into a mixed algal community.

TABLE 3

INITIAL AND FINAL CONCENTRATIONS, C_o and C_t, RESPECTIVELY, OF NUTRIENTS IN THE AMMONIUM UPTAKE EXPERIMENTS

			PO_4		$NO_3 + NO_2$		Si	
DATE	HOUR	VELOCITY (cm sec^{-1})	C_o	C_t	C_o	C_t	C_o	C_t
22 Aug.	4.72	47.6	0.20	0.09	0.54	1.05	7.80	6.25
23 Aug.	7.65	5.6	0.17	0.14	0.56	1.25	8.14	5.30
24 Aug.	6.50	5.6	0.15	0.20	0.43	1.44	8.96	7.65
25 Aug.	7.0	28.5	0.20	0.17	0.63	0.96	7.65	4.48
26 Aug.	3.75	50.6	0.20	0.13	0.50	0.17	9.14	2.99
27 Aug.	6.5	56.9	0.21	0.20	0.29	0.63	8.96	4.10
Control								
29 Aug.	6.72	36.9	0.19	0.13	0.63	0.83	9.62	9.25

of release from the corals was 478 (670/1.4). C : N ratios of host tissue for control corals from this workshop (Muller-Parker et al. 1994) were from 5 to 7; zooxanthellae C : N ratios varied from 7 to 20 depending on the ammonium concentration of the incubation water. If organic substrates of catabolism have C : N ratios of 7, then 98% of N flux is retained within the coral; similarly, if C : N ratios are 20, then 94% of N flux is retained. This calculation was based on the fastest uptake rate constant, assuming ambient concentrations of 0.15 μM. There was only a 2.4-fold change in the uptake rate constant with water velocity, so water velocity had little effect on these conclusions. At least 10-fold changes in N uptake would be required to support the flux of ammonium without large changes in N retention within corals. Rahav et al. (1989) showed that recycled N from host tissue accounts for >90% of the zooxanthellae N demand in *Stylophora pistillata* Esper. Our results corroborate those findings.

Both calcification and respiration rates were not significantly correlated to water velocity. It is not surprising that calcification is not correlated to water velocity. It is difficult to imagine concentration-depleted diffusive boundary layers of Ca, considering that seawater is ca. 10 mM Ca. Furthermore, the production of CO_3 is controlled within corals and is probably not directly related to the pH of the overlying water. Previous observations that community calcification is correlated to water velocity (Kinsey 1985) are probably a result of changes in community structure or some other indirect effect of water velocity, such as increased nutrient uptake.

Respiration did not appear to be affected by water velocity in this community. Apparently in this study the rates of O_2 uptake were not fast enough to develop diffusive boundary layers around the coral (Newton and Atkinson 1991). Note that the first-order rate constants for O_2 uptake (Table 1, Figure 2) are almost three-fold lower than the rate constants for ammonium uptake (Table 2, Figure 1).

N uptake is limited by diffusion through diffusive boundary layers. N uptake is first order and positively correlated to the bulk water velocity. The increase in k_N with water velocity, however, was less than expected. Normally for turbulent flow, the increase would be 0.7–0.8 root, or $\log k = 0.8 \log U_b$ (Bilger and Atkinson 1992). A 10-fold change in water velocity would increase k 6.3-fold, not just 2.1-fold as in these experiments. Given our data, the slope between $\log k_N$ and $\log U_b$ is only about 0.3. This value is too low to conclude definitely that N uptake is mass-transfer limited.

Figure 2 shows our results compared with some previously published results. The solid line is the best line representing P uptake versus water velocity from Atkinson and Bilger (1992). The dashed line connects values of k_N

for the low-velocity experiments of our study with values for the high-velocity experiments. Note that k_N is two to three times above k_P at the lower velocities. This result is to be expected for mass-transfer limited rates, because diffusion of ammonium through water (hence the boundary layers) is three times faster than that of P (Li and Gregory 1974). However, at the higher velocities, N uptake into this coral community is about the same as P uptake into the mixed coral-algal community. There are two plausible explanations for this result. The first is that the concentration of ammonium at the surface of the organisms is a substantial percentage of the concentration in the bulk flow. This would result in a decreased effect of velocity on N uptake. The other explanation is that coral branches force water into more interstitial spaces than would otherwise occur, giving enhanced uptake at lower velocities. This explanation is unlikely, because a two- to threefold uptake rate above P uptake is expected. It is apparent that these explanations need further research to verify whether coral morphology in a mixed community alters the relative uptake of nutrients at different velocities.

An interesting pattern in metabolic rates is illustrated in Figure 2; O_2 uptake is near mass-transfer limited rates but shows no significant effects with changes in velocity; in contrast, N and P uptake have larger rate coefficients and are therefore closer to the mass-transfer limit. We suggest that respiration probably cannot be maintained at a mass-transfer limited rate because low water velocities would then stress or kill microorganisms living in the boundary layer. The reduced flux of O_2 would continually limit the activity of these organisms. Thus the observed community respiration rate is sustained at the highest level without becoming strongly water velocity–dependent. Organisms probably shunt intracellular energy to nutrient-uptake mechanisms that allow the fastest nutrient uptake under all conditions; thus they take advantage of the high water velocity by increasing nutrient uptake. We believe further experimentation will show that other coral or coral-algal communities have similar metabolic patterns.

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Resource Partitioning by Reef Corals as Determined from Stable Isotope Composition II. $\delta^{15}N$ of Zooxanthellae and Animal Tissue versus Depth[1]

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ABSTRACT: The pattern of resource partitioning versus depth for corals collected in February, 1983, from Jamaica was investigated by analyzing their stable nitrogen isotope composition. Observations were made on isolated zooxanthellae and corresponding algae-free animal tissue from nine species of symbiotic corals at four depths over a 50-m bathymetric range, and from a nonsymbiotic coral at 1 m. $\delta^{15}N$ values versus depth ranged from +3.54 to −2.15 ‰ for zooxanthellae and from +4.71 to +0.23 ‰ for animal tissue. In those species that occurred over a 30- to 50-m depth range, both animal tissue and zooxanthellae tended to be depleted in ^{15}N as depth increased to 30 m. In a few species animal tissue was enriched in ^{15}N from 30 to 50 m. Depletion of ^{15}N in zooxanthellae with increasing depth may be the result of depth-dependent differences in their nitrogen-specific growth rates. Animal tissue was consistently more depleted in ^{15}N than for the nonsymbiotic coral *Tubastrea coccinea* (Ellis) at the same depth, but it was still slightly more enriched in ^{15}N than corresponding zooxanthellae in 16 of 25 paired samples. The latter trend was not correlated with depth. A comparison of $\delta^{13}C$ and $\delta^{15}N$ for zooxanthellae and animal tissue over 50 m revealed a tendency toward depletion of heavy isotopes as depth increases. Increased carbon fixation appears to be accompanied by decreased nitrogen fractionation.

RESOURCE UTILIZATION by scleractinian reef corals is profoundly affected by endosymbiotic dinoflagellates (zooxanthellae). Although coral polyps feed on particulate organic carbon and nitrogen (Lewis and Price 1975, Lewis 1976, 1977, Clayton and Lasker 1982), their phototrophic endosymbionts take up and assimilate inorganic carbon (Muscatine and Cernichiari 1969, Schmitz and Kremer 1977, Crossland et al. 1980, Black and Burris 1983) and nitrogen (Franzisket 1974, Crossland and Barnes 1977, D'Elia and Webb 1977, Muscatine and D'Elia 1978, Webb and Wiebe 1978, Muscatine et al. 1979, Muscatine 1980*a*, Burris 1983, D'Elia et al. 1983, Wafar et al. 1985, Summons et al. 1986, Anderson and Burris 1987, Rahav et al. 1989) from the environment and from host catabolism. Organic carbon and nitrogen is translocated from algae to host (Muscatine 1980*b*, Falkowski et al. 1984) and possibly from host to algae (e.g., see Cook 1983, Steen 1986). The symbiotic algae also enable the retention and recycling of carbon and nitrogen atoms within the coral. These features confer an apparent selective advantage on coral animals in oligotrophic environments. There is little information, however, on how depth and light attenuation might influence these potential fluxes and consequently the selective advantage of the symbiosis to the partners.

In previous studies, Davies (1984), McCloskey and Muscatine (1984), and Muscatine et al. (1984) noted that photosynthetic rates by zooxanthellae in shallow-water corals are high and that carbon translocated from algae could meet the daily carbon demand of the animal for respiration and

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growth. In contrast, in deep-water corals, photosynthetic rates are low and much less photosynthetically fixed and translocated carbon is available for animal respiration and growth. McCloskey and Muscatine (1984) predicted that in deep water, reduced input of photosynthetically fixed and translocated carbon may be supplemented by input of carbon from allochthonous sources. Muscatine et al. (1989) attempted to evaluate this prediction by analyzing the stable carbon isotopes of coral animal tissue and zooxanthellae as a function of depth. Their data revealed that $\delta^{13}C$ of zooxanthellae was relatively high in shallow water. These high values were interpreted as the result of diffusion-depletion of internal CO_2 at high rates of photosynthesis and consequent minimal stable isotope discrimination (see also Goreau 1977). Zooxanthellae $\delta^{13}C$ became lower as depth increased and as light for photosynthesis diminished. Animal tissue $\delta^{13}C$ was slightly lower than zooxanthellae $\delta^{13}C$ in shallow water, probably as a result of translocation of photosynthetically fixed carbon from zooxanthellae to animal. As depth increased, the animal tissue exhibited a disproportionately lower $\delta^{13}C$, suggesting that more particulate organic carbon (POC) is taken up in deep water or that at the lower rates of photosynthesis CO_2 was no longer limiting.

To gain further insight into resource utilization by reef corals, we examined the $\delta^{15}N$ of zooxanthellae and animal tissue versus depth in the same coral samples as those analyzed by Muscatine et al. (1989). $\delta^{15}N$ values for marine organisms generally range from about -3 ‰ to about $+20$ ‰ (Owens 1987), but some organisms from hydrothermal vents or hydrocarbon seeps, or possessing endosymbiotic bacteria, exhibit values as low as -12.9 ‰ (Rau 1981, Paull et al. 1985, Brooks et al. 1987, Conway et al. 1989). $\delta^{15}N$ values reflect the nature of the source nitrogen, which may undergo only minimal fractionation when it is limiting (Wada and Hattori 1976, Owens 1987) or when atmospheric nitrogen is fixed by marine cyanobacteria (see references in Macko et al. 1984). Alternatively, source nitrogen may undergo maximal fractionation during assimilation (Wada and Hattori 1978, Minegawa and Wada 1980, Wada 1980, Macko et al. 1986, 1987). $\delta^{15}N$ values are also useful as indicators of trophic level (Van Dover and Fry 1989).

The data presented here show that both the zooxanthellae and the animal tissues in Jamaican scleractinian corals tend to be depleted in ^{15}N, particularly as depth increases. Depth-dependent variables that may contribute to ^{15}N depletion are discussed.

MATERIALS AND METHODS

Methods for sampling corals, separation of zooxanthellae and animal tissue, and analytical techniques were described by Muscatine et al. (1989). Briefly, 10 species of corals (nine symbiotic, one nonsymbiotic) were collected from 1 and 10 m depth from the back reef and from 10, 30, and 50 m depth from the fore-reef at Discovery Bay, Jamaica, in February 1983. Tissue was removed from whole colonies or from large pieces of massive corals to minimize sample heterogeneity within colonies. Algae and animal tissues were separated by a series of careful centrifugations and washings. Algae were recovered as pellets, and animal tissue was deposited on precombusted glass fiber filters (Reeve Angel). Both fractions were dried at 50°C. Samples were combusted as described by Minegawa et al. (1984). Mass spectrometry was performed on a Varian MAT 250 instrument. The $^{15}N/^{14}N$ of the samples is reported as $\delta^{15}N$, in units per mil (‰), where:

$$\delta^{15}N\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 1000$$

and

$$R = {}^{15}N/{}^{14}N$$

Atmospheric nitrogen was the standard. The analytical precision of these measurements was 0.2 ‰.

RESULTS

The values of $\delta^{15}N$ for each species versus depth ranged from $+3.54$ to -2.15 ‰ for

TABLE 1

$\delta^{15}N$ FOR ALGAE AND ANIMAL TISSUE FROM JAMAICAN CORALS OVER A 50-m BATHYMETRIC RANGE

CORALS	DEPTH			
	1 m	10 m	30 m	50 m
Symbiotic corals				
Madracis mirabilis (Duchassaing & Michelotti)				
Algae	+3.54	+3.26	+2.64	*
Animal	+3.90	+3.05	+1.84	*
Acropora cervicornis (Lamarck)				
Algae	+1.76	+1.68	+0.16	*
Animal	+4.11	+1.86	+1.56	*
Agaricia agaricities (Linnaeus)				
Algae	+1.64	+1.85	+1.86	+0.30
Animal	+3.02	**	+1.48	+1.54
Acropora palmata (Lamarck)				
Algae	+1.76	+1.48	*	*
Animal	**	+2.13	*	*
Porites astreoides Lamarck				
Algae	+2.99	+2.30	+1.74	*
Animal	+2.79	+2.10	+2.04	*
Montastrea annularis (Ellis & Solander)				
Algae	+3.00	+1.83	+2.21	−0.16
Animal	+3.32	+2.41	+0.23	+1.87
Montastrea cavernosa (Linnaeus)				
Algae	+0.95	+0.35	−2.15	−1.73
Animal	+2.96	+1.11	+1.16	+3.43
Eusmilia fastigiata (Pallas)				
Algae	+3.45	+2.18	+0.94	*
Animal	+3.45	+2.52	+2.76	*
Dendrogyra cylindrus Ehrenberg				
Algae	*	+2.43	*	*
Animal	*	+2.23	*	*
Nonsymbiotic coral				
Tubastrea coccinea (Ellis)	+4.74	*	*	*

*, not found at depth; **, samples lost.

algae and from +4.11 to +0.23 ‰ for animal tissue (Table 1). In those species that occurred over a 30- to 50-m depth range, both zooxanthellae and animal tissue tended to be depleted in ^{15}N as depth increased, although in *Montastrea annularis* (Ellis & Solander) and *M. cavernosa* (Linnaeus) at 50 m, animal tissue was again enriched in ^{15}N.

There were differences between $\delta^{15}N_{animal}$ and $\delta^{15}N_{algae}$ in each species. Animal tissue was more enriched in ^{15}N than algae in 16 of the 25 paired samples (mean ± SD = 1.51 ± 1.3 ‰; range, 0.30–5.16 ‰). In the remaining nine, the $\delta^{15}N$ animal values were equal to or lower than those of corresponding algae (mean ± SD = 0.46 ± 0.61 ‰; range, 0.18–1.98 ‰).

The $\delta^{15}N$ value for *Tubastrea coccinea* (Ellis) is +4.74 ‰ and is taken as representative of coral animal tissue at 1 m that has acquired particulate and/or dissolved organic nitrogen in the absence of a photosynthetic endosymbiont.

DISCUSSION

$\delta^{15}N$ *of Zooxanthellae*

$\delta^{15}N$ values for zooxanthellae in Jamaican corals range from −2.15 to +3.54 ‰. The data cluster at the low end of the range of values representative of marine organisms. Interpretation of the absolute values of $\delta^{15}N$

for the algae and animal must await data on the $\delta^{15}N$ values of the source nitrogen, which, at present, are unknown. The most important sources of dissolved inorganic nitrogen (DIN) for symbiotic dinoflagellates seem to be nitrate and ammonium from seawater and ammonium from coral animal catabolism (D'Elia 1988, Rahav et al. 1989). Other potential sources include nitrate and ammonium from local sources such as groundwater (D'Elia et al. 1981), nitrification associated with bacteria in sponges (Corredor et al. 1988) and coral skeletons (Szmant-Froelich and Pilson 1977, Wafar et al. 1985), coral head porewater (Risk and Muller 1983), and migrating fishes (see, for example, Meyer and Schultz 1985*a*,*b*).

An interpretation of the trend in depletion of zooxanthellae ^{15}N with depth is suggested by observations of Wada and Hattori (1978; see also Minegawa and Wada 1980) on cultured marine diatoms. They demonstrated that isotope fractionation of nitrate and ammonium was negligible during uptake, but substantial during assimilation, and that fractionation was inversely proportional to growth rate. The highest fractionation occurred when growth was light-limited and N-sufficient. Because the specific growth rate of zooxanthellae in at least one coral species tends to be higher in high-light habitats than in shade (Muscatine et al. 1989), the depth profile for zooxanthellae $\delta^{15}N$ could be interpreted on the basis of relative specific growth rates of light-sufficient, nutrient-limited zooxanthellae in shallow water and light-limited, nutrient-sufficient zooxanthellae in deep water. A few observations support this scenario.

Zooxanthellae in corals are very effective scavengers of nitrogen, often taking up ambient DIN at or below concentrations of 1 μM and effectively retaining host catabolic ammonium so that, under normal conditions, little host catabolic ammonium is released to the environment (Muscatine and D'Elia 1978, Cook and D'Elia 1987, Cook et al. 1988, Rahav et al. 1989, Stambler et al. 1991). However, there is a growing body of evidence that suggests that growth rate or biomass increase of zooxanthellae in shallow-water corals and other symbiotic cnidarians may be nitrogen-limited (Cook and D'Elia 1987, Cook et al. 1988, Dubinsky et al. 1989, Høegh-Guldberg and Smith 1989, Muscatine et al. 1989). N-limited cells in shallow water might assimilate most of the available DIN and consequently might exhibit minimal stable isotope fractionation (Wada and Hattori 1976). In contrast, zooxanthellae in light-limited deep-water corals may grow more slowly and consequently exhibit greater isotope discrimination. Wilkerson et al. (1988) estimated generation times for zooxanthellae from the same set of corals as analyzed in this study. When our $\delta^{15}N$ data are plotted against their growth rate data (as generation times) for zooxanthellae from all species at all depths, no significant correlation emerges ($\delta^{15}N = 1.38 + 0.02$ (generation time) ($n = 26$; $r = 0.01$). This appears to argue against a "growth rate fractionation" hypothesis. However, generation time is derived from zooxanthellae mitotic index and is a function of the standing stock of cells. It does not take into account the translocated nitrogen that does not appear in the standing stock of cell nitrogen. Consequently, the parameter of interest is the nitrogen-specific growth rate (u_N), manifested by the uptake and assimilation of DIN by zooxanthellae and the translocation of dissolved organic nitrogen (DON) to the host. That is, in symbiotic algae, and perhaps even in some free-living algae (see Zehr et al. 1988), nutrient uptake and assimilation is uncoupled from cell growth at the low specific growth rates exhibited by zooxanthellae in hospice. For example, from data on uptake rates of ^{15}N (as ammonium) by the shallow-water Red Sea coral *Stylophora pistillata* Esper, Muscatine et al. (1984) estimated that u_N was 0.31 day^{-1} for light-adapted cells and 0.25 day^{-1} for shade-adapted cells, a 20% decrease. This trend is consistent with our conjecture that u_N for coral zooxanthellae may decrease with decreasing irradiance at greater depths, so that rates of uptake, assimilation, and translocation are lower and the scope for fractionation is higher.

Alternatively, the lower $\delta^{15}N$ values (< 1.00 ‰), especially as depth increases, are

close to the generally accepted value of 0.00 ‰ for dissolved nitrogen in seawater (Owens 1987) and so could be derived from acquisition of DIN from fixed nitrogen sources. The lower values could also result from assimilation of local sources of ^{15}N-depleted ammonium, such as host catabolism, zooplankton excretion (Checkley and Entzeroth 1985), or from greater discrimination by the algae of locally elevated concentrations of ammonium and nitrate.

$\delta^{15}N$ *of Animal Tissue*

$\delta^{15}N$ of animal tissue ranged from +4.71 to +0.23 ‰. Corals living at 1 m depth had consistently lower animal tissue $\delta^{15}N$ values (mean $\pm$ SD = 3.36 $\pm$ 0.49 ‰; range, 2.79–4.11 ‰) than the nonsymbiotic coral *Tubastrea coccinea* (+4.74 ‰), living at the same depth. The reasons for these differences are still obscure, but are undoubtedly related to the fact that the diet of *T. coccinea* is solely allochthonous particulate and DON enriched in ^{15}N (e.g., zooplankton), whereas the diet of the symbiotic corals also includes substrates acquired from the zooxanthellae. Allochthonous substrates may also account for the increase in animal tissue $\delta^{15}N$ in *M. annularis* and *M. cavernosa* at 50 m depth. This interpretation is consistent with the previous interpretation that $\delta^{13}C$ of animal tissue versus depth is influenced by allochthonous POC (Muscatine et al. 1989).

Although the $\delta^{15}N$ values for animal tissue are low relative to those for *T. coccinea*, in the majority of coral species they are still slightly higher than those of their corresponding zooxanthellae. The difference, although not correlated with depth, is consistent with the general observation that, in most cases, the $\delta^{15}N$ of marine invertebrates and vertebrates is greater than their dietary $\delta^{15}N$ by an average of 2.6 $\pm$ 2.1 ‰ (Owens 1987). Coral animal cells could acquire ^{15}N-enriched substrates by translocation from zooxanthellae. Coral zooxanthellae release alanine in vitro (Muscatine and Cernichiari 1969) and may do so in situ (Lewis and Smith 1971). In addition, some zooxanthellae secrete nitrogen-containing macromolecules that may be acquired by the host (Markell and Trench 1993). Animal tissue could also be enriched in ^{15}N relative to zooxanthellae as a result of protein catabolism and excretion of isotopically light ammonium. More than 90% of the ammonium excreted by *Stylophora pistillata* is taken up by resident zooxanthellae (Rahav et al. 1989). Retention of such host excretory ammonium by zooxanthellae may be a general phenomenon among corals (Muscatine and D'Elia 1978) and would exacerbate the tendency of zooxanthellae to be depleted in ^{15}N relative to animal tissue.

Comparison with Other Endosymbioses

Relatively low (i.e., negative) $\delta^{15}N$ values have been reported for some, though not all, endosymbioses involving bacteria in worms and clams from communities associated with hydrothermal vents and deep seeps (Rau 1981, Paull et al. 1985, Brooks et al. 1987, Van Dover and Fry 1989, Rau et al. 1990*a*). The low $\delta^{15}N$ values are attributed to either assimilation of isotopically depleted nitrogen sources, fractionation, or nitrogen fixation (Rau 1985). Bacteria and host tissues in protobranch bivalves (*Solemya reidi*) from shallow-water reducing sediments have similar $\delta^{15}N$ values. The similarity is attributed to assimilation by bacteria of a nonlimiting DIN source, such as porewater ammonium, and translocation of DON to the host (Conway et al. 1989). However, like endosymbiotic algae, although the cell-specific growth rate of endosymbiotic chemoautotrophic bacteria in *S. reidi* is relatively low (see Cavanaugh 1985), the nitrogen-specific growth rate could be relatively high and as such could be a factor that influences $\delta^{15}N$ values.

$\delta^{13}C$ *and* $\delta^{15}N$ *versus Depth*

The analysis of multiple stable isotopes has been used to provide insight into the diet of a variety of organisms and their trophodynamics (see Owens 1987, Rau et al. 1990*a*,*b*, 1991). Figure 1 provides a first glimpse of variation in $\delta^{13}C$ and $\delta^{15}N$ versus depth for animal tissue and zooxanthellae in Jamaican

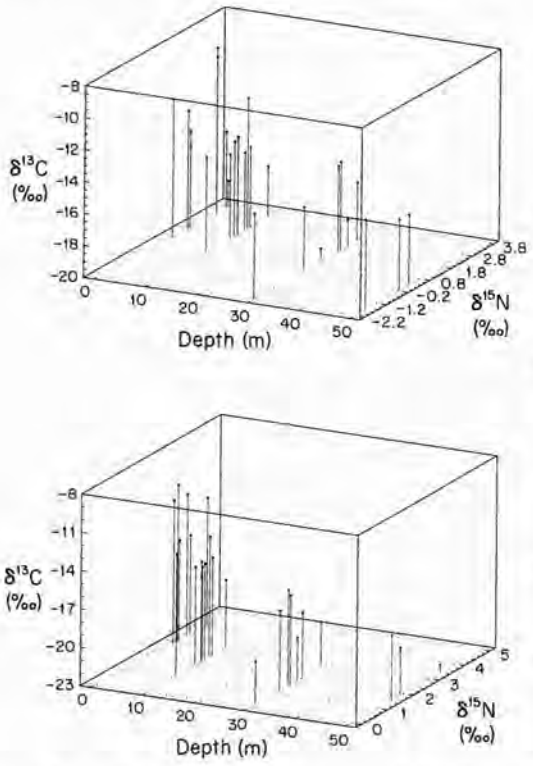

FIGURE 1. $\delta^{15}N$ and $\delta^{13}C$ for zooxanthellae (upper) and animal tissue (lower) versus depth for nine species of Jamaican corals. Data from Table 1 (this paper) and Table 1 of Muscatine et al. (1989).

corals over a 50-m bathymetric range. The data reveal a tendency toward depletion of ^{13}C and ^{15}N in both the zooxanthellae and animal tissue in most species as depth increases. Increased carbon fixation is apparently accompanied by decreased N fractionation. Additional measurements may prove useful in interpreting stable isotope abundances and in elucidating resource partitioning strategies in symbiotic corals.

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Ratio of Energy and Nutrient Fluxes Regulates Symbiosis between Zooxanthellae and Corals[1]

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ABSTRACT: Ambient irradiance levels determine the rate of carbon influx into zooxanthellae at any given time, and thereby the energy available for the whole coral symbiotic association. Long-term photoacclimation of zooxanthellae to the time-averaged light regime at which the host coral grows results in optimization of light harvesting and utilization. Under high irradiance light harvesting is reduced, thereby avoiding photodynamic damage, whereas under low light, photon capture and quantum yield are maximized. Most of the photosynthate produced by the algae is respired. However, the capability of the zooxanthellae and the coral to retain carbon beyond that required to meet their respiratory needs depends on the availability of the commonly limiting nutrients, nitrogen and phosphorus. Therefore, the ratio of the flux of these nutrients into the colony to that of the photosynthetically driven carbon flux will regulate the growth of the zooxanthellae and of the animal. Nutrients acquired by predation of the coral on zooplankton are available first to the animal, whereas those absorbed by the zooxanthellae from seawater as inorganic compounds lead first to growth of the algae.

IT HAS BEEN an accepted dogma in coral biology that the extensive spatial and long-term temporal success of coral reefs in the oligotrophic littoral of tropical seas stems from the symbiotic association between endocellular microalgae (the zooxanthellae) and the host hermatype. It is this association that allows corals and coral reef communities to thrive in spite of the low concentrations of nitrogen and phosphorus in the oligotrophic ambient waters (Muscatine and Porter 1977). It is this oligotrophy that also causes the striking paucity of phyto- and zooplankton in these "blue deserts," limiting the availability of particulate food as an alternate nutrient source.

In this mutualistic symbiosis the algae contribute their capability to harness sunlight to the photosynthetic production of high-energy compounds, mainly carbohydrates. These are produced in great excess of that needed to support the basic metabolic needs of the zooxanthellae. However, because these compounds have a very high C : N ratio, they cannot by themselves support multiplication of the algae. The excess photosynthate, which may reach as much as 95% of the total, is "translocated" to the host animal, a process stimulated by "host factors" that dramatically increase the excretion of assimilated carbon compounds by the algae (Muscatine 1967, Sutton and Høegh-Guldberg 1990). The translocated carbon compounds are more than enough to provide for the respiratory needs of the host (Muscatine et al. 1984), although, as is also the case for the zooxanthellae, they cannot support growth of animal tissue. These energy-rich, nitrogen-poor products of photosynthesis were termed "junk food" because of their insufficiency as food for growth (Falkowski et al. 1984).

In return for fixed carbon, the zooxanthellae gain access to the high nitrogen and phosphorus metabolic waste products of their

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host. Thereby the colony retains and recycles these precious substances, which in the absence of the algae would have been excreted into the sea and lost to the system. In addition to photosynthetic capability, the two partners in the coral symbiosis also differ in that the animal is an avid and efficient predator on zooplankton, an important source of nitrogen, while the zooxanthellae are capable of assimilating inorganic nitrogen and phosphorus compounds inaccessible to the animal. However, it is important to bear in mind that under normal reef conditions both of these sources are very limited. Therefore, although the very efficient uptake by the zooxanthellae of nutrients produced by the host animal assures recycling of these resources and prevents their loss from the association (Rahav et al. 1989), this cannot account for growth or "new production."

The effects of the modulation of the flux of carbon into zooxanthellate corals in response to different light intensities, as those encountered at different depths, has been examined in some detail, mostly in a series of studies of the common Red Sea coral *Stylophora pistillata* Esper (Falkowski and Dubinsky 1981, Muscatine et al. 1983, 1984, Dubinsky et al. 1984, Falkowski et al. 1984, Porter et al. 1984). It has been found that the zooxanthellae photoacclimate within a week to a new irradiance level and that the host also responds to this change. Among the reported differences between high (HL)- and low-light (LL) corals were differences in areal chlorophyll *a*. These were a result of up to four-fold increases in the concentration of this pigment in the LL zooxanthellae. This difference was clearly mirrored on the ultrastructural level, as a corresponding difference in thylakoid area (Dubinsky et al. 1984, Berner et al. 1987).

In most cases, photoacclimation was reported to occur primarily on the cellular level of the zooxanthellae, and their densities remained around 10^6 cells cm^{-2} (but see also Dustan 1979 and Titlyanov 1991). However, under extremely low irradiance the algae are confined to the side of the colony facing the light (Figures 1 and 2).

The strategy of photoacclimation in the zooxanthellae of *S. pistillata* is by change in size, not in the number, of photosynthetic units (Falkowski and Dubinsky 1981), although this may not be true for all zooxanthellae (Chang and Trench 1982). It was also found that the HL zooxanthellae had higher dark respiration and light-saturated photosynthetic rates and lower quantum yields than their LL counterparts (Falkowski and Dubinsky 1981, Dubinsky et al. 1984, Porter et al. 1984). The animal responses to the different light regimes included higher respiration and calcification in the HL corals (Dubinsky et al. 1983, Porter et al. 1984). In those studies it was also concluded that in *S. pistillata*, under high light, photosynthesis is sufficient to provide substrata for both animal and algal respiration, which is not the case in LL colonies, which have to supplement algal photosynthesis by animal predation (Falkowski et al. 1984).

In a subsequent series of studies the effect of added nutrients and of feeding on *Artemia salina* (Linnaeus) nauplii on *S. pistillata* was examined (Muscatine et al. 1989, Dubinsky et al. 1990, Falkowski et al. 1993). Although nutrient-enriched colonies changed within 3 weeks to nearly black, whereas the controls remained ivory colored, making them look like LL and HL colonies, this change resulted not from change in the pigment content in the zooxanthellae as was the case in photoacclimation, but from an up to five-fold increase in algal population. Nutrient enrichment brought about additional changes in the interrelation between the zooxanthellae and the coral. Division rate of the zooxanthellae increased (Høegh-Guldberg 1994), but their photosynthetic rates on a per-cell basis decreased, probably resulting from carbon limitation in the dense algal population (Dubinsky et al. 1990). The fraction of photosynthate translocated to the host also decreased.

In studies on the effect of nutrient enrichment done in Hawaii with *Pocillopora damicornis* (Linnaeus), it was also found that nutrient enrichment resulted in increased algal density (Stambler et al. 1991). In those studies nutrient enrichment also led to significantly reduced calcification rates (Stimson

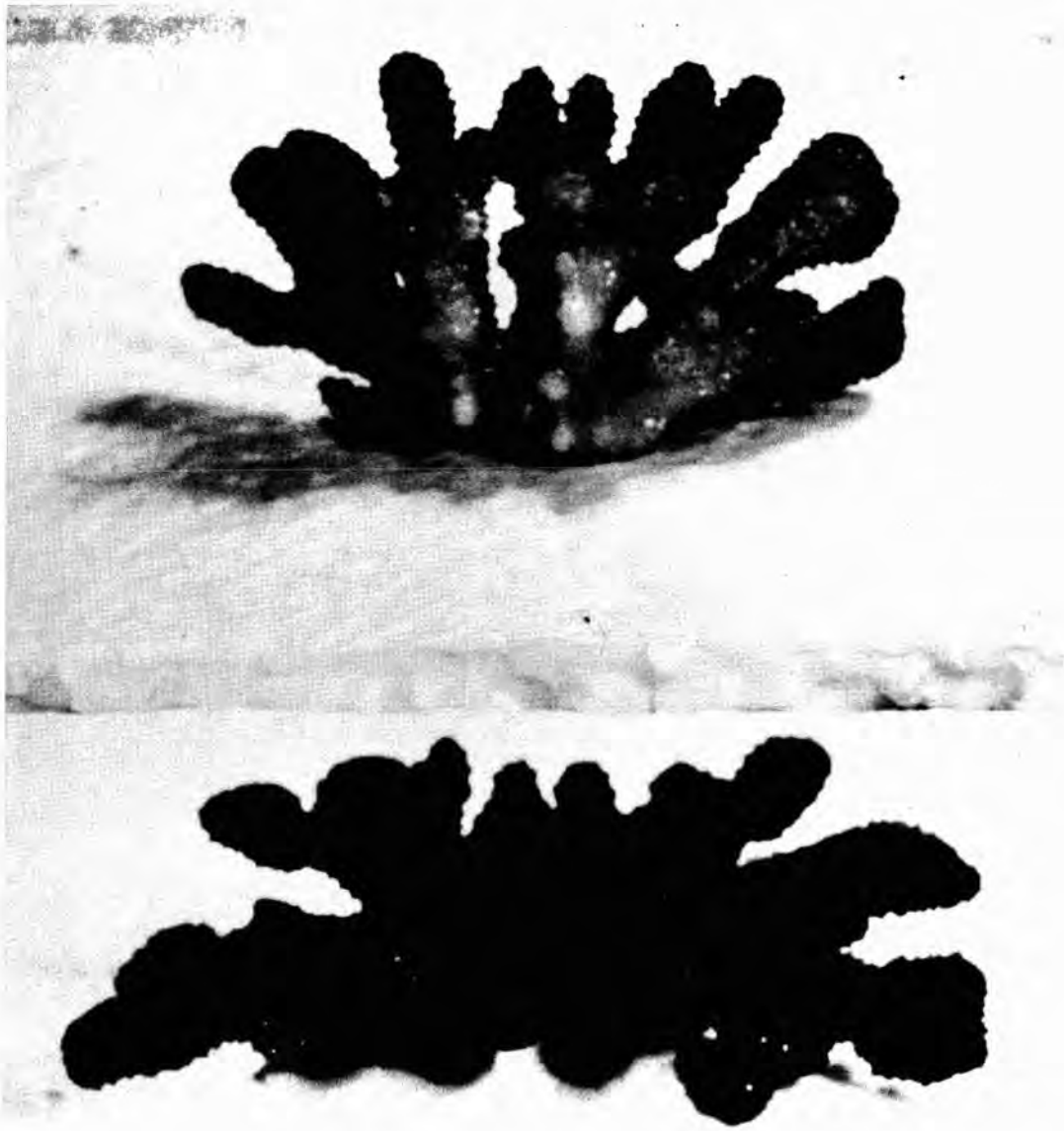

FIGURE 1. *Stylophora pistillata* colony from a deep, shaded crevice. The dark side is the one facing the light; the side facing the rear wall, reflected in the mirror, is nearly white.

1992). Nitrogen enrichment was also reported to weaken the skeletal architecture of corals (Yamashiro 1992) and slow down its growth (Stambler et al. 1991).

In this study we examine the possible interactions between the underwater light and nutrient fields surrounding corals. The effects of light and of nutrients were hitherto studied separately, and we shall attempt to integrate the concepts that emerged from these studies.

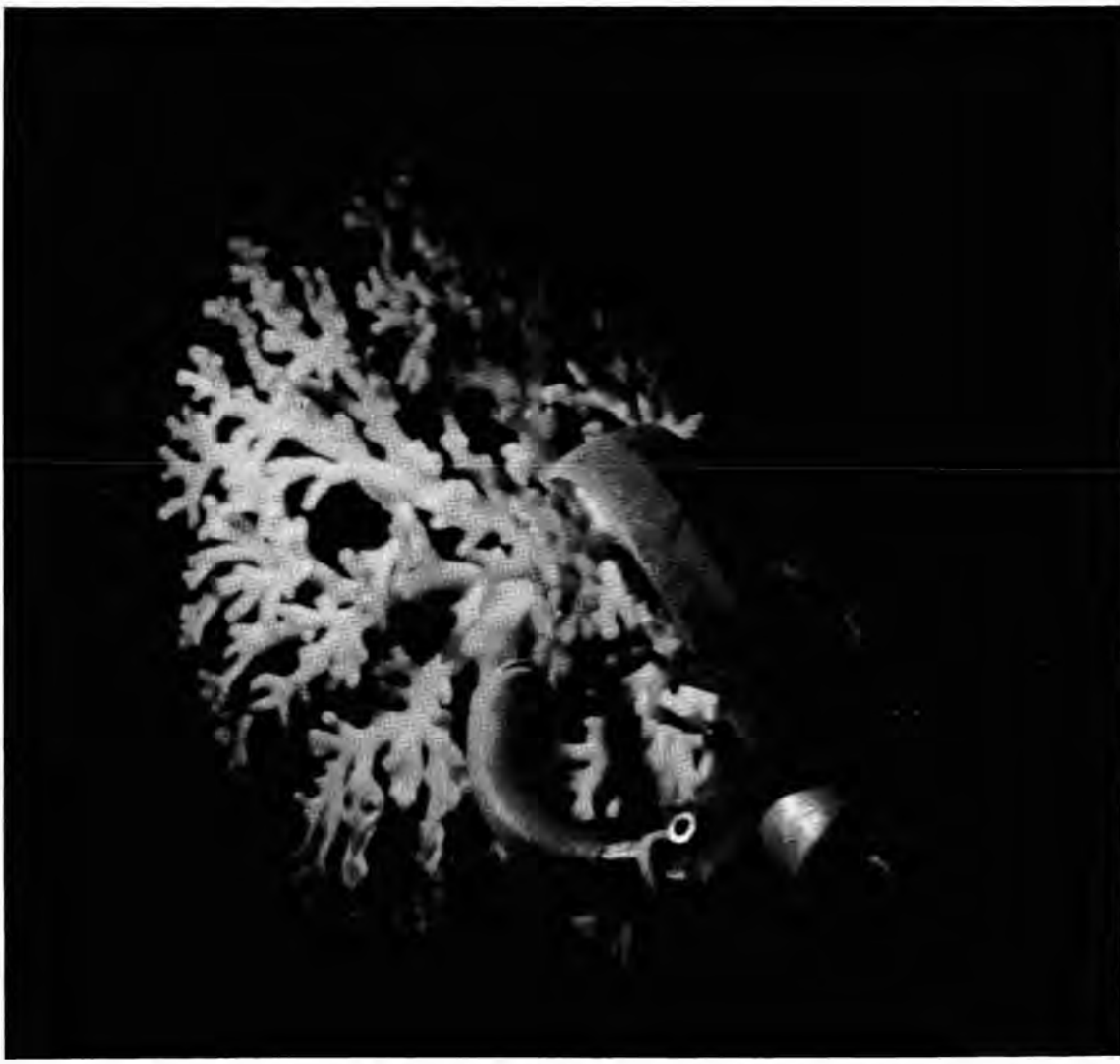

FIGURE 2. *Stylophora pistillata* at 66 m in the Gulf of Elat, northern Red Sea. Zooxanthellae are found only on the up-facing, dark surface; none are on the down-facing side (shown).

Light and Nutrient Flux

When corals exposed to various light intensities are compared, two interesting questions emerge. First, in LL corals, photosynthesis alone cannot account for all of the colony respiration; therefore such colonies have to supplement zooxanthellae autotrophy with heterotrophic animal predation on zooplankton (Falkowski et al. 1984). Indeed, under low light most corals remain with their tentacles extended continuously (Figure 3). Second, why should HL corals, which have more photosynthate produced than that needed to support respiration (Figure 4*a* and *b*), still have to hunt for zooplankton. Although such HL colonies usually extend their tentacles only after sundown, why should they extend them at all? Yonge (1930:54) wrote "Corals as a general rule, expand only

FIGURE 3. *Stylophora pistillata* colony growing under low light. Polyps remain constantly extended.

at night when, as the results of the investigations on plankton will make abundantly clear, their food is most abundant." There is no doubt that these corals are hunting quite efficiently the zooplankton that rises at night from deeper waters.

Because the nutrient concentration to which LL and HL corals are exposed does not differ, we would assume that they do not differ in their nutrient status. However, this seems not to be the case. To grow, both zooxanthellae and coral have to acquire carbon and nitrogen at the same ratios found in their biomass, but assuming that inorganic nitrogen intake by the zooxanthellae is controlled by its concentration in the water, that of carbon is governed by photosynthesis and, thereby, by irradiance. Therefore, although LL colonies acquire carbon and nitrogen at the C : N ratio of 9.97, HL colonies acquire them at a 30.15 ratio (Falkowski et al. 1984, Muscatine et al. 1984). From this follows that while at low light corals may be only slightly nitrogen-limited and, assuming that respiration preferentially uses high C : N compounds, may in fact not be nitrogen-limited at all, HL corals have to be severely nitrogen-limited, although both grow in the same water. In an analysis of the products of photosynthesis in corals growing at different depths, it was indeed found that in deep-water corals a much higher fraction of photosynthetically assimilated ^{14}C was incorporated into amino acids than in shallow-water (HL) corals (Bil' et al. 1992).

We suggest that, unlike LL colonies, HL corals do not hunt zooplankton for their carbon but rather for their nitrogen (Atkinson 1992). Of course, the nutrient requirement of the zooxanthellae population also depends on algal numbers. Indeed, under reduced densities of zooxanthellae, like those occurring in partially bleached coral colonies, the algae are nutrient sufficient (Cook et al. 1992).

Although it is easy to see why LL corals

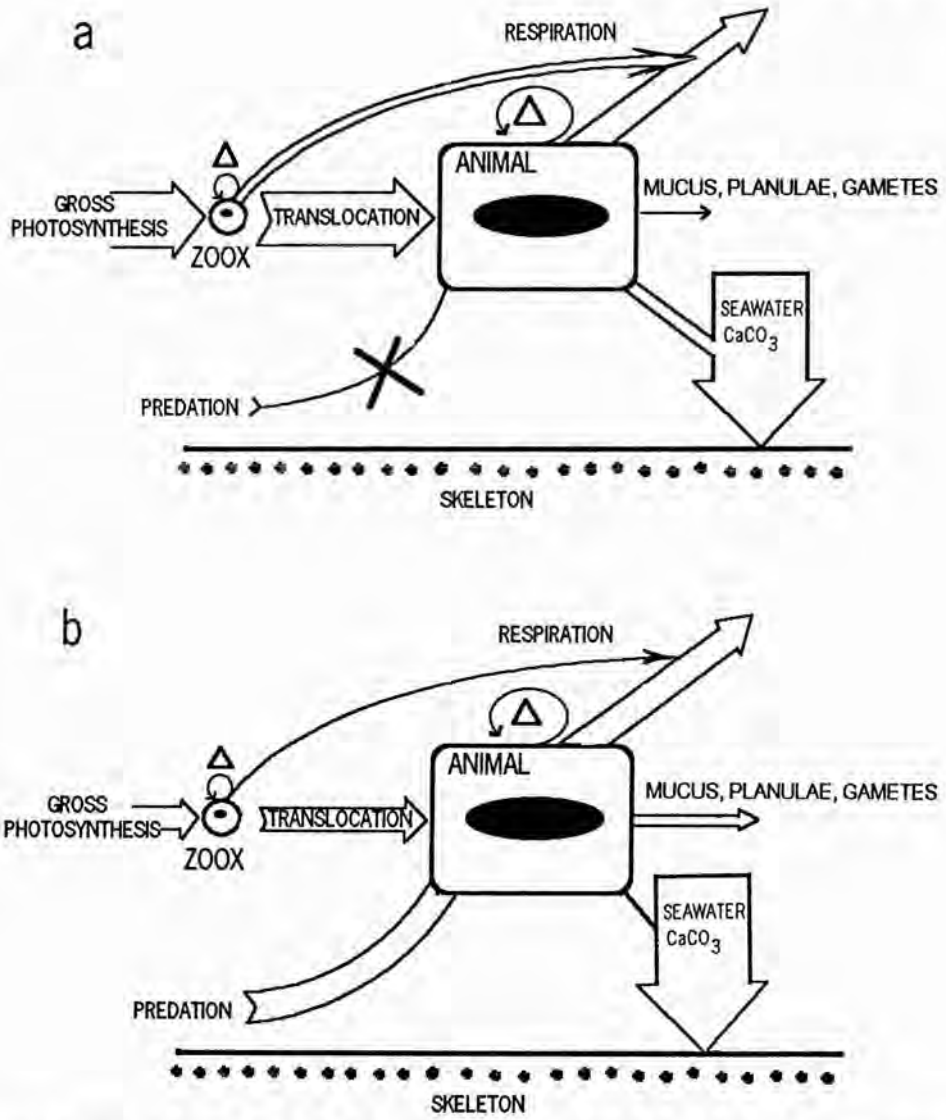

FIGURE 4. Carbon budget for 24 hr in HL (*a*) and LL (*b*) colonies of *Stylophora pistillata* (redrawn after Falkowski and Dubinsky 1981).

would extend their tentacles continuously, to capture as much of the scarce zooplankton as possible, we do not have an explanation of the daylight retraction of tentacles by most (not all) shallow-water corals, as may be seen in Figures 5–7 (Abe 1939). In the Red Sea and in the Caribbean (Porter 1974), this is indeed the rule, with very few exceptions, such as *Goniopora lobata* Edwards & Haime. However, this may not be a universal phenomenon (Lasker 1979) and is not the case in Hawaiian reefs. An analogous situation was described when *S. pistillata* colonies from different depths and irradiance levels were compared for their prey hunting and killing efficiency (E. A. Titlyanov, V. A. Leletkin, and

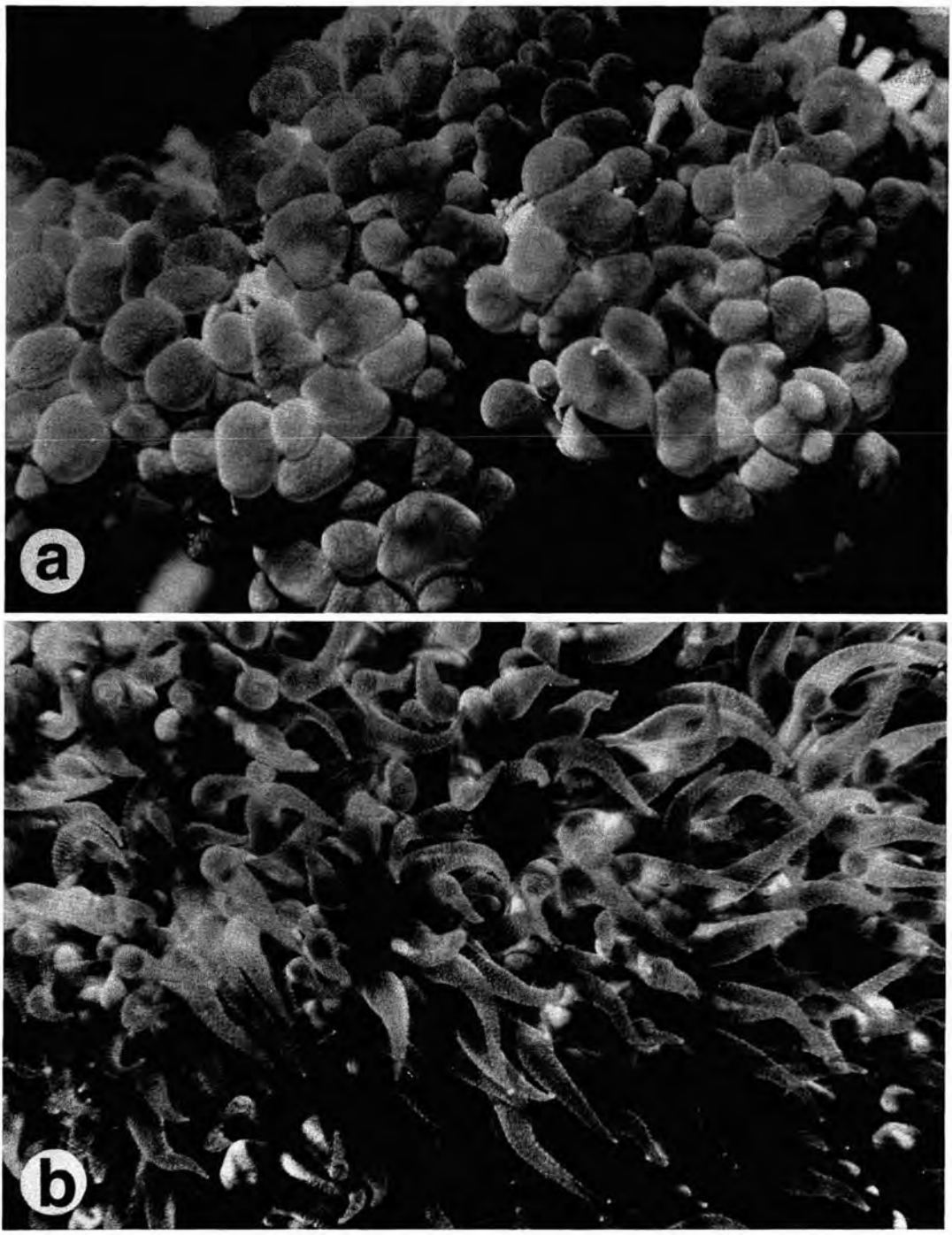

FIGURE 5. The Red Sea coral *Pleurogyra sinuosa* (Dana): (*a*) during daytime, with polyps contracted, vesicles extended; (*b*) at night with polyps fully extended, nematocyte batteries visible, vesicles collapsed.

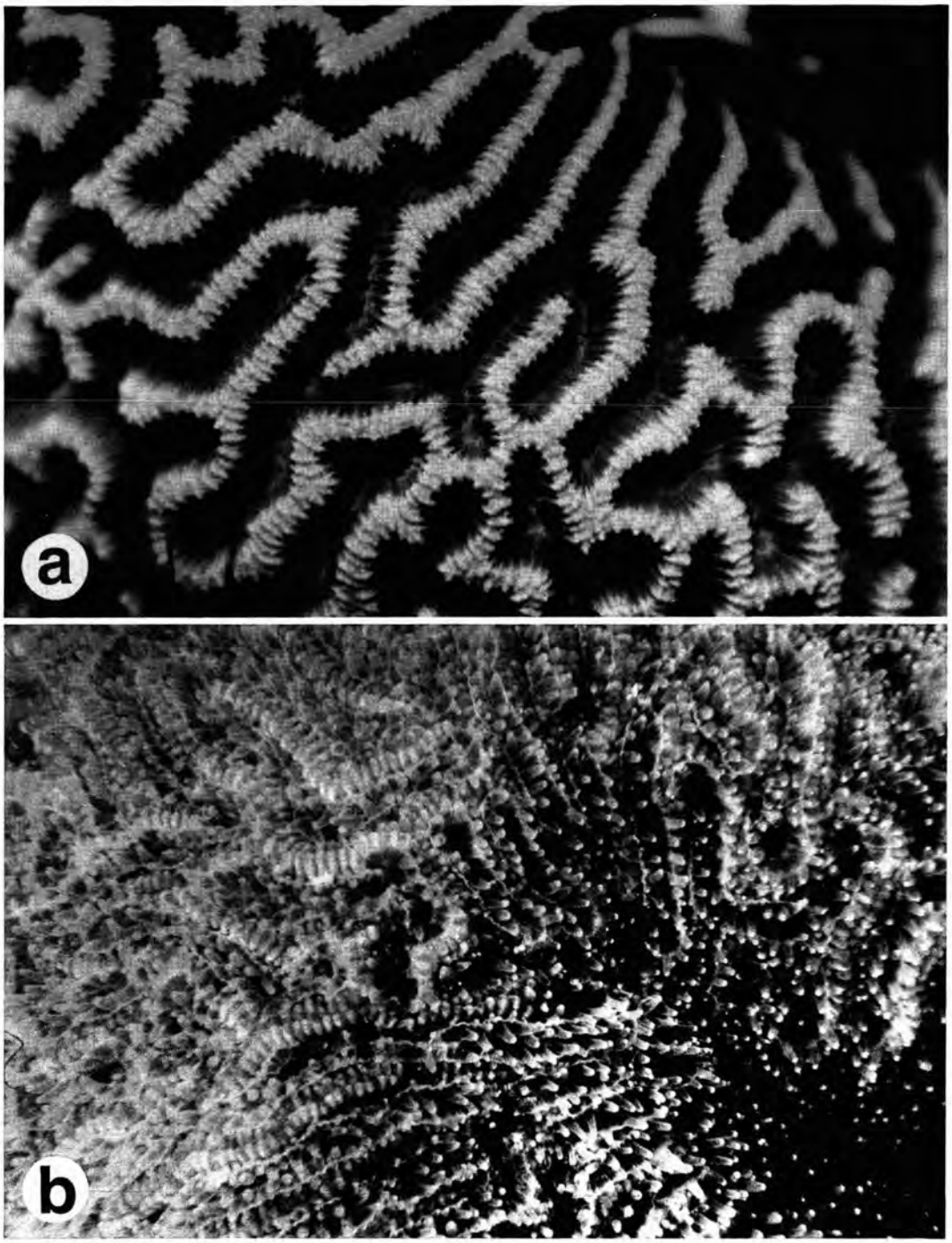

FIGURE 6. The Red Sea coral *Platygyra lamellina* (Ehrenberg): (*a*) during daytime, with polyps contracted; (*b*) at night, with polyps fully extended.

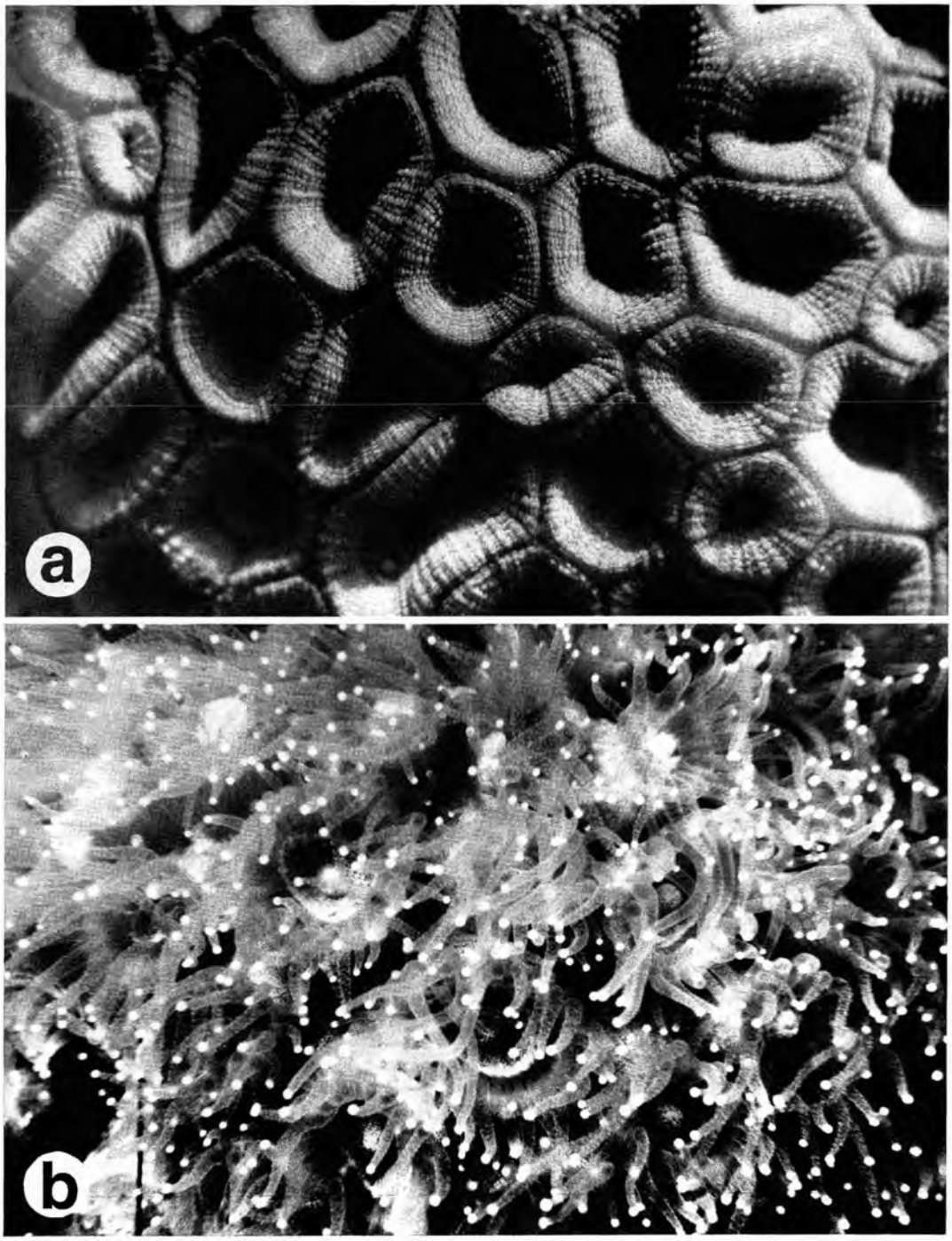

FIGURE 7. The Red Sea coral *Favites flexuosa* (Dana): *a* and *b* as in Figure 6.

Z.D., unpublished data). In all cases HL colonies showed lower prey-hunting efficiency than the deep-water, LL colonies.

The Effects of Nutrient Enrichment and Feeding on Carbon Flux

If we examine the energy and nutrient relationships from the nutrient end, we may find some additional interesting interactions. Because to increase in numbers zooxanthellae have to acquire on the order of one atom of nitrogen for every seven carbon atoms, it follows that any carbon in excess of this ratio will be either respired or translocated to the host. In corals exposed to elevated nutrient levels, the zooxanthellae, instead of acting like a carbon-moving conveyer belt translocating "junk food" to the coral host, retain seven carbon atoms for every nitrogen atom absorbed from the water. This results in two changes in the symbiotic association. Less carbon is translocated to the host, the C : N ratios in the algae decrease (Muscatine et al. 1989, Muller-Parker et al. 1992), and the algae are able to use photosynthetically produced carbon skeletons for the synthesis of nitrogen-containing molecules required for cell multiplication, such as amino acids and nucleotides. The algal population increases two- to five-fold in numbers, with their respiratory needs being satisfied before those of the coral. As a result of this growth in algal population, the increased algal population becomes carbon-limited, producing less photosynthate per cell (Dubinsky et al. 1990); of the total produced, more is respired, and more is retained, ending up as new zooxanthellae cells.

It also was shown that if the coral is fed zooplankton, not only will the coral tissue be able to grow, as is shown in an increase in animal protein (Muscatine et al. 1989), but as a result of enhanced metabolism (Rahav et al. 1989) and digestion the zooxanthellae will be provided with nitrogen by "reverse trans-

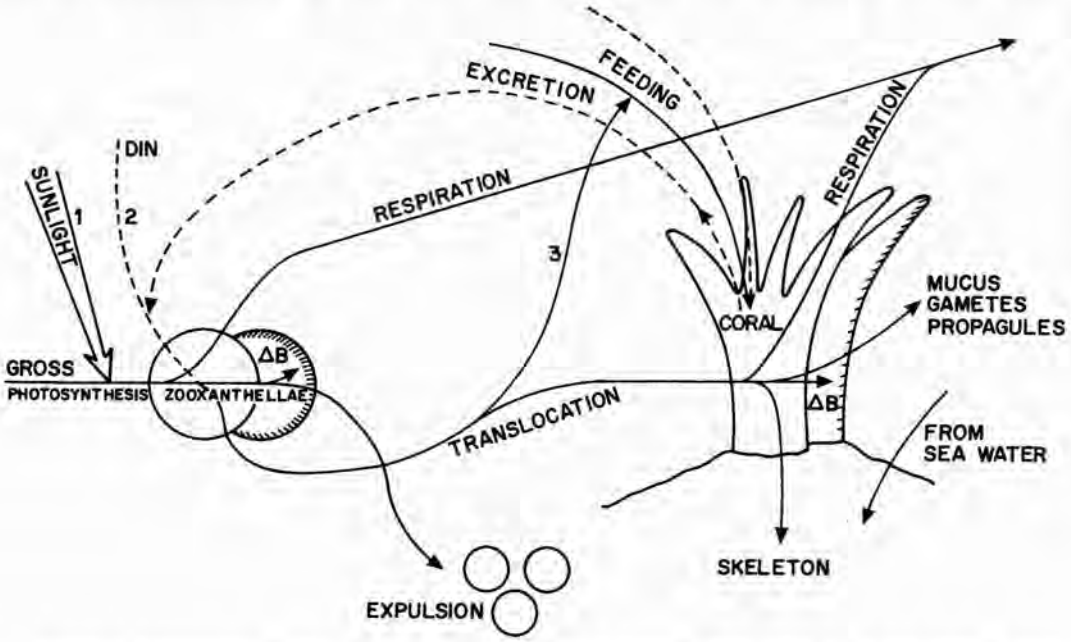

FIGURE 8. Interactions between nitrogen and carbon fluxes in zooxanthellate corals. Light and dissolved inorganic nitrogen control the allocation of carbon to either proliferation of algae or translocation to host. Translocation affects the importance and intensity of feeding. Solid arrows represent carbon fluxes, dashed lines fluxes of nitrogen. 1 and 2 are the main external forcing functions; 3 is an internal feedback loop. Shaded areas represent biomass growth.

location." Feeding on zooplankton, by increasing nitrogen supply, will reduce the uptake of ammonium from the water by the zooxanthellae, as was shown using ^{14}C methylamine, a nonmetabolizable ammonuim analogue (D'Elia and Cook 1988). Al-Moghraby et al. (1992) recently reported that feeding *Galaxea fascicularis* (Linnaeus) polyps reduced their uptake of free amino acids from the water.

Conclusions

Figure 8 summarizes the main interactions and feedback mechanisms connecting light intensity, nutrient level, and feeding in zooxanthellate corals: (1) Under constant nutrient concentration, light intensity determines the onset of nutrient limitation; as light increases, C : N ratios exceed Redfield ratios. (2) The availability of other nutrients, mainly nitrogen, determines the fate of photoassimilated carbon. Under high C : N ratios, most carbon goes into respiration, calcification, and excreted mucus, whereas low C : N ratios favor increases in zooxanthellae density, reduce translocation, and slow down calcification. (3) Feeding on zooplankton by the coral under low light provides carbon for metabolism. Under high light it supplies both algae and animal with nitrogen.

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