

Effects of nutrient enrichment of the cyanobacterium *Lyngbya* sp. on growth, secondary metabolite concentration and feeding by the specialist grazer *Stylocheilus striatus*

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ABSTRACT: Harmful blooms of the benthic cyanobacteria *Lyngbya* spp. are increasing in coastal marine habitats. Nutrient enrichment has been implicated in bloom formation; however, the effects of nutrient enrichment on secondary metabolite concentrations and the resulting palatability of *Lyngbya* spp. are not known. Using nutrient bioassays, we examined the effects of nitrogen (N), phosphorus (P) and chelated iron (Fe) on growth and secondary metabolite concentration in *Lyngbya* sp. collected from reefs in Broward County, Florida. The consequences of these nutrient additions on feeding behavior of a major specialist opisthobranch grazer, *Stylocheilus striatus*, were examined. Chelated Fe additions (+FeEDTA) significantly increased *Lyngbya* sp. growth, while additions of N, P and chelated Fe combined (+All) resulted in significantly lower concentrations of microcolin A than in the control. Overall, there was a negative correlation between growth and total concentrations of microcolins A and B. When crude extracts from the control, +FeEDTA and +All treatments of the *Lyngbya* sp. bioassay were offered to *S. striatus* in artificial food, they consumed greater quantities of the control and +FeEDTA treatments than the +All. These results provide the first evidence that changes in nutrient availability can affect secondary metabolite concentrations in marine *Lyngbya* spp. and support previous studies that show that Fe can stimulate growth in benthic marine cyanobacteria. This study also demonstrates quantifiable changes in feeding behavior by a specialist grazer in response to changes in the nutrient conditions under which *Lyngbya* sp. grows and underscores the need to consider secondary metabolite concentrations, and their effect on grazers, when managing harmful algal blooms.

KEY WORDS: *Lyngbya polychroa* · Microcolins · Cyanobacterium · *Stylocheilus striatus* · Nutrient enrichment · Trade-off · Florida

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INTRODUCTION

Benthic cyanobacterial blooms, including extensive ephemeral mats of cyanobacteria, are becoming more prevalent in shallow marine habitats, often overgrow-

ing seagrass, macroalgae and corals. These blooms tend to be associated with warm water and eutrophication (Dennison et al. 1999, Paul et al. 2005, Paerl & Huisman 2008), and both climate change and anthropogenically derived nutrient additions to the marine

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environment provide the potential for increases in these nuisance blooms (Paerl 2008, Paul 2008). *Lyngbya* spp. blooms (Oscillatoriaceae; see Fig. 1) appear to have been increasing in frequency and distribution in tropical and subtropical marine and estuarine waters in recent decades (Osborne et al. 2001, Paul et al. 2005). This genus is a prolific producer of secondary metabolites, and over 200 compounds have been isolated from *Lyngbya* spp. worldwide (Blunt & Munro 2008). Human contact dermatitis and intoxication have been attributed to *Lyngbya majuscula*, contributing to its status as a harmful bloom (HAB) species (Osborne et al. 2001). The ecological impacts of these secondary metabolites are largely unknown, but blooms of *Lyngbya* spp. are often unpalatable to generalist consumers (Paul et al. 2007) and can contribute to phase shifts from seagrass- and coral-dominated habitats to those overgrown by macroalgae and cyanobacteria (Kuffner et al. 2006).

As many cyanobacterial HAB species are chemically defended, and thus unpalatable to generalist herbivores, it has been proposed that they have a competitive advantage over other autotrophic organisms (Paul et al. 2007). Consequently, investigations into the controls of blooms often focus on bottom-up factors, such as nutrient limitation, rather than top-down control by consumers. However, to facilitate bloom proliferation, HABs require optimal environmental conditions in terms of temperature and light (Paerl 1988), they require access to nutrients, and growth must outweigh loss due to consumption and death. Therefore, the interactions of top-down and bottom-up controls on HABs can be complex, and should be considered in concert (Buskey 2008).

Increased anthropogenic inputs of nitrogen (N) and phosphorus (P) to coastal marine waters have been implicated in the increase in near-shore HABs (Heisler et al. 2008), including *Lyngbya majuscula* (Paerl et al. 2008). *Lyngbya* spp. are non-heterocystous nitrogen fixers, i.e. they can convert atmospheric nitrogen (N_2) into ammonia, a bioavailable form of N (Diaz et al. 1990, Lundgren et al. 2003). Since they have ready access to N, it is commonly thought that nitrogen fixing organisms are limited instead by P (Whitton & Potts 2000). In addition, because iron (Fe) is a constituent of nitrogenase, the enzyme responsible for N-fixation, nitrogen-fixing cyanobacteria may be limited by Fe availability (Fay 1992). Both P and chelated Fe have been demonstrated to be limiting elements for growth and productivity in *L. majuscula* from Australia (Elmetri & Bell 2004, Watkinson et al. 2005, Ahern et al. 2006, 2007, 2008, Bell & Elmetri 2007), while *L. majuscula* growth was enhanced only by phosphate additions in Guam (Kuffner & Paul 2001). In addition, recent work in Florida coastal waters has shown car-

bon fixation in *L. majuscula* to be stimulated by combined N and P additions (Paerl et al. 2008).

While nutrient enrichment and chelated Fe additions have been demonstrated to affect growth and productivity in *Lyngbya* spp., changes in nutrient availability are known to affect toxin production in other cyanobacteria (Yin et al. 1997, Ray & Bagchi 2001, Vuorio et al. 2005, Pyo & Jin 2007), but little is known of the effect of nutrient enrichment on secondary metabolite production in *Lyngbya* spp. In a study on the freshwater species *L. wollei*, low N and P concentrations gave rise to an increase in toxicity (Yin et al. 1997). In contrast, a positive correlation was observed between tissue carbon, N and P concentrations and lyngbyatoxin A in *L. majuscula* from Moreton Bay, Australia, suggesting that increased nutrient availability allowed for greater production of secondary metabolites (O'Neil & Dennison 2005), but no experimental tests were conducted to test this relationship. Secondary metabolite concentrations can be highly variable in environmental samples of cyanobacterial HABs (Arthur et al. 2008) and few studies have attempted to explain this variability. Due to the role these compounds may play in deterring grazing, it is important to understand the environmental factors that may lead to changes in secondary metabolite concentration.

Secondary metabolites produced by *Lyngbya* spp. often deter grazing by generalist herbivores (Nagle et al. 1996, Nagle & Paul 1999). In addition, the abundance of herbivorous fish has been reported to significantly decline in areas where *Lyngbya* spp. are in bloom (Baumberger 2008), suggesting generalist grazers are unlikely to be significant control agents for *Lyngbya* spp. blooms. Specialist grazers such as the opisthobranch mollusk *Stylocheilus striatus* consume *Lyngbya* spp., and in some instances they sequester these compounds, potentially for their own defense against predation (Kato & Scheuer 1974, Pennings & Paul 1993). Although *S. striatus* has been reported to metamorphose on red algae as well as *L. majuscula*, only the latter supported rapid growth of these sea hares (Switzer-Dunlap & Hadfield 1977). Sea hares are known to feed voraciously on *Lyngbya* spp. (Capper et al. 2006), and vast numbers have been described in association with *L. majuscula* blooms in Hawaii (www.turtles.org/limu/limu.htm). Sea hares are the most common and abundant mesograzer associated with *Lyngbya* spp. in reef habitats of southeastern Florida (Capper & Paul 2008). Some secondary metabolites from *Lyngbya* spp. stimulate feeding in *S. striatus* (Nagle et al. 1998, Capper et al. 2006); however, the palatability of these compounds can be concentration dependent (Nagle et al. 1998). The implications for sea hare foraging due to changes in the host cyanobacterial secondary metabolite concentration

resulting from changes in environmental conditions are not known, but may have a significant impact on bloom proliferation and persistence.

Blooms of *Lyngbya* spp. have been described on the Broward County reefs of Florida since 2002 (Paul et al. 2005) and consist of 4 chemotypes that are closely related morphologically but have differing chemical compositions. These include *L. confervoides*, *L. majuscula* and 2 species that are morphologically consistent with *L. polychroa*: *Lyngbya* sp. (A) and *Lyngbya* sp. (B) (Sharp et al. 2009). *Lyngbya* sp. (A) produces microcolins A and B (Fig. 1), compounds that have previously been described from a collection of *L. majuscula* from Venezuela. Microcolin A has immunosuppressive and antiproliferative activity (Koehn et al. 1992, Zhang et al. 1997), while microcolin B deterred feeding in *S. striatus* at natural concentrations (Nagle et al. 1998). *Stylocheilus striatus* preferentially feeds on *Lyngbya* sp. (A) on the Broward County reefs (Capper & Paul 2008), but it is not known what characteristics of *Lyngbya* spp. drive this selectivity.

Although many of the secondary metabolites produced by cyanobacteria act as feeding deterrents in generalist grazers, the consequences of changes in secondary metabolite production resulting from nutrient enrichment and eutrophication have not been closely examined and little is known about the interactions of bottom-up and top-down controls of HABs (Buskey 2008). In this study, we assess the impact of N, P and bioavailable Fe enrichment in *Lyngbya* sp. from the hard-bottom reefs of Broward County, Florida, in terms of their effects on growth and secondary metabolite concentration. We also endeavor to put these changes into an ecological context by assessing the resultant effects of nutrient enrichment on the feeding behavior of the specialist opisthobranch grazer *Stylocheilus striatus* in response to changes in secondary metabolite concentrations in *Lyngbya* sp.

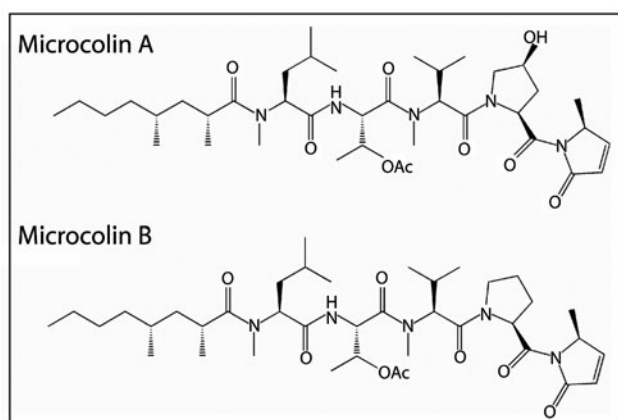


Fig. 1. Microcolins A and B

MATERIALS AND METHODS

Sample collection. *Lyngbya* sp. (Fig. 2) was collected from the hard bottom reefs located parallel to the coast ~1 km off-shore from Dania Beach (26° 04' N, 80° 06' W), in an area previously described by Paul et al. (2005). *Lyngbya* sp. was collected by hand on SCUBA from 8 to 10 m depth in plastic Ziplock® bags filled with seawater. It was subsequently rinsed, separated from other benthic algae and cyanobacteria and divided into a bulk collection for chemical analysis, voucher specimens for morphological observations, initial (T_0) samples for chemical analysis, and samples for the *in situ* nutrient-enrichment bioassay.

Lyngbya sp. was examined under a phase contrast light microscope (Zeiss, Germany) with a 40× non-immersion objective and a 10× ocular lens with a calibrated eyepiece micrometer. Filament width, cell width and cell length were measured for each of 10 filaments in the collection.

***In situ* nutrient bioassay.** Nutrient-enrichment bioassays were conducted at Nova Southeastern University Oceanographic Center in Fort Lauderdale, Florida in May 2007. Bioassays were run following the general methods of Paerl et al. (2008). Briefly, *Lyngbya* sp. was divided into equal sized portions and spun 20 times in a salad spinner to remove excess water. It was then weighed (~ 1 g wet wt) and immediately placed in the bioassay chamber (Fig. 2B) with filtered (10 μm) ambient seawater collected off-shore at the *Lyngbya* sp. collection site. All containers were pre-washed with 0.1 N HCl, rinsed 3 times with deionized (DI) water and then rinsed with sample water prior to the bioassay.

Water was changed every 48 h and nutrient additions were made on a daily basis (Table 1) with +N added as potassium nitrate (KNO₃), +P added as

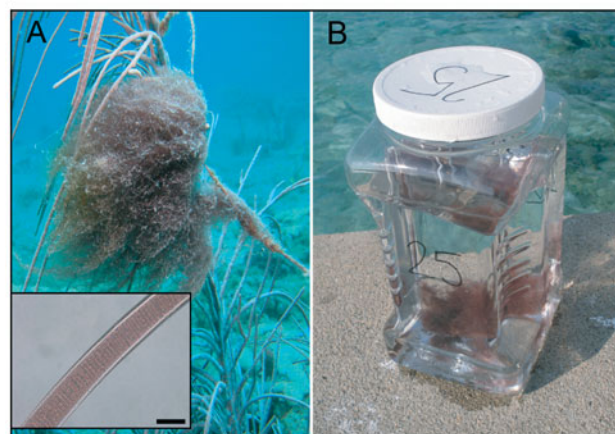


Fig. 2. *Lyngbya* sp. from near-shore hard-bottom reefs in Broward County, Florida. (A) *In situ* overgrowing a gorgonian. Inset: Micrograph of filament. Scale bar: 30 μm. (B) Specimen in nutrient-enrichment experimental container

Table 1. Nutrient treatment experimental design for *Lyngbya* sp. bioassay. Five replicates were used for each treatment and nutrient additions were made daily

Treatment	Nutrient addition
Control	No nutrient additions
+N	+ 10 μM N-KNO ₃
+P	+ 5 μM P-K ₂ HPO ₄
+N&P	+ 10 μM N-KNO ₃ + 5 μM P-K ₂ HPO ₄
+FeEDTA	+ 1 μM FeCl ₃ + 1 μM C ₁₀ H ₁₄ N ₂ Na ₂ O ₈
+All	+ 10 μM N-KNO ₃ + 5 μM P-K ₂ HPO ₄ + 1 μM FeCl ₃ + 1 μM C ₁₀ H ₁₄ N ₂ Na ₂ O ₈

monobasic potassium phosphate (K₂HPO₄) and +FeEDTA as chelated iron chloride in the presence of ethylenediaminetetraacetic acid (EDTA) (FeCl₃-C₁₀H₁₄N₂Na₂O₈). Nutrient concentrations reflected potential nutrient-loading events in estuarine and coastal waters of Florida. Nutrient stock solutions were made up with DI water in sterilized polyethylene (Nalgene) bottles. A concurrent control group was run using filtered ambient seawater with no nutrient additions. Each treatment and the control consisted of 5 replicate samples. *Lyngbya* sp. was incubated for 4 d at ambient water temperatures in an *in situ* corral used to house the bioassay chambers. The corral was covered with shade cloth to mimic light conditions at the collection site at 8 to 10 m depth, which was found to be 20% surface irradiation as measured with an underwater quantum light sensor (LI-COR, Lincoln, Nebraska). Growth, or change in biomass, was calculated as the difference between the wet weight of *Lyngbya* sp. at the beginning and the end of the 4 d bioassay. A 4 d bioassay was chosen to allow observable growth and potential changes in chemical composition while minimizing artifacts such as container effects (Paerl 2002).

Secondary metabolite isolation and quantification.

Major secondary metabolites were isolated and identified as described by Meickle et al. (2009). The bulk *Lyngbya* sp. collection was lyophilized (80.0 g dry wt) and extracted 3 times with 1:1 methanol:ethyl acetate (1 L) for 24 h to yield 11.6 g of extract. The crude extract was fractionated by 3 combined separations on a C18 flash column (Varian Mega Bond Elut, 60 ml) using a step gradient of MeOH-H₂O-acetone. Final purification was achieved by reverse phase HPLC (Econasil C18, 10 μm , 1 \times 25 cm, absorbance 254 nm, flow rate 2.0 ml min⁻¹) with a MeOH/H₂O gradient to obtain microcolins A and B. This extraction technique yielded 133.0 mg pure microcolin A and 43.8 mg pure microcolin B.

Frozen *Lyngbya* sp. samples from the bioassay and initial collection (*T*₀) were lyophilized and weighed. Samples (0.14 to 0.34 g dry wt) were soaked in 10 ml 1:1 methanol:ethyl acetate, sonicated and extracted

overnight. Solvent was removed and the extraction repeated 5 times. All extract solvent was combined, filtered, dried and weighed. The crude organic extract yield, or proportion of the sample weight accounted for by the crude extract, was determined by dividing the crude extract weight by the total *Lyngbya* sp. sample dry weight.

The concentrations of microcolins A and B were determined by HPLC using a Perkin Elmer Quaternary LC Pump Model 200Q/410 with Series 200 auto sampler (Shelton, USA). Bioassay sample crude extracts were resolubilized in 1:1 methanol:water at a concentration of 1 mg ml⁻¹. A 50 μl injection was used for each sample. Samples were separated using a Waters Symmetry C 18 analytical column (Milford, Massachusetts) with HPLC elution conditions consisting of 1:1 methanol: water for 10 min followed by a linear gradient to 100% methanol over 20 min. The system was held at 100% methanol for a further 5 min before being flushed with 100% ethyl acetate for 8 min between samples. Compound peaks were detected using a PE Series 200 Diode Array Detector (Shelton, USA) scanning at 229 nm (maximum peak detection for microcolins) and 254 nm. Under these conditions, microcolin A eluted at 19.5 min and microcolin B at 20.6 min. The peak area obtained for each compound was integrated and compared with the calibration curve using TotalChrom Workstation version 6.2.0 (PerkinElmer). Standard curves for both microcolins A and B were prepared using pure compound isolated from the bulk *Lyngbya* sp. sample. Standard curves consisted of 7 levels ranging from 0.001 to 0.1 mg ml⁻¹ pure compound, with compound concentrations obtained from serial dilutions of 3 separate pure compound weights. Standard curves were fitted using the least squares method (microcolin A: $r^2 = 0.97$; microcolin B: $r^2 = 0.99$). Under these conditions the detection limit was 0.001 mg ml⁻¹. Samples were run in triplicate to ensure within sample reproducibility (mean SD between 3 samples for microcolin A: 0.0003 mg ml⁻¹, microcolin B: 0.0001 mg ml⁻¹). The final concentrations of microcolins A and B were calculated by averaging the 3 replicate injections to determine the amount of microcolins A and B per mg of extract, multiplying by the total weight of the crude extract and dividing by the sample dry weight to give microcolins as a function of the dry weight of *Lyngbya* sp. extracted.

***Stylocheilus striatus* feeding trials.** To examine how a major specialist grazer would be affected by changes in secondary metabolite composition resulting from nutrient additions in the *Lyngbya* sp. bioassay, we undertook feeding trials using the sea hare *S. striatus*. This opisthobranch mollusk is a relatively small (>5 g) circumtropical specialized herbivore that feeds voraciously on *Lyngbya* spp. (Switzer-Dunlap & Hadfield

1977). Individual *S. striatus* were collected in association with *Lyngbya* sp. from the hard bottom reefs of Broward County. *Lyngbya* sp. was placed in aerated aquaria with coastal Atlantic Ocean seawater and the water was changed daily until *S. striatus* could be found (since *S. striatus* were small and difficult to detect amongst *Lyngbya* sp. filaments at the time of collection) and moved to individual aquaria (~1 wk). In the individual aquaria, water was maintained at consistent ambient temperature (22.4 to 25.9°C) with 12:12 h light and dark cycles. Prior to feeding trials, *S. striatus* were fed a diet of the *Lyngbya* sp. on which they were collected. At the start of each feeding trial, *S. striatus* were blotted dry and a wet weight obtained for each animal.

A multiple choice feeding assay was used to determine *Stylocheilus striatus* feeding preference for extracts of *Lyngbya* sp. from the nutrient bioassay samples. Artificial diets were made following the methods of Capper & Paul (2008). Crude extracts were obtained from the bioassay samples as described above. The crude extracts from replicates of the control, +FeEDTA and +All treatments from the bioassay were separately pooled to make the artificial foods for the palatability test. They were resolubilized in 1:1 ethyl acetate:methanol, and coated onto lyophilized, ground red algae, *Gracilaria tikvahiae*, at concentrations observed in the *Lyngbya* sp. at the end of the bioassay (average for each treatment). Solvent was removed by rotary evaporation. The dried *G. tikvahiae* was then incorporated into an agar-based artificial food that could be molded onto a plastic screen to make food strips consisting of 10 × 10 mesh squares. In this case, the control food was made using the *Lyngbya* sp. extract from the control treatment of the bioassay rather than being a solvent control as described in previous studies (Nagle et al. 1998, Cruz-Rivera & Paul 2007, Capper & Paul 2008). *S. striatus* individuals (wet wt: 1.4 to 3.9 g) were housed in individual aquaria and were offered the 3 artificial foods simultaneously. The preference test was stopped when >50% of the total available food had been consumed, alleviating effects of size differences between individuals. Replicates in which <10% or >90% of total food was consumed were not included in analyses to ensure preference was measurable (Lockwood 1998).

To determine whether the concentration of microcolin A in the crude extract affected *Stylocheilus striatus* food preference, additional palatability tests were run using artificial food containing pure microcolin A at concentrations of 0.073, 0.350 and 0.864% on a dry weight basis, reflecting the minimum, average and highest concentrations observed in bioassay samples. In this experiment, individual *S. striatus* (wet wt: 0.4 to 2.8 g) were offered a choice between artificial food

containing one of the microcolin A concentrations and a control food that was made using the same methods and solvents as treatment food, but without any pure compound (Nagle et al. 1998, Cruz-Rivera & Paul 2006, Capper & Paul 2008). The feeding trial was repeated 3 times to test the 3 different microcolin A concentrations. To confirm that microcolin A was not lost during food preparation or over the duration of the test, remaining food was re-extracted using 100% methanol, dried and then partitioned between ethyl acetate and water. The resulting ethyl acetate fraction was placed on a thin liquid chromatography (TLC) plate and run in 90% ethyl acetate 10% methanol and compared with pure microcolin A.

Statistics. All results are presented as the mean +1 standard error (SE). Statistical analyses were conducted using Sigma Stat V3.11 (Systat Software, California, USA). All data were tested for normal distribution and homogeneity of variance. Where data were not normally distributed, they were \log_{10} transformed. Bioassay effects were tested by comparing initial (T_0) samples with bioassay end (T_4) control samples using a *t*-test. Nutrient addition treatment effects were assessed using a 1-way analysis of variance (ANOVA) of T_4 samples. Where a significant result was obtained, a Tukey's posthoc pairwise comparison was used to assess differences between treatment groups. The relationship between total microcolin concentration and growth was analyzed using Pearson's product moment correlation. *Stylocheilus striatus* feeding preference tests were compared using a Friedman's repeated measures ANOVA. The microcolin A palatability tests were analyzed using paired *t*-tests.

RESULTS

The *Lyngbya* sp. used in these bioassays (Fig. 2) was identified based on morphological traits in accordance with Littler & Littler (2000) as *Lyngbya* cf. *polychroa*, with an average filament width of 38.8 ± 3.4 µm, average cell width of 30.3 ± 3.4 µm and average cell length of 3.0 ± 1.1 µm. The identification of this cyanobacterium was later revised to *Lyngbya* sp. (A) to reflect genetic diversity within this morphological grouping (Sharp et al. 2009). In this study, we refer to the study organism as *Lyngbya* sp., although we have previously described it as *Lyngbya* cf. *polychroa* (Paul et al. 2005, Meickle et al. 2009) and *Lyngbya* sp. (A) (Sharp et al. 2009) in other studies. Environmental collections of this cyanobacterium have been genetically and chemically consistent through time (Sharp et al. 2009).

Lyngbya sp. appeared healthy in the bioassay chambers with gas bubbles present at the end of each day, indicating it was photosynthetically active. *Lyngbya* sp.

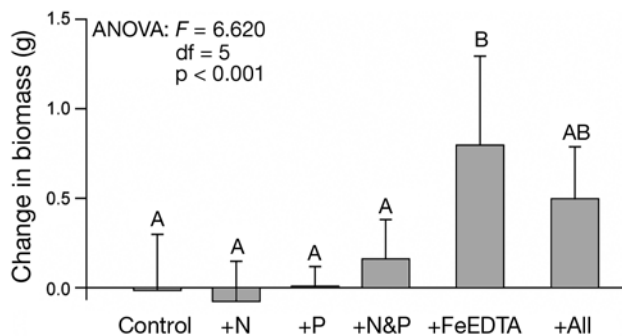


Fig. 3. *Lyngbya* sp. Change in biomass over the course of the 4 d bioassay showing growth within nutrient-enrichment chambers in comparison with control (n = 5). Error bars represent +1 SE. Different letters represent treatment groups that are significantly different from one another. Treatments as in Table 1

did not bleach during the experiment and samples generally grew well over the course of the 4 d experiment. There was a significant nutrient treatment effect in terms of the change in biomass with the +FeEDTA treatment growing significantly more than the control, +N, +P and +N&P treatments (ANOVA: $p < 0.001$; Fig. 3).

Microcolins A and B were the only detectable secondary metabolites produced by this species of *Lyngbya*, and both compounds were present in all *Lyngbya* sp. samples included in this experiment. There was a similar concentration of microcolin A in the control samples at the beginning and the end of the bioassay; however, there was more microcolin B in control samples at the end of the bioassay (t -test: $p = 0.049$; Fig. 4A). At the end of the 4 d bioassay, there were significant differences between nutrient treatment groups in both microcolin A and B (ANOVA: microcolin A: $p < 0.001$; microcolin B: $p = 0.004$). There was significantly less microcolin A in the +All treatment than in the controls, while there was significantly less microcolin B in the +FeEDTA and +All treatments than in the +N&P treatment, but these did not differ significantly from the controls (Fig. 4B). In addition, there was a negative correlation between the concentration of total microcolins (A+B) in *Lyngbya* sp. at the end of the bioassay and the change in biomass of *Lyngbya* sp. samples over the course of the 4 d bioassay (Pearson's product moment correlation: $p = 0.002$; Fig. 5).

Stylocheilus striatus consumed significantly less of the artificial food containing the +All *Lyngbya* sp.

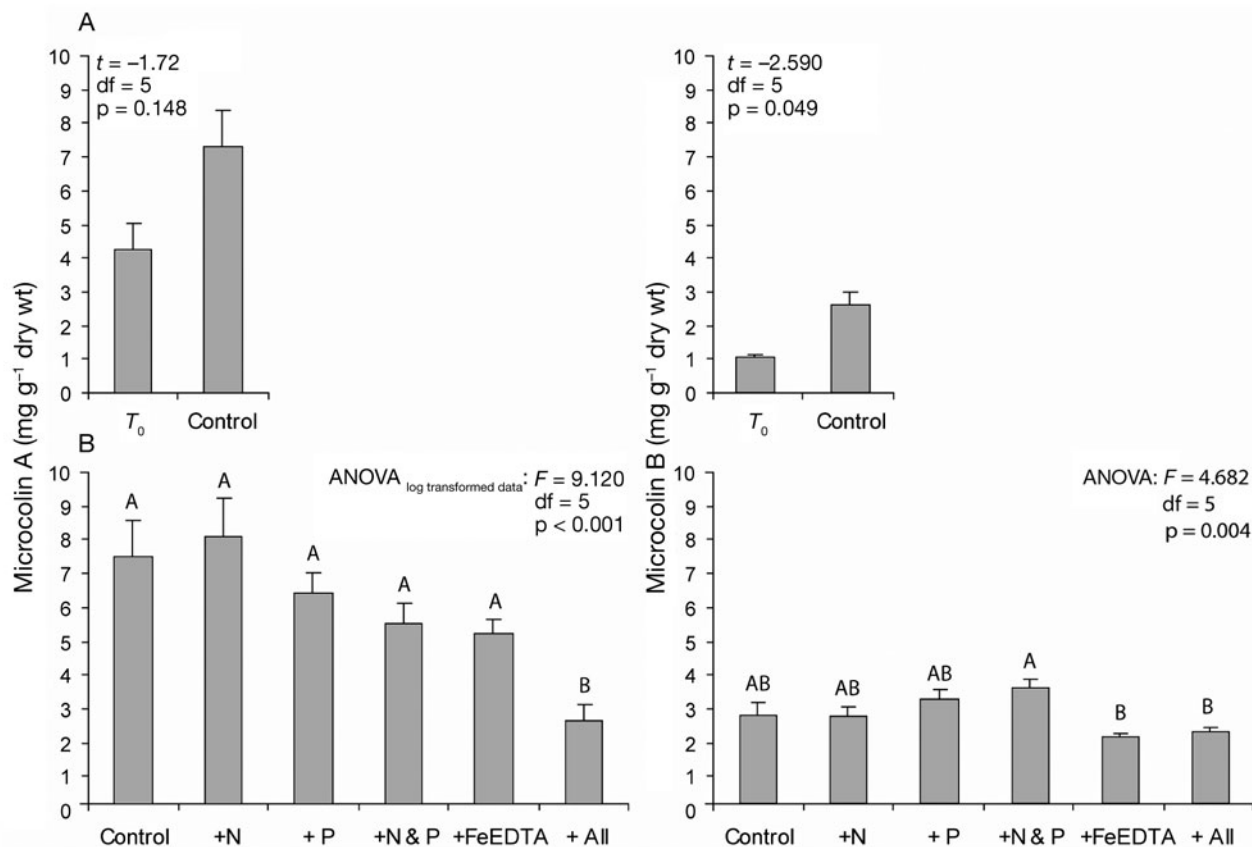


Fig. 4. *Lyngbya* sp. Microcolin A and B concentrations in (A) initial (T_0) samples compared with control treatment at the end of the 4 d bioassay and (B) bioassay treatment groups at the end of the bioassay. Error bars: +1 SE. Different letters represent treatment groups that are significantly different from one another, as determined by the Tukey's posthoc test. Treatments as in Table 1

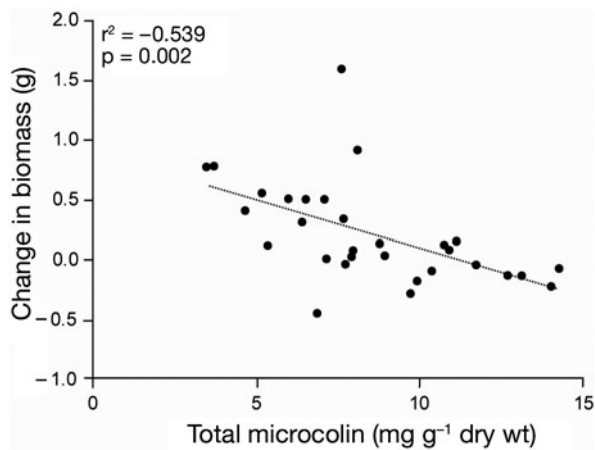


Fig. 5. *Lyngbya* sp. Total microcolins (A+B) compared with growth at the end of the 4 d bioassay

crude extract when compared with the control and +FeEDTA treatments (Friedman's ANOVA: $p < 0.001$; Fig. 6A). There was no significant effect of microcolin A on the palatability of artificial food at any of the concentrations tested (Fig. 6B). *S. striatus* consumed similar amounts of control and treated foods, suggesting they did not show a preference for or against microcolin A at concentrations observed in *Lyngbya* sp. from the bioassay (Fig. 6B). Microcolin A was confirmed to be present in the artificial food at the end of the feeding trial via TLC.

DISCUSSION

In this study, we were able to demonstrate significant changes in the secondary metabolite concentrations of a marine *Lyngbya* sp. in response to nutrient

additions. Furthermore, we demonstrated that the addition of combined N, P and FeEDTA (+All treatment) made extracts of the *Lyngbya* sp. less palatable to one of the major consumers of this HAB, potentially increasing its capacity to bloom. While numerous studies have looked at the impact of nutrient enrichment on HAB growth (e.g. Cloern 2001, Anderson et al. 2002), few have assessed the impact of nutrient availability on secondary metabolite concentrations, and rarely are these changes considered in an ecological context of the effect on potential consumers.

In the current study, we found that the addition of combined N, P and FeEDTA (+All treatment) resulted in significantly lower concentrations of microcolin A than observed in the control (ambient seawater) treatment. This finding probably represents a trade-off with growth since there was a strong negative correlation between change in biomass and total microcolins concentration, with higher growth rates associated with lower concentrations of microcolins (Fig. 5). The trade-off between secondary metabolite production and growth represents a metabolic cost of chemical defense that has also been described in *Lyngbya wollei* (Bevis 2003), a freshwater species that produces saxitoxins (Carmichael et al. 1997). However, this trade-off relationship does not hold for all toxin-producing cyanobacteria and may be dependent on consumer abundance, specific nutrient availability or other environmental factors. For example, in a separate study of *L. wollei*, the highest biomass and toxicity were found concurrently when N and P were limiting, but there was an excess of calcium available, giving a positive association between growth and toxicity (Yin et al. 1997). In the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, greater amounts of saxitoxins were produced with increased growth (Pomati et al. 2003), and microcystin concentration in *Microcystis aerugi-*

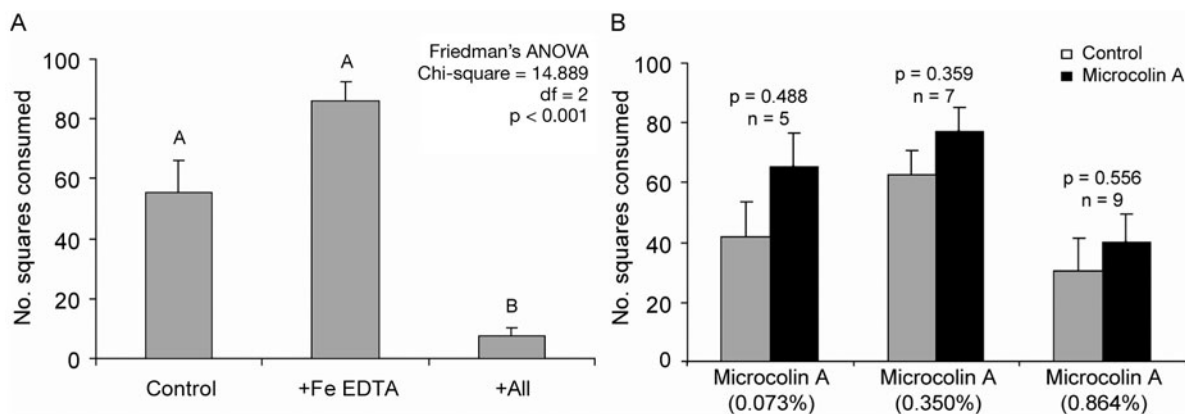


Fig. 6. *Stylocheilus striatus* feeding trials. (A) Bioassay treatment preference test (n = 8). (B) Microcolin A palatability tests. Each control treatment pair was run separately and tested using a paired *t*-test. Only sea hares that consumed >10% or <90% offered food were included in the analysis. Error bars: +1 SE

nosa was also positively correlated with specific growth rate (Long et al. 2001, Downing et al. 2005). The positive relationship between microcystin and *M. aeruginosa* growth rate was suggested to be correlated with N:P ratio-dependent assimilation of nitrogen. Both microcolin A and B have immunosuppressive activity (Koehn et al. 1992, Zhang et al. 1997), but these tests were undertaken on mammalian models rather than ecologically relevant organisms. The palatability of microcolins A and B for generalist grazers has not been tested, but many cyanobacterial secondary metabolites deter feeding by generalist grazers (Nagle & Paul 1999). Assuming the microcolins act as feeding deterrents, then in the current study, the trade-off suggests that when there is an abundance of nutrients there is greater benefit to the cyanobacterium to increase biomass rather than to produce anti-feedants. However, it should be noted that the current experiment excluded grazers and therefore did not consider the potential stimulatory effects of grazing pressure on secondary metabolite production.

Stylocheilus striatus consumed artificial foods containing the extracts from the *Lyngbya* sp. control and +FeEDTA bioassay treatments in preference to those from the +All treatment (Fig. 6A). This leads us to suggest that either the crude extract from the control and +FeEDTA treatments stimulated the *S. striatus* to feed, or the +All treatment crude extract contained compounds that deterred feeding. The control and +FeEDTA treatments contained significantly higher concentrations of microcolin A than the +All treatment (Fig. 4B), suggesting that the microcolin A may have stimulated the *S. striatus* to feed on these foods. However, when we tested the palatability of pure microcolin A against artificial food without *Lyngbya* sp. compound, there was no significant difference between the microcolin A and control food at any of the concentrations observed in the bioassay samples, although at all concentrations they did consume more of the treatment food (Fig. 6B). We had insufficient microcolin B to repeat the palatability tests using pure microcolin B, but this compound has previously been tested in a similar *S. striatus* palatability study in Guam, which showed feeding deterrence at much lower concentrations of pure microcolin B (0.022% dry wt) than observed in the current study (Nagle et al. 1998). The minimum concentration of microcolin B observed in the bioassay was 0.121%, with an average of 0.343% and maximum of 0.572% on a dry weight basis. At the lower concentration of 0.022% tested by Nagle et al. (1998), microcolin B inhibited feeding when compared with a control, but there was no effect of microcolin A at 0.05%. It should be noted that in the Nagle et al. (1998) study, the *S. striatus* had not previously been exposed to microcolins because these compounds are

not produced by *Lyngbya* spp. in Guam (V. J. Paul pers. obs.), whereas *S. striatus* individuals used in this study were collected with—and have been documented to preferentially feed on (Capper & Paul 2008)—the *Lyngbya* sp. that produces microcolins. In addition, because the highest concentrations of microcolin B were observed in the control and +FeEDTA bioassay treatments, and because these concentrations were much higher than those tested by Nagle et al. (1998), it seems unlikely that microcolin B was driving the observations in the *S. striatus* preference test in this study. Instead, it is possible that other biochemical changes occurred in the bioassay *Lyngbya* sp. that made the control and +FeEDTA crude extracts more palatable to *S. striatus* than the +All treatment. The experiments reported here demonstrate that changes in the availability of nutrients in the marine environment can not only change growth and secondary metabolite production in *Lyngbya* sp., but also affect the palatability of this organism to specialized grazers.

If nutrient and trace metal additions can affect grazing behavior of the top-down biological control organisms as well as stimulate growth, then these conditions may increase *Lyngbya* sp. bloom potential in the near-shore environment. Here, not only did we demonstrate a change in the feeding behavior of a major *Lyngbya* sp. consumer, but there was also increased growth in the chelated Fe treatment, demonstrating that growth of *Lyngbya* sp. on the Broward County hard-bottom reefs is iron limited. While there was no effect of N or P on the change in biomass, the addition of chelated Fe during the 4 d bioassay caused a significant increase in growth over and above that observed in control samples and those to which N and P were added. This is similar to the results of *in situ* growth experiments with *L. majuscula* in Moreton Bay, Australia, where chelated Fe additions led to significant increases in biomass above that of control samples (Dennison et al. 1999, Watkinson et al. 2005, Ahern et al. 2007, 2008). In the Australian studies, phosphorus also stimulated growth, but there was no significant P effect in the current study (Fig. 3). The lack of N or P stimulation in this nutrient-limitation bioassay suggests that there was sufficient N and P available in the near-shore waters of Broward County to facilitate *Lyngbya* sp. growth. Indeed, blooms of this genus have been a regular summer event at this site when light and temperature conditions have been conducive to cyanobacterial growth (Paul et al. 2005). Instead, it appears that levels of trace elements such as bioavailable Fe are limiting growth of this cyanobacterium in these coastal waters. Ambient nutrient (N and P) concentrations are monitored at the *Lyngbya* sp. collection site over the summer months (July to November) by the Broward County Environmental Protection and Growth Management Depart-

ment. In 2007, the average (\pm SD) for monthly sampling was $\text{NH}_4 = 0.32 (\pm 0.14) \mu\text{M N}$, $\text{NO}_3 = 0.46 (\pm 0.13) \mu\text{M N}$, $\text{NO}_x = 0.51 (\pm 0.14) \mu\text{M N}$, and $\text{Tp} = 0.20 (\pm 0.05) \mu\text{M P}$. These values are an order of magnitude lower than our experimental enrichment values, but still provide sufficient nutrients for cyanobacterial growth. Bioavailable Fe concentrations are rarely measured during regular marine monitoring, but should be considered in areas where *Lyngbya* spp. blooms are becoming a predominant part of the seascape.

The experiments reported here demonstrate that changes in the availability of nutrients in the environment can not only change growth and secondary metabolite production in *Lyngbya* sp., but can also affect the palatability of this organism to specialized grazers. Specifically, the addition of chelated Fe significantly increased growth of *Lyngbya* sp. from the Broward County reefs, while the addition of N, P and chelated Fe combined made the *Lyngbya* sp. extract less palatable to the major consumer of this cyanobacterium, potentially increasing the bloom potential of this species. As such, the management of near shore *Lyngbya* sp. blooms should consider the effects of nutrient enrichment on secondary metabolite production, and the associated effects on potential grazers, as well as the role nutrient additions play in cyanobacterial growth.

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