Marine Pollution Bulletin 64 (2012) 602–613

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Phosphorus and nitrogen effects on microbial euendolithic communities and their bioerosion rates

M. Carreiro-Silva [a,*], W.E. Kiene [b], S. Golubic [c], T.R. McClanahan [d]

[a] Chesapeake Biological Laboratory, University of Maryland, Center for Environmental Science, Solomons, MD 20688-0038, USA
[b] NOAA/Office of National Marine Sanctuaries Southeast, Gulf of Mexico and Caribbean Region, Galveston, TX, USA
[c] Biological Science Center, Boston University, Boston, MA, USA
[d] Marine Programs, Wildlife Conservation Society, Bronx, NY, USA

ARTICLE INFO

Keywords:
Calcium carbonate balance
Cyanobacteria
Eutrophication
Green algae
Microbial euendoliths
Nutrient limitation

ABSTRACT

Cages and fertilizers were used at Glover's Atoll, Belize to test the relative importance of nitrogen (N) and phosphorus (P) to microbial euendolithic communities (bacteria, algae and fungi) and their bioerosion rates of *Strombus gigas* shells after 56-days of exposure. By the end of the experiment, the abundance of green algae was higher than cyanobacteria and fungi in N and N + P treatments, although green algae did not increase proportionally with increasing N concentrations, suggesting that green algae were co-limited by P and N. In contrast, cyanobacteria abundance increased with increasing P concentration, suggesting that cyanobacteria were P-limited. Fungi were not significantly affected by the addition of nutrients. Microbioerosion rates in the N and N + P treatments were 2-times greater than rates in the P treatment and 15-times greater than the control treatment. Results demonstrate that increased nutrient concentrations on coral reefs may increase microbioerosion rates, and variations in nutrient ratios can modify microborers community composition.

1. Introduction

Euendolithic algae and cyanobacteria are ubiquitous inhabitants of tropical environments (Golubic and Schneider, 2003), colonizing every available carbonate substrata (e.g. limestone rocks, sediment grains, corals, coralline algae, mollusk shells: Tribollet, 2008a). They play important ecological and geological roles in reef environments. Experimental work by Tribollet et al. (2006) have demonstrated that cyanobacteria and algae, which are euendolithic phototrophs, are major primary producers in coral reef ecosystems, with rates of net photosynthesis more than 2 g C m^{-2} day^{-1}. Microbial euendoliths are also important agents of microbioerosion of dead carbonate substrates (e.g. Chazottes et al., 1995, 2002; Tribollet et al., 2002; Tribollet and Golubic, 2005; Tribollet, 2008a,b) representing a significant destructive force that can modify a reef's calcium carbonate budget. Chazottes et al. (1995) showed that microborers are the main agents of bioerosion at the early stage of the bioerosion process ($\leqslant$1 year), and represent 60% of total bioerosion (microborers, macroborers and grazers) on the reef at Tiahura (Moorea, French Polyensia). Tribollet et al. (2002) and Tribollet and Golubic (2005) showed that after 3 years, euendoliths still contributed between 20% and 40% of the total bioerosion of coral substrates that were exposed to grazers in oligotrophic waters of the northern Great Barrier Reef.

Rates of microbioerosion vary depending on the species composition of the boring microflora (Chazottes et al., 1995; Tribollet, 2008b). At early stages of bioerosion and in shallow waters, the pattern of bioerosion depends on the most abundant euendoliths with high density networks and/or largest boring diameters, generally the cyanobacterium *Mastigocoleus testarum* and the chlorophyte *Phaeophila dendroides* (Chazottes et al., 1995). After one year of exposure, the later colonizing euendolithic chlorophyte *Ostreobium quekettii* becomes the principal agent of microbioerosion due to its higher depth of penetration within substrates (Chazottes et al., 1995; Tribollet, 2008b). The composition of the boring microflora can be modified by epilithic algal cover and excavating grazers, and the interaction between these processes largely determines the overall rates of microbioerosion of carbonate substrates (Schneider and Torunski, 1983; Chazottes et al., 2002; Carreiro-Silva et al., 2005; Tribollet and Golubic, 2005).

Investigations on the relative importance of nitrogen versus phosphorus limitation of algal communities in coral reefs have produced varying results (Lapointe, 1987; Lapointe et al., 1992; Fong et al., 1993; Larned, 1998; Kuffner and Paul, 2001; McClanahan et al., 2007). Based on comparisons of atomic concentrations of inorganic nitrogen and inorganic phosphorus (N:P ratios) in seawater and in algal tissues, some studies have suggested that

* Corresponding author. Current address: Departamento de Oceanografia e Pescas, Universidade dos Açores PT-9901-862 HORTA, Portugal. Tel.: +351 292 207 839; fax: +351 292 207 811.
E-mail address: mcsilva@uac.pt (M. Carreiro-Silva).

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doi:10.1016/j.marpolbul.2011.12.013

phosphorus is more often the limiting nutrient in carbonate environments, where carbonate sediments can adsorb phosphorus (Lapointe, 1987; Littler et al., 1991; Lapointe et al., 1992). However, results of nitrogen- and phosphorus-enrichment bioassays using tropical macroalgae indicate that nitrogen limitation is also common (Fong et al., 1993; Delgado and Lapointe, 1994; Larned, 1998), but may be taxa- or habitat-specific (McClanahan et al., 2007). Less is known about the relative importance of nitrogen and phosphorus to microphytobenthic turfs, although microcosm studies on benthic or mat-forming cyanobacteria suggest that cyanobacterial mats may be phosphorus limited, whereas diatoms are mainly limited by nitrogen (Fong et al., 1993; Kuffner and Paul, 2001).

Results from our previous fertilization and herbivory experiments in the Glover's Reef Atoll, Belize showed that bioerosion rates by microbial euendoliths were enhanced eight to ten times by fertilization with inorganic nitrogen and phosphorus (Carreiro-Silva et al., 2005, 2009), but the inclusion of grazers reduced the abundance of microborers and measurable microbioerosion rates by half when compared to ungrazed substrates (Carreiro-Silva et al., 2005). These studies have, however, tested the effects of elevated nutrient additions by adding inorganic nitrogen and phosphorus simultaneously. Therefore, the present study was specifically conceived to distinguish between the particular effects of phosphorus vs. nitrogen on microbial euendolithic community composition and microbioerosion rates.

In this study, we measured the response of microbial euendoliths living within *Strombus gigas* shells to different proportions of nitrogen and phosphorus. Our aim was to determine whether microbial euendolithic organisms were limited by a single nutrient or co-limited by N and P, and whether changes in responses of microbial euendoliths were proportional to the concentrations of nutrients in the mixture.

2. Materials and methods

2.1. Study site

This study was conducted at Glover's Reef Atoll, Belize from June to August 2004 (see map in Carreiro-Silva et al., 2009). Experimental substrata were placed at 2-m depth on the windward side of a patch reef in the Conservation Zone of the atoll's lagoon, where resource extraction is prohibited. The reef is remote and experiences no significant local organic pollution apart from seasonal inputs from distant rivers into this larger oceanic region (Cherubin et al., 2008). The waters in this area are calm with a small (<0.5 m) tidal range and slow current speeds (<1 m s^{-1}). No large waves, currents, or other physical disturbances such as hurricanes were experienced during the study period.

2.2. Experimental design

A "mixture-experiment" design with closed cages and fertilizers was used to test for the relative importance of nitrogen (N) and phosphorus (P) to microbial euendolithic communities and their bioerosion rates after a 56-day period. In a mixture experiment, the independent factors are in proportion, where the sum of the mixture must equal to 1 (Cornell, 2002). We used the simplest case of a mixture-experiment design with only 2 components or factors. The factors studied were inorganic nitrogen and phosphorus in a 4-treatment structure: (1) a control treatment without fertilizer addition, therefore the treatment consisted of environmental background conditions; (2) the addition of 1.5 kg of nitrogen (N) fertilizer; (3) the addition of 1.5 kg of phosphorus (P) fertilizer; and (4) the addition of 0.75 kg of N and 0.75 kg of P.

The experiment used 16 cages (4 cages per treatment, 50 × 50 × 20 cm) constructed with PVC frames and 3-cm mesh plastic caging material. Cages were tied to cement masonry blocks that kept them solidly to the reef bottom. The cage mesh size allowed for good water flow and light penetration and conditions in the cage were expected to resemble natural conditions. Nevertheless, extrapolation of the results of actual microbioerosion rates should be interpreted with caution due to possible caging effects and the limited length of the study.

Experimental substrata were made of *S. gigas* mollusk shells. Shells were used instead of coral blocks because their less-porous structure produces better casts of the boring organisms of interest, improving measurements of area colonized and depth of penetration (Carreiro-Silva et al., 2009). By using the interior parts of undamaged shells in this study, pre-existing microborings were avoided. There are differences in the density of coral skeletons and mollusk shells substrata, so microbioerosion rate estimates for shells provided here may not directly correspond to rates for corals. However, the objective of the study was to investigate how inorganic nutrients interact and affect microbioerosion of carbonate substrata by comparing species composition and microbioerosion rates among treatments, and not to determine absolute microbioerosion rates for coral reefs.

We placed two pieces (~12 × 6 cm) of *S. gigas* shell fragments in each cage, making a total of eight replicate shell samples exposed to each of the four treatments. A hole was drilled in each of the conch shell pieces and threaded with a plastic cable tie to attach them to the bottom of the cages such that the shell interiors faced upward. Cages were placed >1 m apart in a line aligned 90° to the dominant current direction such that neighboring cages would not slow the currents experienced by the other cages and fertilizer would not influence the non-fertilized treatments. We used wire brushes to clean all cages of algae and other settling organisms every other day to reduce caging artifacts such as decreased light and obstruction of local water flow associated with increased algal growth on the mesh of cages.

Cages excluded large herbivorous fishes and large predators but allowed small fishes such as damselfishes (*Stegastes* spp.), wrasses, and small parrotfish (*Sparisoma aurofrenatum* and *Scarus iserti*) to enter and forage (McClanahan et al., 2007). The experimental site was not inhabited by sea urchins, thus not affected by their foraging. No gastropods or crustaceans were observed inside cages throughout the experiment. The numbers of damselfish, parrotfish, and wrasses that occupied each cage over a 3-min period were counted on days 11, 25, 39, and 53 of the experiment. Following the count, we arbitrarily selected and watched one fish from each cage and recorded the number of bites it took during a 1 min period. These data were presented in McClanahan et al. (2007).

2.3. Nutrient enrichment and sampling

We placed 1.5 kg each of solid high phosphate and high nitrogen slow-release fertilizer in the phosphorus and nitrogen cages, respectively. The phosphorus fertilizer was a rock fertilizer (46% phosphate by weight). The nitrogen fertilizer was an Osmocote fertilizer (11.5% nitrogen as nitrate, 11.5% as ammonium, 0.5% sodium, and 3.3% calcium by weight). The mixed fertilizer treatment contained 0.75 kg of high nitrogen fertilizer and 0.75 kg of high phosphate fertilizer. We fertilized the cages with the above quantities on the 1st and 28th day of the experiment by placing fertilizer bags underneath cages as described in McClanahan et al. (2007). There was fertilizer remaining beneath the cages at the time of re-fertilization after 1 month, suggesting that the original fertilizer was still diffusing out when it was replenished.

Water samples were collected from each cage on days 4 and 32 of the experiment, such that 32 water samples were taken equally

between the four treatments. Samples were taken from each cage by opening 1-L acid-washed Nalgene bottles approximately 1-cm above the surface of the substratum. Concentrations of nitrite, nitrate, ammonium, and soluble phosphates were measured the same day with a Hach DR/2500 spectrophotometer, using the cadmium-reduction method for nitrate and ascorbic acid method for phosphorus (Parsons et al., 1984). Due to the high variability and uncertainty of ammonium measurements, only concentrations of nitrogen as nitrate and nitrite were used to determine nitrogen concentration. Variability in the ammonium results was attributed to problems with reagents packets used for the ammonium analysis. These data were presented in McClanahan et al. (2007).

2.4. Sample preparation

Immediately after collection from the cages, shell fragments were fixed in 4% formaldehyde in sea water solution. Epilithic algal communities on shells ($n = 8$ per treatment) were studied by taking photographs of the shell's upper surface and estimating the percent surface area covered by different algal groups (turfs, crustose coralline algae and macroalgae) using the image analysis software ImageJ (available at the National Institute of Health website).

Two approaches were used to document the composition and abundance of microbial endoliths in each treatment: (1) casts of the boring traces in the experimental samples were observed under scanning electron microscopy (SEM) to document the composition and abundance of microbial euendolith community, and allowing the quantification of their bioerosion rates; (2) observation of microbial euendoliths under light microscope for detailed identification and confirmation of organisms that produce the traces seen in the SEM casts.

Casts were prepared by cutting and trimming two 1-cm^3 cubes from the middle portion of each shell fragment with a diamond-blade rock saw, removing organic matter remains with sodium hypochlorite, and using the cast-embedding technique (modified after Golubic et al., 1970), as described in Carreiro-Silva et al. (2009). Casts of shells used in the experiment and casts of unexposed shells were investigated by scanning electron microscopy. Examination of unexposed shell fragments ($n = 8$) confirmed that there were no borings prior to the experiment. A total of eight shell samples per treatment were analyzed, and two 1-cm^3 sub-samples per shell.

In order to investigate the organisms by light microscopy, the soft epilithic overgrowth of shell pieces ($n = 20$) was removed under a dissecting microscope and diluted HCl dissolved the remaining calcareous incrustation (coralline algae) and substratum. The emerging microbial endoliths were mounted on microscope slides and examined with a Leica DM6000 digital microscope with differential interference contrast (DIC) at 400–1000× magnification.

We applied a dual nomenclature to microbial euendoliths and refer to them using both their ichnotaxonomy as a morphological classification of the traces and the biological nomenclature for classifying the euendolithic organisms that produced those traces. Identification of the microbial organisms and their boring cast followed descriptions in Le Campion-Alsumard (1979), Le Campion-Alsumard and Golubic (1985), Radtke (1993), Radtke and Golubic (2005), and Wisshak et al. (2005).

2.5. Microbioerosion rates

The resin-cast method described above results in three-dimensional casts of the tunnel systems produced by euendoliths within the shells. Based on their structure, microboring traces were classified into morphological types (filament networks, spherical chambers, filament clusters). Scanning electron micrographs with several examples of boring percent area colonized of the shell's inner upper surface were prepared and the surface areas of boring traces were carefully measured using ImageJ. The percentage abundance measurements were based on keys presented in Flügel (1982) used in sedimentary geology and the microscopic analysis of grain percentages in rock thin-sections. Microborers percent area colonized was determined by comparing the scanning electron images to this key of different abundances (Kiene et al., 1995; Vogel et al., 2000). By comparing these key images to the small areas viewed with the SEM on each sample's inner upper surface, the areas could be rapidly classified as to their type and percent area colonized by borings without having to measure these variables for every area observed. The depth of boring was measured in each sample by observing the sides of boring casts under SEM and measuring the height of boring tunnel networks ($n = 20$). Although it is not an absolute measure of bioerosion, the results obtained from this procedure provide an adequate method for comparing relative bioerosion rates between samples and treatments (Vogel et al., 2000).

Abundance of different microborers and rate of microboring was measured by classifying 20 1-mm^2 areas of the 1 cm^2 sample's inner upper surface using the keys described above. The volume of calcium carbonate removed by the microborers in each sample was calculated using the percent area colonized by the boring traces multiplied by their depth of penetration. Rates of calcium carbonate removed by microborers ($g\ m^{-2}$) were calculated using the volume removed multiplied by the shell density ($2.65\ g\ cm^{-3}$). Bioerosion rates over the 56-day experiment were converted to $g\ m^{-2}\ y^{-1}$to present them in the form most commonly reported in previous studies.

2.6. Statistical analysis

A mixed model nested analysis of variance (ANOVA) was used to test for the effects of nitrogen and phosphorus treatments on bioerosion rates by all microborings and on microborer depth of penetration within the substratum, and to examine the variation in microbioerosion rates and depths of penetration among shells and among cages within a treatment (Mixed procedure, SAS Institute, 2004). The treatments were treated as fixed effects, whereas cages within treatments and shells within cages and treatments were treated as random effects in the model. Fixed effects were tested using the approximate F-tests of this procedure and the random effects were tested using the variance component approach (Littell et al., 2006). The residual variance component was interpreted as variability among sub-samples within each shell (the basal unit of replication). The percent variation explained by the nested factor relative to the total variation of the random terms was estimated by dividing the variance component of the nested factor by the total variance (cages within treatments variance + shells within cages and treatments variance + residual variance). The analysis was performed on the log-transformed data to correct for lack of homogeneity of variance.

Treatment effects on the percent area colonized by epilithic algae (algal turfs and crustose coralline algae) and microboring groups (green algae, cyanobacteria, and heterotrophs) were tested with a nested ANOVA using the generalized linear mixed model "Proc GLIMMIX" procedure of (SAS Institute, 2004; Littell et al., 2006) for proportion data. Predicted values of percent cover were logit-transformed to linearize data and models were fit to the data using residual pseudo-likelihood. This generalized linear mixed model procedure assumed a pseudo-binomial error distribution because the data were recorded on a scale from 0 to 1, and a logit-link function (SAS Institute, 2004). Fixed and random effects in the model were the same as described above.

Tukey's test (Sokal and Rohlf, 1995) was used to perform post hoc comparisons of means for significant effects. We tested

whether the change in the microbial euendoliths' area colonized and microbioerosion rates were proportional to changes in the nitrogen and phosphorus ratios in treatments, by comparing the simple average value of the response in the N and P treatments and the response measured for the N + P treatment. According to mixture design theory, when the effects of both components in a mixture are additive, the change in the mean response is proportional to changes in treatments and the response variable is best represented by a straight line that joins the responses of the single component treatments (Cornell, 2002). In our case, if the response to the N + P treatment is higher or lower than the simple average response of the N and P treatments (above or below the additive line), then there is an interaction between nitrogen and phosphorus, and the mean response is not proportional to changes in treatments.

A Detrended Correspondence Analysis (Sall et al., 2001) was performed to characterize the main patterns of variation in the abundance and composition of euendolithic microorganisms with respect to the treatments.

Data on area coverage by organisms, depths of penetration and bioerosion rates are presented as mean ± standard deviation (mean ± SD) or as standard error of the mean (mean ± SE) of samples studied ($n = 8$).

3. Results

3.1. Epilithic algal cover on experimental substrata

Epilithic algal communities on experimental substrata were mainly composed of algal turfs, which covered 27–64% of total surface area of the experimental substrata. Crustose coralline algae covered <2% of the substrata in all treatments (Fig. 1). Frondose macroalgae were not observed in any treatment. Algal turf cover was two times greater in the N and N + P treatments (63.8 ± 10.7% and 61.4 ± 19.4% respectively, mean ± SD) than in the P and control treatments (35.5 ± 10.5% and 27.1 ± 6.2% respectively; Table 1). In contrast, crustose coralline algae cover was not significantly different among treatments. An estimation of the variance components indicated that 87–98% of the total variance of the random terms was due to differences among shells within treatments and 2–13% to differences among sub-samples within shells for turf algae.

3.2. Microborers within experimental substrata

3.2.1. Microborer species composition

We identified a total of 15 different microborer traces in *S. gigas* shells corresponding to seven species of cyanobacteria, three species of green algae, four species of fungi, and one unidentified heterotroph (Table 2; Figs. 2 and 3). Boring traces identified as *Rhopalia catenata* produced by the green alga *Phaeophila* sp. were dominant in all treatments (Fig. 2a and b). The second most abundant trace, especially in the P treatment, corresponded to initial stages of *Fascichnus* ispp., produced by the cyanobacteria *Hyella* spp. (9.7 ± 8.9%, mean ± SD; Fig. 2c and d). Percent area colonized by other microborer traces was variable and generally less than 2%.

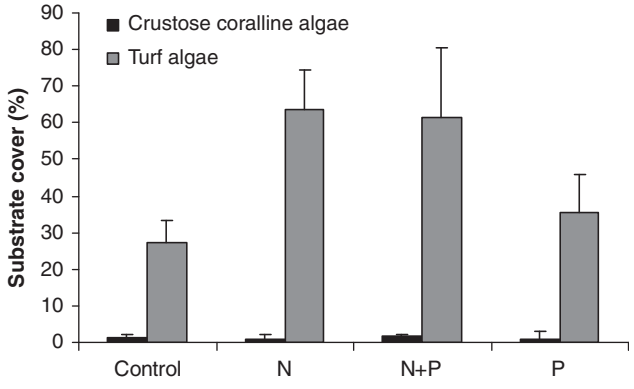

Fig. 1. Epilithic algal cover (mean% ± SD) observed on *Strombus gigas* shells exposed to different nutrient treatments during a period of 56 days.

Table 1
Nested ANOVA (mixed model) results on the effects of nitrogen and phosphorus mixture treatments on on epilithic algae logit-transformed means.

	Effect	DF	Variance component	*F*-value	*P*-value
Crustose Coralline Algae					
Treatment	Fixed	3		0.67	0.5912
Cage (treatment)	Random		0.7980		
Residual	Random		0.0119		
Turf Algae					
Treatment	Fixed	3		7.79	0.0093
Cage (treatment)	Random		0.1209		
Residual	Random		0.0181		

Note: For random effects the variance components are reported, while for fixed effects the F-ratios and their probabilities are reported. DF are ordinary least of squares degrees of freedom.

Results of the Detendred Correspondence Analysis (Fig. 4) showed that the P treatment was distinguished from other treatments by the presence of the trace *Fascichnus* ispp., produced by *Hyella* spp. (Fig. 2b and c and Fig. 3a), the trace *Eurygonum nodosum*, produced by *Mastigocoleus testarum* (Fig. 2e and f), the boring trace *Planabola* isp. (Fig. 3b)produced by *Cyanosaccus* sp. (all cyanobacteria) and *Saccomorpha spherula* produced by the fungus *Lithopythium gangliiforme*. The N and N + P treatments were most similar and were characterized by the presence of the boring trace *Scolecia filosa,* produced by the cyanobacterium *Plectonema terebrans* (Fig. 2g, h), the boring trace *Fascichnus grandis* (Fig. 3c), produced by rizhoids of the green alga *Acetabularia*, and a high abundance of boring traces of *Rhopalia* produced by the chlorophyte *Phaeophila* sp. (Fig. 3d). The control treatment was very similar in composition to the N and N + P treatments but with lower abundance of boring traces of *Phaeophila* sp. (compare Fig. 2a with 3d).

The addition of fertilizers significantly affected colonization by green algae and cyanobacteria, but did not affect heterotroph colonization (Table 3). Colonization by green algae increased by a factor of four with the addition of nitrogen (in both N and N + P treatments) compared with the control and P treatments (Tukey T-test $p < 0.0001$; Table 3; Fig. 5a). There was no difference in colonization by green algae between the control and P treatments (Tukey T-test $p > 0.05$), which contained the lowest green algal colonization. Green algal colonization in the N + P treatment was significantly different from the average of the N and P treatments (planned comparison $p < 0.01$), indicating that a change in algal abundance was not proportional to the change in fertilizer proportions (interaction of N and P; Fig. 6a).

The addition of P alone increased colonization by cyanobacteria 20 times above control values, while the addition of N + P increased cyanobacterial colonization five to seven times above the N and control treatments respectively (Tukey T-tests $p < 0.001$; Table 3; Fig. 5a). Colonization by cyanobacteria was the lowest and not statistically different between the control and N treatments (Tukey T-tests $p > 0.05$). We did not find a significant difference between colonization by cyanobacteria in the N + P treatment and the simple average in the N and P treatments (planned comparison $p > 0.1$; Fig. 6b). Thus, cyanobacterial abundance increased proportionally with increased P in the mixture.

The variance components for green algal colonization indicated that ~39% of the total variance of the random terms was due to

Table 2
Percent surface area colonized by microboring traces (ichnotaxa) and their producers (bio-species) in experimental substrata made from *Strombus gigas* shell and exposed to different treatments for 56 days. Values are Mean (Standard deviation).

Ichnotaxa (Bio-species)	Control	P	N	P + N
Cyanobacteria				
Scolecia filosa (Plectonema terebrans)	0.03 (0.08)	1.5 (2.1)	0.9 (1.0)	5.1 (4.5)
Fascichnus dactylus (Hyella caespitosa)	0.4 (0.3)	1.1 (1.5)	0.4 (0.7)	1.1 (1.8)
Fascichnus frutex (Hyella gigas)	0.08 (0.24)	2.2 (2.1)	0.05 (0.15)	0.1 (0.4)
Fascichnus parvus (Hyella pyxis)	–	0.3 (1.2)	–	–
Fascichnus isp. (Hyella sp.)	–	9.7 (8.9)	0.1 (0.5)	0.3 (0.8)
Eurygonum nodosum (Mastigocoleus testarum)	0.3 (0.5)	4.9 (6.0)	–	0.8 (1.2)
Planabola isp. (Cyanosaccus sp.)	0.1 (0.15)	0.6 (1.8)	–	–
Green algae				
Fascichnus grandis (Acetabularia rizhoid)	–	–	2.0 (1.2)	1.0 (1.9)
Ichnoreticulina elegans (Ostreobium quekettii)	0.6 (1.17)	0.4 (0.9)	–	–
Rhopalia catenata (Phaeophila sp.)	13.7 (2.8)	17.7 (8.7)	63.1 (13.2)	62.6 (17.9)
Heterotrophs				
Saccomorpha sphaerula (Lithopythium gangliiforme)	0.3 (0.4)	2.5 (2.8)	0.2 (0.3)	0.1 (0.2)
Saccomorpha clava (Dodgella priscus)	0.8 (0.9)	–	–	0.04 (0.12)
Polyactina araneola (Conchyliastrum enderi)	0.02 (0.05)	–	–	0.0005 (0.0002)
Orthogonum fusiferum (Ostracoblabe implexa)	0.3 (0.5)	0.4 (0.7)	0.7 (0.9)	0.4 (0.7)
Orthogonum isp. (Unknown heterotroph)	0.3 (0.6)	–	–	–
Total	17 (3.0)	41 (16)	67.5 (14.2)	71.5 (20.1)

differences among cages within treatments, 13% was due to differences among shells within treatments, and 48% due to differences among sub-samples within shells (Table 3). Variance components for cyanobacteria and heterotroph colonization indicated that most of the total variance of the random terms was due to differences among cages within treatments (62% for cyanobacteria and 99% for heterotrophs). Differences in cyanobacterial colonization among shells within treatments explained 37% of the total variance in random terms, whereas differences among sub-samples within shells explained 1% of the total variance. Differences in heterotroph colonization among shells within treatments and among sub-samples within shells explained only 1% of the total variance.

3.2.2. Microborer depth of penetration

Depths of penetration of euendolithic filaments in experimental shells were significantly greater in fertilized treatments (46.7 ± 8.6 μm in N, 48.1 ± 7.3 μm in N + P, and 33.2 ± 18 μm in P treatments, mean ± SD) than in the control treatment (12.6 ± 4 μm; Fig. 7), but did not differ significantly among fertilized treatments (Table 3). An estimation of the variance components indicated that 56% of the total variance of the random terms was due to differences among shells within treatments and 44% to differences among sub-samples within shells.

3.3. Microbioerosion rates

N and P addition either alone or in combination increased microbioerosion rates above control values (Table 3, Fig. 8). Microbioerosion rates were ~14–15 times greater in treatments with added N (544 ± 39 g $CaCO_3$ m^{-2} y^{-1} and 593 ± 72 g $CaCO_3$ m^{-2} y^{-1} in the N and N + P treatments, respectively, mean ± SE) than in the control treatment (40 ± 7 g $CaCO_3$ m^{-2} y^{-1}) and 6 times greater in the P (235 ± 33 g $CaCO_3$ m^{-2} y^{-1}) treatment than in the control treatment (Tukey T-tests, $p < 0.0001$; Table 3). Rates were two times greater in the N than in the P treatment (Tukey T-test, $p < 0.001$). We found a significant difference in microbioerosion rates in the N + P treatment when compared to the simple average of microbioerosion rates in the N and P treatments (planned comparison $p < 0.01$), indicating that a change in microbioerosion rates was not proportional to a change in fertilizer proportions (interaction of N and P; Fig. 6c).

Variance component estimates indicated that microbioerosion rate estimates were consistent among cages within treatments and did not contribute much to the total variance of the random terms (Table 3). Differences in microbioerosion rates among shells within treatments explained 37% of the total variance, and differences among sub-samples within shells explained 63% of the total variance.

4. Discussion

4.1. Effectiveness of treatments

Nutrient concentrations were doubled in nitrogen and phosphorus fertilized treatments compared with control treatment (see data in McClanahan et al., 2007), and were within levels expected for nutrient-enriched reef environments (Lapointe, 1999). The mixture experiment used proportions of nitrogen and phosphorus while the total amount of fertilizer was maintained constant in all fertilized treatments. This experimental design added twice the N and P in the single factor than in the mixture treatment (N + P treatment). Accordingly, we expected that the concentration of nitrate + nitrite and phosphate in the N and P treatments would be double the mixture treatment concentrations. While this was true for the phosphorus treatment, the nitrate + nitrite concentrations in the N alone were not different from the mixture treatment. Given that half of the nitrogen added to the nitrogen fertilized treatments was in the form of ammonia and we were unsuccessful in measuring it, the total available nitrogen in the nitrogen-fertilized treatments was most likely greater than what we measured. Ammonia is often the preferred inorganic nitrogen source by algae (Graham and Wilcox, 2000), and was likely to have made the greatest contribution to the measured response of algae in nitrogen treatments.

Herbivore exclusion cages were used to minimize the possible effects of large grazers on our experiment. Grazers were previously found to reduce the abundance of microborers and measurable microbioerosion rates, potentially masking the effects of nutrients on microbioerosion (Carreiro-Silva et al., 2005). The caging was, therefore, expected to maximize the response to the nutrients, although small grazers were able to enter the cages. The herbivory rates did not differ among treatments, eliminating any indirect effects of unequal herbivory in our treatments (McClanahan et al., 2007). The small size of the cage mesh permitted only juvenile parrotfish to enter cages and graze on the experimental substrata.

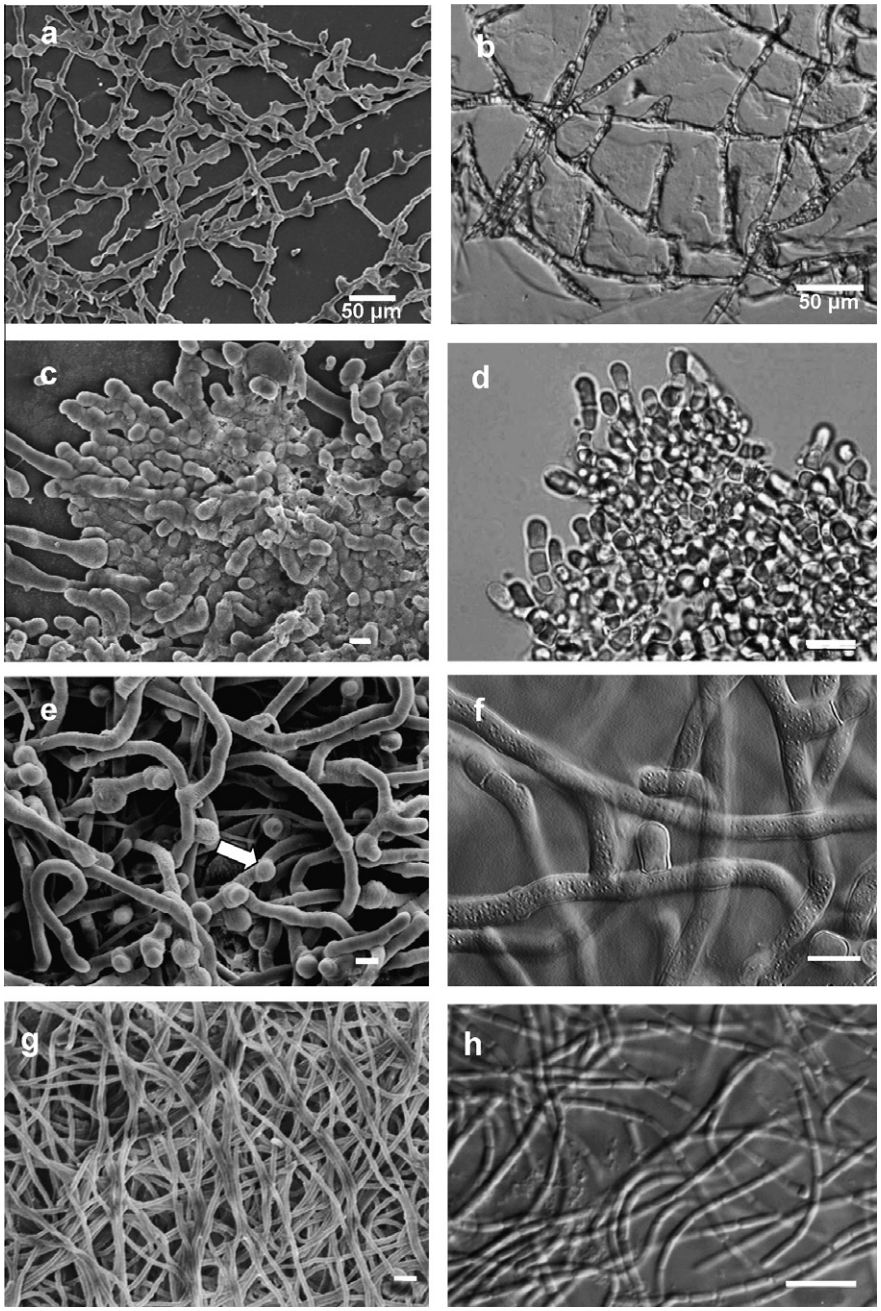

Fig. 2. Traces produced by boring euendolithic microorganisms (SEM of resin casts) and corresponding producers (DIC) observed in *Strombus gigas* shells exposed at 2 m depth, Glover's Reef, Belize for 56 days. (a) Boring trace *Rhopalia catenata* (b) produced by the chlorophyte *Phaeophila* sp. in the control treatment. (c) Boring trace *Fascichnus* isp. produced by (d) early stages of the cyanobacterium *Hyella* sp. (e) Boring trace *Eurygonum nodosum* produced by (f) the cyanobacterium *Mastigocoleus testarum*; notice the laterally positioned heterocysts (arrow). (g) Boring trace *Scolecia filosa* and (h) its producer, the cyanobacterium *Plectonema terebrans*. SEM = scanning electron microscopy; DIC = differential interference contrast microscopy. Scale bar = 10 µm if not marked otherwise.

Studies of ontogenic changes in parrotfish food selection and food intake have demonstrated that juvenile parrotfish feed mainly on epilithic algae, while larger fish feed increasingly on crustose corallines and endolithic algae (Bruggemann et al. 1994a,b). Therefore, the presence of juvenile parrotfish inside cages is likely to have only negligible affected the studied colonization processes.

4.2. Epilithic algae on experimental substrata

Filamentous turf algae were the only algal group that responded to the addition of fertilizers during this short experiment. Turf algal cover increased in treatments with nitrogen (N and N + P) but were unaffected by the addition of phosphorus alone. Crustose coralline algae remained very low (<2%), and frondose macroalgae were absent from all treatments. Findings are in agreement with reports in previous fertilization studies at Glover's Reef (McClanahan et al., 2002, 2003, 2005, 2007; Carreiro-Silva et al., 2009). These studies consistently show that inorganic nutrients increased filamentous turf algae and either did not affect or decreased crustose coralline and brown frondose algae colonization and cover.

Epilithic algal communities are an important factor influencing the abundance and composition of euendolithic communities by changing the light conditions and influencing grazers. It is,

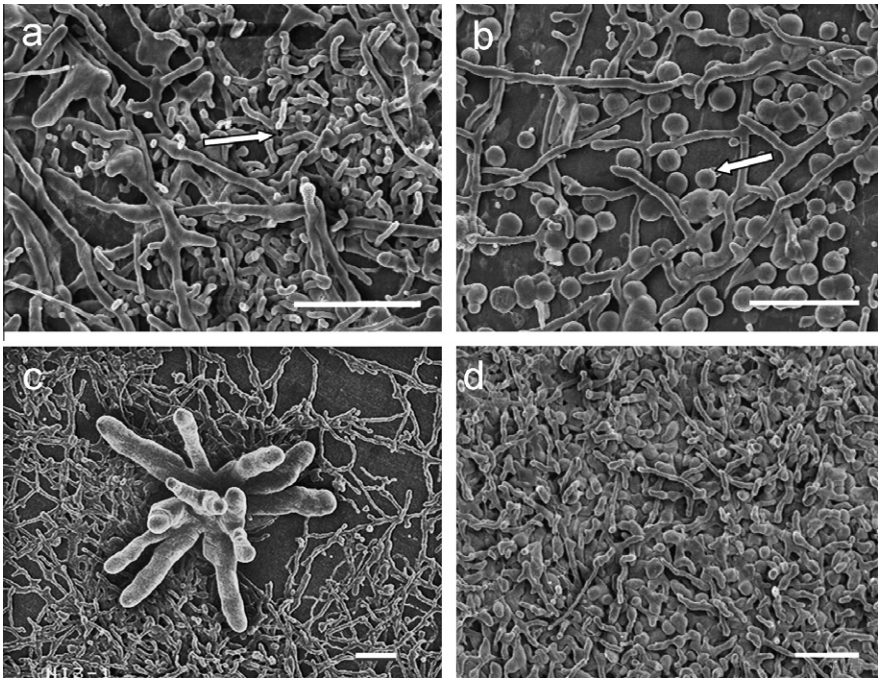

Fig. 3. Traces produced by boring euendolithic microorganisms (SEM of resin casts) in *Strombus gigas* shells exposed at Glover's Reef (2 m depth for 56 days) (cont.). (a) Boring traces *Rhopalia catenata* and *Fascichnus dactylus* (arrow) produced by chlorophyte *Phaeophila* sp. and the cyanobacterium *Hyella caespitosa* respectively; (b) boring trace *Planabola* isp (spherical chambers) produced by the cyanobacterium *Cyanosaccus sp.*; (c) boring trace *Fascichnus grandis* presumably produced by rizhoids of the green alga *Acetabularia*; (d) high abundance of *Rophalia catenata* supported by N treatment. SEM = scanning electron microscopy; Scale bar = 100 μm.

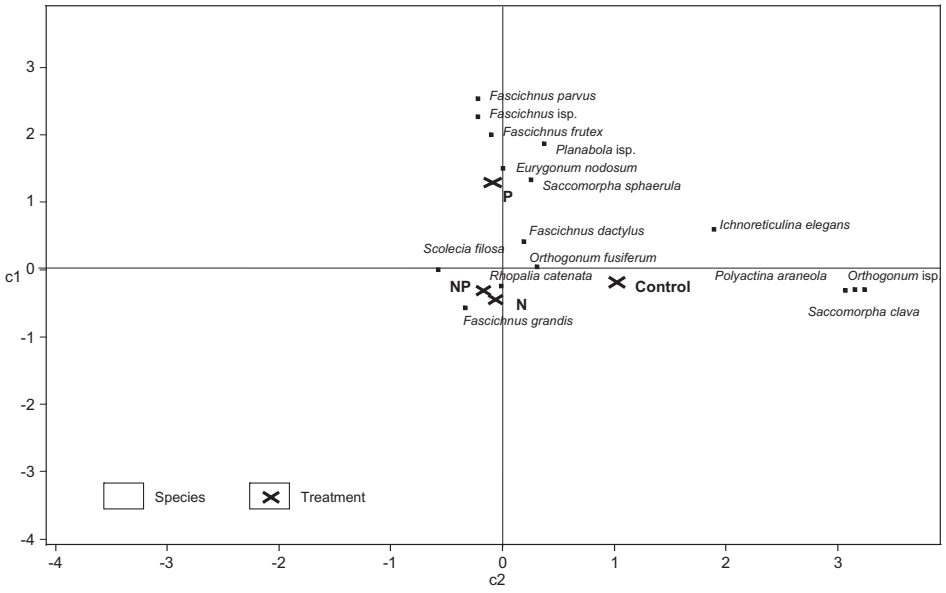

Fig. 4. Detendred correspondence analysis of the microboring community composition in the four experimental treatments. Treatments are shown as C = control, P = phosphorus addition, N = nitrogen addition, and NP = the nitrogen and phosphorus addition.

therefore, important to control epilithic growth in microbioerosion experiments (Gektidis, 1999; Vogel et al., 2000; Chazottes et al., 2002). However, the algal groups able to cause the greatest reduction in light within substrata, i.e. crustose coralline and erect algae, had low cover or were absent during our experiment. Algal turfs were composed by thin sparse filamentous algae and responded equally strong to fertilizer addition as euendolithic communities. The interactions between epilithic and euendolithic communities, reported in other studies (Gektidis, 1999; Chazottes et al., 2002) that are generally found on substrata exposed for longer than 6 months, were not observed.

4.3. Microborers within experimental substrata

4.3.1. Microborer community composition

Nutrient addition significantly increased microbial euendolith colonization on shells above control levels, suggesting that the euendoliths were nutrient-limited. Nitrogen addition increased

Table 3
Nested ANOVA (mixed model) on the effects of nitrogen and phosphorus mixture treatments on microbial euendoliths logit-transformed mean surface area cover (%), depth of penetration (μm) and log-transformed microbioerosion rates (g $CaCO_3$ m^{-2} y^{-1}) by all microborers.

	Effect	D.F.	Variance component	*F*-value	*P*-value
Green algae					
Treatment	Fixed	3		39.40	<0.0001
Cage (treatment)	Random	1	0.0424		
Shell (cage * treatment)	Random	1	0.0144		
Residual	Random	23	0.0527		
Cyanobacteria					
Treatment	Fixed	3		17.60	0.0004
Cage (treatment)	Random	2	0.2572		
Shell (cage * treatment)	Random	2	0.1521		
Residual	Random	15	0.0060		
Heterotrophs					
Treatment	Fixed	3		1.47	0.2874
Cage (treatment)	Random	6	0.9223		
Shell (cage * treatment)	Random	1	0.0039		
Residual	Random	27	0.0049		
Depth of penetration					
Treatment	Fixed	3		36.56	<0.0001
Cage (treatment)	Random		0		
Shell (cage * treatment)	Random		0.0095		
Residual	Random		0.0077		
Microbioerosion rates					
Treatment	Fixed			57.31	<0.0001
Cage (treatment)	Random		0		
Shell (cage * treatment)	Random	5	0.0185		
Residual	Random	24	0.0310		

Note: For random effects the variance components are reported, while for fixed effects the F-ratios and their probabilities are reported. DF are ordinary least of squares degrees of freedom.

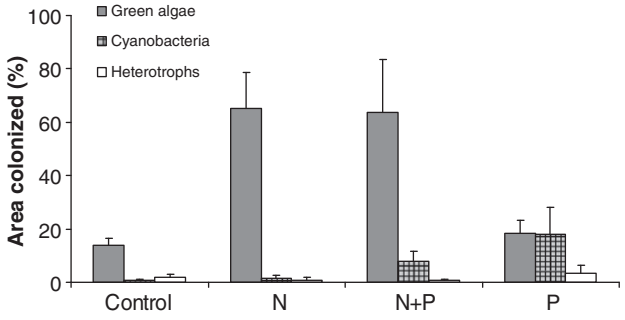

Fig. 5. Surface area colonized by green algae, cyanobacteria, and heterotrophs (mean% ± SD) in *Strombus gigas* shells exposed 56 days to different treatments.

green algal cover by a factor of four compared with control and P treatments. In contrast, the addition of P alone caused an increase in cyanobacteria abundance relative to green algae, while heterotrophic fungi did not respond to inorganic nutrient addition. Thus, the relative availability of N and P and differential response of green algae and cyanobacteria were the responsible factors for the different communities found in each treatment.

The proportional increase of cyanobacteria with increasing P concentration in the experimental treatments indicated P limitation. In contrast, green algae were lowest in the P treatment and did not increase proportional to N concentrations in the experimental treatments. According to mixture design theory, a response to a mixture treatment (in this case N + P) significantly above the additive line indicates a synergistic effect of the components in the mixture (N and P) on the response variable (Cornell, 2002). Green algal abundance was above the additive line and this suggests co-limitation by nitrogen and phosphorus.

The positive response of euendolithic cyanobacteria to phosphorus additions recorded in this study is consistent with results from fertilization studies in both tropical and temperate environments. For example, Fong et al. (1993) found increased biomass of cyanobacterial mats with phosphorus additions compared with other phototrophs in microcosm experiments representing shallow coastal lagoons in Southern California. Microcosm experiments by Kuffner and Paul (2001) also demonstrated phosphorus limitation of two benthic mat-forming cyanobacteria from Cocos Lagoon, Guam. In temperate environments, nutrient addition bioassays in intertidal cyanobacterial mat communities showed increased cyanobacterial growth relative to diatoms when phosphorus was added (Pinckney et al., 1995). In addition, Camacho and de Wit (2003) reported that phosphorus additions favored the development of benthic cyanobacterial mats relative to diatoms in a benthic microbial community in a hypersaline lake in NE Spain. Cyanobacterial abundance is often limited by the availability of nutrients, such as phosphorus and iron, both of which are required for the synthesis of nitrogenase (Paerl, 1990). Low N:P ratios often favor cyanobacteria in fresh and saltwater systems because of the organism's ability to fix nitrogen (Sellner, 1997).

The relative proportions of green algae and cyanobacteria in our different treatments are in agreement with the resource ratio theory (Tilman, 1982), which predicts community changes associated with changing resource supply ratios. The resource-ratio theory assumes a dynamic relationship between resources and consumers, competition for potentially limiting resources, and long-term coexistence of competing species when the growth rate of each species is limited by a different nutrient. Thus, a change in resource ratios should result in a directional shift in their competitive dominance. In our experiment, directional changes caused by inorganic nitrogen and phosphorus additions were reflected in a directional shift in the competitive dominance of cyanobacteria and green algae. Cyanobacteria were most abundant when phosphorus was added alone (low N:P ratio), while green algae dominated in treatments with added nitrogen (higher N:P ratio). When the N:P ratio is low, nitrogen should limit the growth of most of the microbial euendolithic species present. Cyanobacteria were dominant under low N:P ratio (P treatment) probably because of their ability to fix

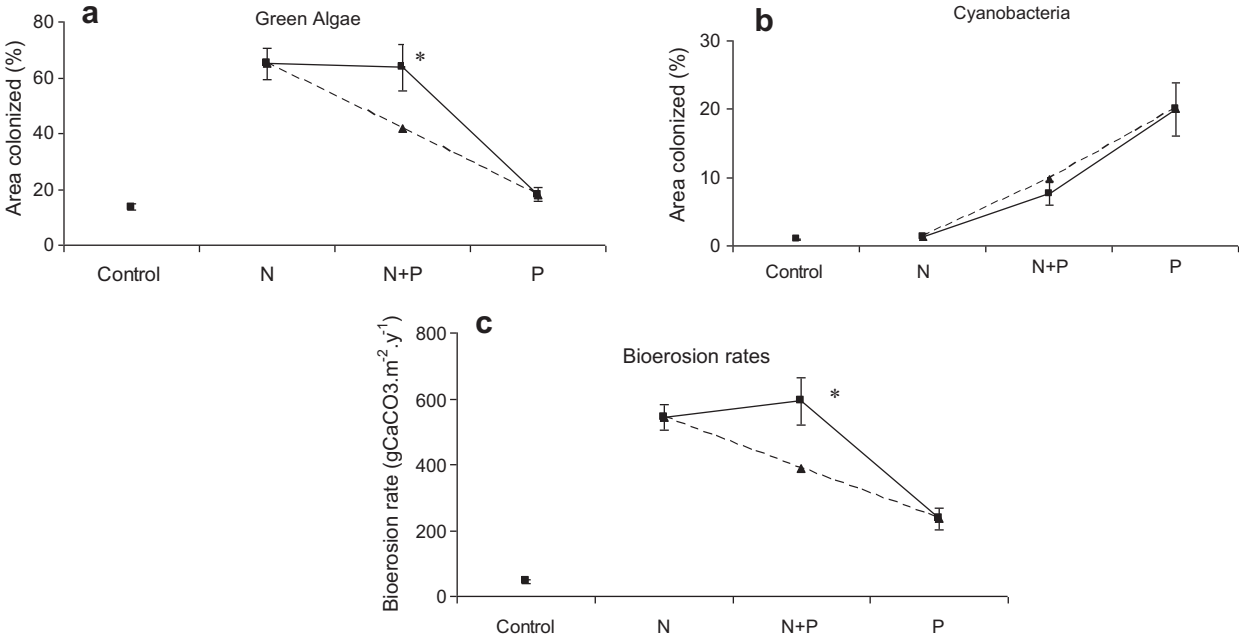

Fig. 6. Shell surface area colonized by green algae (a), cyanobacteria (b) and total microbioerosion rates (c) for experimental exposure to different proportions of phosphorus and nitrogen. Dashed lines indicate the expected abundances (providing that the change in abundances were proportional to an increase in either N or P). The asterisk indicates significant difference from the expected simple average. Values are mean% ± SE.

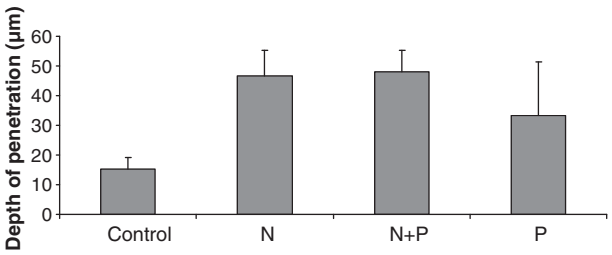

Fig. 7. Depth of penetration (mean% ± SD) by all microbial euendoliths in *Strombus gigas* shells exposed to different treatments for a period of 56 days.

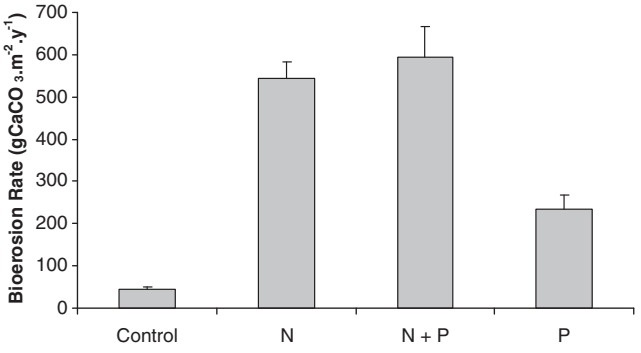

Fig. 8. Microbioerosion rates (mean% ± SE) resulting from the whole microboring communities in *Strombus gigas* shells exposed to different P and N treatments for a period of 56 days.

nitrogen. In contrast, under high N:P ratios phosphorus becomes limiting. The dominance of green algae in treatments with higher N:P ratios (N treatment) suggests that green algae were superior competitors for phosphorus than cyanobacteria.

Although fungal abundance was slightly higher in the P than other treatments, their percent area cover was very low (<3%) and not statistically different among treatments. Results from another organic matter and inorganic nutrients enhancement study (Carreiro-Silva et al., 2009) showed that fungi were stimulated by the addition of organic matter suggesting carbon-limitation, but were not affected by the addition of N and P.

4.3.2. Species-specific responses to nutrients

The green alga *Phaeophila* sp. was the dominant species in all treatments. Percent area colonized by this species increased up to 60% in the N + P and N treatments as compared with 14% in the control treatment. *Phaeophila* sp. is a pioneer short-lived species that typically dominates early boring communities (Kiene et al., 1995; Gektidis 1999; Vogel et al., 2000). We observed the same response by *Phaeophila* sp. to the addition of N + P in our previous fertilization experiments (Carreiro-Silva et al., 2005, 2009). In contrast, the large response of the cyanobacteria *Hyella* spp. to the P treatment is possibly a direct response to low N:P ratios as *Hyella* spp. were uncommon in other treatments and in our previous experiments (Carreiro-Silva et al., 2005, 2009).

Despite the dominance of *Hyella* spp. in the P treatment, the other cyanobacterium *P. terebrans* was more abundant in the N + P than the P treatment (5.1 ± 4.5% compared with 1.5 ± 2.1%, mean ± SD). Its response was also found to be variable in our two previous fertilization experiments (Carreiro-Silva et al., 2005, 2009), despite similar lengths of time, season and location. These differences may be due to variable recruitment of this cyanobacterium as suggested by Kiene et al. (1995). The high variability in cyanobacterial abundance, among cages within treatments and among shells within treatments suggests patchy recruitment at this small scale. Similarly high variability of green algae at the sub-sample level applies to patchy distribution of the dominant green alga, *Phaeophila* sp. Once settled, this alga spreads horizontally by shallow tunnels maintaining repeated connections to the substrate surface, as we have commonly observed in several patches of *Phaeophila's* boring trace *Rophalia catenata*.

It is likely that we have underestimated the full diversity of prokaryotic as well as eukaryotic microbial euendoliths, which was based on the morphological identification of their cellular

structures and boring traces. Differentiating among different *Hyella* species is difficult (Radtke and Golubic, 2005, 2011; Chacón et al., 2006) and future studies using molecular genetic techniques are expected to help uncover their true species richness.

4.4. Microbioerosion rates

Microbioerosion rates in dead substrates depend on the depth of penetration of euendolithic filaments and their abundance in substrates, with those parameters varying with the species community composition (Tribollet, 2008a,b). In this study, the addition of inorganic nutrients, both separately and combined, significantly increased microbioerosion rates in relation to control levels. Higher abundance of euendolithic microorganisms and higher depths of their penetration in fertilized experimental substrata resulted in higher microbioerosion rates over the time of the experiment.

The fast-growing early boring green algae *Phaeophila* sp was the main agent of erosion in treatments with added nitrogen, covering as much as 63% of the experimental substrate. In contrast, cyanobacteria were the main agent of erosion in the P treatment. Here, although the substrate cover by cyanobacteria was very similar to the cover by green algae (~20%, Fig. 5), they were able to remove a larger volume of calcium carbonate than green algae, mainly because cyanobacterial colonies (in particular of the genus *Hyella*) grew perpendicular to the substrate and deeper. For example, the species *Hyella gigas* penetrated as much as 200 μm within the substrate, in comparison with a maximum of 50 μm penetration by *Phaeophila* sp. Euendolithic fungi had a relative minor contribution to microbioerosion rates because of low abundance (<3%) in all treatments and the small borings they produce.

The increase in microbioerosion rates in treatments with added nitrogen was slightly higher than those recorded in our previous experimental studies in Belize (Carreiro-Silva et al., 2005, 2009): In the present study we found an increase in microbioerosion rates of 15 times over the controls, as compared with the eight to ten times increase in our previous studies. These differences are related to larger average percent area covered as well as to deeper penetration achieved in the present study (area colonized = 71.5 ± 20.1%, depth of penetration = 48.1 ± 7.3 μm in the N + P treatment), as compared with the results of the previous study (area colonized = 58.1 ± 25.6%, depth of penetration = 34.6 ± 11.4 μm, mean ± SD; Carreiro-Silva et al., 2009).

The 56-day period of our experiment was insufficient to determine the effects of N and P enrichment on the succession of the microborers community and whether the observed increases in microbioerosion rates were maintained over time. Species we report here are characteristic of early boring communities and more than 6-months exposure may be required to document the full succession (Gektidis, 1999; Tribollet and Golubic, 2005; Tribollet, 2008b). Mature communities dominated by cyanobacterium *P. terebrans* and the chlorophyte *Osterobium quekettii* are generally established after 1 year exposure (Gektidis, 1999; Tribollet and Golubic, 2005; Tribollet, 2008b). These species can grow under low light intensities and thus we would expect them to become more abundant in substrates as these become covered by epilithic organisms.

Determining the long-term effects of N enrichment on microboring communities is particularly important because of the major role of the chlorophyte *O. quekettii* as an agent of microbioerosion in coral reefs (Tribollet, 2008b). Given the strong positive effect of nitrogen on chlorophytes, it is reasonable to assume that mature communities dominated by the chlorophyte *O. quekettii* would result in even greater microbioerosion rates under N-enrichment than those measured here. However, long-term experiments are necessary to confirm this hypothesis.

Differences in experimental substratum, depth, methods, and length of exposure make comparisons between this and other reported microbioerosion rates difficult (Kiene et al., 1995; Vogel et al., 2000). Consequently, variable responses have been recorded by microborers in relation to nutrient conditions. For example, studies on the Great Barrier Reef (the ENCORE experiment – Kiene, 1997; Koop et al., 2001) did not report any significant effects of fertilizers on microbioerosion. However, microbioerosion rates in fertilized treatments reported here and in previous fertilization experiments (Carreiro-Silva et al., 2005, 2009) are comparable to microbioerosion rates measured in coral blocks after 2 months of exposure on reefs with low herbivory and the occasional elevation of nutrients at Moorea Island (Wolanski et al., 1993; Chazottes et al., 1995; Peyrot-Clausade et al., 1995). In contrast, higher microbioerosion rates have been measured from coral blocks exposed for 1–3 years in reefs located in the Coral Sea, far from the Great Barrier Reef, which experience very little or no anthropogenic influence (Tribollet et al., 2002; Tribollet and Golubic, 2005). Factors such as temperature, salinity, wave energy, epilithic algal cover and grazing by herbivorous fishes and sea urchins strongly affect microbioerosion, which may interact with nutrient supply and responses. Therefore, such studies require controls organized in accordance with local ecological conditions.

In this study as in previous experimental studies on Glover's Atoll (Carreiro-Silva et al., 2005, 2009), the bioerosion rates were extrapolated to yearly rates, which is customary in such studies. The recorded rates, including extrapolations, are the closest measurements of "primary" or "net" microbioerosion, and reflect the capacity and the initial removal of carbonate by euendoliths alone, given that efforts were made to exclude most of the grazing. Several studies have observed that microbioerosion rates do not increase linearly in time. In the absence of grazing, the activity of microborers would tend towards stabilization at the point where they reach their compensation depth (photosynthesis = respiration) (Schneider and Torunski, 1983). However, in conjunction with grazing activity, as noted by Schneider (1976), microboring is continuous. Consequently, bioerosion is a major cumulative process over time (Tribollet and Golubic, 2005; Wisshak et al., 2005; Tribollet, 2008b), and the actual rates should be extrapolated with caution.

5. Conclusions

Results from this and previous experiments (Carreiro-Silva et al., 2005, 2009) consistently show a significant positive effect of nutrients on microbial euendolith colonization and microbioerosion rates of carbonate substrata. The effect is specifically different for eukaryotic chlorophytes and prokaryotic cyanobacteria. High N:P ratios in the water column caused the greatest increase by stimulating the fast-growing pioneer green alga *Phaeophila* sp. Changes in nutrient ratios induced a shift in microbial euendoliths: the addition of nitrogen alone (or in combination with phosphorus) stimulated mostly green algae, whereas the addition of phosphorus alone stimulated cyanobacteria. Results from shells cannot be easily extrapolated to coral substrata, but experiments demonstrate a clear, direct effect of nutrient enrichment on increasing microbioerosion of carbonates during the early stages of colonization. The length of the experiment was, however, too short to see the full succession of microboring communities and how nutrient enrichment affects changes in the community composition over time.

Microbial euendoliths contribute to the erosion of reef framework not only through their microboring activity, but also by supporting an array of grazers and facilitating recruitment and settlement of filter-feeding macroborers (worms, sponges, mollusks) (Chazottes et al., 1995; Pari et al., 1998; Tribollet and

Golubic, 2005). Results of this study suggest that an increased eutrophication of coastal waters is likely to result in changes in composition, increase in abundance as well as in the total rates of carbonate removal by euendoliths and grazers that feed upon them – in addition to attract filter-feeding macroboring organisms. Thus increase in nutrient availability initiates a feed-back mechanism where different bioerosion agents reinforce each other, resulting in the accelerated erosion of the reef framework. The effect of nutrients on the bioerosion loop and the calcium carbonate budget (accretion-bioerosion) is further aggravated by the global change-related increases in seawater temperature and acidification. Rising seawater temperature contributes to coral mortality (Baker et al., 2008), reducing the capacity for biological carbonate accretion and simultaneously increasing substrate surface area available for colonization by boring organisms. While ocean acidification reduces calcification rates of reef framebuilders (e.g. Langdon and Atkinson, 2005; Kuffner et al., 2008), it also increases microbioerosion of carbonate substrates by boring microflora (Tribollet et al., 2009). Ultimately, the synergy between these different impacts may lead to the net erosion and deterioration of the reef framework.

Acknowledgements

The research received financial support from Wildlife Conservation Society (WCS) (T.R. McClanahan) and Fundação para a Ciência e a Tecnologia, Portugal, and European Social Fund under the III Communitary Support Board (M. C-S, SFRH/BD/789/2000 and SFRH/BPD/34634/2007). We thank the Smithsonian National Museum of Natural History and Dr. Ian McIntyre and Jonathan Wingerath for their assistance in preparing samples. Brie Cokos of WCS, and Michael Dilorenzo and Jean Lee of the Columbia University Environmental Research Consortium assisted with the conch shells, cages and fixing samples. L.W. Douglass provided statistical advice. The thoughtful comments of J.A. Mihursky and V.S. Kennedy improved earlier versions of this manuscript. International collaboration was supported by Hanse Institute for Advanced Studies, Delmenhorst (S. Golubic), Permission to undertake the work was provided by the Belize Fisheries Department. This is publication number 39 of the Glover's Reef Research Station.

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