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

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ABSTRACT: Branches of the hermatypic coral *Pocillopora damicornis* (Linnaeus) were exposed for 2, 4, 6, and 8 weeks to ammonium concentrations of $<1 \mu M$ (nutrient-stripped), $2 \mu M$ (seawater as a control), $20 \mu M$, and $50 \mu M$ (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, on-going 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 sea-water 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
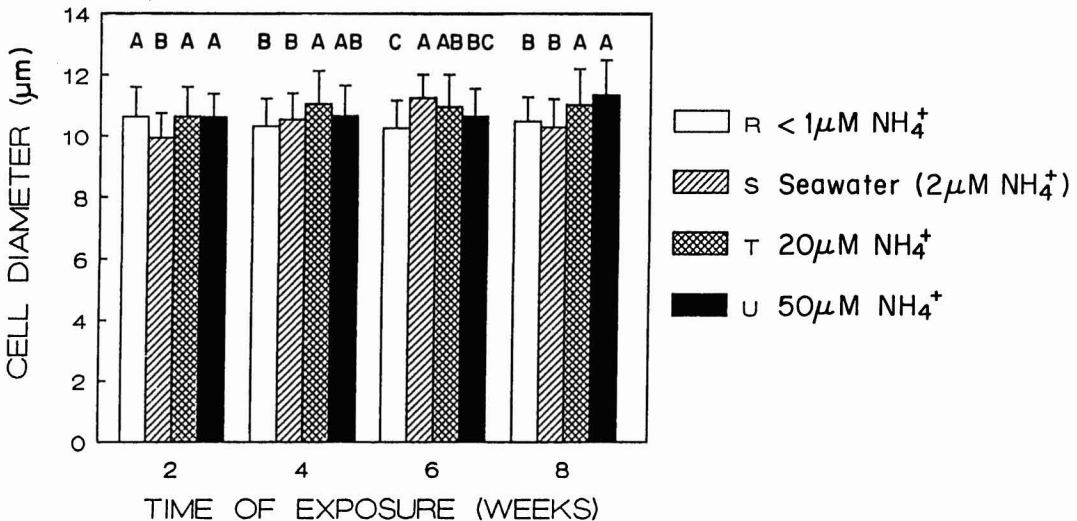

FIGURE 1. Cell diameter as a function of time of exposure to different levels of nitrogen. Bars represent standard errors. Different letters above columns show that means are significantly different according to the Duncan multiple range test; $n = 40$.
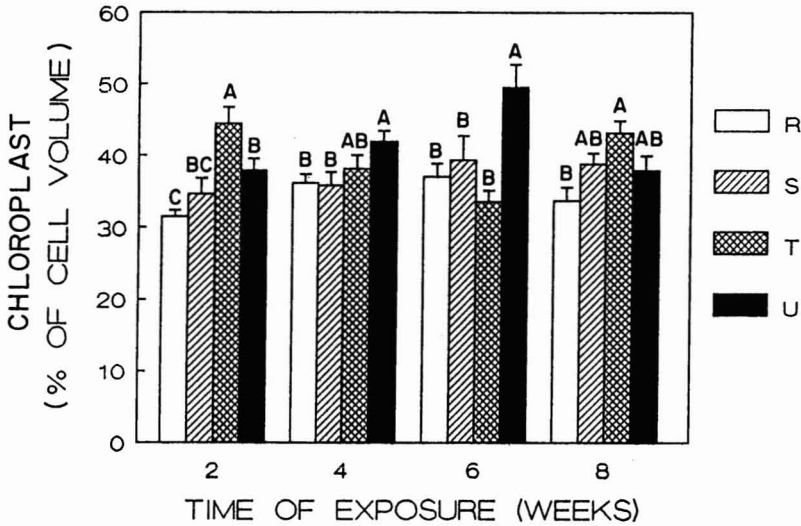
FIGURE 2. Volume ratio of chloroplast to cell as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

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 photo-acclimation (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 differ-

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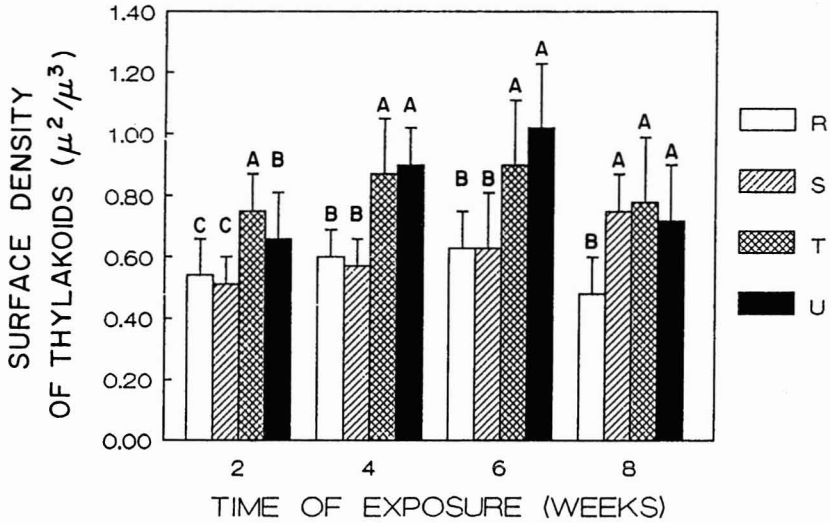
FIGURE 3. Surface density of thylakoids in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.
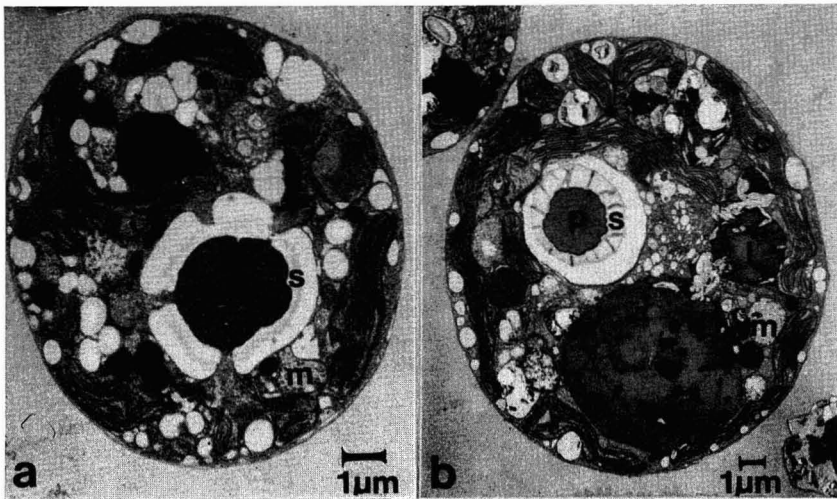

FIGURE 4. Electron micrographs of low (*a*) and high (*b*) nitrogen concentrations. c, chloroplast; l, lipids; m, mitochondria; n, nucleus; p, pyrenoid; s, starch.

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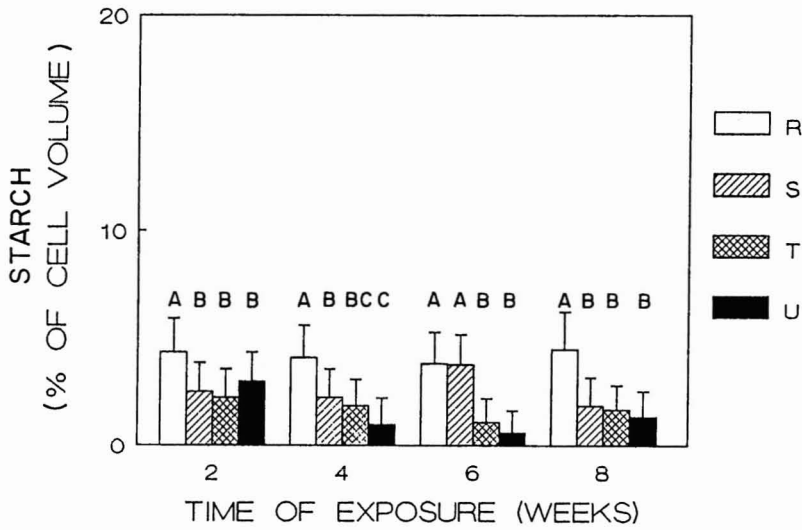
FIGURE 5. Volume ratio of starch to cell in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.
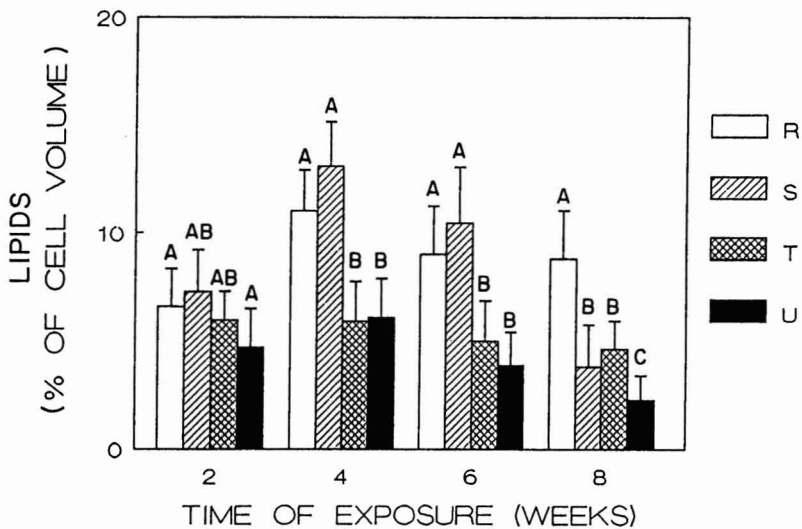

FIGURE 6. Volume ratio of lipids to cell in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

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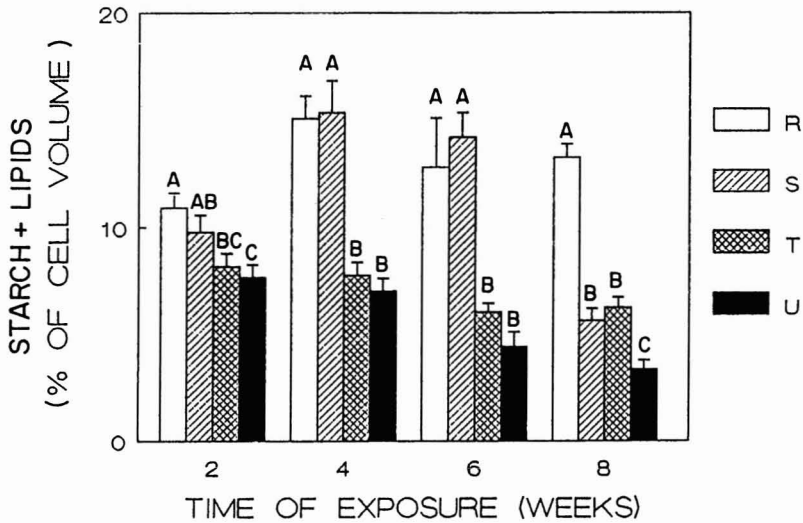
FIGURE 7. Combined volume ratio of starch and lipids to cell in zooxanthellae as a function of time of exposure to different levels of nitrogen. For explanation of legend, see Figure 1.

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.

ACKNOWLEDGMENTS

Bar-Ilan University: Zvy Malik and Yehudith Chanaya. The critical discussion with Zvy Dubinsky and Noga Stambler and their thoughtful comments on the manuscript were most crucial.

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