

Nitrate uptake in the scleractinian coral *Stylophora pistillata*

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Abstract

We assessed the uptake rates of nitrate by the scleractinian coral *Stylophora pistillata* by following ¹⁵N from seawater into the coral tissue. Two sets of corals were first prepared, with “nitrate-enriched” corals grown in 5 μmol L⁻¹ NO₃⁻ and control corals grown in ≤1 μmol L⁻¹ NO₃⁻. Uptake rates at 0.3 and 3 μmol L⁻¹ [¹⁵N]NO₃⁻ were then measured. Most of the %¹⁵N enrichment occurred in the zooxanthellae fraction. Uptake rates were not significantly different between nitrate-enriched and control corals, suggesting that they were not dependent on a nitrate acclimation. These rates increased with the in situ nitrate concentration and varied from 1.2 ± 0.2 ng h⁻¹ cm⁻² N to 6.1 ± 1.1 ng h⁻¹ cm⁻² N in the algal fraction at 0.3 and 3 μmol L⁻¹ [¹⁵N]NO₃⁻, respectively. In a second experiment, two sets of corals were prepared, with “ammonium-enriched” corals grown in 5 μmol L⁻¹ NH₄⁺ and control corals grown in <1 μmol L⁻¹ NH₄⁺. Uptake rates at 3 μmol L⁻¹ [¹⁵N]NO₃⁻ were measured. These rates were significantly lower with high NH₄⁺ concentrations in seawater. In the algal fraction, they ranged from 0.1 to 0.6 ng h⁻¹ cm⁻² N in NH₄⁺-enriched corals and from 2.2 to 4.5 ng h⁻¹ cm⁻² N in control corals. Nitrate can therefore be considered as an important source of nitrogen for corals, at least when ammonium concentrations are low in seawater.

Nitrate and ammonium are the major sources of nitrogen in the marine environment for primary production (Codispoti 1989). Reef waters usually contain low levels of inorganic nutrients. The ranges of nitrogen concentrations are typically of 0.3 to 1 μmol L⁻¹ nitrate and 0 to 0.4 μmol L⁻¹ ammonium (Bythell 1990, D’Elia and Wiebe 1990, Furnas 1991). Scleractinian corals thriving in these nutrient-poor waters have developed adaptations for conserving nitrogen. They live in symbiosis with dinoflagellates called zooxanthellae that can take up and retain dissolved inorganic nitrogen from the surrounding seawater (Muscatine 1980; Wilkerson and Trench 1986; Falkowski et al. 1993; Wang and Douglas 1998) or recycled from the host (Rahav et al. 1989).

Many studies have investigated the uptake of ammonium by cultured and freshly isolated zooxanthellae (D’Elia et al. 1983; Domotor and D’Elia 1984; Yellowlees et al. 1994) as well as by the entire association (Muscatine and D’Elia 1978; Burris 1983; Wilkerson and Trench 1986; Bythell 1990; Hoegh-Guldberg and Williamson 1999; Grover et al. 2002). Ammonium is assimilated into glutamine through the action of glutamine synthetase. This enzyme is present in both the host and algal fractions. Corals are able to efficiently take up and retain ammonium from seawater, even when concentrations are as low as those measured in reef waters

(Bythell 1990; Grover et al. 2002). The uptake rates range from 1 to 35 nmol nitrogen (μg chlorophyll *a*)⁻¹ h⁻¹ or 1 to 20 nmol cm⁻² h⁻¹ depending on the flow rate experienced by the corals and the level of ammonium in seawater (Atkinson et al. 1994).

Fewer studies have, however, assessed the uptake of nitrate by corals or other symbiotic anthozoans and produced equivocal results. Some anthozoans appear to remove nitrate from seawater (Franzisket 1974; D’Elia and Webb 1977; Webb and Wiebe 1978; Bythell 1990), whereas others, and all symbiotic anemones, do not (Muscatine and Marian 1982; Muscatine et al. 1984; Wilkerson and Muscatine 1984; Wilkerson and Trench 1986). Miller and Yellowlees (1989) have questioned the methodology of these experiments and they suggested that depletion could result from bacterial assimilation. However, later studies based on physiological measurements confirmed that nitrate was actually taken up by the symbiotic association (Marubini and Davies 1996; Cook et al. 1997; Shepherd et al. 1999; Ferrier-Pagès et al. 2001). Nitrate is reduced to ammonium through the action of the nitrate and nitrite reductases (Miller and Yellowlees 1989). These enzymes have not been isolated in animals but only in plants. Crossland and Barnes (1977) detected them once in zooxanthellae. In phytoplankton, the induction of this enzyme normally occurs after a time lag of a few hours (Vergara et al. 1998) and when ammonium is limiting in seawater (Syrett 1981; Berges et al. 1995; Vergara et al. 1998). However, these properties have not been assessed with corals.

This study therefore investigated for the first time the ability of the scleractinian coral *Stylophora pistillata* to take up

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Table 1. Nitrogen concentrations ($\mu\text{mol L}^{-1}$) in the different experiments.

Treatment	NH_4^+	NO_3^-	NO_2^-
Culture tanks	0.4–0.8	0.8 ± 0.2	0.2 ± 0.2
First experiment			
Control tanks	0.5–0.9	0.6 ± 0.3	0.2 ± 0.2
Experimental tanks	0.5–0.9	5.8 ± 0.2	0.2 ± 0.2
Second experiment			
Control tanks	0.5–0.8	0.8 ± 0.2	0.2 ± 0.2
Experimental tanks	3.2–4.1	0.7 ± 0.2	0.3 ± 0.2

nitrate under different environmental conditions. For this purpose, nitrate uptake was measured (1) with corals acclimated or not to high ambient nitrate concentration, to test if there is a need of an activation of the nitrate reductase; (2) under high ($3 \mu\text{mol L}^{-1}$) and low ($0.3 \mu\text{mol L}^{-1}$) [^{15}N]nitrate concentrations in the incubation medium; (3) under different light intensities; and (4) with and without high ambient ammonium concentrations. We have not investigated the effect of flow rates on the nitrate uptake in this series of experiments, since we first wished to assess conditions in which corals were able to take up this form of nitrogen.

Materials and methods

Biological material—Experiments were performed in the laboratory using colonies of the scleractinian coral *S. pistillata* (Esper 1797), which were collected in the Gulf of Aqaba (Red Sea, Jordan) and maintained several months in the laboratory under conditions described below. Nubbins of about the same size (4 cm long, 2 cm wide) were obtained by cutting terminal portions of branches of eight parent colonies and were used only when the animal tissue entirely regenerated over the skeleton to avoid isotopic exchanges between seawater and bare skeletons. They were maintained in aquaria supplied with oligotrophic Mediterranean seawater with low amounts of nutrients (Table 1). Metal halide lamps (400 W) (Philips, HPIT) provided a constant irradiance of $350 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (photoperiod 12:12 h). Salinity and irradiance were measured using a conductivity meter (Meter LF196), and a 4π quantum sensor (Li-Cor, LI-193SA) respectively. Temperature (precision: $\pm 0.05^\circ\text{C}$) was logged at 10-min intervals using a Seamon[®] temperature recorder and varied between 26.5°C and 27.5°C . During the period of healing, nubbins were slightly fed once a week with the same amount of *Artemia salina* nauplii (ca. $1,500 \text{ artemia L}^{-1}$). During the experiments, however, nubbins were not fed, since feeding interacts with nitrogen uptake (Muller-Parker et al. 1988; Grover et al. 2002).

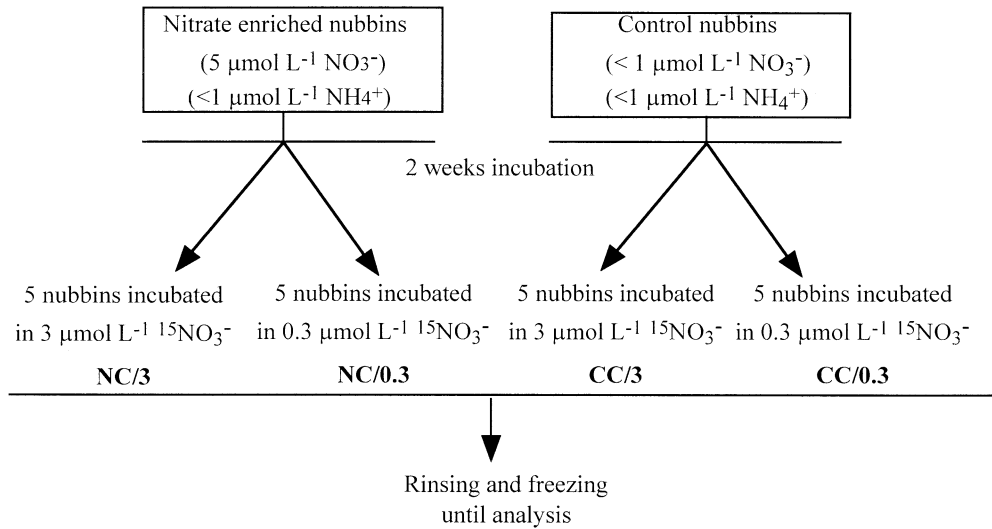
First set of experiments: Effect of nitrate past history and nitrate concentration—The first experiment was designed to assess the ability of *S. pistillata* to use nitrate as a nitrogen source, depending on the nitrate past history of the corals. For this purpose, 20 nubbins were randomly divided into four tanks, maintained under the same conditions as above (Fig. 1). Two tanks were enriched with a solution of sodium

nitrate (NaNO_3) continuously pumped from a stock solution via a peristaltic pump. The stock solution was made of seawater where NaNO_3 has been added to reach a final concentration of $5.8 \pm 0.2 \mu\text{mol L}^{-1} \text{NaNO}_3$. This solution was renewed every day, and was added to the tanks with a constant flow of 1 L h^{-1} . The two remaining tanks (control tanks) were kept at in situ concentrations of nitrate. Seawater renewal was the same as in the nitrate-enriched tanks. Nutrient concentrations were monitored in the tanks every 2 d and the mean concentrations are presented in Table 1. Corals were maintained during 2 weeks under the above conditions. They will be called NC and CC for the nitrate-enriched and control corals respectively.

After pretreatment into these low- and high-nitrate media, nubbins were sampled and incubated in [^{15}N]NO₃⁻-enriched seawater. The incubation with the ^{15}N tracer allowed us to measure the uptake rates of [^{15}N]NO₃⁻ by the different colonies according to their nitrate past history. Two [^{15}N]NO₃⁻ concentrations were also tested (0.3 or $3 \mu\text{mol L}^{-1}$ [^{15}N]NO₃⁻) to assess the effect of the nitrate concentration on the uptake rates. For this purpose, a stock solution of ^{15}N was prepared by dissolving 0.3 or $3 \mu\text{mol L}^{-1}$ [^{15}N]NO₃⁻ in seawater (original product: $\text{Na}^{[15}\text{N}]\text{O}_3^-$ [98% atom, CEA, France]). The final nitrate concentration (corresponding to the amount of nitrate present in seawater and added as ^{15}N) was measured. It varied between 1.0 ± 0.2 and $3.8 \pm 0.3 \mu\text{mol L}^{-1}$ whether 0.3 or $3 \mu\text{mol L}^{-1}$ [^{15}N]NO₃⁻ was added. For each condition, five nubbins were taken in the morning a few minutes before the lights switched on and were incubated during 12 h in individual 250-ml beakers filled with the solution of [^{15}N]NO₃⁻. To avoid depletion in the beakers, solutions of [^{15}N]NO₃⁻ were continuously pumped with a peristaltic pump from the batch solution to the beakers. The flow rate was equal to 7 ml min^{-1} . Beakers contained a magnetic stirrer to homogenize the medium and were immersed in a water bath maintaining a constant temperature of 26.5°C . Irradiance was kept at $350 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. At the end of the 12 h, nubbins were rinsed in a large volume of filtered seawater during 30 min to wash the coelenteron (Tambutté et al. 1995) and were processed as described below.

Second set of experiments: Effect of light and ammonium concentration—The second experiment was designed to investigate the effect of irradiance and ammonium concentrations on the uptake rates of nitrate (Fig. 1). For this purpose, 30 nubbins were incubated during 1 week in four different tanks as described above. Two tanks were enriched with a solution of ammonium (NH_4Cl) continuously pumped from a stock solution via a peristaltic pump. The stock solution was made of seawater where NH_4Cl has been added to reach a final concentration of $5.4 \pm 0.5 \mu\text{mol L}^{-1}$. This solution was renewed every day, and was added to the tanks with a constant flow of 1 L h^{-1} . The two remaining tanks (control tanks) were kept at in situ concentrations of ammonium. Seawater renewal was the same as in the ammonium-enriched tanks. Nutrient concentrations were monitored in the tanks every 2 days and the mean concentrations of nutrient are presented in Table 1. Ammonium is taken up faster than nitrate by all the microorganisms present in seawater. There-

First experiment: Effect of nitrate past-history and nitrate concentration



Second experiment: Effect of light and ammonium concentration

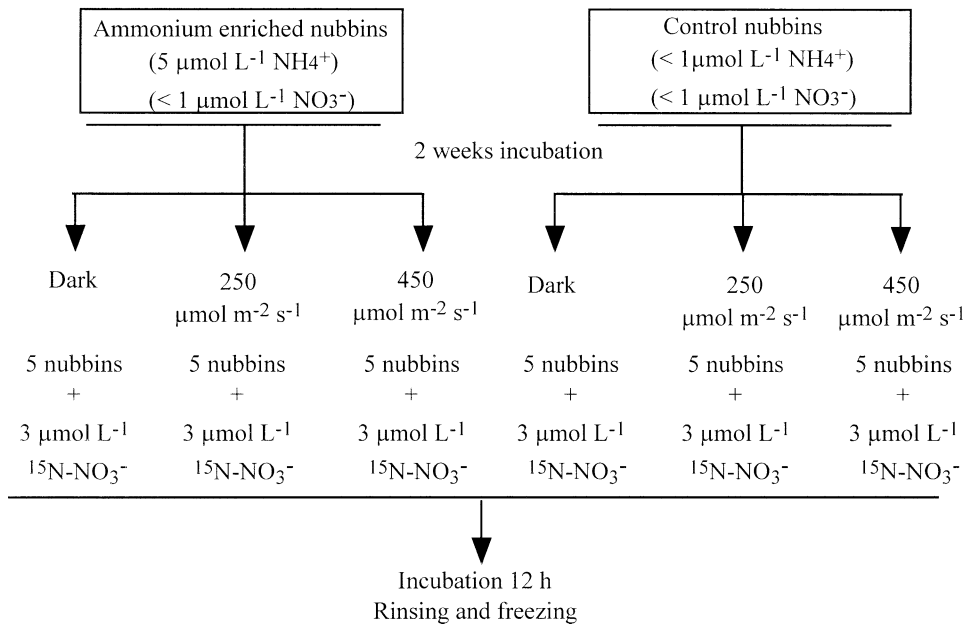


Fig. 1. Schematic description of the experiments.

fore, the concentration in the experimental tanks varied between 3.2 and 4.1 $\mu\text{mol L}^{-1}$, which remained three to four times higher than in the control tanks. After 2 weeks, nubbins (15 in each condition) were sampled and incubated during 12 h in individual beakers continuously supplied with 3 $\mu\text{mol L}^{-1}$ [^{15}N] NO_3^- as described above (and containing low and high ammonium concentrations for the control and NH_4^+ -enriched corals respectively). For each treatment, five corals were incubated under 0 (dark), 250, and 450 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ respectively. Corals for the 250 and 450

$\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ incubation were sampled just before the lights switch on, whereas incubations at 0 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ were performed during the night. At the end of the incubation, nubbins were rinsed to avoid the residual inorganic nitrogen that could remain in the coelenteron, as underlined in Tambutté et al. (1995), and processed as described below.

Analysis—Tissue extraction: At the end of the incubation with $^{15}\text{NO}_3^-$, tissues were completely removed from the skel-

eton with an "air pick" (air under pressure) and homogenized with a Potter tissue grinder. The homogenate was centrifuged at $2,000 \times g$ for 10 min at 4°C to pellet the zooxanthellae. The supernatant was centrifuged again at least two times to pellet residual zooxanthellae (Muscatine et al. 1989) and transferred into 25-ml polypropylene tubes. Pellets of zooxanthellae were resuspended and washed three times with filtered seawater to avoid tissue contamination. Tubes containing tissue and zooxanthellae were then immersed in liquid nitrogen and freeze-dried using a Heto lyophilizer (CT 60).

Nitrate uptake rate determination: $^{15}\text{N}/^{14}\text{N}$ isotopic ratios of the animal tissues and zooxanthellae, as well as carbon and nitrogen contents, were determined using a Flash EA 1112 elemental analyzer coupled to a Thermofinnigan Delta plus mass spectrometer via a ConFlo III interface. The ^{15}N enrichment of the samples was recorded as at. % excess: Atom % excess $^{15}\text{N} = (\text{at. \% } N_{\text{mes}}) - (\text{at. \% } N_{\text{natural}})$. An enrichment of the coral tissue in ^{15}N shows that there is a transfer of nitrogen from seawater to the coral compartment. This is a qualitative result since this enrichment depends on the initial seawater enrichment and on the experimental parameters (light, nutrients, etc.). To quantify the nitrogen fluxes between the two compartments (i.e., calculate the uptake rates, ρ), we used the equation of Dugdale and Wilkerson (1986), which takes into account several parameters such as the ^{15}N enrichment in the coral tissue and initially in seawater, the incubation length, and the coral biomass (NOP), which can be different from one sample to another. In this case, results expressed as % ^{15}N enrichment or as uptake rates might be completely different.

Since it has been shown, either for isolated zooxanthellae (Domotor and D'Elia 1984) or for intact symbiosis (Wilkerson and Trench 1986), that the uptake of nitrate is constant during the incubation, ρ is expressed in $\text{ng N h}^{-1} \text{cm}^{-2}$ in this study.

$$\rho = \frac{N_{\text{mes}} - N_{\text{natural}}}{(N_{\text{enr}} - N_{\text{mes}})t_{\text{inc}}S} \times M_{\text{sample}} \times M_{\text{N}} \times 10^6$$

where N_{mes} is % ^{15}N measured in the sample; N_{natural} is natural abundance ^{15}N in control nubbins; N_{enr} is ^{15}N enrichment of the incubation medium; t_{inc} is incubation time of the nubbins (h); S is nubbin surface area (cm^2); M_{sample} is mass of the freeze-dried sample (mg); M_{N} is particulate nitrogen mass (mg) per milligram of tissue or zooxanthellae.

Surface area of the colonies was measured according to the wax recovering technique (Stimson and Kinzie 1991).

Measurement of nutrient concentrations in the experimental tanks: Nitrite, nitrate, and ammonium concentrations in the culture tanks were measured every day using a Technicon Autoanalyzer (Alliance Instruments) according to Tréguer and Le Corre (1975).

Statistical treatments: Differences between treatments were assessed using one- or two-factor analysis of variance (ANOVA). When a significant effect was found, means were compared with a Bonferroni/Dunn post hoc test. Statistical analyses were performed using StatView 4.01 (Abacus Con-

cept). Data are reported as mean \pm standard deviation of the mean (SE).

Results

Since the results expressed in % ^{15}N enrichment and in uptake rates might be different, we have presented both types of results in the following section.

First set of experiments: Effect of nitrate concentration and nitrate past history—There was a significant difference between the nitrate-enriched and control corals concerning the nitrogen content of the zooxanthellae ($F_1 = 9.56$, $P = 0.01$) and of the animal tissue ($F_1 = 11.14$, $P = 0.006$). There was also a significant difference in the carbon content of the animal tissue ($F_1 = 9.32$, $P = 0.01$). The zooxanthellae contained $5.5 \pm 0.3 \text{ mg cm}^{-2} \text{ N}$ and $33.1 \pm 4.4 \text{ mg cm}^{-2} \text{ C}$ in the nitrate-enriched corals versus $3.3 \pm 0.1 \text{ mg cm}^{-2} \text{ N}$ and $25.3 \pm 2.0 \text{ mg cm}^{-2} \text{ C}$ in the control corals. The animal tissue contained $12.5 \pm 2.1 \text{ mg cm}^{-2} \text{ N}$ and $73.7 \pm 16.6 \text{ mg cm}^{-2} \text{ C}$ in the nitrate-enriched corals compared to $7.3 \pm 2.1 \text{ mg cm}^{-2} \text{ N}$ and $41.7 \pm 6.2 \text{ mg cm}^{-2} \text{ C}$ in the control corals. The C/N ratios remained unchanged between the two conditions, and were equal to 6.8 for the zooxanthellae fraction and to 6.0 for the animal tissue.

Results expressed in % ^{15}N enrichment or in uptake rates are represented in Fig. 2. Most of the ^{15}N enrichment occurred in the zooxanthellae fraction, which was 10 times enriched compared with the animal fraction (Fig. 2a). Uptake rates therefore remained three to four times higher in the zooxanthellae than in the animal fraction (Fig. 2b). Considering the nitrate past history, there was only a significant effect on the % ^{15}N enrichment of the algal fraction (Table 2). The algae of corals pre-incubated into $5 \mu\text{mol L}^{-1}$ nitrate were indeed significantly less ^{15}N -labeled than algae extracted from control corals (1 against 1.5% ^{15}N enrichment). There was, however, no effect of the nitrate past history either on the % ^{15}N enrichment of the animal fraction or on the results expressed in uptake rates (Table 2, Fig. 2). These rates were therefore comparable in control corals and in corals pre-incubated in $5 \mu\text{mol L}^{-1}$ nitrate.

Results of the two-factor ANOVA also showed that there was a strong effect of the nitrate concentration on both the % ^{15}N enrichments or on the uptake rates (Table 2). For each set of corals (control or nitrate enriched), incubations in $3 \mu\text{mol L}^{-1}$ $^{15}\text{N}[\text{NO}_3^-]$ led to significantly higher enrichments and uptake rates than the incubations in $0.3 \mu\text{mol L}^{-1}$ $^{15}\text{N}[\text{NO}_3^-]$, both in the zooxanthellae and animal fractions. In the algal fraction, these rates were equal to $1.2 \pm 0.2 \text{ ng h}^{-1} \text{cm}^{-2} \text{ N}$ when corals were incubated with $0.3 \mu\text{mol L}^{-1}$ $^{15}\text{N}[\text{NO}_3^-]$. They were six times higher when corals were incubated with $3 \mu\text{mol L}^{-1}$ $^{15}\text{N}[\text{NO}_3^-]$ ($6.1 \pm 1.1 \text{ ng h}^{-1} \text{cm}^{-2} \text{ N}$).

Second set of experiments: Effect of light and ammonium concentration—There was no effect of the 1-week ammonium enrichment on the C and N content of the nubbins ($F_1 = 1.409$, $P = 0.25$). These contents were comparable with those measured in the previous experiment. Results obtained with corals incubated with and without ammonium enrich-

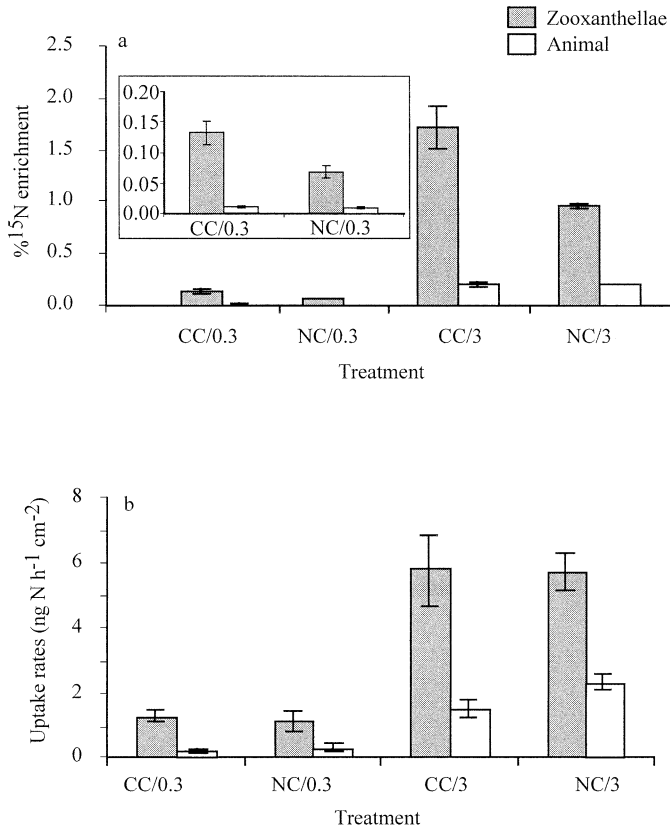


Fig. 2. Effect of the nitrate past history and the nitrate concentration on the %¹⁵N enrichments and on the uptake rates of nitrate. (a) %¹⁵N enrichments; (b) uptake rates (ng h⁻¹ cm⁻² N). CC, control corals; NC, nitrate-enriched corals, i.e., corals maintained during 2 weeks in 5 μmol L⁻¹ nitrate; 0.3 and 3: incubation performed with 0.3 and 3 μmol L⁻¹ [¹⁵N]NO₃⁻.

ment are represented in two separate figures (Figs. 3, 4) because of the great difference in the %¹⁵N enrichments and in the uptake rates measured in the two conditions. In this experiment, the major part of the enrichment was also found in the zooxanthellae that were 2 to 10 times enriched compared to the animal fraction. Results of the two-factor ANOVA showed a significant effect of the presence of ammonium on the %¹⁵N enrichments or on the uptake rates of nitrate (Table 3, Figs. 3, 4). Both results were significantly lower with high ammonium concentrations (3–4 μmol L⁻¹) in the incubation medium. In the algal fraction, uptake rates ranged from 0.1 to 0.6 ng h⁻¹ cm⁻² N in presence of ammonium and from 2.2 to 4.5 ng h⁻¹ cm⁻² N in control corals. In the animal fractions, these rates ranged from 0.05 to 0.4 ng h⁻¹ cm⁻² N in presence of ammonium and from 1.2 to 2.2 ng h⁻¹ cm⁻² N in control corals. High ammonium concentrations in seawater therefore inhibited the uptake of nitrate.

According to the two-factor ANOVA, there was no effect of light on the uptake rates of nitrate, or in the control or in the ammonium-enriched corals (Table 3). However, each result (obtained with low and high ammonium concentrations) can be considered separately and tested for the effect of light. In this case, the one-factor ANOVA showed a significant effect of light on the uptake rates measured with ammonium-enriched corals, either in the zooxanthellae ($F_2 = 10.55$, $P = 0.002$) or in the animal ($F_2 = 4.81$, $P = 0.03$) fraction. The uptake rates of nitrate indeed increased with the increase in the light level. In the zooxanthellae fraction, they were six times higher when corals were incubated at 450 μmol quanta m⁻² s⁻¹ (0.57 ± 0.1 ng h⁻¹ cm⁻² N) than when they were incubated in the dark (0.05 ± 0.02 ng h⁻¹ cm⁻² N).

Discussion

Nitrate uptake has been investigated in this work using the ¹⁵N technique according to the protocol described in

Table 2. Results of the two-factor analysis of variance testing the effects of the “nitrate past history” and nitrate concentrations on the uptake of ¹⁵N-NO₃⁻.

	df	F value	P	Effect
% ¹⁵ N enrichment in the algal fraction				
1. Nitrate past history effect	1	14.59	0.0024*	Significant
2. Nitrate concentration effect	1	138.64	<0.0001*	Significant
3. 1×2 effect	1	10.25	0.0080*	Significant
% ¹⁵ N enrichment in the animal fraction				
1. Nitrate past history effect	1	0.20	0.66	
2. Nitrate concentration effect	1	334.30	<0.0001*	Significant
3. 1×2 effect	1	0.10	0.76	
Uptake rates in the algal fraction				
1. Nitrate past history effect	1	0.04	0.85	
2. Nitrate concentration effect	1	49.93	<0.0001*	Significant
3. 1×2 effect	1	0.12	0.91	
Uptake rates in the animal fraction				
1. Nitrate past history effect	1	5.48	0.06	
2. Nitrate concentration effect	1	68.18	<0.0001*	Significant
3. 1×2 effect	1	3.35	0.09	

* $P < 0.05$.

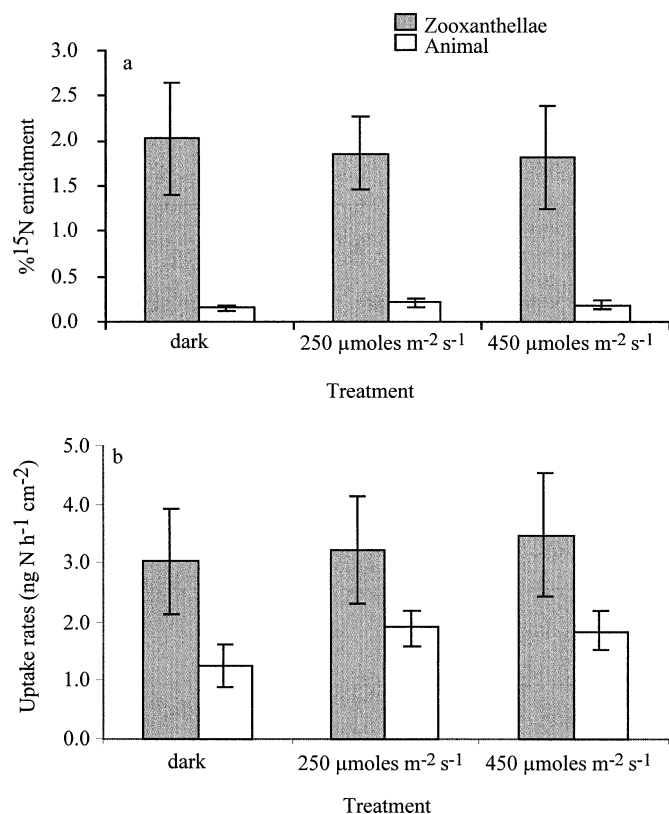


Fig. 3. (a) %¹⁵N enrichment and (b) uptake rates (ng h⁻¹ cm⁻² N) for corals incubated in 3 μmol L⁻¹ [¹⁵N]NO₃⁻ and three different light levels. DLC, dark; MLC, 250 μmol m⁻² s⁻¹; HLC, 450 μmol m⁻² s⁻¹. Ammonium concentrations were kept low (<1 μmol L⁻¹) during the 2 weeks of culture as well as during the uptake experiment.

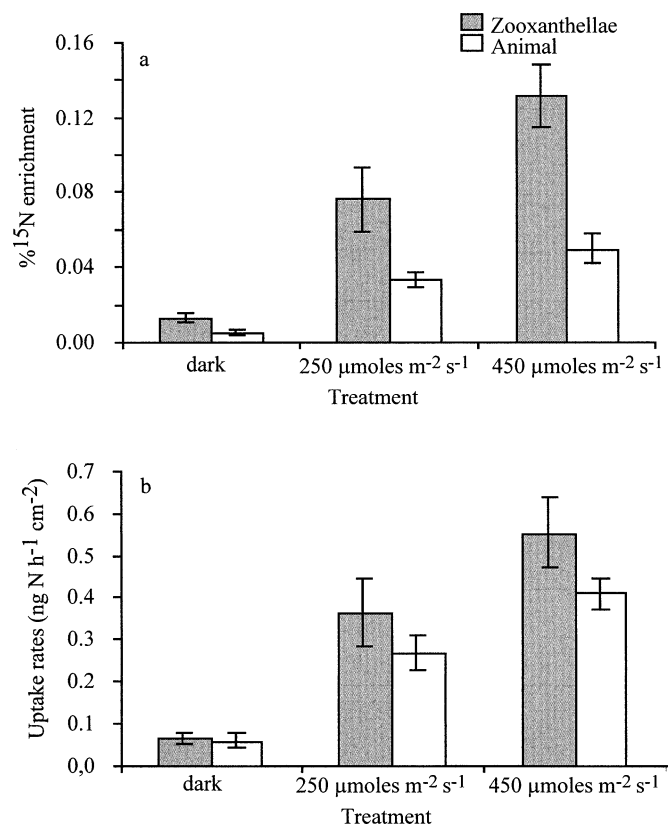


Fig. 4. (a) %¹⁵N enrichment and (b) uptake rates (ng h⁻¹ cm⁻² N) for corals incubated in 3 μmol L⁻¹ [¹⁵N]NO₃⁻ and three different light levels. DLC, dark; MLC, 250 μmol m⁻² s⁻¹; HLC, 450 μmol m⁻² s⁻¹. Ammonium concentrations were kept high (3–5 μmol L⁻¹) during the 2 weeks of culture as well as during the uptake experiment.

Table 3. Results of the two-factor analysis of variance testing the effects of the ammonium concentration and the light on the uptake of ¹⁵N-NO₃⁻. *P*<0.05 significant (*).

	df	<i>F</i> value	<i>P</i>	Effect
% ¹⁵ N enrichment in the algal fraction				
1. high ammonium concentration	1	33.64	<0.0001*	Significant
2. light effect	2	0.99	0.99	
3. 1×2 effect	2	0.09	0.9	
% ¹⁵ N enrichment in the animal fraction				
1. high ammonium concentration	1	50.21	<0.0001*	Significant
2. light effect	2	1.75	0.19	
3. 1×2 effect	2	0.34	0.71	
Uptake rates in the algal fraction				
1. high ammonium concentration	1	27.23	<0.0001*	Significant
2. light effect	2	0.31	0.73	
3. 1×2 effect	2	0.07	0.9	
Uptake rates in the animal fraction				
1. high ammonium concentration	1	40.21	<0.0001*	Significant
2. light effect	2	2.67	0.09	
3. 1×2 effect	2	0.45	0.64	

* *P*<0.05.

Grover et al. (2002). ^{15}N has not been used extensively to assess nitrogen uptake by anthozoans (Muscatine and D'Elia 1978; Burris 1983; Roberts et al. 1999; Lipschultz and Cook 2002) despite its powerful application. Most of the previous studies on nitrate uptake by symbiotic associations have reported uptake from experiments in which nitrate depletion was monitored, usually from a high initial concentration ($10 \mu\text{mol L}^{-1}$), except in three studies where in situ concentrations were investigated (D'Elia and Webb 1977; Webb and Wiebe 1978; Bythell 1990). We therefore present one of the first attempts to assess nitrate uptake rates by the scleractinian coral *S. pistillata* at near-natural concentrations and under different environmental conditions using the ^{15}N technique. Results expressed in % ^{15}N enrichment and in uptake rates generally led, in this paper, to the same conclusions, except for the first experiment, where there was an effect of the nitrate past history on the % ^{15}N enrichment but not on the results expressed in uptake rates.

The first set of experiments clearly demonstrated for the first time that there was no need to activate the nitrate reductase of our coral samples by incubating them several days in high nitrate concentrations. This result suggests either that the enzyme is induced by nitrate concentrations $\leq 1 \mu\text{mol L}^{-1}$ or is constitutive. This does not seem to be the case for all anthozoans, since Wilkerson and Muscatine (1984) found no uptake of nitrate by *Aiptasia pulchella*, even when animals were pretreated for 24 h or 1 month with $10 \mu\text{mol L}^{-1} \text{NO}_3^-$.

Results obtained have also shown a concentration-dependent nitrate uptake, since uptake rates increased with the increase in the concentration of nitrate in seawater. Rates were five to seven times lower at 0.3 than at $3 \mu\text{mol L}^{-1} \text{NO}_3^-$ enrichment, and varied between 1.4 and $8.0 \text{ ng h}^{-1} \text{ cm}^{-2} \text{ N}$. This pattern has often been described for the uptake of ammonium by corals (Muscatine and D'Elia 1978; Wilkerson and Trench 1986). As already observed previously in different marine invertebrates, the ^{15}N enrichment of the algal fraction was up to 12 times greater than the ^{15}N enrichment of the host (Wilkerson and Kremer 1992; Hawkins and Klumpp 1995; Swanson and Hoegh-Guldberg 1998; Roberts et al. 1999), suggesting that the zooxanthellae are the primary site of accumulation of nitrogen. The role of the host in the assimilation of nitrate is not obvious, since no nitrate reductase activity has ever been observed. Moreover, we also measured both higher % ^{15}N enrichments and higher uptake rates in the algal fraction, suggesting that the algae indeed drive the uptake. As far as ammonium is concerned, the relative role of the host or the zooxanthellae in its assimilation is still controversial. Some studies are in favor of a zooxanthellae assimilation of ammonium (Swanson and Hoegh-Gulberg 1998; Roberts et al. 1999; Grover et al. 2002), because they measured a 10-times-higher % ^{15}N enrichment in the zooxanthellae compared with the host. Conversely, some works suggest that the host is primarily involved (Miller and Yellowlees 1989; Szmant et al. 1990; Wang and Douglas 1998; Lipschultz and Cook 2002). In this latter theory, zooxanthellae only provide carbon and photosynthates to the host, which uses both this carbon and the acquired nitrogen to synthesize amino acids (Wang and Douglas 1998). Lipschultz and Cook (2002), in favor of a

host assimilation, demonstrated that a higher % ^{15}N enrichment in the zooxanthellae is only due to a lower nitrogen content of the algae compared with the host. They concluded that the preponderance of nitrogen in host tissue balances a lower degree of labeling. This is not the case for the results obtained in our experiment, since both the % ^{15}N enrichment and the uptake rates (which take into account the amount of particulate nitrogen) are higher in the zooxanthellae than in the host fraction. Nevertheless, considering that all nitrogen sources have to cross at least two animal membranes to reach the zooxanthellae, the host has therefore a role in nitrate assimilation, whether directly or indirectly. This role remains to be determined.

We also clearly demonstrated that there is an ammonium-dependent uptake of nitrate in the entire coral-algae association, at least in the scleractinian coral *S. pistillata*. We indeed found that the rates of nitrate uptake were much higher under low ($< 1 \mu\text{mol L}^{-1}$) than under high ammonium concentrations ($3\text{--}4 \mu\text{mol L}^{-1}$). This suggests that, conversely to macroalgae, under high ammonium levels, zooxanthellae cannot use ammonium and nitrate simultaneously (Syrett 1981) because their nitrate reductase may be repressed by ammonium (Guerrero et al. 1981). Except in the study of D'Elia et al. (1983), most of the other works performed on freshly isolated or cultured zooxanthellae have shown a similar repression of nitrate uptake by ammonium (Crossland and Barnes 1977; Domotor and D'Elia 1984; Taguchi and Kinzie 2001). These observations have several implications. Several authors have suggested that in the host, zooxanthellae are exposed to elevated tissue ammonium concentrations due to host catabolism (Crossland and Barnes 1977; Wilkerson and Muscatine 1984). In this case, this ammonium should have inhibited nitrate uptake unless (1) zooxanthellae are not directly exposed to this catabolic ammonium, (2) corals have low rates of ammonium excretion (Szmant-Froelich and Pilson 1984), and (3) zooxanthellae in hospite are not subject to ammonium inhibition. Conversely, D'Elia and Cook (1988) also suggested that intracellular nutrient concentrations could be very low and that zooxanthellae are in fact N-limited in the host (Cook and D'Elia 1987). Results obtained in this work are in good agreement with this hypothesis.

This ammonium-dependent uptake of nitrate also seems to affect the relation between light levels and nitrate uptake. Under ambient ammonium concentration in seawater ($< 1 \mu\text{mol L}^{-1}$), there was no significant effect of light on the uptake rates of nitrate. These rates remained unchanged from dark to $450 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photons and equal to 3.0 ± 1.7 and $1.8 \pm 0.3 \text{ ng h}^{-1} \text{ cm}^{-2} \text{ N}$ for the zooxanthellae and animal fraction respectively. The effect of darkness on nitrate uptake has been only tested once on isolated zooxanthellae of the clam *Tridacna gigas* (Wilkerson and Trench 1986). Here also, there was no noticeable effect of irradiance on nitrate depletion. With ammonium as a nitrogen source, corals also did not show differences between light and dark uptake rates (D'Elia and Webb 1977; Muscatine and D'Elia 1978; Burris 1983; D'Elia et al. 1983) unless they received a period of dark preconditioning well in excess of that of the natural daily cycle (Wilkerson and Trench 1986). Under high ammonium concentration in seawater, however, there

was a significant effect of light on the nitrate uptake rates, with higher uptake under high light. This result suggests that when the fluxes of nitrate are low, the enzyme might not be saturated and might show a light effect. Conversely, when corals experience high fluxes of nitrate (under low ammonium concentrations), all the sites of the enzyme might be saturated, even under low light.

It is rather difficult to compare ammonium and nitrate uptake rates, because few studies have investigated the two nitrogen sources under the same environmental conditions and with the same coral species (D'Elia and Webb 1977; Wilkerson and Trench 1986; Bythell 1990). When maximal uptake rates of nitrate (measured in this study) are compared with the uptake rates of ammonium obtained for the same species under comparable conditions (Grover et al. 2002), we found a preferential uptake of ammonium compared with nitrate (uptake rates of one order of magnitude higher). D'Elia and Webb (1977) as well as Wilkerson and Trench (1986) reached the same conclusions for other scleractinian species and the same trend was also noticed for the symbiotic medusae, *Linuche unguilata* (Wilkerson and Kremer 1992). Bythell (1990) was the only one to find contrary results, with mean net rates of nitrate uptake exceeding that of ammonium by a factor of two. He explained these results by a variable direction of ammonium flux at low concentrations. His nitrate uptake rates were also 10 times higher than those measured in this study, but a different coral species was investigated, and a different technique was used for uptake rate measurements (in situ depletion). Moreover, the flow might also have been different. Atkinson et al. (1994) indeed demonstrated that the uptake of ammonium may vary by two times during a 10-fold change in water velocity.

On the basis of the nitrogen content of zooxanthellae, and assuming 0.7×10^6 zoox cm⁻² (Grover et al. 2002), it is possible to calculate if an external concentration of nitrate equal to $0.3 \mu\text{mol L}^{-1}$ can sustain the growth of the zooxanthellae population. Nitrate uptake rate by the zooxanthellae fraction was estimated equal to $2 \text{ fmol h}^{-1} \text{ zoox}^{-1} \text{ N}$ or $48 \text{ fmol d}^{-1} \text{ zoox}^{-1} \text{ N}$. The nitrogen content of these zooxanthellae was found to be equal to $2.3 \text{ pmol zoox}^{-1} \text{ N}$. The external nitrogen supply would therefore support a generation time of 43 d for these algae. Most of the generation times calculated for symbiotic zooxanthellae are lower than 43 d (38 d for Rahav et al. 1989 and 20 d for Szmant et al. 1990), suggesting that nitrate alone, at a low concentration, can not sustain entirely the growth of the algae. However, when combined with the uptake of ammonium (at in situ concentrations, Grover et al. 2002), these two nitrogen sources seem to completely satisfy the algal growth requirements. Zooxanthellae have therefore developed adaptations for surviving in their oligotrophic environments (Szmant-Froelich and Pilson 1984; Bythell 1990) and take advantage of all nitrogen sources available in the surrounding waters.

We made here a first attempt to look at the relation between ammonium and nitrate in the entire symbiosis. The results obtained have shown strong interactions between these two nitrogen sources, but they remain to be further investigated. We have also shown that the uptake of nitrate might be dependent on light when corals are taking both ammonium and nitrate. However, here again, the interactions

between the three parameters remain to be further investigated.

References

- ATKINSON, M. J., E. KOTLER, AND P. NEWTON. 1994. Effects of water velocity on respiration, calcification, and ammonium uptake of a *Porites compressa* community. *Pac. Sci.* **48**: 296–303.
- BORGES, J. A., W. P. COCHLAN, AND P. J. HARRISON. 1995. Laboratory and field responses of algal nitrate reductase to diel periodicity in irradiance, nitrate exhaustion, and the presence of ammonium. *Mar. Ecol. Progr. Ser.* **124**: 259–269.
- BURRIS, R. H. 1983. Uptake and assimilation of ¹⁵NH₄⁺ by a variety of corals. *Mar. Biol.* **75**: 151–155.
- BYTHELL, J. C. 1990. Nutrient uptake in the reef building coral *Acropora palmata* at natural environmental concentrations. *Mar. Ecol. Progr. Ser.* **68**: 65–69.
- CODISPOTI, L. A. 1989. Phosphorus versus nitrogen limitation of new and export production, p. 377–408. *In* Berger et al. [eds.], *Productivity of the ocean: Present and past*, Dahlem Conf. Wiley.
- COOK, C. B., AND C. F. D'ELIA. 1987. Are natural populations of zooxanthellae ever nutrient-limited? *Symbiosis* **4**: 19–212.
- , G. MULLER-PARKER, AND M. D. FERRIER. 1997. An assessment of indices of nutrient sufficiency in symbiotic dinoflagellates. *Proceedings of the 8th International Symposium on Coral Reefs* **1**: 903–908.
- CROSSLAND, C. J., AND D. J. BARNES. 1977. Nitrate assimilation enzymes from two hard corals, *Acropora acuminata* and *Goniastrea australensis*. *Comp. Biochem. Physiol.* **57B**: 151–157.
- D'ELIA, C. F., AND C. B. COOK. 1988. Methylamine uptake by zooxanthellae/invertebrate symbioses: Insights into host ammonium environment and nutrition. *Limnol. Oceanogr.* **33**: 1153–1165.
- , S. L. DOMOTOR, AND K. L. WEBB. 1983. Nutrient uptake kinetics of freshly isolated zooxanthellae. *Mar. Biol.* **75**: 157–167.
- , AND K. L. WEBB. 1977. The dissolved nitrogen flux of reef corals. *In* D. L. Taylor [ed.], *Proceedings of the 3rd International Symposium on Coral Reefs* **1**: 325–330.
- , AND W. J. WIEBE. 1990. Biogeochemical nutrient cycles in coral reef ecosystems, p. 49–74. *In* Z. Dubinsky [ed.], *Coral Reefs: Ecosystems of the world series*. Elsevier.
- DOMOTOR, S. L., AND C. F. D'ELIA. 1984. Nutrient uptake kinetics and growth of zooxanthellae maintained in laboratory culture. *Mar. Biol.* **80**: 93–101.
- DUGDALE, R. C., AND F. P. WILKERSON. 1986. The use of ¹⁵N to measure nitrogen uptake in eutrophic oceans; experimental considerations. *Limnol. Oceanogr.* **31**: 673–689.
- FALKOWSKI, P. G., Z. DUBINSKY, L. MUSCATINE, AND L. MCCLOSKEY. 1993. Population control in symbiotic corals. *Bioscience* **43**: 606–611.
- FERRIER-PAGÈS, C., V. SCHOELZKE, J. JAUBERT, L. MUSCATINE, AND O. HOEGH-GULDBERG. 2001. Response of a scleractinian coral *Stylophora pistillata* to iron and nitrate enrichment. *J. Exp. Mar. Biol. Ecol.* **259**: 249–251.
- FRANZISKET, L. 1974. Nitrate uptake by reef corals. *Int. Revue ges. Hydrobiol.* **59**: 1–7.
- FURNAS, M. J. 1991. Nutrient status and trends of the waters in the Great Barrier Reef Marine Park, p. 162–179. *In* D. Yellowlees [ed.], *Land uses patterns and nutrient loadings of the Great Barrier Reef region*. James Cook Univ.
- GROVER, R., J. F. MAGUER, S. REYNAUD-VAGANAY, AND C. FERRIER-PAGÈS. 2002. Uptake of ammonium by the scleractinian

- coral *Stylophora pistillata*: Effect of feeding, light and ammonium concentrations. *Limnol. Oceanogr.* **47**: 782–790.
- GUERRERO, M. G., J. M. VEGA, AND M. LOSADA. 1981. The assimilatory nitrate-reducing system and its regulation. *A. Rev. Plant Physiol.* **32**: 169–204.
- HAWKINS, A. J. S., AND D. W. KLUMPP. 1995. Nutrition of the giant clam *Tridacna gigas*. II. Relative contributions of filter-feeding and the ammonium acquired and recycled by symbiotic alga towards total nitrogen requirements for tissue growth and metabolism. *J. Exp. Mar. Biol. Ecol.* **190**: 263–290.
- HOEGH-GULDBERG, O., AND J. WILLIAMSON. 1999. Availability of two forms of dissolved nitrogen to the coral *Pocillopora damicornis* and its symbiotic zooxanthellae. *Mar. Biol.* **133**: 561–570.
- LIPSCHULTZ, F., AND C. B. COOK. 2002. Uptake and assimilation of ¹⁵N-ammonium by the symbiotic sea anemones *Bartholomea annulata* and *Aiptasia pallida*: Conservation versus recycling of nitrogen. *Mar. Biol.* **140**: 489–502.
- MARUBINI, F., AND P. S. DAVIES. 1996. Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals. *Mar. Biol.* **127**: 319–328.
- MILLER, D. J., AND D. YELLOWLEES. 1989. Inorganic nitrogen uptake by symbiotic marine cnidarians: A critical review. *Proc. R. Soc. (Ser B)* **237**: 109–125.
- MULLER-PARKER, G., C. F. D'ELIA, AND C. B. COOK. 1988. Nutrient limitation in zooxanthellae: Effects of host feeding history on nutrient uptake by isolated algae. *Proceedings of the 6th International Symposium on Coral Reef* **3**: 15–19.
- MUSCATINE, L. 1980. Uptake, retention and release of dissolved inorganic nutrients by marine alga-invertebrate associations, p. 229–244. *In* C. B. Cook et al. [eds.], *Cellular interactions in symbiosis and parasitism*. Ohio State.
- , AND C. F. D'ELIA. 1978. The uptake, retention and release of ammonium by reef corals. *Limnol. Oceanogr.* **23**: 725–734.
- , AND R. E. MARIAN. 1982. Dissolved inorganic nitrogen flux in symbiotic and nonsymbiotic medusae. *Limnol. Oceanogr.* **27**: 910–917.
- , P. G. FALKOWSKI, Z. DUBINSKY, P. A. COOK, AND L. R. MCCLOSKEY. 1989. The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. *Proc. R. Soc.* **236**: 311–324.
- , ———, J. W. PORTER, AND Z. DUBINSKY. 1984. Fate of photosynthetically fixed carbon in light- and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. *Proc. R. Soc. (Ser B)* **222**: 181–202.
- RAHAV, O., Z. DUBINSKY, Y. ACHITUV, AND P. G. FALKOWSKI. 1989. Ammonium metabolism in the zooxanthellate coral *Stylophora pistillata*. *Proc. R. Soc. Lond. B* **236**: 325–337.
- ROBERTS, J. M., P. S. DAVIES, L. M. FIXTER, AND T. PRESTON. 1999. Primary site and initial products of ammonium assimilation in the symbiotic sea anemone *Anemonia viridis*. *Mar. Biol.* **135**: 223–236.
- SHEPHERD, D., W. LEGGAT, T. A. V. REES, AND D. YELLOWLEES. 1999. Ammonium, but not nitrate, stimulates an increase in glutamine concentration in the haemolymph of *Tridacna gigas*. *Mar. Biol.* **133**: 45–53.
- STIMSON, J., AND R. A. KINZIE. 1991. The temporal pattern and rate of release of zooxanthellae from the reef coral *Pocillopora damicornis* (Linnaeus) under nitrogen-enrichment and conditions. *J. Exp. Mar. Biol. Ecol.* **153**: 63–74.
- SWANSON, R., AND O. HOEGH-GULDBERG. 1998. Amino acids synthesis in the symbiotic sea anemone *Aiptasia pulchella*. *Mar. Biol.* **131**: 83–93.
- SYRETT, P. J. 1981. Nitrogen metabolism of microalgae. Physiological bases of phytoplankton ecology. *Can. Bull. Fish. Aquat. Sci.* **210**: 182–210.
- SZMANT, A. M., L. M. FERRER, AND L. M. FITZGERALD. 1990. Nitrogen excretion and O:N ratios in reef corals: Evidence for conservation of nitrogen. *Mar. Biol.* **104**: 119–127.
- SZMANT-FROELICH, A. M., AND M. E. Q. PILSON. 1984. Effects of feeding frequency and symbiosis with zooxanthellae on nitrogen metabolism and respiration of the coral *Astrangia danae*. *Mar. Biol.* **81**: 153–162.
- TAGUCHI, S., AND R. A. KINZIE III. 2001. Growth of zooxanthellae in culture with two nitrogen sources. *Mar. Biol.* **138**: 149–155.
- TAMBUITTE, E., D. ALLEMAND, I. BOURGE, J. P. GATTUSO, AND J. JAUBERT. 1995. An improved ⁴⁵Ca protocol for investigating physiological mechanisms in coral calcification. *Mar. Biol.* **122**: 453–459.
- TRÉGUER, P., AND P. LECORRE. 1975. Manuel d'analyse des sels nutritifs dans l'eau de mer (utilisation de l'Autoanalyseur II Technicon). Lab d'Océanographie Chim Univ Bretagne Occidentale, 2^e ed. Brest.
- VERGARA, J. J., J. A. BERGES, AND P. G. FALKOWSKI. 1998. Diel periodicity of nitrate reductase activity and protein levels in the marine diatom *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycol.* **34**: 952–961.
- WANG, J. T., AND A. E. DOUGLAS. 1998. Nitrogen recycling or nitrogen conservation in an alga-invertebrate symbiosis? *J. Exp. Biol.* **201**: 2445–2453.
- WEBB, K. L., AND W. J. WIEBE. 1978. The kinetics and possible significance of nitrate uptake by several alga-invertebrate symbioses. *Mar. Biol.* **47**: 21–27.
- WILKERSON, F. P., AND P. KREMER. 1992. DIN, DON and PO₄ flux by a medusa with algal symbionts. *Mar. Ecol. Prog. Ser.* **90**: 237–250.
- , AND L. MUSCATINE. 1984. Uptake and assimilation of dissolved inorganic nitrogen by a symbiotic sea anemone. *Proc. R. Soc. (Ser B)* **221**: 71–86.
- , AND R. K. TRENCH. 1986. Uptake of dissolved inorganic nitrogen by the symbiotic clam *Tridacna gigas* and the coral *Acropora* sp. *Mar. Biol.* **93**: 237–246.
- YELLOWLEES, D., T. A. V. REES, AND W. K. FITT. 1994. 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. *Pac. Sci.* **48**: 291–295.

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