2006-18

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Impact of toxic cyanobacterial blooms (*Lyngbya* spp.) on coral reef habitats off southeastern Florida and the Florida Keys

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Summary

Lyngbya spp. are bloom-forming, toxic cyanobacteria that have been demonstrated to impact coral reefs through smothering of corals and other benthic organisms and by producing biologically active secondary metabolites that impact specialist and incidental grazers. Using an in situ bioassay approach we were able to show that nutrient enrichment, particularly the addition of iron, can increase growth and secondary metabolite in *Lyngbya polychroa*. In addition, we demonstrated that the specialist grazer *Stylocheilus striatus* can sequester secondary metabolites produced by *Lyngbya* spp. in Florida. Finally, we assessed the potential role of *Lyngbya* secondary metabolites in fibropapilloma in green turtles in Florida waters and found that the tumor promoting compounds produced by *Lyngbya majuscula* in the Pacific are not present in *Lyngbya* spp. in Florida.

Project Objectives

The objective of this project was to assess the prevalence and impact of toxic cyanobacterial blooms of *Lyngbya* spp. upon coral reefs and the organisms associated with those reefs off of southeastern Florida and the Florida Keys.

I. Sample Collection

Between May 2007 and June 2008, 21 *Lyngbya* spp. samples were collected and added to our previous collections. These samples came from six sites and represented six putative species. Collection sites and the *Lyngbya* species present are provided in Figure 1. Blooms generally occurred in summer months and Table 1 provides a summary of the blooms sampled, the dominant cyanobacterial species present and their morphological characteristics. *Lyngbya polychroa* morphotypes are discussed in more detail below and are distinguished by the color of the organism, the cell length and the gross morphology of the filament bundles.

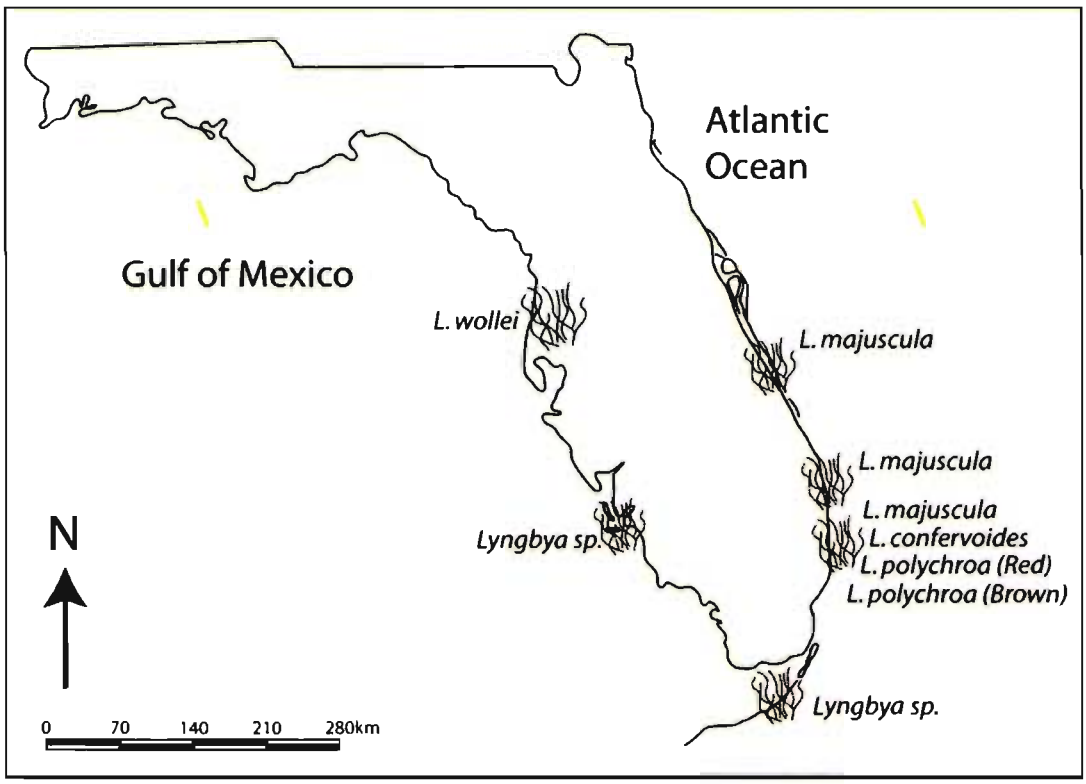


Figure 1: *Lyngbya* spp. blooms observed in Florida during 2007-2008.

Table 1: *Lyngbya* spp. collected in Florida marine waters May 2007-June 2008. Cell morphology data are provided as the mean (range) of average widths / lengths for each sample (n = number of samples collected at each site).

				Cell morphology (µm)		
Site	**Species**	**Months observed**	**n**	**Filament width**	**Cell width**	**Cell length**
Keys – Kemp's Channel	*Lyngbya sp.*	May 2007	1	19.0	17.3	3.8
Indian River Lagoon	*L. majuscula*	May – Sep 2007	5	37.9 (32.5-43.3)	33.6 (25.5-39.6)	3.7 (3.1-5.3)
Broward County Reefs	*L. confervoides*	May- Jul 2007	3	14.5 (13.1-16.0)	13.0 (12.8-13.3)	4.9 (4.1-5.8)
Broward County Reefs	*L. polychroa* (red)	May – Jul 2007	3	40.7 (38.8-44.0)	33.1 (30.3 – 36.1)	3.1 (2.5-3.8)
Broward County Reefs	*L. polychroa* (brown)	Jun 2007	1	43.8	34.8	7.4
Broward County Reefs	*L. majuscula*	May 2007	1	25.0	21.9	2.8
Crystal River	*L. wollei*	Jan 2008	1	47.3	36.6	6.4
Sanibel Island	*L. majuscula*	Jul 2007	3	27.3 (25.5-28.8)	25.3 (22.5-28.3)	3.2 (2.8-4.0)
Lake Worth Lagoon	*L. majuscula*	Aug 2007*	3	27.6 (25.3-30.3)	21.8 (20.5-23.8)	3.9 (3.1-4.9)

*Not present in Nov 2007

Broward Country Reefs

Four co-occurring chemotypes of *Lyngbya* were found on the Broward County Reefs. There appears to be a temporal shift in the presence of these chemotypes with *L. confervoides* as the dominant species early in the summer (May-July). *Lyngbya polychroa* (red) increased in abundance during the mid summer months (June- September), and *L. polychroa* (brown) appeared to be most abundant in the late summer and fall months. *Lyngbya majuscula* was observed throughout the summer in lower quantities. Underwater the gross morphology of the four chemotypes is distinctly different from one another. *Lyngbya majuscula* formed dense, dark grey or black mats, and both morphs of *L. polychroa* occurred as long, thick filaments with the two morphs only distinguishable from one another underwater through color and texture. *L. polychroa* (brown) colonies appeared in clumps, whereas *L. polychroa* (red) was more evenly dispersed. *Lyngbya confervoides* was much finer and appeared silkier than *L. polychroa* underwater (Figure 2). Microscopically, *L. polychroa* (brown) and *L. polychroa* (red) appeared to be similar in cell width, but *L. polychroa* (brown) had longer cells, was dark brown, and often covered in epiphytic dinoflagellates. *Lyngbya majuscula* had narrower and shorter cells than either *L. polychroa* color morphs. *L. confervoides* had the narrowest filaments of the four chemotypes (Table 1; Figure 2). In addition to morphometric differentiation, these four putative species could be differentiated by the major compounds they produced (Table 2) and based on 16S gene sequences they were also genetically distinct.

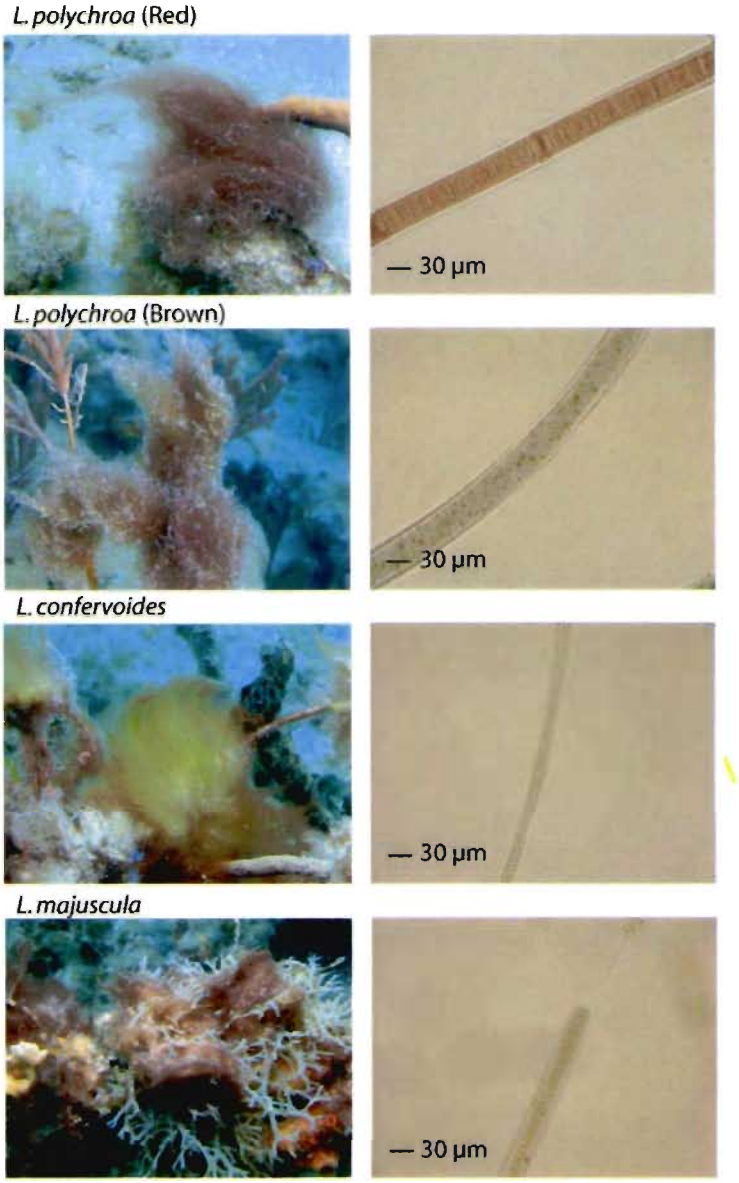


Figure 2: *Lyngbya* spp. collected in Fort Lauderdale, Florida. Each species, as identified based on morphological features, are shown both i*n situ* at the collection and as micrographs from light microscopy (400X).

II. Chemical extraction & identification of Lyngbya spp. *secondary metabolites*

All samples collected during this study were freeze-dried and extracted in ethyl acetate:methanol (1:1) and ethanol:water (1:1) to provide a lipophilic and hydrophilic extract. Extracts were then screened using bioactivity fractionation. Where bioactivity was detected, extracts were fractionated using silica gel (Mega Bond-Elut) column for the lipophilic fraction and a C18 column for the hydrophilic fraction. All crude fractions were subsequently chromatographed using HPLC (silica and RP C18) to provide pure compounds where possible.

Nuclear Magnetic Resonance (NMR) spectroscopy was used to compare spectra with literature values for compounds from taxonomically similar organisms. Compounds identified from these collections of Lyngbya are provided in Table 2.

Table 2: Major compounds produced by *Lyngbya* spp. collected in marine and estuarine habitats of Florida.

Location of bloom	**Species**	**Major compounds identified**
Keys – Kemp's Chanel	*Lyngbya sp.*	Lyngbyastatin 7, Kempopeptins A & B
Indian River Lagoon	*L. majuscula*	Malyngolide
Broward County Reefs	*L. confervoides*	Largamides and Lyngbyastatins 4-6
Broward County Reefs	*L. polychroa* (red)	Microclins A & B
Broward County Reefs	*L. polychroa* (brown)	Curacin D, Dragonamides C & D
Sanibel Island	*L. majuscula*	Malyngolide

III. Assessing cyanobacterial growth and secondary metabolite production in response to nutrient inputs

Lyngbya growth and secondary metabolite production in response to nutrient enrichment was assessed using the in situ bioassay technique. Briefly, *Lyngbya* was collected from Broward Country reefs and grown in individual 3 L polyethylene clear jars and grown at ambient light and temperature (Figure 3). Measurements of growth (change in wet weight biomass), acetylene reduction (proxy for nitrogen fixation) and concentration of major secondary metabolites were made at the beginning and end of the five day bioassay.

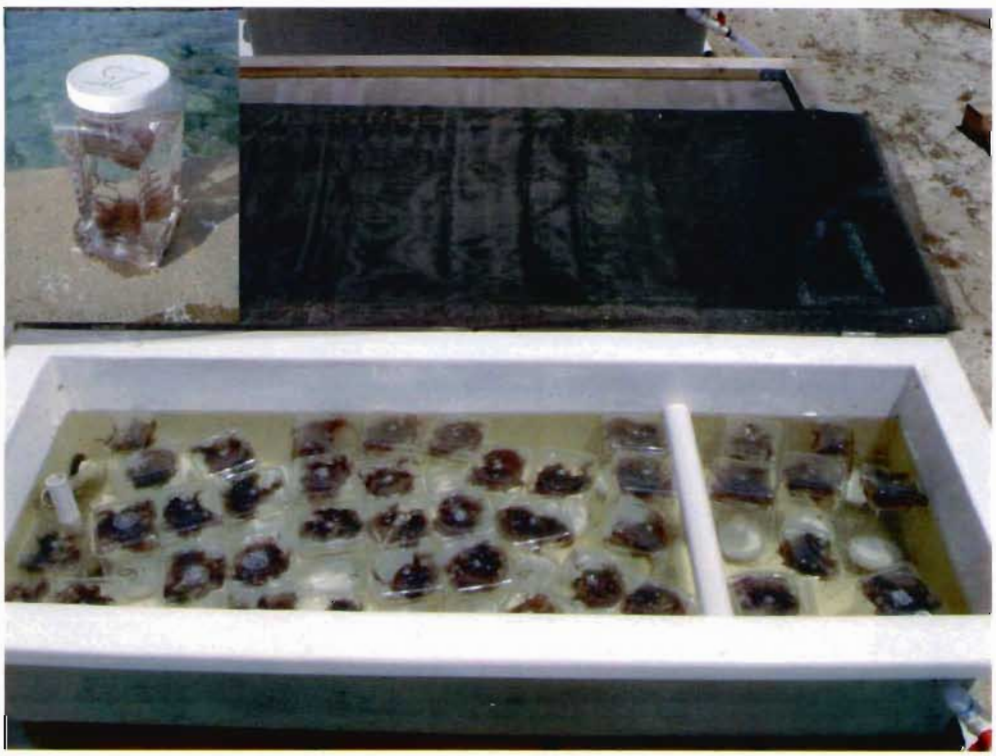


Figure 3: Nutrient bioassay set up. *Lyngbya* is placed in 3L clear polyethylene jars and then incubated under ambient temperature and light conditions. Nitrogen, phosphate and iron are added to containers daily and Lyngbya is allowed to grow for 5 days.

Change in Biomass:

Lyngbya polychroa demonstrated a significant increase in biomass in treatments enriched with chelated iron (Figure 4). However, *L. confervoides* found growing contemporaneously with *L. polychroa* did not elicit a significant growth response to any of the nutrient treatments.

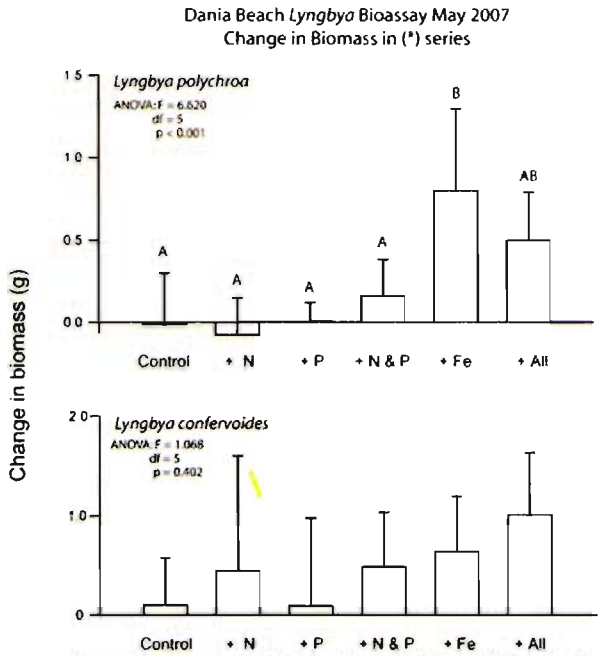


Figure 4: Change in biomass in *L. polychroa* and *L. confervoides* collected on Broward Country reefs in May, 2007. *Lyngbya polychroa* showed a significant increase in growth in samples enriched with chelated iron. Error bars represent one standard deviation and letters above the bars represent significant differences between treatments using a Tukey post-hoc pairwise test (n=5 for all treatments).

To determine whether the significant increase in growth in the May 2007 bioassay in chelated iron treatments was caused by iron enrichment or the presence of the chelator, we ran a follow up bioassay in July 2007 using *Lyngbya polychroa* again collected from the reefs at Fort Lauderdale. In this instance we tested the effects of iron ($FeCl_3$) on its own, chelator (EDTA) and a combination of the two on the growth and secondary metabolite production. Although this experiment did not yield a statistically significant result, all treatments had higher mean growth rate than the controls and it is likely that the addition of both iron, chelator and a combination allow *Lyngbya polychroa* a growth advantage (Figure 5).

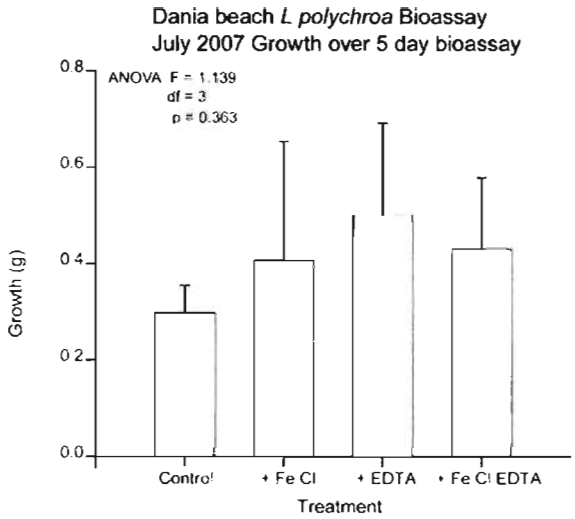


Figure 5: *Lyngbya polychroa* collected from Broward County reefs did not show a significant increase in growth with the addition of iron on its own, EDTA on its own and a combination of the two.

Secondary metabolite production:

Microcolins A and B were found to be the major compounds produced by *L. polychroa* collected on Broward County reefs. The change in concentration of both microcolin A & B was quantified using HPLC. All bioassay samples were immediately frozen at the end of the bioassay. They were then lyophilized and extracted using 1:1 ethyl acetate: methanol. Crude extracts were compared with standard curves of microcolin A and B prepared from pure compounds isolated from bulk collections of *L. polychroa* (R^2 = 0.98 & 0.99 respectively).

Although there was more microcolins present in samples at the end of the bioassay than found in the initial samples, this was not statistically significant (Figure 6). However, at the end of the bioassay both microcolin A and microcolin B appeared to be inversely proportional to growth observations (Figure 4) with significantly less secondary metabolites in +Fe and +All treatments (Figure 6).

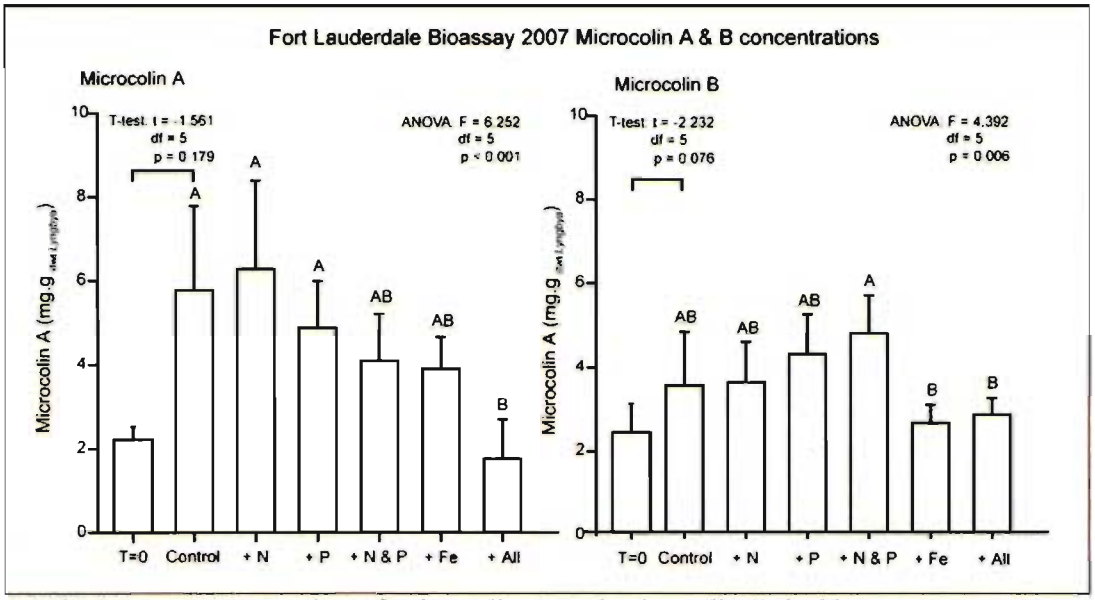


Figure 6: Average concentration of microcolin A and microcolin B in bioassay treatments at the beginning of the bioassay (T=0), the control, nitrogen addition (+N), phosphate addition (+P), iron EDTA addition (+Fe) and a combination of all nutrients (+All). Error bars represent one standard deviation and letters above the bars represent significant differences between the mean secondary metabolite concentrations of each treatment (n=5).

When the production of microcolins was considered as a function of the crude extract (i.e. independent of the mass of *Lyngbya* extracted) there was still a significant decrease in the amount of microcolin A in the +All nutrient addition treatment. However, there was not a significant difference between the control and treatment groups in terms of microcolin B concentration (Figure 7).

In the follow up bioassay testing the effects of iron, EDTA and a combination of the two, there was not a significant difference between the concentration of Microcolin A in any of the treatments; however, their was a significant increase in microcolin B in samples in which EDTA alone had been added and iron in the presence of EDTA (Figure 8).

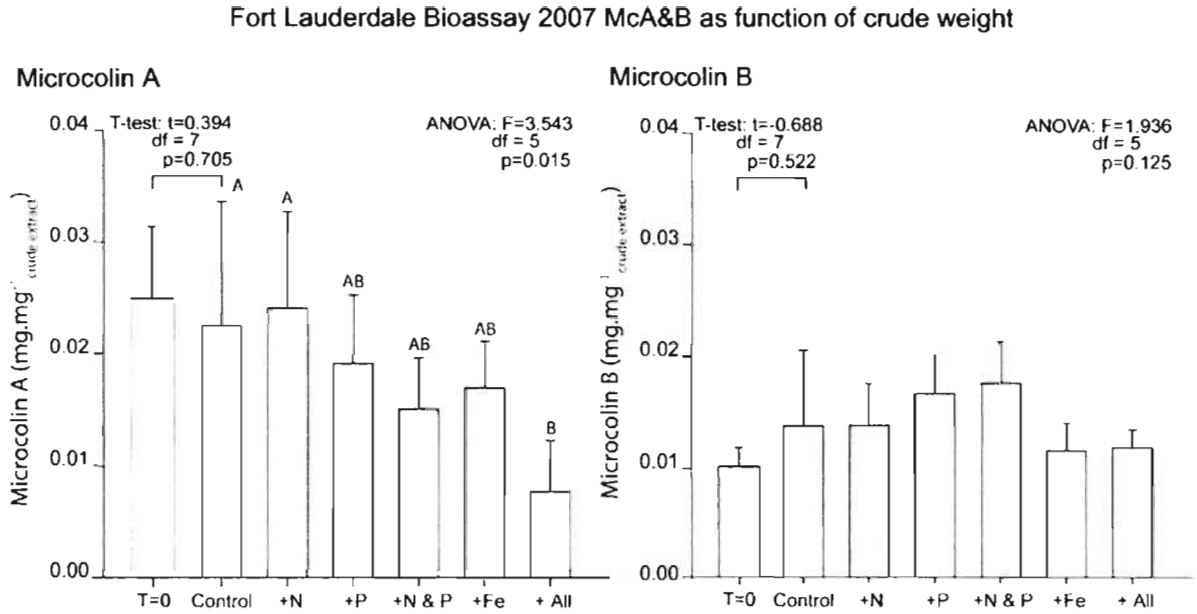


Figure 7: Microcolin A & B concentration as a function of crude extract weight extracted from the *Lyngbya polychroa* samples.

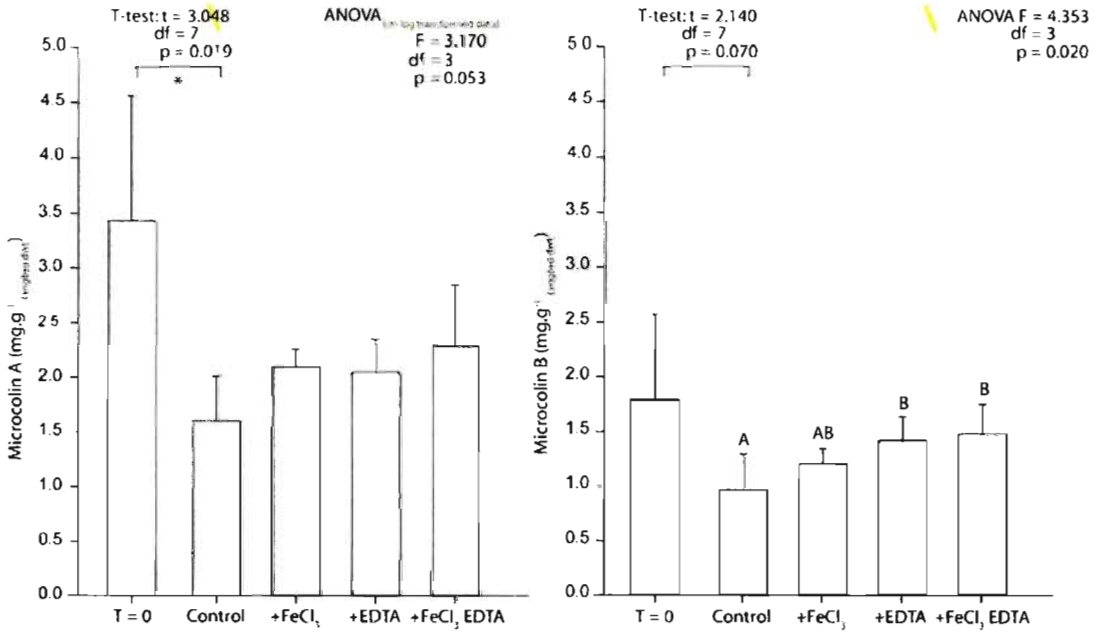


Figure 8: Microcolin A & B concentration in the follow up bioassay assessing the impacts of iron alone, EDTA alone and iron and EDTA combined on the secondary metabolite production.

Patterns in *Lyngbya* sp. Tissue $\delta^{15}N$ and $\delta^{13}C$ Values

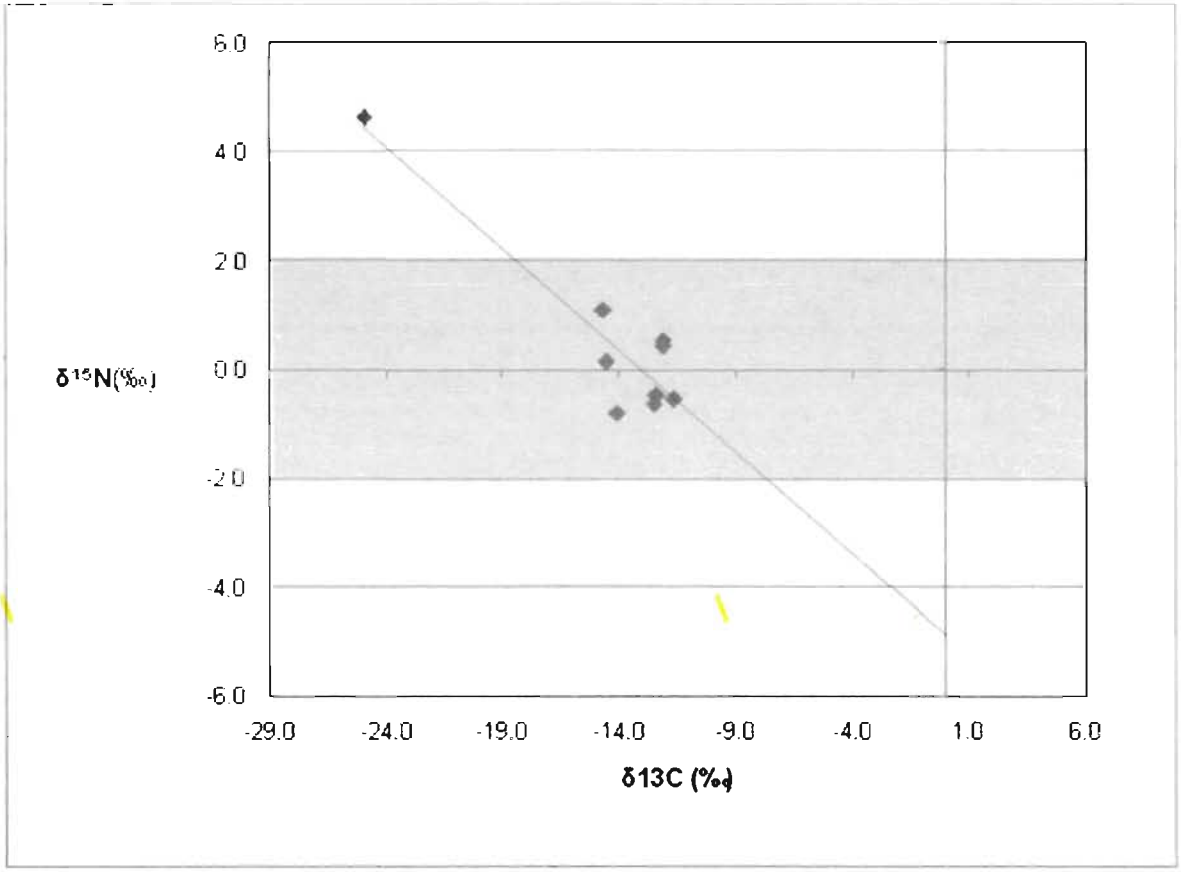
Stable isotope analysis ($\delta^{15}N$ and $\delta^{13}C$) was conducted to provide a quantitative measure of C and N content in *Lyngbya* tissue and as tracer isotopes to identify the source of C and N incorporated into *Lyngbya* tissue. *Lyngbya* sp. tissue was preliminarily collected from across South Florida benthic habitats, including Fort Pierce (Little Jim Island, IRL), Sanibel Island, and Hollywood Beach. A summary of percent by weight tissue carbon and tissue nitrogen, as well as resulting $\delta^{15}N$ and $\delta^{13}C$, is included in Table 1. All but one sample (FLL Hollywood Beach) fell within the range of accepted $\delta^{15}N$ values for pure N_2-fixing primary producers. This outlier's high $\delta^{15}N$ value (4.6 ‰) reflects N uptake from a terrestrial source resembling organic N in soils and manure (LaPointe et al., 2004). $\delta^{13}C$ values analyzed in all collected *Lyngbya* tissue reflect C fixation of ^{13}C-enriched bicarbonate or CO_2 in ambient ocean waters. The negative correlation between $\delta^{15}N$ and $\delta^{13}C$ suggests a possible connection between C and N sources in the system.

Lyngbya sample collections are ongoing, and efforts have expanded to include reef sites around Summerland Key, Biscayne National Park, and Fort Lauderdale inlets and sewage outfalls. Collected tissue values will be compared with stable isotope signatures of water samples (porewater, outfall water, upwelled water, coastal water, and ambient water) to identify sources of pollution potentially contributing to bloom formation.

Table 3. Data collection summary for 2006-2007.

Species	Location	Date	wt% C	wt% N	d13C	d15N
L. majuscula	Little Jim Island	10/25/2006	0.035599	0.006115	-12.1	0.4
L. majuscula	Little Jim Island	5/11/2007	0.061353	0.009331	-12.1	0.5
L. majuscula	Sanibel Island	7/10/2006	0.042422	0.006134	-14.7	1.1
L. majuscula	Sanibel Island	7/14/2006	0.214332	0.034188	-14.6	0.1
L. majuscula	Sanibel Island	7/26/2007	0.178354	0.024091	-12.4	-0.5
L. majuscula			0.158651	0.021196	-11.6	-0.5
L. majuscula	Sanibel Site 2	7/23/2007	0.121159	0.015573	-12.5	-0.6
L. majuscula	N. Causeway IRL	8/7/2006	0.068217	0.009461	-14.1	-0.8
Lyngbya sp.	FLL Hollywood Beach	8/3/2006	0.24984	0.027552	-25.0	4.6

Figure 9. Bivariate plot of $\delta^{15}N$ and $\delta^{13}C$ values for *Lyngbya* sp. collected from across South Florida. Most values fell within the shaded region, which reflects the accepted range in $\delta^{15}N$ values for pure N2-fixing primary producers (0.0 ± 2.0). $\delta^{15}N$ and $\delta^{13}C$ values are negatively correlated (Pearson's r = -0.92118).



IV. Fate of Lyngbya compounds in marine food chains

Specialist grazer – *Stylocheilus striatus*

The seahare *Stylocheilus striatus* is a specialist grazer that has evolved to feed on *Lyngbya* spp. and is known to sequester toxins produced by cyanobacteria, potentially as defense against predators. We tested whether *S. striatus* feed on *L. polychroa* and whether they are able to take up the microcolins produced by *L. polychroa*. Ten sea hares were fed *L. polychroa* exclusively for ten days. At the end of the ten day trial the seahares and the *L. polychroa* they had been feeding on was frozen, extracted and quantified as per bioassay samples.

There was no relationship between the amount of microcolins present in the *L. polychroa* at the end of the feeding trial and the concentration of microcolins observed in the seahares (Figure 10).

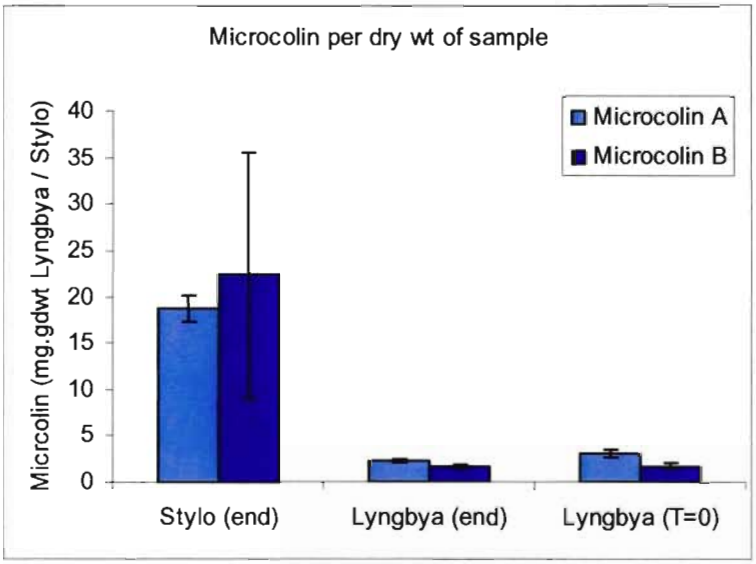


Figure 9: Microcolin A & B concentration in the sea hare *Stylocheilus striatus* and *Lyngbya polychroa* at the end (end) of the 10 day feeding trial compared with concentrations in the *Lyngbya polychroa* on the first day of the feeding trial (T=0).

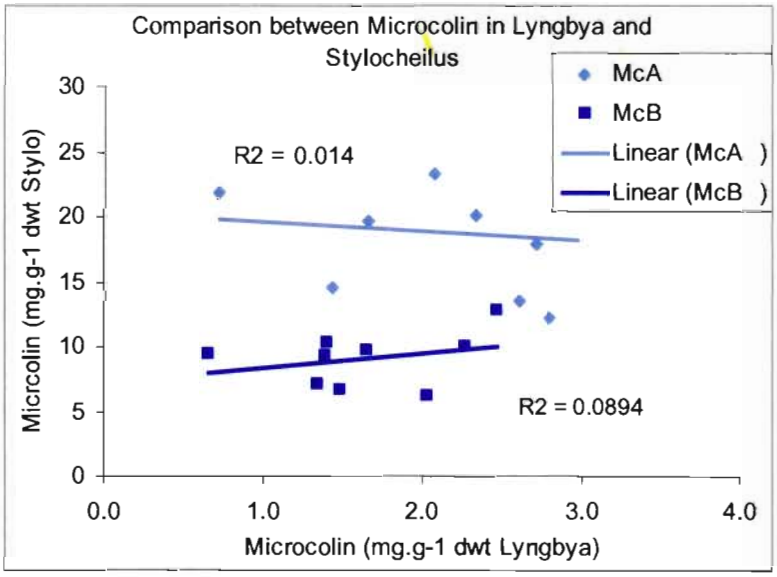


Figure 10: There was no relationship between the amount of microcolins in the Lyngbya and the concentration observed in the Stylocheilus samples (n=10).

Generalist Herbivore – The green turtle (*Chelonia mydas*)

Research undertaken in Australia and the Hawaiian Islands suggested that the tumor promoting compounds lyngbyatoxin A and debromoaplysiatoxin produced by *Lyngbya majuscula* in these areas may be related to the presence of fibropapilloma lesions in green turtles (Arthur *et al.*

2008). It was hypothesized that the high prevalence of turtles suffering from fibropapillomatosis in Floridian waters (Figure 11) may be related to blooms of *Lyngbya*.

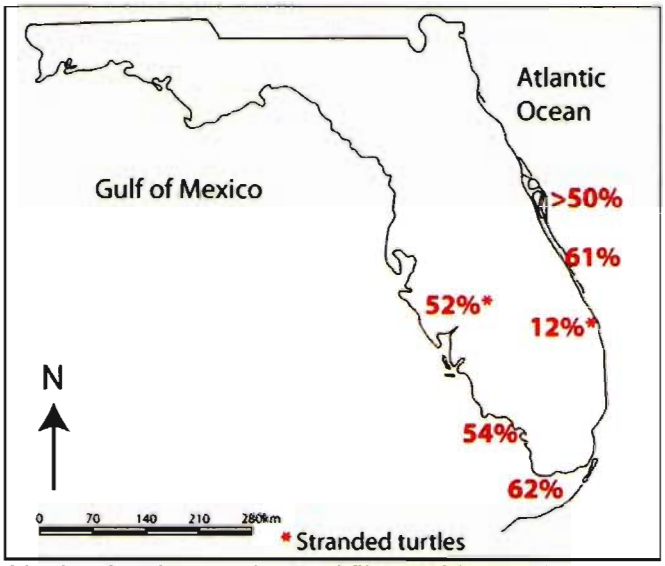


Figure 11: Map of Florida showing the prevalence of fibropapillomatosis among green turtle populations in Florida. Data source: Foley & Schroeder et al. 2005; Witzell & Schmid 2004; Bresette, et al. 2000; Schroeder et al. 1998.

Through this study we have determined that neither lyngbyatoxin A, nor debromoaplysiatoxin (the two tumor promoting compounds implicated in fibropapillomatosis in Australia) are produced by any of the *Lyngbya* spp observed in Florida. In addition to this, we sampled muscle, stomach and fecal material from 12 stranded green turtles. These samples were extracted and compared with pure lyngbyatoxin A, malyngolide and microcolin A using thin layer chromatography (Figure 12). No samples were found to be positive for these compounds, however there are some currently unidentified compounds of interest present in these samples.

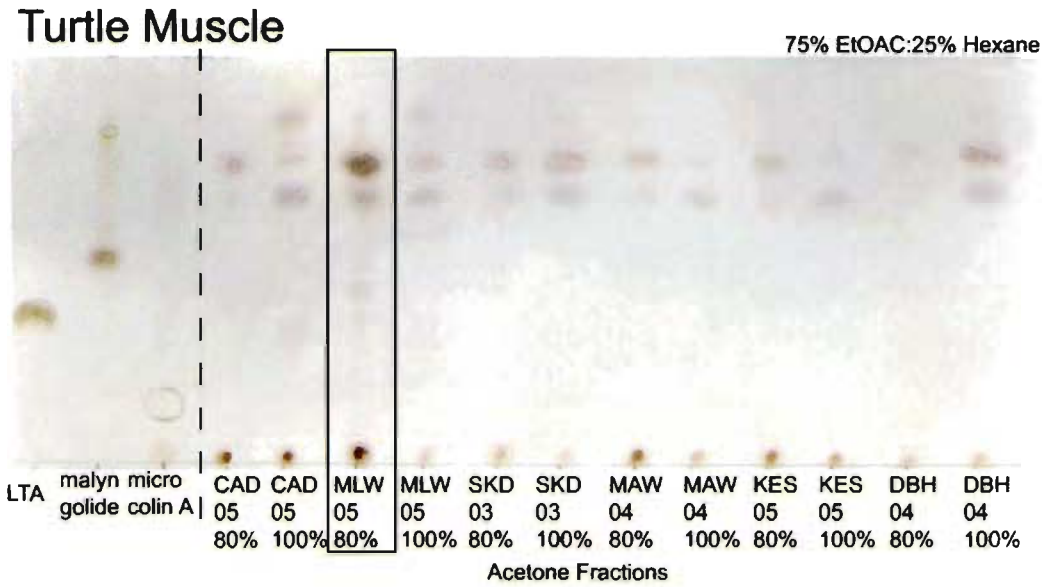


Figure 12: Thin layer chromatography comparing green turtle sample extracts with known compounds produced by *Lyngbya*.

V. Student Training

Ms. Genelle Harrison worked as a student intern with us between June – December 2007. Genelle assisted us with the extraction of many of the *Lyngbya* samples described in this study and under the guidance of Dr Koty Sharp (Postdoctoral Fellow Smithsonian Marine Station Fort Pierce), Genelle did much of the laboratory work involved with the characterization of the 16S gene sequences for the four chemotypes of *Lyngbya* we have been able to describe for the Broward County Reefs.

VI. Outputs based on this research

Publications:

Data arising from this Florida Reef grant work is being prepared for the following scientific literature publications:

Arthur, K. E., Paul, V. J., Paerl, H. W., O'Neil, J. M., Joyner, J., Ross, C., Capper, A., Erickson, A. A., Gunasekera, S., Meickle, T., Luesch, H. and Matthew, S. Distribution of *Lyngbya* spp. in Florida, USA. *In Prep for Harmful Algae*

Sharp, K., Arthur, K. E., Gu, L., Sherman, D., Ross, C., Harrison, G., Meickle, T., Luesch, H., Matthew, S. and Paul, V. J. Phylogenetic and chemical diversity of four chemotypes of bloom-forming *Lyngbya* (Cyanobacteria: Oscillatoriales) from reefs of South Florida. *In Prep for Applied and Environmental Microbiology*

Conference Presentations

These results have / will be presented at the following meetings:

- *Smithsonian Marine Science Symposium*, Washington DC, 15-16 November 2007
  - Oral presentation: Arthur KE & Paul VJ *"Ecology of the toxic cyanobacteria* Lyngbya *sp. in Florida, USA"*
- International Coral Reef Symposium, Fort Lauderdale, 7-11 July 2008
  - Oral presentation: Arthur, K. E., Paul, V. J., Paerl, H. W., O'Neil, J. M., Joyner, J., Ross, C., Gunasekera, S., Meickle, T., "*The role of nutrients in* Lyngbya *growth and chemical defense*"
- *Ocean Sciences Meeting* – American Society of Limnology and Oceanography, Orlando, FL 2-7 March 2008.
  - Poster presentation: Arthur, K. E., Paul, V. J., Paerl, H. W., O'Neil, J. M., Joyner, J., Joyner, A. R., Ross, C. and Gunasekera, S. P. "*The role of nutrients in* Lyngbya majuscula *growth and chemical defense.*"
  - Poster presentation: Paul, V.J., Ross, C., Ritson-Williams, R., Walters, L.J., Arthur, K., Gunasekera, S., Meickle, T., *"Impacts of benthic cyanobacterial blooms on coral reefs"*
- International Coral Reef Symposium, Fort Lauderdale, 7-11 July 2008
  - Oral presentation: Arthur, K. E., Paul, V. J., Paerl, H. W., O'Neil, J. M., Joyner, J., Ross, C., Gunasekera, S., Meickle, T., "*The role of nutrients in* Lyngbya *growth and chemical defense*"
  - Oral presentation: Paul, V.J., Ross, C., Ritson-Williams, R., Walters, L. Arthur, K., Gunasekera, S., Meickle, T., *"Ecological consequences of cyanobacterial blooms on coral reefs"*

Invited Seminar Presentations

Data resulting from this grant has been presented as part of the following invited seminars:

- Karen Arthur: University of Florida School of Veterinary Medicine – 2nd October 2007 "*Lyngbya* - a toxic cyanobacterium and its potential impacts on marine turtles"
- Karen Arthur: Jacksonville University Marine Seminar Series – 27th November 2007 "Blooms of the toxic cyanobacterium *Lyngbya majuscula* and its impact on green sea turtles"
- Karen Arthur: Florida International University – 13th February 2008 "The impacts of the toxic cyanobacterium *Lyngbya majuscula* on green turtles"
- Karen Arthur: Disney's Animal Kingdome Brown Bag Lunch Seminar Series – 19th February 2008 "The impacts of toxic blue-green algae *Lyngbya* on green turtles"
- Karen Arthur: Palm Beach Department of Environmental Resource Management Technical Presentation – 26th February 2008 "*Lyngbya* in Florida, ecological consequences of toxic algal blooms"
- Valerie Paul: Invited lecture for Department of Biology, Lewis and Clark College, Portland, OR – 14th March 2008 "Algal natural products mediate multiple ecological interactions on coral reefs"
- Valerie Paul: Invited presentation for Southeast Florida Coral Reef Initiative land Based Sources of Pollution Technical Advisory Committee Meeting May 2, 2008 "Studies of ecology and toxicity of *Lyngbya* on Florida reefs"

VII. References

Arthur, K., Limpus, C., Balazs, G., Capper, A., Udy, J., Shaw, G., Keuper-Bennett, U. and Bennett, P. (2008). The exposure of green turtles (*Chelonia mydas*) to tumour promoting compounds produced by the cyanobacterium *Lyngbya majuscula* and their potential role in the aetiology of fibropapillomatosis. *Harmful Algae* **7**, 114-125.

Bresette, M. J., Gorham, J. C. and Peery, B. D. (2000). Preliminary survey of sea turtles in the southern region of the Indian River Lagoon System. *Florida Scientist* **63**, 43-44.

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