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Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals

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Abstract Very little information exists on the effects of nitrate on corals, although this is the major form in which nitrogen is present in tropical eutrophic coastal waters. In this study we incubated nubbins of *Porites porites* and explants of *Montastrea annularis* in laboratory photostats illuminated by halide lamps, with concentrations of nitrate of 0, 1, 5 and 20 μM, for 40 and 30 d, respectively. At the end of this period it was found that the population density of the zooxanthellae had increased significantly with increased nitrate concentration, suggesting nitrogen limitation of the growth rate of zooxanthellae in the control group. There were also significant increases in the amount of chlorophyll *a* and c_2 per algal cell, in the volume of the algal cells, and in the protein per cell. Overall, the protein per unit surface increased, but this was attributable solely to increased algal protein: there was no significant change in host protein. Maximum gross photosynthesis normalized to surface area was enhanced by nitrate addition, reflecting the increase in algal population density. There was no change when normalized on a per cell basis. Respiration rate normalized to protein content was decreased by nitrate. The most dramatic change was in the rate of skeletogenesis, which decreased by ≃50% in both species when exposed to nitrate enrichment. A model is presented which suggests that the diffusion-limited supply of CO_2 from surrounding seawater is used preferentially by the enlarged zooxanthellae population for photosynthesis, thereby reducing the availability of inorganic carbon for calcification. It is concluded that enhanced nitrate levels in tropical coastal waters will have a hitherto unrecognized effect on the growth rate of tropical coral reefs.

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Introduction

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

Although effects in terms of changes to community structure have been documented, very little is known of the causal mechanisms involved and of the specific response of individual organisms to nutrient enrichment. Experimental work on hermatypic corals has largely centred on the effects of elevated levels of ammonia: it has been shown that exposure to 20 μM NH_4^+ for 3wk results in a doubling of the population of zooxanthellae, a decrease in the rate of photosynthesis per algal cell (Muscatine et al. 1989; Hoegh-Guldberg and Smith 1989; Dubinsky et al. 1990; Stimson and Kinzie 1991), and a decrease in the rate of skeletogenesis (Stambler et al. 1991; C. Ferrier-Pagès personal communication). However, in situ on reefs, corals are not normally exposed to elevated levels of ammonia. Inorganic nitrogen in its reduced form is rapidly removed, and it is in the oxidised form, as nitrate, that concentrations build up. Very little information is available on the effects of elevated levels of nitrate on corals. Earlier studies on the uptake of nitrate by symbiotic anthozoans produced equivocal results. Some corals appear to remove nitrate from seawater (e.g. Franzisket 1974; Webb and Wiebe 1978; Bythell 1990), while others, and all symbiotic

anemones do not (Wilkerson and Trench 1986). The methodology of these nitrate-depletion experiments has been questioned by Miller and Yellowlees (1989), who suggested that depletion could result from bacterial assimilation. On theoretical grounds, the likelihood of nitrate uptake has been questioned by Miller and Yellowlees, who pointed out that uptake into the host cytoplasm would require the presence of a specific nitrate-carrier mechanism in the host-cell membranes. Furthermore, nitrate assimilation by the algae would require the induction of nitrate reductase, and this normally only occurs when ammonia is limiting (Syrett 1981). It is perhaps because of the ambiguities in the published accounts of nitrate uptake, coupled with theoretical considerations, that there is a dearth of information on the effects of nitrate on coral physiology.

In this paper, which forms part of a wider study on the ecotoxicology of some Caribbean corals, we investigate the effects of a 3 wk exposure to elevated levels of nitrate on photosynthesis and respiration, on algal and host biomass, and on the rate of skeletal deposition.

Materials and methods

Corals and their preparation

Branches from several adjacent colonies (>10) of *Porites porites* and two colonies of the massive coral *Montastrea annularis* were collected from the fringing reefs on the west coast of Barbados, and transferred to aquaria in the Bellairs Research Institute. Branch tips of *P. porites* were made into nubbins by cementing them on to 3 cm × 3 cm acrylic tiles, whilst 25 mm cores ("explants") of *M. annularis* were cemented into PVC cups as described by Davies (1995). The coral preparations were then transferred on racks to the reef at a water depth similar to that of the original "parent colonies" (≃ 2 m), and were allowed to recover for 3 wk before use in experiments.

Experimental chambers and treatment regimes

During the experiments, the corals were maintained in 7-litre glass chambers, termed photostats, in a constant-temperature water bath maintained at 27 °C ± 0.5 C°. In order to create turbulent water flow, each photostat was fitted with a glass tray forming a false floor, beneath which were two tubes with fan-shaped openings connected to an aquarium air pump. The trays had the effect of trapping the air bubbles until these were large enough to escape around the sides. The rate of water flow was judged to be 37% of that of corals on the fringing reef, based on measurements of the comparative rates of dissolution of plaster of Paris clod cards (Doty 1971). The photostats were illuminated by two metal halide lamps, providing 250 ± 50 μmol photons $m^{-2}s^{-1}$ for 13 h per day.

Four photostat chambers were used and moved around the waterbath at 3 d intervals to minimize any difference due to position relative to lamps and air pumps. Seawater was supplied to each chamber by a peristaltic pump. The control chamber received oligotrophic water (PO_4^{2-} = 0.05 μ*M*; NO_3^{2-} = 0.2 μ*M*) freshly collected from 3 km offshore. The three experimental chambers received seawater enriched with KNO_3 to give inflow concentrations of 1, 5 and 20 μ*M*. Residence time in the chambers was ≃ 20 h. The actual nitrate concentration in the chambers was measured at intervals of 4 d, by the method of Strickland and Parsons (1972). Corals were maintained under these experimental regimes for 40 d (*Porites porites*) or 30 d (*Montastrea annularis*). The physiological measurements at the end of the experiment took 8 d to accomplish for each species. Within each species, the sequence of analysis was random across treatments, with no more than two corals from the same treatment analysed sequentially. The longer exposure duration for *P. porites* was to accomodate these measurements on *M. annularis* which was sampled first. On every fourth day, the corals were removed for 15 min to aquaria containing the same concentrations of nitrate in order to allow the photostat chambers to be cleaned and refilled.

Measurement of photosynthesis and respiration

Rates of photosynthesis and respiration were measured at the end of the experimental period in a twin-chambered respirometer constructed of transparent acrylic. The 90 ml chambers were surrounded by a waterjacket maintained at 27 °C ± 0.5 C°, and were fitted with microcathode oxygen electrodes which were connected to oxygen meters (Strathkelvin Instruments, Glasgow) whose output was displayed on a two-channel chart recorder. The water in the chambers had the same nitrate concentration as the treatment water, and was stirred with a magnetically coupled spinbar. The respirometer was located within an aluminium foil-lined hood with an overhead bank of high-energy fluorescent light tubes (Philips high-frequency regulation luminaires). The power to the lights was varied using a Philips LPS100 control potentiometer, to provide illumination levels in the chambers of 25, 50, 80, 120, 200, 300 and 440 μmol photons $m^{-2}s^{-1}$. Dark respiration was measured over a period of ≃ 30 min during which the oxygen in the chambers was depleted to ~ 70% saturation. The lights were then switched on at the lowest level of irradiance and the illumination was increased sequentially. The oxygen level in the chambers at the end of a photosynthesis measurement run was not more than 110% saturation. Gross photosynthesis (P_{gross}) values were obtained from values of net photosynthesis (P_{net}) plus dark respiration, and plotted vs irradiance using a hyperbolic tangent curve-fitting program.

Measurement of skeleton growth

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

Biometry and biomass measurements

The surface area of *Porites porites* nubbins was calculated from calliper measurements of the height and diameter, considering each nubbin to comprise a cylinder surmounted by a terminal half sphere (see Rinkevich and Loya 1983).

Tissue-biomass values were measured following sacrifice of the nubbins and explants at the termination of the experiment. The tissues of five corals of each species were removed using a Waterpik with a 35‰ w/v solution of NaCl. This was used in preference to seawater, to avoid interference of Ca^{2+} ions with sodium dodecyl sulphate (SDS) used in the protein assay. The slurry was homogenised in a hand-held Potter homogeniser. Total protein content of the homogenate was determined on two 0.5 ml aliquots, using the Markwell et al. (1978) modification of the Lowry et al. (1951) method. Two 1.0 ml samples of homogenate were fixed and stained with Lugol's iodine, and zooxanthellae density was determined from ten counts per sub-sample using a haemocytometer. A subsample of fresh homogenate was used for the determination of zooxanthellae volume by measuring the cell diameter at a magnification of × 1250 under bright-field illumination on × 25 zooxanthellae per coral.

The remaining homogenate was used to prepare a clean suspension of zooxanthellae by differential filtration. The homogenate

was first passed through a 12 µm Nuclepore membrane filter, which retained nematocysts and cell debris but allowed zooxanthellae to pass through. The filtrate was then added to a 5 µm Nuclepore filter which retained the zooxanthellae. The zooxanthellae were resuspended by repeated forceful expulsion from a 2 ml syringe. Examination of the suspension, using oil-immersion light microscopy during an evaluation of this zooxanthellae isolation procedure, showed that the suspension was virtually free from contaminating host-cell components, and was considerably cleaner than suspensions achieved by the standard centrifugation method (Muscatine 1967; Davies 1984).

From the zooxanthellae suspension, two 1 ml aliquots were removed for counts of zooxanthellae density using a haemocytometer. These values were used to normalize the subsequent measurements of chlorophyll *a*, chlorophyll c_2 and protein. Zooxanthellae protein content was measured on two 1 ml aliquots by the method given in the foregoing subsection for total coral protein. The remaining suspension was filtered on to precombusted 27 mm GF/C filters before extracting and measuring chlorophyll pigments by the method of Jeffrey and Humphrey (1975).

Statistical analysis

The question posed by this study can be addressed in two sequential steps: (1) does nitrate addition to oligotrophic seawater have any effect on coral physiology, and if it does, then (2) what is the critical threshold along the nitrate concentration gradient (treatment variable) at which the significant change in the physiological response occurs. The effect of nitrate on each parameter measured was tested using ANOVA. The second stage of the analysis in those cases where the null hypothesis was rejected, would normally use a multiple-comparison test such as Tukey's honest-significant-difference (HSD) test. However, treating data in this manner neglects the fundamental ordinal nature of the treatment variable (i.e. 0 µ*M* <1 µ*M* <5 µ*M* <20 µ*M*). It was also judged inappropriate to fit the data to a linear regression model because in practice the four nitrate concentrations did fluctuate over time (see Table 2) and therefore cannot be precisely reduced to four points on the independent variable (i.e. 0, 1, 5 and 20 µ*M* NO_3 are their nominal values rather than the exact concentration in each treatment).

A solution was found by coding the four treatment levels using three "dummy" variables (Zar 1984) and analysing the resulting distribution by multiple regression (Walter et al. 1987). The coding scheme adopted is given in Table 1. Since in this multiple-regression model the response variable is dependent upon the three dummy variables, their relationship between response and treatment level is expressed by the following equation:

$$Y = a + b_1D_1 + b_2D_2 + b_3D_3 \quad . \qquad (1)$$

Substituting the appropriate dummy variable value for each treatment level (e.g. for treatment 1, $D1 = 0$, $D2 = 0$ and $D3 = 0$) into Eq. (1), it then follows that: for Treatment 1, $Y_1 = a + b_1 \cdot 0 + b_2 \cdot 0 + b_3 \cdot 0 = a$; for Treatment 2: $Y_2 = a + b_1 \cdot 1 + b_2 \cdot 0 + b_3 \cdot 0 = a + b_1$ etc.

The difference between Y_2 and Y_1 therefore is b_1, and the difference between Y_3 and Y_2 is b_2 and so on. The multiple-regression analysis then employs the Student *t* statistic to test that the regression coefficients b_1, b_2, and b_3 are different from 0, and this corresponds to testing for differences between treatments (i.e. if $b_1 \neq 0$ then Treatment 1 ≠ Treatment 2, if $b_2 \neq 0$ then Treatment 2 ≠ Treatment 3, and if $b_3 \neq 0$ then Treatment 3 ≠ Treatment 4). If the interaction term between species and treatments was significant in the initial two-way ANOVA, the two species were treated separately. If there was no significant interaction, the two species were tested in the multiple regression above, using a fourth dummy variable to identify them.

Table 1 Coding scheme for four treatment levels used in multiple-regression analyses (see "Materials and methods – Statistical analysis" for details of statistical procedure)

Treatments		Dummy variables		
Levels	NO_3 (µ*M*)	D_1	D_2	D_3
1	0	0	0	0
2	1	1	0	0
3	5	1	1	0
4	20	1	1	1

Results

Experimental chambers and treatment regimes

The actual concentration of nitrate in the chambers was determined at 4 d intervals immediately before the chambers were cleaned and refilled with stock solutions. In each outflow from the three experimental chambers, the measured concentration of nitrate was below that of the inflow (Table 2), probably as a result of the uptake activity by both the corals and the surface film of algae which developed between cleaning, and the long residence time (20 h). Therefore over each 4 d period between refillings, nitrate concentration fluctuated within each chamber. In subsequent sections, each treatment is identified by the notional inflow concentration of 0, 1, 5 and 20 µ*M* NO_3, but it should be kept in mind that the actual average concentration experienced by the corals is somewhat lower in each treatment.

Measurement of photosynthesis

The photosynthesis vs irradiance curves for *Porites porites* and *Montastrea annularis* after 40 and 30 d exposure to the treatments is shown in Fig. 1, and all photosynthetic parameters are presented in Table 3. In *P. porites*, values for maximum gross photosynthesis ($P_{gross\ max}$) normalised to surface area, increased from 44.2 µl O_2 cm^{-2} h^{-1} in the control to 61.8 in the 20 µ*M* nitrate group. In *M. annularis*, the values increased from 39.5 to 49.5 µl O_2 cm^{-2} h^{-1}. ANOVA showed that there was no significant interaction between species and treatments, and so the two species were combined for analysis of treatment effects. There was a significant positive relationship between $P_{gross\ max}$ and treatment

Table 2 Range of nitrate concentrations of inflow stock solutions and as measured in photostats at 4 d intervals immediately before chambers were cleaned and refilled

	Treatment No.			
	1	2	3	4
NO_3 inflow (µmol l^{-1})	0.1	1	5	20
NO_3 outflow (µmol l^{-1})	0.1–0.3	0.2–0.5	0.5–3	5–17

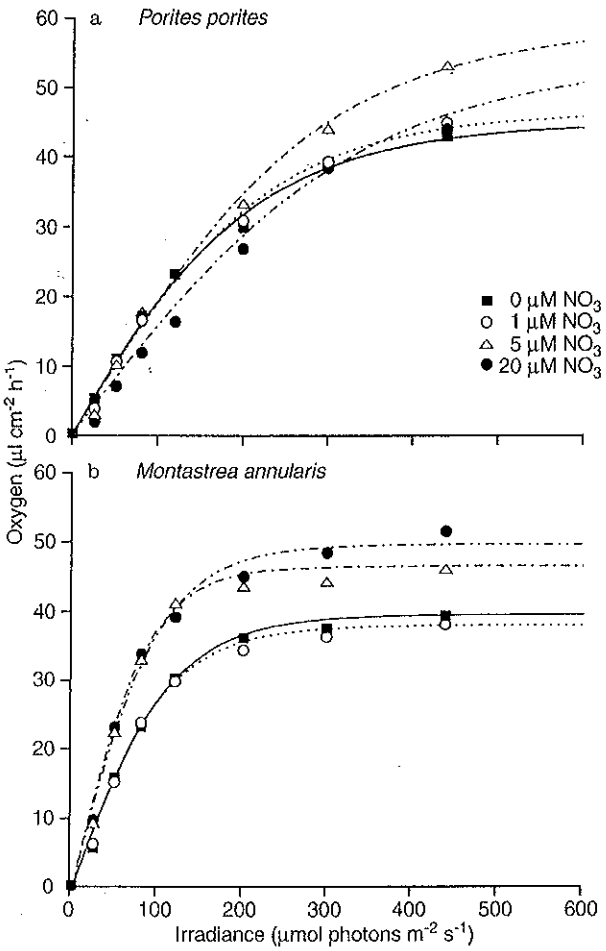

Fig. 1 *Porites porites* and *Montastrea annularis*. Gross photosynthesis vs irradiance after incubation for 40 and 30 d, respectively, in photostat chambers with four different nitrate concentrations. Line fits were derived from hyperbolic tangent function. Error bars omitted for clarity; $n = 6$ for each treatment

levels of nitrate ($F_{4,41} = 10.21$; $P < 0.0001$). While no significant change in response was observed between control and 1 µ*M* NO_3-treated corals, a highly significant increment of $P_{\text{gross max}}$ occurred with 5 µ*M* NO_3 (Student's $t = 3.347$, $P < 0.001$). There was no further increase in response in those kept at 20 µ*M* nitrate. Since an increase in maximum photosynthetic rate corresponds to an increase in the number of photosynthetic units (Prézelin1987), the analysis was repeated using photosynthetic measurements normalised to number of zooxanthellae. In this case, there was no significant difference in $P_{\text{gross max}}$ ($F_{3,35} = 1.675$, $P > 0.05$), suggesting that the increase in photosynthesis had not resulted from an increase in the number of photosynthetic units or thylakoid membranes per algal cell, but from an increase in algal cells per surface area. The second essential parameter of the *P* vs *I* curve is alpha, the slope of the tangent to the initial part of the curve, which is a measure of photosynthetic efficiency. ANOVA showed a significant interaction between species and treatments ($F_{3,42} = 13.5$, $P < 0.001$), so separate multiple-regression

Table 3 *Porites porites* and *Montastrea annularis*. Photosynthetic parameters and respiration after 40 and 30 d exposure, respectively, to control oligotrophic seawater and three concentrations of nitrate. Values are means ± SD, $n = 6$ in each treatment. Data were analysed using multiple-regression model (see "Materials and methods – Statistical analysis"). Position of asterisks indicates where significance between nitrate treatments first appeared (* significant at $P < 0.05$; ** significant at $P < 0.01$; *** significant at $P < 0.001$). In Analysis A; ANOVA interaction between species and treatments is significant, and each species is analysed seperately; in Analysis B, ANOVA interaction is not significant, so common multiple regression obtained by combining data of the two species is used, and in such case, asterisks are shown between rows for the two species

Parameter	Analysis	Species	Treatment (µmol l^{-1} NO_3)				
			0	1		5	20
Photosynthesis							
Max. gross photosynthesis per surface area (µl O_2 $cm^{-2}h^{-1}$)	B	*P. porites*	44.2 ± 13.47	45.8 ± 4.62	***	58.6 ± 5.8	61.8 ± 14.32
		M. annularis	39.5 ± 5.10	37.9 ± 5.17		46.4 ± 6.40	49.5 ± 4.66
Max. gross photosynthesis per algal cell (µl O_2 cell × 10^{-6} h^{-1})	B	*P. porites*	14.75 ± 4.789	20.33 ± 3.669		16.98 ± 3.00	15.83 ± 4.72
		M. annularis	10.19 ± 1.542	10.38 ± 1.445		10.58 ± 1.661	10.44 ± 0.753
Alpha (µl O_2 cm^{-2} h^{-1} µmol photons^{-1} m^2 s^{-1})	A	*P. porites*	0.207 ± 0.0276	0.199 ± 0.0246		0.198 ± 0.0392	0.165 ± 0.0170
		M. annularis	0.338 ± 0.0511	0.346 ± 0.0309	***	0.529 ± 0.0611	0.491 ± 0.0975
I_k (µmol photons m^{-2} s^{-1})	A	*P. porites*	215 ± 67.0	232 ± 37.4		304 ± 63.1	* 382 ± 116.2
		M. annularis	119 ± 25.4	111 ± 22.9		88 ± 10.4	104 ± 21.5
Respiration							
Respiration rate per surface area (µl O_2 cm^{-2} h^{-1})	A	*P. porites*	10.9 ± 1.26	10.2 ± 1.07		9.6 ± 2.23	8.5 ± 0.99
		M. annularis	14.8 ± 1.12	13.6 ± 0.52		14.4 ± 1.20	15.0 ± 1.40
Respiration rate per protein content (µl O_2 mg^{-1} h^{-1})	B	*P. porites*	3.78 ± 0.30	3.55 ± 0.37		3.44 ± 0.47	2.93 ± 0.52
		M. annularis	2.64 ± 0.27	2.68 ± 0.32		2.19 ± 0.49	1.73 ± 0.22

Table 4 *Porites porites* and *Montastrea annularis*. Daily calcification rate (mg cm^{-2} d^{-1}) after 40 and 30 d exposure, respectively, to control oligotrophic seawater and three concentrations of nitrate. Values are means ± SD; n= 6 in each treatment. ANOVA interaction was not significant, so a common multiple regression is used. Further details as in legend to Table 3

Species	Treatment (μmol l^{-1} NO_3)			
	0	1	5	20
Porites porites	1.24 ± 0.186	0.91 ± 0.061	0.65 ± 0.122	0.68 ± 0.107
Montastrea annularis	1.14 ± 0.14	0.53 ± 0.085	0.43 ± 0.229	0.51 ± 0.074
	***	**		

analyses were used to assess the effect of nitrate addition in the two species. In *P. porites*, values of alpha ranged from 0.207 to 0.165 μl O_2 cm^{-2} h^{-1}μmol^{-1} photons m^2 s^1, with no significant differences between treatments. In *M. annularis*, however, nitrate had a very significant effect on alpha ($F_{3,20}$ = 14.35, P <0.001), with significance appearing at 5 μm NO_3 (Student's t = 4.987, P <0.001).

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

Respiration rate

Dark respiration rates were normalised to surface area and also to protein content, in view of the possibility of biomass per unit surface area changing during the course of the experiment (Table 3). Since no significant interaction between species and treatments was found, data for both species were analysed together. Multiple-regression analysis showed no significant relationship between nitrate concentration and respiration rate when data were normalised to surface area. However, when the biomass normalised data were tested, respiration rate was found to fall from 3.78 to 2.93 μl O_2 cm^{-2} h^{-1} in *Porites porites* and from 2.64 to 1.73μl O_2 cm^{-2} h^{-1} in *Montastrea annularis*, as nitrate concentration increased ($F_{4,35}$ = 30.24, P <0.001), with a very significant decrease occurring in the corals treated with 20 μM NO_3 (Student's t = 9.358, P <0.01).

Measurement of skeleton growth

Growth rate decreased significantly with increasing nitrate levels ($F_{4,45}$ = 36.42, P <0.001), and there was no interaction between treatments and species, indicating that nitrate addition affected the growth rate of both species in the same way. In *Porites porites*, the growth rate declined from 1.24 mg cm^{-2} d^{-1} in the control to 0.68 in those exposed to 20 μM NO_3, whilst in *Montastrea annularis*, the decline was from 1.14 to 0.51 mg cm^{-2} d^{-1} (Table 4). The negative relationship obtained was not linear, but was most strongly affected by the lower nitrate concentrations (Fig. 2): a significant difference appeared at 1 μM NO_3 (Student's t = 6.965, P <0.001) and again at 5 μM NO_3 (t = 2.648, P = 0.011) where calcification was half that of control corals, but no further suppression was obtained with 20 μM NO_3 (t = 0.023, P > 0.05).

Biometry and biomass measurements

In all biomass measurements there was no interaction between species and treatment in the initial ANOVA, so that the relationship between physiological response measured and nitrate concentration was the same for both species (Table 5). In all cases, algal biomass was positively correlated with nitrate concentration.

Chlorophylls a and c_2. Photosynthetic pigment concentration increased with nitrate concentration ($F_{4,35}$ = 23.56,

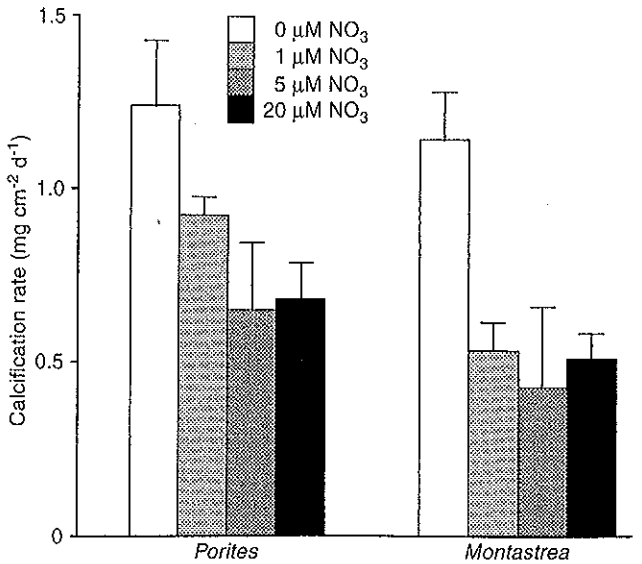

Fig. 2 *Porites porites* (n = 32) and *Montastrea annularis* (n = 18). Daily calcification rate during incubation for 40 and 30 d, respectively, in photostat chambers with four different nitrate concentrations. (bars = 1 SD)

Table 5 *Porites porites* and *Montastrea annularis*. Biomass characteristics after 40 and 30 d exposure, respectively, to control oligotrophic seawater and three additional concentrations of nitrate. Values are means ± SD; n = 5 in each treatment. In all cases ANOVA interaction is not significant, so common multiple regression obtained by combining data of the two species is used, and asterisks are shown between rows for the two species. Further details as in legend to Table 3

Parameter	Species	Treatment (μmol l^{-1} NO_3)			
		0	1	5	20
Chlorophyll *a* (pg $cell^{-1}$)	*P. porites*	2.95 ± 0.281	2.93 ± 0.712	4.06 ± 1.013	3.93 ± 0.671
	M. annularis	1.62 ± 0.285	1.79 ± 0.113 ***	2.53 ± 0.475 ***	2.71 ± 0.220
Chlorophyll c_2 (pg $cell^{-1}$)	*P. porites*	0.82 ± 0.084	0.83 ± 0.312	1.20 ± 0.421	1.22 ± 0.280
	M. annularis	0.39 ± 0.131	0.46 ± 0.055 *	0.60 ± 0.168 *	0.68 ± 0.076
Zooxanthella protein (pg $cell^{-1}$)	*P. porites*	170.4 ± 7.309	164.8 ± 30.826	191.8 ± 27.908	230.7 ± 18.121
	M. annularis	90.3 ± 27.96	126.4 ± 58.62	122.8 ± 22.26 ***	216.3 ± 71.56 ***
Zooxanthella volume (μm^3)	*P. porites*	491.3 ± 27.84	527.3 ± 42.94	547.7 ± 28.66	627.2 ± 35.76
	M. annularis	406.9 ± 28.87	413.1 ± 47.74 *	457.1 ± 39.57 ***	510.5 ± 21.93 ***
Zooxanthellae density ($\times 10^6$ cm^{-2})	*P. porites*	2.93 ± 0.348	2.30 ± 0.487	3.63 ± 0.934	3.96 ± 0.274
	M. annularis	3.86 ± 0.392 *	3.61 ± 0.198 ***	4.29 ± 0.194 *	4.86 ± 0.466 *
Coral protein (mg cm^{-2})	*P. porites*	4.23 ± 0.568	3.77 ± 0.550	4.58 ± 0.503	4.96 ± 0.199
	M. annularis	3.96 ± 0.066	3.84 ± 0.434 *	4.25 ± 0.445 **	5.19 ± 1.037 **
Host protein (mg cm^{-2})	*P. porites*	3.73 ± 0.504	3.39 ± 0.535	3.89 ± 0.492	4.04 ± 0.221
	M. annularis	3.62 ±0.091	3.38 ±0.535	3.73 ± 0.392	4.13 ± 0.818

P <0.001 for chlorophyll *a*, $F_{4,35}$ = 16.59, P <0.001). It did not show a difference between control and 1 μ*M* NO_3, but was significantly enhanced by the addition of 5 and 20 μ*M* NO_3 (Student's t = 3.936, P <0.001 for chlorophyll *a*; t = 2.562, P <0.02 for chlorophyll c_2).

Zooxanthellae protein content. This was also significantly affected by nitrate ($F_{4,35}$ = 23.56, P <0.001), but an addition of 20 μ*M* NO_3 was required to observe a significant increase of 66.22 pg protein $cell^{-1}$ above the content of zooxanthellae isolated from corals treated with 0, 1 and 5 μ*M* NO_3 (Student's t = 3.754, P <0.001).

Zooxanthellae volume. The volume of zooxanthellae increased with nitrate concentration ($F_{4,35}$ = 38.97, P <0.001): the average cell size in the control chamber was 490 μm^3 when isolated from *Porites porites*, and 400 μm^3 when isolated from *Montastrea annularis* (for species: Student's t = 9.315, P <0.001). The regression model shows a non-significant increase in size of 21 μm^3 between control and 1 μ*M* NO_3 (t = 1.369, P = > 0.05); a first significant increase of 32.2 μm^3 occurs with 5 μ*M* NO_3 (t = 2.092, P <0.05), and a final very significant increase of 66.4 μm^3 appears with 20 μ*M* NO_3 (t = 4.312, P <0.001), suggesting a linear positive relationship between algal volume and nitrate.

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

Protein content of total coral tissue. The treatment also had a significant effect in enhancing the protein content per unit surface area of coral ($F_{4,35}$ = 7.787, P <0.001): corals treated with 5 μ*M* NO_3 showed a significantly higher protein content than control and 1 μ*M* NO_3 - treated corals (Student's t = 2.547, P <0.02), and a further enhancement was found in those treated with 20 μ*M* NO_3 (t = 2.748, P <0.01). Coral protein per surface area is the measure of both animal and algal protein content: since nitrate was positively correlated both with algal protein per cell and with the density of zooxanthellae, in order to study the effect of nitrate on animal protein only the protein content due to algal biomass was subtracted from the measurement of total coral protein. However, once the zooxanthellae contribution to the total protein content was removed, no relationship was found between host protein and nitrate concentration.

Discussion

In our experiments, *Porites porites* and *Montastrea annularis* were cultured in oligotrophic oceanic water enriched with four different levels of nitrate (0, 1, 5 and 20 μ*M* NO_3). At the end of the 30 or 40 d incubation, all physiological parameters measured showed a significant

A P S M

correlation with nitrate concentration in the seawater. To the authors knowledge, this is the first study that analyses the physiological effects of elevated nitrate on hermatypic corals. Previous research has focused on rates of nitrate depletion from enriched seawater by hermatypic corals and clams (Franzisket 1974; D'Elia and Webb 1977; Webb and Wiebe 1978; Wilkerson and Trench 1986) or from unenriched seawater (Bythell 1990). In our experiments, nitrate depletion occurred in the experimental chambers, but even though some of this could be attributed to the growth of algae and bacteria on the surfaces of the photostat, it is obvious from the physiological results obtained that nitrogen was being taken up by the corals. In *Acropora cervicornis* and *M. annularis*, nitrate depletion was found to occur during the daytime and to continue for 20 h of darkness, after which it ceased (Szmant et al. 1990). This suggests that depletion is associated with assimilation by zooxanthellae during photosynthesis. This in turn suggests that nitrate is in fact transported across host-cell membranes (cf. Miller and Yellowlees 1989), and that the algae are equipped with the nitrate reductase enzymes required for its assimilation. Syrett (1981) showed that in phytoplankton, nitrate reductase was only expressed when ammonia was limiting. In *Acropora acuminata* and *Goniastrea australensis*, Crossland and Barnes (1977) showed that nitrate reductase of the symbiotic algae was induced by light and high exogenous nitrate (500 μM NO_3) and depressed by incubation with ammonia. Muscatine et al. (1984) failed to demonstrate nitrate reductase activity in *Stylophora pistillata* from the Red Sea, suggesting that this may have been correlated with the low nitrate concentration in seawater at the time of sampling. Wilkerson and Kremer (1992) measured $^{15}NO_3$ incorporation in the coronate scyphozoan *Linuche unguiculata* at a rate an order of magnitude less than ammonium uptake rates, whilst Wilkerson and Muscatine (1984) showed that the temperate anemone *Aiptasia pallida* would not deplete nitrate from enriched seawater unless starved for over one month. It is likely that some of the inconsistency (see review by Miller and Yellowlees 1989) in the literature regarding nitrate depletion could be explained by variation in the expression of nitrate reductase by zooxanthellae, which would in turn depend upon the availability of ammonia, from heterotrophic feeding or from the seawater in which the symbiotic association had been living.

Photosynthesis

Maximum gross photosynthesis normalized to coral surface area increased with increased in nitrate in both species (Table 1, Fig. 1). This can largely be explained by the associated increase in the population density of zooxanthellae expressed on a surface area basis. Hence, when $P_{\text{gross max}}$ is normalized to number of algal cells, no significant change with nitrate concentration is observed. In *Stylophora pistillata* and *Seriatopora hystrix*, Hoegh-Guldberg and Smith (1989) showed a similar increase in $P_{\text{gross max}}$ following incubation in 20 μM NH_4^+, which was also attributed to increase in population density of zooxanthellae; however, $P_{\max}$ per cell was reduced, and this was attributed to light limitation induced by shading of the deeper algae by the more superficially located ones. In *Stylophora pistillata*, Dubinsky et al. (1990) demonstrated an increase in $P_{\max}$ normalized to surface area when the corals were treated with 100 μM ammonium, 10 μM phosphate + 100 μM ammonium or fed with *Artemia sp.* nauplii for 18 d. The $P_{\max}$ per algal cell declined, and the authors suggested that this was due to competition among the enlarged population of algae for CO_2.

Photosynthetic efficiency, as measured by the value of alpha (the initial slope of the P vs I curve), increased with increasing nitrate concentration in *Montastrea annularis* but not in *Porites porites*. The increased alpha of *M. annularis* is similar to that observed in the ammonium-enrichment experiments on *Stylophora pistillata* and *Seriatopora hystrix* (Hoegh-Guldberg and Smith 1989), where it was interpreted as a photoadaptational response by the deeper-dwelling zooxanthellae to the attenuated light resulting from increased population density. We are not able to explain why this was not observed in *P. porites* in the present experiments.

Respiration

There was no change in the rate of colony dark respiration expressed on a surface-area basis with different concentrations of nitrate. This is surprising in view of the increase in protein biomass per unit surface area with increase in nitrate levels (Table 5), since respiration rate is usually closely correlated with actual biomass. Recalculating the respiration rates on a unit protein-biomass basis showed that in both species the respiration rate fell with increasing nitrate and, by extrapolation, with increasing biomass. This is probably attributable to the limitation of respiration by oxygen diffusion (Harland and Davies 1995), which is exacerbated as the biomass per unit area increases.

Biomass changes

After a 30 to 40 d exposure to nitrate, both species appeared to be markedly darker in colour. This is attributable to an increase in both the population density and the concentration of chlorophylls a and c_2 in each algal cell. This is the first record of increased population density resulting from exposure to nitrate, although similar increases have been observed with incubation of corals and anemones in seawater with elevated levels of NH_4^+ (e.g. Cook et al. 1988; Hoegh-Guldberg and Smith 1989; Stambler et al. 1991; Stimson and Kinzie 1991; Muller-Parker et al. 1994b). In these cases, the increased population density was interpreted as a re-

sponse to nitrogen limitation of zooxanthellae growth in the control corals in normal seawater. An increase in the concentration of chlorophyll *a* was also observed by Muscatine et al. (1989) following incubation of *Stylophora pistillata* in 20 μM NH_4^+ for 14 d.

In both *Porites porites* and *Montastrea annularis*, there was an increase in the protein biomass per unit surface area, with increasing nitrate. However, this was almost wholly associated with increased protein of the symbionts: the host protein did not change. Similar increases in total protein were found in *Stylophora pistillata* (Muscatine et al. 1989), *S. pistillata* and *Seriatopora hystrix* (Hoegh-Guldberg and Smith 1989), and in *Pocillopora damicornis* (Muller-Parker et al. 1994a) following incubations in elevated NH_4^+. In *P. damicornis*, there was also an increase in host protein. Increases in the protein and nitrogen content of zooxanthellae in ammonia-treated corals were also recorded in the experiments of Snidvongs and Kinzie (1994) and Muller-Parker et al. (1994b) with *P. damicornis*, and Muscatine et al. (1989) with *Stylophora pistillata*. This probably reflects an increase in structural protein, since Berner and Izhaki (1994) demonstrated an increase in thylakoid membranes of zooxanthellae of *P. damicornis* after exposure to elevated NH_4^+. It is clear that the changes in the biomass characteristics of *Porites porites* and *M. annularis* following exposure to elevated nitrate are very similar to the responses of symbionts in other corals to increased NH_4^+.

Calcification

In both *Porites porites* and *Montastrea annularis*, the rate of skeletal growth fell abruptly with increasing nitrate concentrations in the photostats. A significant effect was observed with as little as 1 μM nitrate, and the rate in 5 and 20 μM NO_3 was $\simeq$50% of that of the controls in oligotrophic water. Inhibition of calcification by ammonia in *Pocillopora damicornis* was reported by Stambler et al. (1991) and by Stimson (1992). The growth rate of *Stylophora pistillata* determined by buoyant weighing is similarly depressed by NH_4^+ (C. Ferrier-Pagès, personal communication). Stambler et al. (1991) interpreted the lower growth rate as due to either a reduced translocation of organic carbon from algae to host, or to increased competition for inorganic carbon between algae, (for photosynthesis) and host (for skeletogenesis)

In order to explain the inhibition of calcification by nitrate, we adopt the second hypothesis of Stambler et al. (1991), that is that algae and host compete for inorganic carbon. There is abundant evidence for CO_2 limitation of photosynthesis in corals from studies of carbonic anhydrase activity (Weis et al. 1989; Lesser et al. 1994), of ^{13}C fractionation (Muscatine et al. 1989), of stimulation following enrichment of seawater with HCO_3^- (Burris et al. 1983), and of an increased rate of photosynthesis associated with light-enhanced respiration (Harland and Davies 1995). The problem is exacerbated in situations of low water movement by the increase in the thickness of the boundary layer, which decreases the rate of inward diffusion of CO_2 (Dennison and Barnes 1988; Lesser et al. 1994). Following incubation in elevated levels of nitrate, we have seen that maximum gross photosynthesis increases as a result of the increase in the population of zooxanthellae. If photosynthesis and skeletogenesis are two processes drawing upon the same pool of dissolved inorganic carbon, it follows that if one process is enhanced the other will be proportionally limited. We postulate that photosynthesis has a competitive advantage over calcification because zooxanthellae located in the gastrodermal cells are closer to the CO_2 diffusing into the tissues from seawater then the calicoblastic epithelial cells responsible for calcification.

To test this hypothesis, the requirement for carbon for net photosynthesis was compared with the carbon required for skeletal formation in the control group and those kept in 20 μM NO_3, using the data of Tables 3 and 4. (Net photosynthesis was used since it was assumed that respiratory carbon dioxide was recycled.) The results of this inorganic carbon budget are shown in Table 6. In *Montastrea annularis*, the inorganic carbon used in net photosynthesis increased by 70 $\mu g\ C\ cm^{-2}\ d^{-1}$, whilst that used in calcification fell by 85 $\mu g\ C\ cm^{-2}\ d^{-1}$. A similar pattern is seen in *Porites porites*, although here the decline in calcification was greater than the increased utilization in photosynthesis. This endogenous carbon-limitation hypothesis does not fit the case of the corals subjected to 1 μM nitrate, where calcification was reduced without any change in photosynthesis. On the other hand, it could help to explain why corals growing at high levels of nitrogen (~5 μM dissolved inorganic nitrogen) in the Waikiki Aquarium (Atkinson et al. 1995) maintained a high calcification rate: in their study (with no controls and no information on zooxanthellae population density or photosynthesis) the pCO_2 (partial pressure of CO_2) of the inflow water was abnormally high and could have counterbalanced any intracellular

Table 6 *Porites porites* and *Monstastrea annularis*. Mean values for net inorganic carbon fixed in photosynthesis and in skeletogenesis after 40 and 30 d exposure, respectively, to oligotrophic water and to 20 μM NO_3

Species	Treatment ($\mu mol\ l^{-1}\ NO_3$)		
	0	20	difference
Porites porites			
Daily net photosynthesis ($\mu g\ C\ cm^{-2}\ d^{-1}$	105	141	+36
Daily calcification ($\mu g\ C\ cm^{-2}\ d^{-1}$)	149	82	−67
Montastrea annularis			
Daily net photosynthesis ($\mu g\ C\ cm^{-2}\ d^{-1}$)	75.5	145.3	+70
Daily calcification ($\mu g\ C\ cm^{-2}\ d^{-1}$)	136.8	61.6	−75

136.8
− 61.6
75.2

carbon limitation. Obviously, a specific experiment set out to directly test the hypothesis of carbon limitation and its relationship with nutrient levels and coral growth in situ is urgently called for.

Information on the effects of elevated nutrients on reefs is still sparse (Hawker and Connell 1992). The eutrophication responses of the reef system of Kanehoe Bay, Hawaii, following the discharge of domestic sewage, resulted in decreased community calcification (Kinsey 1987). However, the response in Kinsey's study was complicated by other eutrophication-related features including algal overgrowth and destruction by infaunal boring. Tomascik and Sander (1985) found that the skeletal extension rate of *Montastrea annularis* was negatively correlated with nutrient levels of a eutrophication gradient along the west coast of Barbados. In an earlier study on artificial fertilisation of a patch of reef at One Tree Island, Australia, with 2 μM phosphate and 20 μM urea plus ammonia, there was an increase in production and a 50% decline in calcification (Kinsey and Davies 1979). This decreased calcification was attributed to the effects of phosphate. However, it is now clear from studies on ammonia and from our laboratory study on nitrate with *Porites porites* and *M. annularis* that nitrogen elevation is also responsible for the decline in calcification, and that increased levels of nitrate in seawater of coral reefs can result in a dramatic decrease in skeletogenesis and, by extrapolation, in the rate of growth of reefs as a whole.

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