AN ABSTRACT OF THE THESIS OF Wendy L. Milonovich for the Master of Science in Biology presented June 20, 2003.

Title: Nutrient Effects on Growth and Natural Products Chemistry in the Cyanobacteria *Lyngbya majuscula* and *Lyngbya bouillonii*

Approved: 

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This research aimed to look at the key role of nutrients, specifically nitrate and phosphate enrichment, on growth and natural products in two cyanobacteria commonly found around Guam. *Lyngbya majuscula* from Cocos Lagoon and Piti Bomb Holes, and *Lyngbya bouillonii* from Fingers Reef in Apra Harbor were treated with varying concentrations of nitrate, phosphate, and a combination of nitrate and phosphate. Growth rates; rates of uptake of nutrients; percent dry mass of carbon, nitrogen and phosphorus in treated cyanobacteria; and cytotoxicity of lipid and aqueous extracts of treated cyanobacteria against four tumor cell lines were measured. In growth studies, *L. majuscula* from both sites showed significant increases in growth when treated with 6.7 μM phosphate, a level well above ambient. *L. majuscula* from Piti Bomb Holes showed significant increases in growth when treated with 20 μM nitrate, also well above the ambient level. However, these results were not consistent in all studies, suggesting that growth is also influenced by other factors such as light levels, herbivory, seasonality, and physical disturbances. Rates of uptake were measured using time course studies. Uptake of nitrate and phosphate by *L. majuscula* from Piti Bomb Holes was rapid. When treated with 20 μM nitrate, 1 μM phosphate or a combination of 20 μM nitrate and 1 μM

phosphate, cyanobacteria took up most of the available nutrients from the water column within 90 minutes. Chemical analyses for carbon, nitrogen and phosphorus showed nitrogen content in *L. bouillonii* was significantly higher in samples treated with nitrate or a combination of nitrate and phosphate than in controls or samples treated with phosphate, but *L. majuscula* from Piti Bomb Holes and Cocos Lagoon showed no significant differences between control and treatment samples, suggesting that *L. bouillonii* may be more nitrogen-limited than *L. majuscula.* Cyanobacteria from all three sites showed significantly higher levels of phosphorus when treated with phosphate or a combination of nitrate and phosphate compared to controls or samples treated only with nitrate. This demonstrates the ability of cyanobacteria to take up and store phosphorus during times when there is excess phosphorus available, supporting the theory of "luxury consumption." Cytotoxic effects were not significantly different among aqueous or lipid extracts of the cyanobacteria when tested against four tumor cell lines. From these studies, it is apparent that variations in growth and uptake of nutrients are probably not attributable to one single factor such as levels of available nutrients, but instead, to a combination of nutrient levels, seasonality, site specificity, species physiology and various environmental factors such as light, water temperature, and herbivory.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

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NUTRIENT EFFECTS ON GROWTH AND NATURAL PRODUCTS CHEMISTRY IN THE CYANOBACTERIA *LYNGBYA MAJUSCULA* AND *LYNGBYA BOUILLONII*

BY

WENDY L. MILONOVICH

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Table of Contents

Acknowledgments ii
List of Tables iv
List of Figures v

Introduction 1

Materials and Methods 11
- Collection of cyanobacteria 11
- Sample preparation 11
- Laboratory microcosm and tank design 11
- Nutrient enrichment for laboratory studies 13
- Field study design 18
- Nutrient enrichment for field study 18
- Daily-growth calculations for laboratory studies 19
- Water sampling for laboratory studies 19
- Analysis of water for nitrate and phosphate 20
- Uptake of nitrate and phosphate 20
- Carbon, hydrogen, nitrogen (CHN) analyses 21
- Total phosphorus analysis 21
- Preparation of lipid and aqueous extracts 23
- *In vitro* solid tumor selective assays 23
- Zone assay methodology 24

Results 26
- Growth studies 26
- Nutrient uptake 30
- Carbon, nitrogen, and phosphorus 33
- *In vitro* solid tumor selective assays 36

Discussion 40
- Growth 40
- Uptake of nutrients 42
- Natural products chemistry 43

References Cited 46

List of Tables

Table 1. Environmental factors influencing cyanobacterial growth and bloom formation 8

Table 2. Ambient levels of nitrate and phosphate at sites around Guam 16

Table 3. Daily growth of cyanobacteria for studies conducted May 2001-March 2002 22

Table 4. Carbon, nitrogen, and phosphorus content of cyanobacteria 34

List of Figures

Figure 1. Map of collection and study sites around Guam....12

Figure 2. Microcosm and tank design....14

Figure 3. Screening bioassay....25

Figure 4. Effects of nutrients on growth of *Lyngbya majuscula* from Piti Bomb Holes and Cocos Lagoon....27

Figure 5. Effects of nutrients on growth of *Lyngbya majuscula* and *Lyngbya bouillonii*....28

Figure 6. Effects of phosphate on growth of *Lyngbya bouillonii* (September 2001)....29

Figure 7. Nitrate uptake by *Lyngbya majuscula* from Piti Bomb Holes (October 2002)....31

Figure 8. Phosphate uptake by *Lyngbya majuscula* from Piti Bomb Holes (October 2002)....32

Figure 9. Carbon, nitrogen and phosphorus content in cyanobacteria under different treatment regimes....35

Figure 10. Cytotoxic effects of lipid extracts on four tumor cell lines....37

Figure 11. Cytotoxic effects of aqueous extracts on four tumor cell lines....38

Introduction

As earth's first oxygenic phototrophs, cyanobacteria were responsible for the transition from anaerobic to aerobic life (Paerl, 1996). They are also thought to be the main contributors to primary production (Sellner, 1997) and nitrogen fixation in the marine environment (Paerl et al., 1996). Laminated benthic microbial mats in nitrogen-deficient habitats exhibit relatively high rates of carbon (CO_2)- and nitrogen (N_2)- fixation and may account for a substantial portion of new primary production and biologically available nitrogen (Paerl et al., 1996). Furthermore, the planktonic cyanobacterial genus *Trichodesmium* supplies more than half of the new nitrogen used for primary production in tropical waters (Sanudo-Wilhelmy et al., 2001) making it a marine cyanobacterium of global importance (Stihl et al., 2001).

However, increased dominance of cyanobacteria on coral reefs has had a detrimental impact on many near-shore tropical environments (Kuffner and Paul, 2001). Cyanobacterial dominance can be devastating to a coral reef community for many reasons. Benthic mats of cyanobacteria sometimes cover thousands of square meters of reef or shore habitat (Paul et al., 2001; Paul et al., 2005). Corals, macroalgae, and other marine organisms compete with cyanobacteria for space, nutrients, and light (Thacker and Paul, 2001). Because cyanobacteria grow quickly in contrast to other marine organisms, they often out-compete other species for these resources. Additionally, several species of cyanobacteria produce secondary metabolites that are toxic to humans and marine organisms (Nagle and Paul, 1999; Paul et al., 2001; Sellner, 1997; Thacker and Paul, 2001). For example, *Lyngbya majuscula* produces debromoaplysiatoxin, a potent inflammatory substance responsible for incidents of severe dermatitis among swimmers

in Hawaii and Okinawa (Yasumoto and Murata, 1993; Sellner, 1997) and lyngbyatoxin A, which is responsible for severe oral and gastrointestinal inflammations in humans (Paerl et al., 2001; Paul et al., 2001; Sellner, 1997).

Interestingly, some of the same cyanobacteria that cause dermatitis and some human cancers, and are toxic to marine organisms, may also have beneficial medicinal qualities. For example, several bioactive compounds extracted from a collection of cyanobacteria at the University of Hawaii are now being tested as anti-cancer compounds (Duchemin, 2001). Among them are the cryptophycins, a family of antimitotic agents isolated from *Nostoc* species of cyanobacteria.

Those compounds that do not make it as anti-cancer drugs may still be useful in treating other maladies. Astaxanthin, derived from algae, is currently being marketed as a dietary supplement for humans and livestock (Duchemin, 2001). So, it is important to acknowledge the potential benefits from cyanobacteria, even though a shift from a coral-dominated to a cyanobacteria-dominated community imposes a great change on the existing habitat of many marine organisms that live and feed on coral.

The benthic filamentous non-heterocystous cyanobacteria *L. majuscula* and *L. bouillonii* are commonly found in abundance in the coastal water around Guam. *L. majuscula* is normally brown or greenish-brown, whereas *L. bouillonii* is reddish-brown. They grow in tufts or mats loosely attached to the substrate or around the base of soft corals. When they produce large quantities of gases, the cyanobacteria lift up from the substrate and rise to the surface of the water as floating mats. During optimum conditions, *L. majuscula* and *L. bouillonii* proliferate and reach nuisance levels (Paerl et al., 2001). These blooms are termed "nuisance" because the result is often severe

degradation of water quality demonstrated by anoxia, hypoxia, toxicity and bad odor (Paerl, 1996)

Many scientists believe that cyanobacterial blooms occur most frequently in areas of coastal eutrophication (over-enrichment of surface waters with mineral nutrients) (Paerl, 2000; Sellner, 1997) or areas that have low levels of herbivory (Correll, 1998; Dennison et al., 1999; Paerl, 1998; Schaffelke and Klumpp, 1998). Anthropogenic factors, such as pollution from sewage outfalls, fossil fuel combustion, agricultural emissions, fluxes of greenhouse gases (carbon dioxide, methane, and nitrogen dioxide), and over-fishing are implicated as the most common causes of coastal eutrophication and decreased herbivory (Paerl, 1998; Schaffelke and Klumpp, 1998; Thacker and Paul, 2001). Watershed systems that deliver nutrients to coastal water from nearby terrestrial areas may be responsible for many blooms of N_2-fixing cyanobacterial nuisance genera (Paerl, 2000). Land-based nutrient discharges, from local and regional pollution sources, are increasingly affecting coastal and oceanic waters. The concept that "dilution is the solution" no longer applies, as demonstrated by the regionalization and globalization of eutrophication caused by increasing magnitudes of human pollution (Paerl, 1998).

The rise in number and magnitude of harmful algal blooms caused by human pollution creates an imbalance in coastal ecosystems (Paerl, 2000). Algae and cyanobacteria in normal abundance are routinely kept in check by top-down control, or herbivory. Herbivory in some benthic marine environments is so intense that herbivores can remove almost all of the daily production of biomass by marine macroalgae (Paul et al., 2001). However, many generalist herbivores find certain species of cyanobacteria unpalatable due to secondary metabolites they produce and use as chemical

defenses. Therefore, top-down control may be insufficient to control large blooms of cyanobacteria (Thacker and Paul, 2001; Thacker et al., 2001).

Scientists hypothesize that availability of nutrients in the water column greatly affects growth and secondary metabolite chemistry of cyanobacteria (Dennison et al., 1999; Kuffner and Paul, 2001). Cyanobacteria, like macroalgae, acquire nutrients from the water column (Paul et al., 2001), and growth of many species of cyanobacteria may be limited by the availability of nitrogen and phosphorus (Kuffner and Paul, 2001; Paerl, 2000). Not only concentrations of nitrogen and phosphorus, but ratios of nitrogen to phosphorus, have been implicated as major limiting factors for the presence of N_2-fixing cyanobacteria in aquatic environments (Berman, 2001).

Secondary metabolites produced by cyanobacteria often contain nitrogen. This is probably due to the ability of some cyanobacteria to fix atmospheric nitrogen (Paerl, 1990). Rates of fixation in cyanobacterial mats in the oceans range from 1.32 g N m^{-2} yr^{-1} at the Great Barrier Reef, Australia to 76 g N m^{-2} yr^{-1} in Kaneohe Bay, Hawaii (Howarth et al., 1988). Nitrogen in the marine environment is limited (and thought by some to be the limiting nutrient for growth and productivity) due to the high biological demand for it, and the relatively low level of denitrification – conversion of atmospheric nitrogen into usable nitrates (Paerl, 1998; Paerl, 2000). While geologists argue that nitrogen is not the limiting nutrient in the ocean, due to the unlimited genetic potential of many marine organisms to obtain nitrogen from the atmosphere (Paerl, 2000), biologists contend that nitrogen fixation rates in the marine environment are limited because they depend on many environmental factors: oxygen tension, turbulence,

and the availability of nutrients and energy to sustain the process of nitrogen fixation (Paerl, 1990).

Only prokaryotes (Bacteria and Archaea) are capable of fixing nitrogen (Paerl, 2000), but not all prokaryotes are able to fix nitrogen. Ideas on whether *L. majuscula* and *L. bouillonii* are "nitrogen-fixers" are conflicting. *L. majuscula* and *L. bouillonii* do not contain heterocysts, specialized structures where photosynthesis does not take place. Therefore, some scientists believe they are incapable of fixing nitrogen (Paerl, 1998). Because oxygen inactivates nitrogenase, nitrogen fixation processes must be separated from photosynthetic processes by either time or space (Paerl, 2000). Thus, some non-heterocystous nitrogen-fixing species of cyanobacteria have developed methods for compartmentalizing nitrogen fixation in different areas (spatially), or by allowing nitrogen fixation to occur at night during light-dark cycles (temporally) when photosynthetically-derived oxygen is not present (Kuffner and Paul, 2001; Paerl, 2000) or when the ambient O_2 pressure is low (Janson et al., 1994). These spatial and temporal separations support the theory that *L. majuscula* and *L. bouillonii* are, in fact, capable of fixing nitrogen. So, it is no surprise to find the proliferation of a nitrogen-fixing cyanobacterium under potentially nitrogen-limiting conditions (Watkinson, 2000).

Recent investigations of nutrient-limited productivity in coral reef macroalgae have led to the conclusion that phosphorus, not nitrogen, is the primary limiting nutrient in nearshore areas (LaPointe, 1987; Larned, 1998). So, while nitrogen is believed to be the key mineral nutrient controlling primary production in the ocean, excessive phosphorus may be particularly important in controlling production in estuaries and continental shelf waters (Correll, 1999; Paerl, 2000).

Phosphorus is a mineral nutrient and an essential element for all life forms. Phosphorus occurs only in the pentavalent form in aquatic systems. Examples are: orthophosphate, pyrophosphate, longer-chain polyphosphates, organic phosphate esters and phosphodiesters, and organic phosphonates. Orthophosphate is the only form of phosphorus that autotrophs can assimilate, and therefore is the only P source taken up by cyanobacteria (Stihl et al., 2001). It is delivered to aquatic systems as a mixture of dissolved and particulate forms. Phosphorus is very dynamic and biologically active; dissolved orthophosphate in eutrophic surface waters turns over every few minutes (Correll, 1999). Because cyanobacteria can overcome nitrogen-depleted conditions by fixing nitrogen (Paerl, 2000; Steppe et al., 1996), and because phosphorus is in such high demand (for ATP production), some think that available phosphorus may be the limiting factor controlling cyanobacterial growth (Steppe et al., 1996; Watkinson, 2000).

In addition to surviving in a nitrogen-depleted environment, cyanobacteria may have also found a way to survive phosphorus-limited conditions. During phosphorus-loading events, cyanobacteria are able to take up phosphate, in excess of their metabolic and growth needs. They store reserves of the nutrient in the form of cytoplasmic polyphosphates, known as polyphosphate bodies. This excess uptake is termed "luxury consumption" and appears to be an adaptation by cyanobacteria that allows them to survive in otherwise phosphorus-deplete aquatic ecosystems. Some suspect that neither nitrogen nor phosphorus by itself is responsible for controlling cyanobacterial growth (Fuhs et al., 1972).

Several studies of nutrient effects on macroalgal and cyanobacterial growth show positive correlations between high nutrient levels and growth. For example, Shaffelke

and Klumpp (1998) demonstrated that pulses of 8 μM ammonium and 1 μM (or higher) phosphate were taken up rapidly, and significantly increased the tissue mass of the brown alga *Sargassum baccularia*. In the red alga *Gracilaria tikvahiae*, phosphorus-enriched water accounted for more than 60 % of the winter growth and 80 % of the summer growth above normal growth in ambient seawater (Lapointe, 1987). *L. majuscula*, from the coastal waters of Guam, also showed increased growth in phosphate-enriched seawater (Kuffner and Paul, 2001).

Abiotic factors (light, salinity, rainfall) and ambient water quality (dissolved inorganic nutrients and trace metals) have also been attributed to cyanobacterial proliferation (Paerl et al, 2001; Watkinson, 2000). Table 1 shows impacts and cyanobacterial responses to various physical, chemical and biological factors (Paerl et al., 2001). In laboratory experiments where light and nutrient levels were manipulated, higher light levels significantly affected the growth of cyanobacteria, the concentration of organic extract, and secondary metabolite production, whereas enhanced nitrogen and phosphorus did not have any effect. This suggests that light may be the major factor influencing growth of cyanobacteria and secondary metabolite production in *L. majuscula* on Guam (Pangilinan, 2000).

Table 1. Environmental factors influencing cyanobacterial growth and bloom formation.

Factor	Impacts and Cyanobacterial Responses
Physical	
Temperature	Temperatures > 15°C favor cyanobacterial growth, many species have optima at >20°C
Light	Many bloom genera prefer/tolerate high light, while others are shade-adapted
Turbulence and mixing	Many bloom genera prefer low turbulence over a range of spatial scales, poorly mixed conditions are favorable.
Water residence time	Long residence times are preferred by all genera.
Chemical	
Major nutrients (N and P)	Both N and P enrichment favor non-N_2-fixing genera. Low N:P ratios (i.e., high P enrichment favors N_2-fixers.
Micronutrients (Fe, metals)	Fe required for photosynthesis, NO^-_3 utilization, and N_2 fixation; evidence for periodic Fe limitation. Other metals (e.g., Cu, Mo, Mn, Zn, Co) required but not limiting.
Dissolved inorganic C (DIC)	DIC can limit phytoplankton growth, but cyanobacteria can circumvent this; DIC limitation and high pH may provide competitive advantages to cyanobacterial bloom taxa.
Dissolved organic C (DOC)	Many cyanobacterial bloom taxa are capable of utilizing DOC; blooms often flourish in DOC-enriched waters.
Salinity	Not restrictive to cyanobacteria *per se*, but some bloom-forming genera (*Anabaena, Microcystis*) do not thrive in saline waters. Other genera (*Nodularia*) are salt-tolerant.
Biological	
Grazing	Selective factor, favoring large inedible filamentous and colonial, as well as toxic (to zooplankton) genera
Microbial interactions	Consortial cyanobacterial-bacterial interactions may promote growth and bloom formation/persistence. Interactions may be chemically mediated (i.e., role for "toxins"?). Some cyanobacterial-protozoan interactions may also be mutually beneficial. Evidence for viral and bacterial antagonism (i.e., lysis) towards cyanobacteria. However, does not appear to be a common mechanism for bloom control. Cyanobacterial-microbial competition for nutrients exists and may be a competitive mechanism.
Symbioses with higher plants and animals	Cyanobacteria are epiphytic/epizoic and form endosymbioses with algae, ferns, and vascular plants. Many are obligate and involve N_2-fixing cyanobacterial genera.

Table reproduced from Harmful Freshwater Algal Blooms, With an Emphasis on Cyanobacteria. (2001). Hans W. Paerl, Rolland S. Fulton, III, Pia H. Moisander, and Julianne Dyble. The Scientific World. 1, 76-113.

Metals also play a role in cyanobacterial growth and bloom formation. Iron and molybdenum are especially important for metabolic activity. Iron, a major component of ferridoxin (one of the primary constituents of Photosystem I) provides the much needed energy for nitrogen fixation (Watkinson, 2000). Molybdenum, a co-factor of nitrogenase, the enzyme complex mediating nitrogen-fixation, could also play a regulatory role in diazotrophic cyanobacteria (Paerl et al., 2001).

Because past studies of cyanobacteria have shown conflicting results with respect to nutrient requirements and abiotic factors, it is still unclear which resource, if any, is most important for growth of cyanobacteria and production of secondary metabolites in *L. majuscula* and *L. bouillonii*. Furthermore, if nutrient levels and abiotic factors are limiting, it is not clear exactly how much light, which metals, and what concentrations of nutrients are optimal. Cyanobacteria respond to these environmental factors in much the same way as many terrestrial and aquatic plants (Pangilinan, 2000).

This research aimed to investigate the effects of nitrogen and phosphorus on growth and natural products chemistry of *L. majuscula* and *L. bouillonii*. Of particular interest was the uptake of nitrate and phosphate from the water column. This provided information about the ability of *L. majuscula* and *L. bouillonii* to load nutrients ("luxury consumption") while excess nutrients were available.

Additionally, I looked at carbon:nitrogen:phosphorus ratios in cyanobacteria treated with nitrate, phosphate and a combination of nitrate and phosphate. From this, I hoped to determine how much nitrogen and phosphorus had been taken up from the water column and stored for future metabolic requirements.

The final goal of this study was to see if the natural products chemistry of cyanobacteria was influenced by nutrient levels in the water column. Lipid and aqueous extracts of *L. majuscula* and *L. bouillonii* were tested on four tumor cell lines to see if they had increased cytotoxic effects based on treatments of nitrate, phosphate and a combination of nitrate and phosphate.

Materials and Methods

Collection of cyanobacteria

The cyanobacteria, *Lyngbya majuscula* and *Lyngbya bouillonii*, were collected using snorkel or SCUBA equipment at three sites around Guam (Figure 1). *L. majuscula* was collected at Cocos Lagoon and Piti Bomb Holes, at depths of 1-15 m and 1-10 m, respectively, and *L. bouillonii* was collected at depths of 1-10 m at Fingers Reef, in Apra Harbor.

Tufts or mats of cyanobacteria were pulled from the substrate by hand and gently shaken to remove excess debris. While underwater, the cyanobacteria were placed in plastic bags with seawater and sealed. The bags were transported to the marine laboratory in coolers. At the lab, cyanobacteria were immediately removed from the bags and placed into shaded, flowing-seawater tanks containing air stones.

Sample preparation

At least one day but not more than 3 days later, cyanobacteria were cleaned by hand of visible debris and small marine invertebrates. Samples were generated by teasing small pieces of cyanobacteria from larger clumps, spinning them 10 times in a salad spinner, and weighing them. Samples weighed from 1.0 – 2.2 g, depending on the study.

Laboratory microcosm and tank design

Each sample was attached by one end to a plastic grid (8.5 cm^2) using a plastic cable tie. The grid was wedged into the bottom of a labeled, clear, 1-liter plastic cup (microcosm). Each microcosm was filled with 750 ml of seawater from the intake pipe

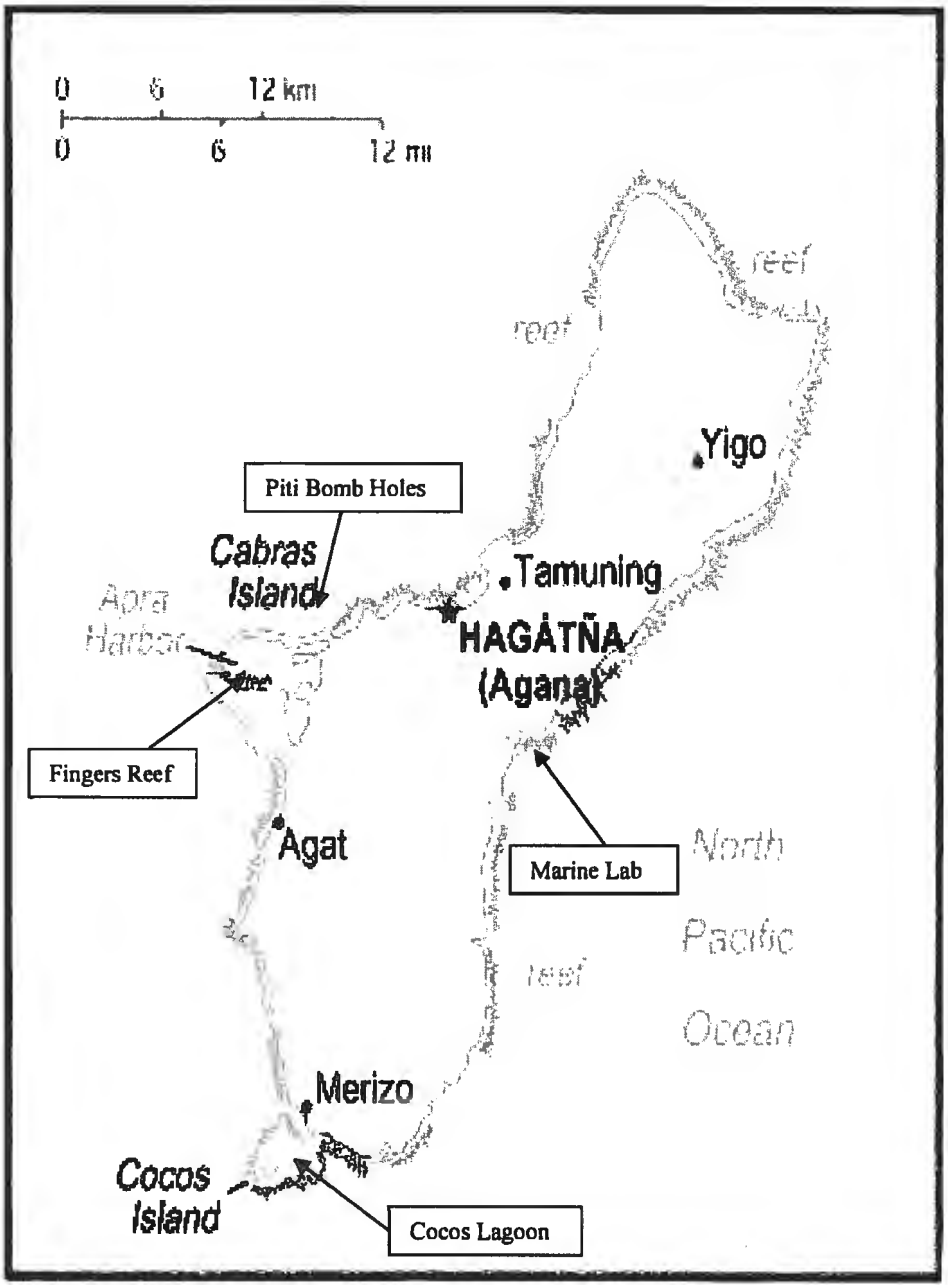

Figure 1. Map of collection and study sites around Guam.

on the lanai at the Marine Lab. Air stones provided gentle aeration to each microcosm. The microcosms were covered with translucent plastic lids to keep out rainwater and minimize evaporative losses.

Microcosms were randomly placed on top of a grate in a large concrete tank with flowing seawater. The water level in the tank was almost to the level of water in the microcosms. This ensured a fairly constant water temperature in the microcosms throughout the experimental period, ranging from approximately 26°C in the early morning to approximately 29°C in the late afternoon. A black mesh screen was placed over the entire tank to reduce light by approximately 50% (Figure 2). According to Pangilinan (2000), light levels at mid-day on a clear day ranged from 135 – 169 μmol photon m^{-2} s^{-1} but were reduced to 78 – 84 μmol photon m^{-2} s^{-1} using the mesh screen. A preliminary study conducted with *L. majuscula* from Cocos Lagoon indicated that ambient light at the laboratory was too intense and caused cyanobacteria to stress (indicated by bleaching) after several days, indicating the need for the mesh screen.

Nutrient enrichment for laboratory studies

Treatments of varying concentrations of nitrate (N), phosphate (P), and nitrate + phosphate (N+P) were used in the enrichment studies. Stock solutions of N and P were made from $NaNO_3$ and NaH_2PO_4, respectively. Compounds were dissolved in MiliQ DI-H_2O (18.2 milli-ohms).

Final test concentrations of treatments, after adding 1 mL stock solution to each microcosm, were approximately: 20 μM and 15 μM for the N treatment; 1 μM, 2 μM,

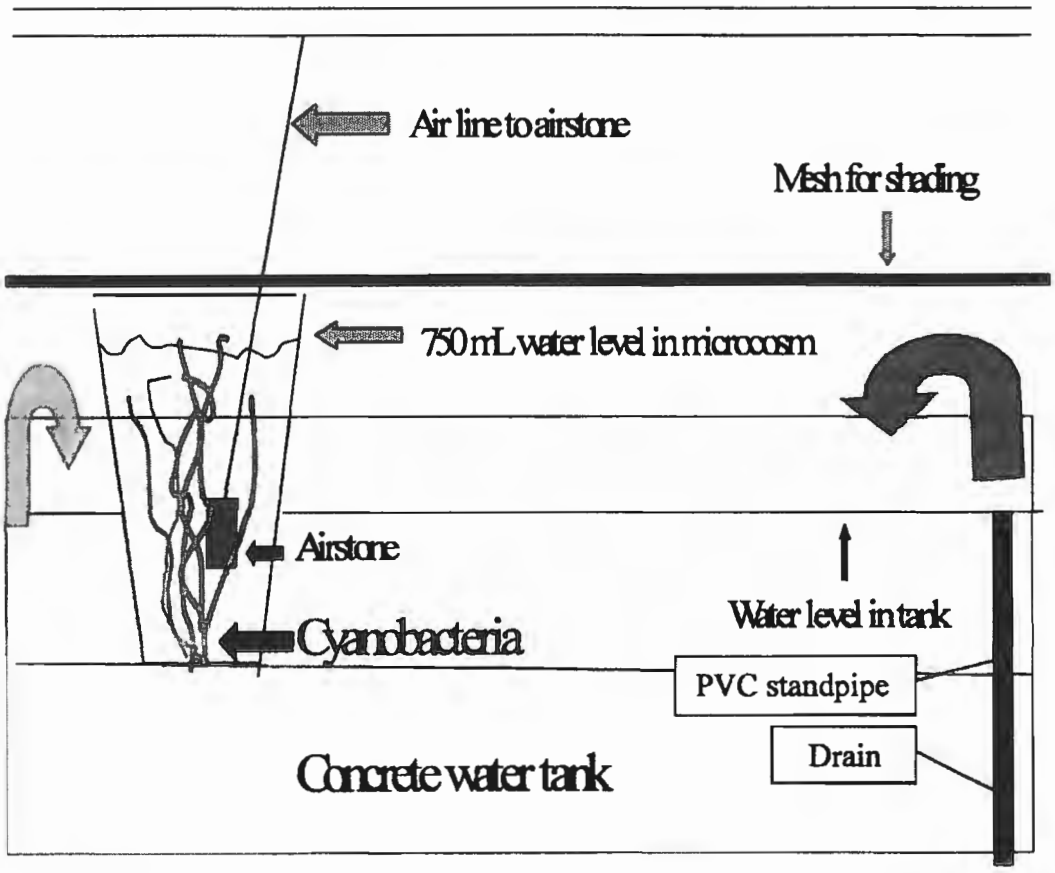

Figure 2. Microcosm and tank design

4 μM, and 6.7 μM for the P treatment; and 20 μM N + 1 μM P and 15 μM N + 6.7 μM P for the N+P treatment. The Water and Environmental Research Institute verified stock solution concentrations for accuracy. Controls received 1 ml DI-H_2O only. Treatment concentrations well above ambient levels were chosen to simulate nutrient-enriched environments (Table 2).

Table 2. Ambient levels of nitrate and phosphate at sites around Guam.

Site	Source Date of Sampling	Average Nitrate conc. (μM)	Average Phosphate conc. (μM)
Marine Lab Influent Seawater	Matson, unpublished data 1993	1.19 (n=29)	0.06 (n=29)
Cocos Lagoon *	July 30, 2002 - One week following the last day of 4 days of heavy rain	0.27 (n=16)	0.25 (n=16)
Cocos Lagoon	Thacker et al., 2001	0.67	0.09
Cocos Lagoon	Kuffner and Paul, 2001 1998-1999	0.24 (n=12)	0.06 (n=12)
Cocos Lagoon	Kuffner and Paul, 2001 16 May 2000	0.23 (n=1)	<0.03 (n=1)
Cocos Lagoon	Matson, 1991 Spring 1989	0.086 (n=17)	0.096 (n=17)
Piti	Belliveau and Paul, 2002	0.59 ± 0.10	BDL of 0.03
Piti *	July 24, 2002 - Sampled immediately following 4 days of heavy rain	0.07 (n=10)	0.68 (n=12)
1-2 km N of Apra Harbor	Matson, 1991 August 1990	0.29 (n=18)	0.35 (n=25)

* Data collected for this research
BDL = below detection limit.

water column within minutes. Controls were tufts of cyanobacteria secured to plastic grids in the absence of a fertilizer bag.

Fertilizer bags of treated samples were placed underneath the plastic grids with attached cyanobacteria and secured to the coral rubble in the same manner. After 7 days, samples were collected, brought back to the laboratory, and weighed. Growth estimates were hampered by fish grazing and were not attempted.

Daily-growth calculations for laboratory studies

At the end of each growth study, samples were removed from the microcosm and carefully detached from the plastic grid and cable tie so as not to lose any filaments. They were measured using the same spinning and weighing method as in sample preparation. Wet weights were recorded and daily growth was calculated by the equation:

$$\frac{\text{final wet wt. (g)} - \text{initial wet wt. (g)}}{\text{\# of days in study}}$$

Samples were then frozen individually to be used for chemical analyses at a later time.

Data analyses included 1-way ANOVA or non-parametric Kruskall-Wallis 1-way AOV for growth by treatment followed by Tukey's (HSD) comparison of means of growth by treatment using average change in weight (growth) over the study period and treatment as factors.

Water sampling for laboratory studies

Water samples were taken at different times during each of the studies depending on the purpose of the study. Water samples were always 15 ml and were removed from microcosms with a 3-ml syringe. They were stored in 15-ml acid-washed (HCl) polypropylene tubes. During sampling, the syringe was rinsed 3 times in DI-H_2O

between samples to avoid cross-contamination. The tubes were capped and placed in the refrigerator until all samples were ready for analysis, usually within 3 days.

Analysis of water for nitrate and phosphate

Water analysis was performed using the Lachat QuickChem+ 8000 Series Flow Injection Analyzer (FIA) from Lachat Zellweger Analytics with RP-100 Series reagent pump. The method used for nitrate analysis was the Standard Cadmium Reduction method which determined a combination of nitrate and nitrite in the sample. Standards were prepared from a bulk 1000 mg/L NO_3N standard from Environmental Resource Associates (ERA). The method used for phosphate analysis was the QuickChem Method 31-115-01-3-A, a standard Molybdate-Ascorbate method for measuring reactive phosphorus or ortho-phosphate. Standards were prepared from a bulk 1000 mg/L PO_4P standard from ERA. The FIA automatically diluted 7 standards for each nutrient. The method used was previously tested for this type of analysis using the following parameters: method cycle period, 60 sec.; time for sample to reach first valve, 28 sec.; load time, 20 sec.; load period, 14 sec. These parameters ensured proper timing of sample-loading onto the column and opening and closing of the valves. Blanks consisted of DI-H_2O and quality control (QC) checks consisted of seawater.

Uptake of nitrate and phosphate

Uptake of nitrate and phosphate was determined for *L. majuscula* (Piti Bomb Holes) by collecting water samples from the microcosm and measuring the amounts of nutrients (N + P) every 10 minutes for 90 minutes following a one-time treatment. Treatments were control (DI-H_2O), 20 μM nitrate (N), 1 μM phosphate (P), or 20 μM nitrate + 1 μM phosphate (N+P). Because the microcosms were closed environments, it

was assumed that any decrease in nutrient was due to uptake by cyanobacteria. Control microcosms received the same treatments but did not have any cyanobacteria present.

Carbon, hydrogen, nitrogen (CHN) analyses

Four samples of each treatment (N, P, N + P and control) were selected from each of the three studies conducted during January, February and March 2002 (Table 3) for carbon, hydrogen and nitrogen (CHN) analyses. Frozen samples of cyanobacteria were placed in aluminum pans and dried at 60° C for 48 hours. They were ground to 60-mesh using a Wiley Mill and stored in glass scintillation vials. Dried and ground cyanobacteria were weighed into 8x5 mm-tin cups. The cups were crimped together and placed in a 96-well plate for analysis in the Perkin-Elmer Series II CHNS/O Analyzer 2400. Sample weights ranged from 2.00-4.00 mg. Standards of 1.00-4.00 mg of acetanilide were used as quality controls.

Total phosphorus analysis

Four samples of each treatment (N, P, N + P and control) were selected from each of the three studies conducted during Jan, Feb and Mar 2002 (Table 3) and analyzed for total phosphorus. Samples of dried, ground cyanobacteria weighing 0.1 g were subjected to the dry-ashing/acid-digest method of Aspila et al. (1976). They were charred for 1 hour at 250° C and ashed for 24 hours at 475° C. Next, the samples were digested in 1M HCl for 8 hours and filtered through 0.45 μM Millipore filters. Total phosphorus was determined by the ascorbic acid colorimetric assay method (Parsons et al., 1984). Peach leaves (obtained from NIST) were used as a reference standard.

Table 3. Daily growth of cyanobacteria for studies conducted May 2001 – March 2002. Daily growth was calculated as (final wt. – initial wt.) / # of days in study. Values are means of n (SD). Symbols indicate results of comparison tests.

Study Dates	Site (n) Species	Treatment	Cyanobacteria growth (g wet wt./ day)
5/24/01-6/8/01	Cocos Lagoon (n = 10)	C	.052 (.011)
	Lyngbya majuscula	N (15 μM)	.042 (.012)
		P (6.7 μM)	.097 (.015)
	Piti Bomb Holes (n = 10)	C	.058 (.023)
	Lyngbya majuscula	N (15 μM)	.059 (.015)
		P (6.7 μM)	.100 (.053)
6/24/01-7/8/01	Cocos Lagoon (n = 10)	C	.105 (.017)
	Lyngbya majuscula	N (20 μM)	.089 (.024)
		P (1 μM)	.090 (.029)
	Piti Bomb Holes (n =10)	C	.053 (.020)
	Lyngbya majuscula	N (20 μM)	.032 (.023)
		P (1 μM)	.060 (.031)
9/3/01-9/7/01	Fingers Reef (n = 3)	C	.212 (.041)
	Lyngbya bouillonii	P (1 μM)	.143 (.167)
		P (2 μM)	.135 (.131)
		P (4 μM)	.270 (.080)
1/21/02-1/29/02	Piti Bomb Holes (n = 10)	C	.094 (.024)
	Lyngbya majuscula	N (20 μM)	.125 (.018)
		P (1 μM)	.092 (.020)
		N+P (20 μM+1 μM)	.097 (.008)
2/18/02-2/26/02	Cocos Lagoon (n = 10)	C	.078 (.018)
	Lyngbya majuscula	N (20 μM)	.068 (.072)
		P (1 μM)	.061 (.024)
		N+P (20 μM+1 μM)	.057 (.030)
3/4/02-3/12/02	Fingers Reef (n = 10)	C	.077 (.023)
	Lyngbya *bouillonii*	N (20 μM)	.104 (.032)
		P (1 μM)	.062 (.024)
		N+P (20 μM+1 μM)	.093 (.018)

Data analyses on C, N and P content in cyanobacteria included 1-way ANOVA (*L. majuscula*) and Kruskall-Wallis 1-way ANOVA (*L. bouillonii*) using factors of % dry mass and treatment.

Preparation of lipid and aqueous extracts

Freeze-dried samples of cyanobacteria were first extracted with a solvent of 1:1 ethyl acetate:methanol. Samples were placed in flasks, layered with solvent, covered with aluminum foil, and left to extract for at least 24 hours. The liquid extract was filtered through #1 Whatman filter paper into a scintillation vial and placed in the Savant speed-vac to remove solvent. The process was repeated using the same vial, pooling lipid extract material. The samples were allowed to dry and then extracted twice with a solvent of 3:7 ethanol:DI-H_2O, pooling the aqueous extracts into separate vials. After all samples were extracted and dried, a portion of each extract (approximately 10 mg) was placed into a small vial and shipped to F. Valeriote's laboratory to be assayed for *in vitro* solid tumor selectivity.

***In vitro* solid tumor selective assays**

The method of Valeriote et al. (2002) was used to conduct *in vitro* solid tumor selective assays. Both lipid and aqueous extracts of 56 cyanobacteria samples and lipid extracts only of 4 samples were tested for differential cell killing among five cancer cell types: three solid tumor cells (murine Colon 38, human colon H-116, and human lung H-125M) and two leukemia cell types (murine L1210 and human CCRF-CEM), as well as one human normal cell type (hematopoietic progenitor cell, CFU-GM). Murine L1210 and human CCRF-CEM were reference tumors.

Lyophilized lipid and aqueous extracts weighing approximately 10 mg each were solubilized in 0.25 ml DMSO. For aqueous extracts, 0.25 ml distilled water was added, and the next day, 0.25 ml DMSO was added, for a water:DMSO ratio of 70:30.

Zone assay methodology

A volume of 15 μL of each sample was dropped onto a 6.5 mm disk (Baxter filter disks). The disks were allowed to dry overnight and then placed close to the edge of the petri dish. The plates were incubated for 7-10 days (depending on cell type) and examined by stereomicroscope (10X) for measurement of the zone of inhibition, measured from the edge of the filter disk to the beginning of normal-sized colony formation (Figure 3).

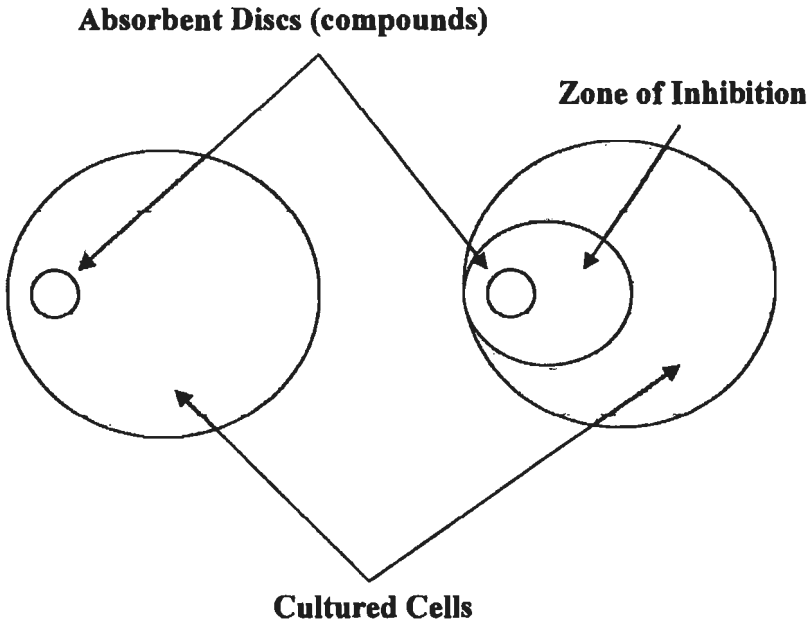

Figure 3. Screening bioassay.

Results

Growth studies

Growth rates of *L. majuscula* and *L. bouillonii* were highly variable with respect to treatment, concentration of treatment, site, and time of year. *L. majuscula* from Piti Bomb Holes and Cocos Lagoon both showed significant increases in daily growth with treatments of 6.7 μM phosphate (Kruskal-Wallis $p=0.003$ and ANOVA $p<0.01$, respectively) compared to treatment with 15 μM nitrate or controls (Table 3, Figure 4). However, neither species of cyanobacteria showed a significant increase in growth when treated with 1 μM phosphate versus 20 μM nitrate or controls (Table 3, Figure 4).

Growth of *L. majuscula* with treatment of nitrate and controls did not differ significantly for cyanobacteria from either site in studies conducted during the summer months (May – July 2001) (Table 3, Figure 4), but *L. majuscula* from Piti Bomb Holes showed a significant increase in daily growth with 20 μM nitrate treatment ($p=0.002$) compared to controls and N+P-treated cyanobacteria during the study conducted in the winter (January 2002) (Table 3, Figure 5). *L. bouillonii*, from Fingers Reef in Apra Harbor, did not show any significant increases in daily growth when treated with 1, 2, or 4 μM phosphate or control (Figure 6). However, daily growth of *L. bouillonii* treated with 1 μM phosphate was significantly lower ($p=0.003$) than *L. bouillonii* treated with 20 μM nitrate, 20 μM nitrate + 1 μM phosphate, or control (Table 3, Figure 5). Growth of cyanobacteria from the field study at Cocos Lagoon could not be calculated because of observations of fish grazing on the samples at the time of collection.

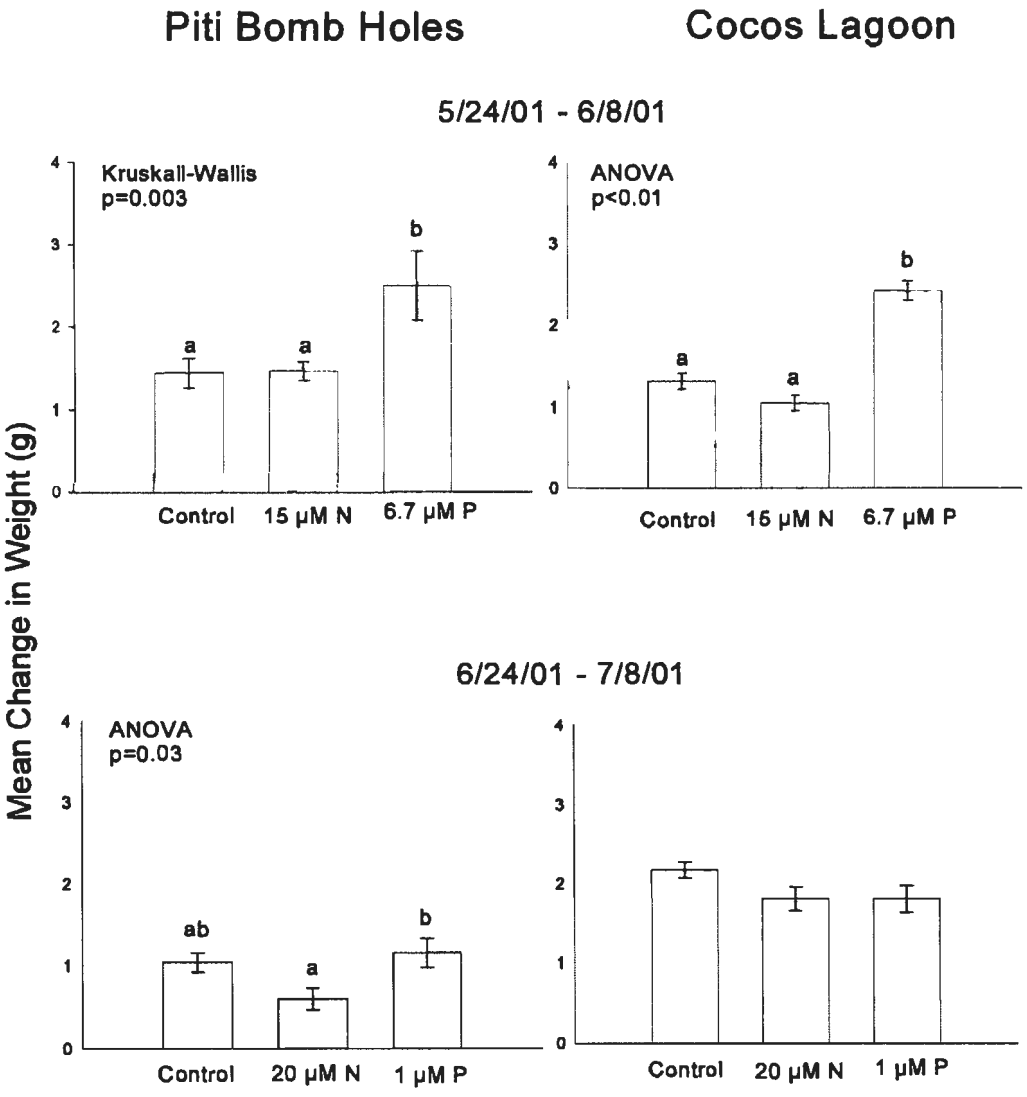

Figure 4. Effects of nutrients on growth of *Lyngbya majuscula* from Piti Bomb Holes and Cocos Lagoon. Error bars represent standard error, n = 10.

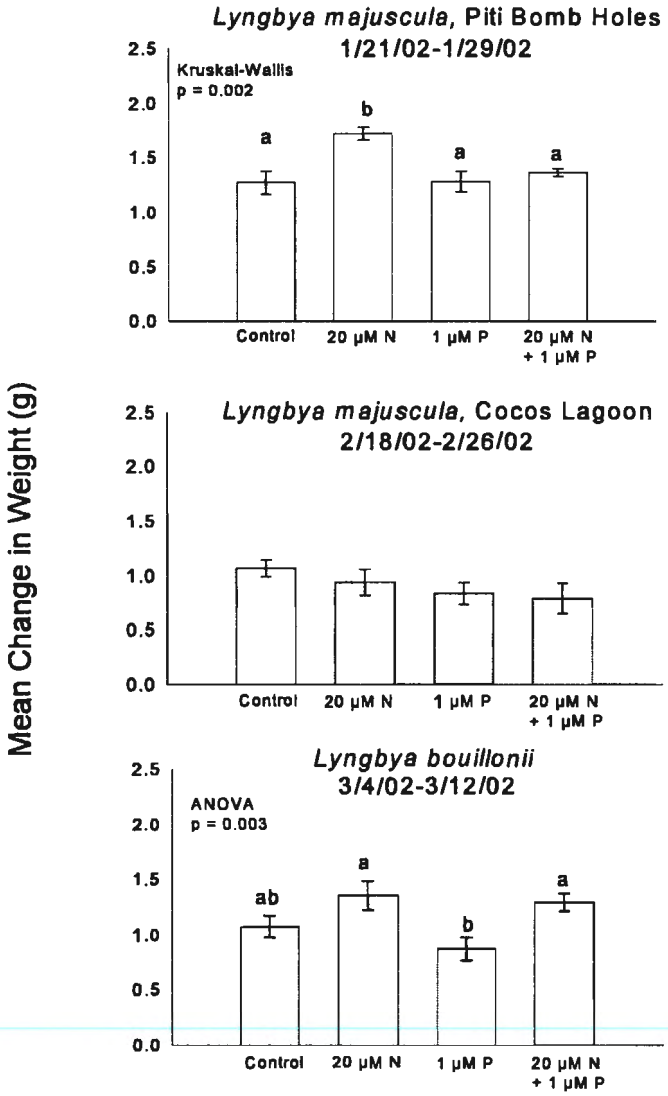

Figure 5. Effects of nutrients on growth of *Lyngbya majuscula* and *Lyngbya bouillonii*. Error bars represent standard error, n = 10.

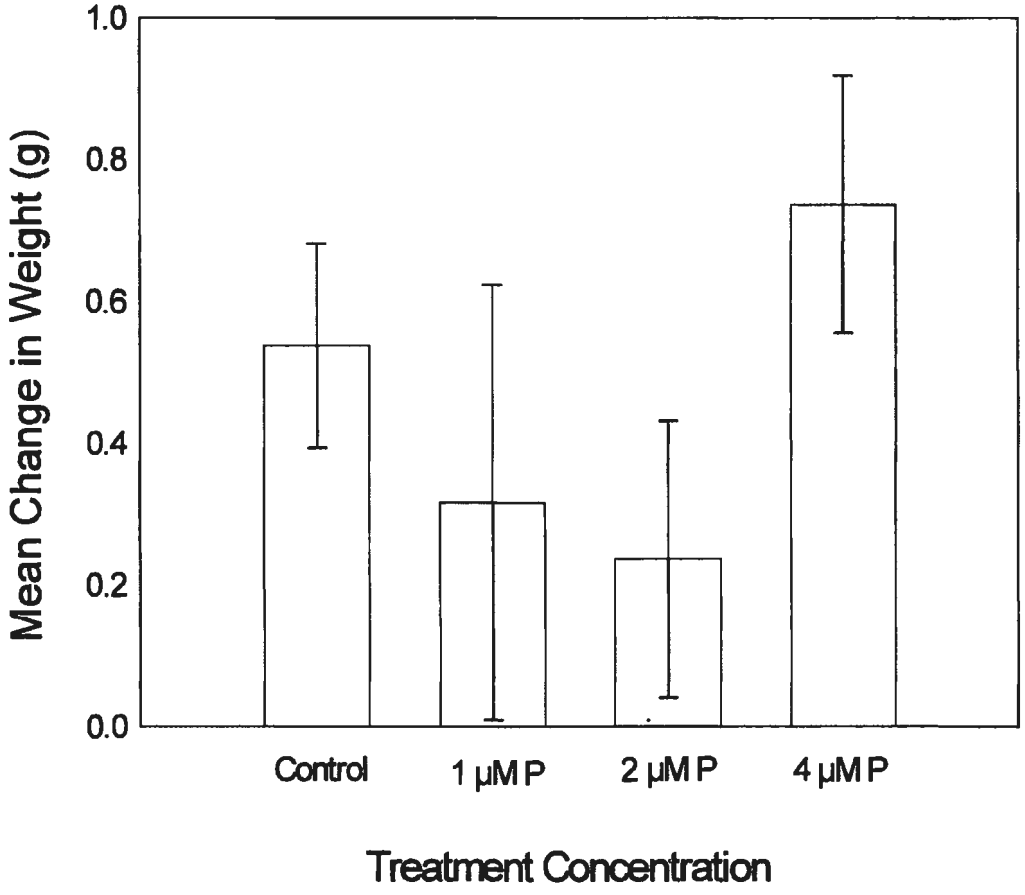

Figure 6. Effects of phosphate on growth of *Lyngbya bouillonii* (September 2001). Error bars represent standard error, n = 3.

Nutrient uptake

Uptake of nitrate and phosphate were measured for *L. majuscula* from Piti Bomb Holes in October 2002. Almost all available nitrate and phosphate were taken up from the water columns within the 90-minute study period. At t=0, the average concentration of nitrate in the N-treated microcosms was 30.15 μM. The concentration dropped to 4.67 μM after 90 minutes (Figure 7a). Average concentrations of nitrate in the N+P-treated microcosms went from 32.49 μM to 4.29 μM during the 90-minute study period (Figure 7b). At t=0, the average concentration of phosphate in the P-treated microcosms was 1.39 μM. The concentration dropped to 0.12 μM after 90 minutes (Figure 8a). Average concentrations of phosphate in the N+P-treated microcosms went from 1.16 μM to 0.75 μM during the 90-minute study period (Figure 8b).

Concentration of nitrate in control and P-treated microcosms, and concentration of phosphate in control and N-treated microcosms (with and without cyanobacteria) remained relatively stable and low. For example, in microcosms containing cyanobacteria, nitrate concentrations in control and P-treated microcosms showed slight decreases, from 3.62 μM to 1.55 μM and from 2.73 μM to 1.31 μM, respectively, over the 90- minute study period. Phosphate concentrations in control and N-treated microcosms remained relatively stable ranging from below detection limits to 0.42 μM and 0.17 μM to 0.18 μM, respectively. Slight fluctuations throughout the study period may be attributed to uptake and then partial re-release of nutrients back into the water column. Similar trends were observed in microcosms not containing cyanobacteria.

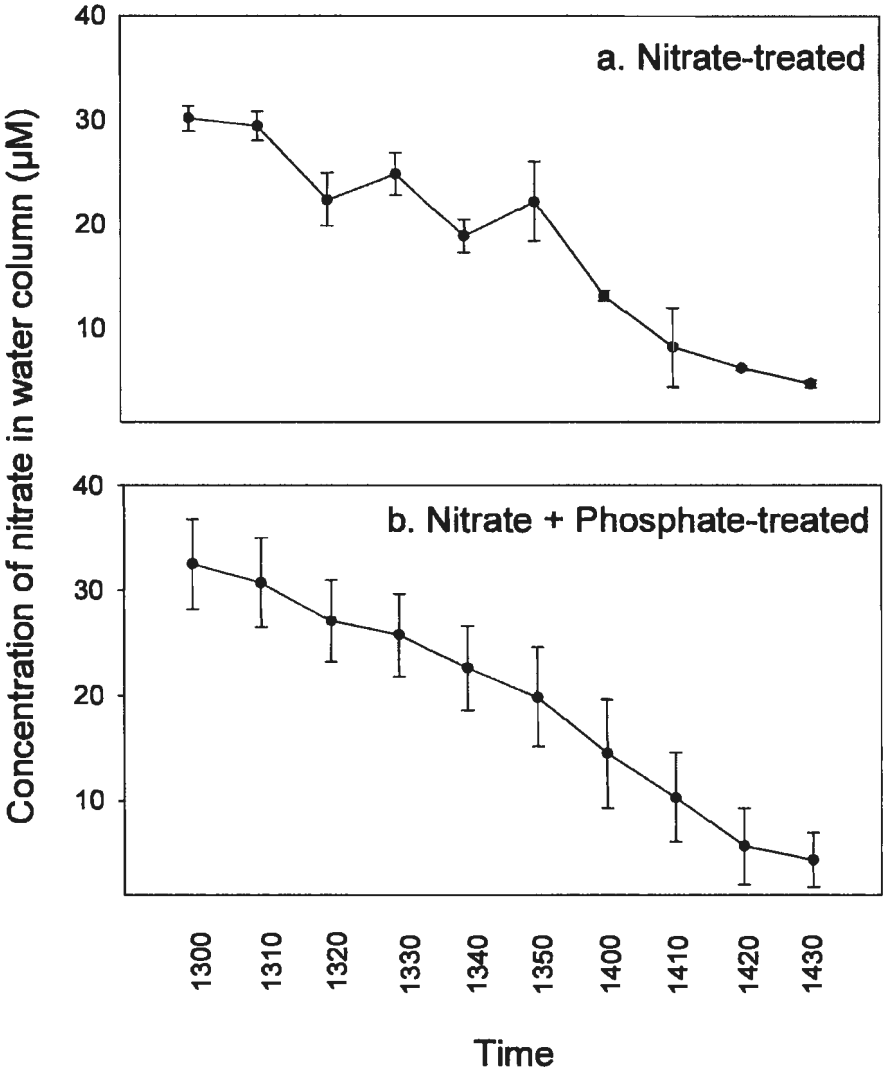

Figure 7. Nitrate uptake by *Lyngbya majuscula* from Piti Bomb Holes (October 2002). Error bars represent standard error, n = 3.

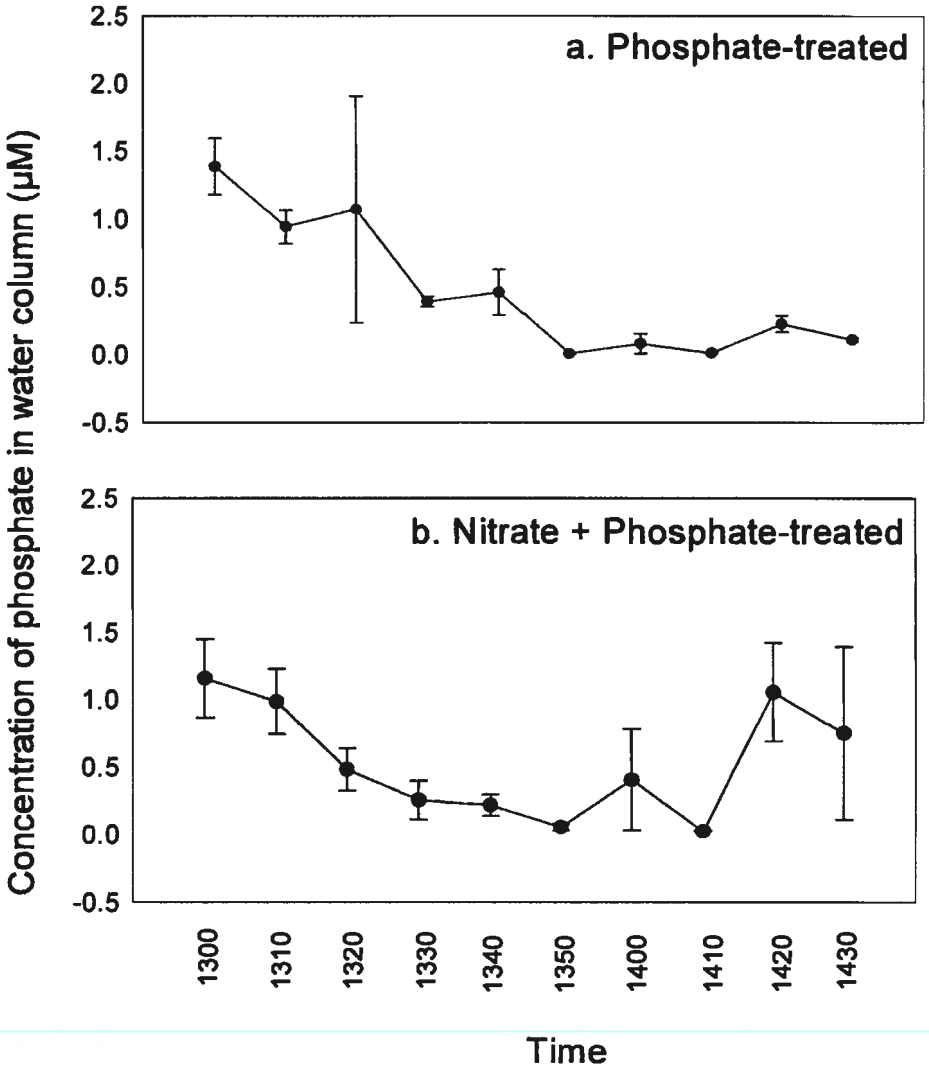

Figure 8. Phosphate uptake by *Lyngbya majuscula* from Piti Bomb Holes (October 2002). Error bars represent standard error, n = 3.

Carbon, nitrogen, and phosphorus

Carbon:nitrogen:phosphorus (C:N:P) ratios differed depending on available nutrients (treatment regimes), species and site of cyanobacteria. Table 4 shows mean percentage C:N:P ratios for four different treatment regimes (n=4) for cyanobacteria from three different sites around Guam.

For all three sites, C:P ratios were much lower in P-treated and N+P-treated cyanobacteria than in controls or N-treated. In all cases, C:P ratios were highest for cyanobacteria treated with nitrate (Table 4).

C:N ratios for *L. majuscula* from both Piti Bomb Holes and Cocos Lagoon ranged from 10:1 to 11:1 for all treatments in laboratory studies and from 9:1 to 10:1 for the field study. However, C:N ratios for *L. bouillonii* were 16:1 and 11:1 for control and P-treated, respectively, and 8:1 for both N-treated and N+P-treated. N:P ratios were much higher in controls and N-treated than in P-treated or N+P-treated samples for cyanobacteria from all three sites.

Phosphorus content was significantly higher in both P- and N+P- treated cyanobacteria compared with control and N-treated cyanobacteria (Piti Bomb Holes and Cocos Lagoon, ANOVA $p < 0.0001$; *L. bouillonii*, Kruskal-Wallis $p = 0.007$). In *L. bouillonii*, nitrogen content was significantly higher in N+P-treated cyanobacteria compared to control and P-treated cyanobacteria (Kruskal-Wallis $p = 0.004$) and significantly higher in N-treated when compared to control (Figure 9).

Table 4. Carbon, nitrogen and phosphorus content of cyanobacteria. Treatments were: DI-H_2O (C), nitrate (N), phosphate (P), combination of nitrate and phosphate (N+P). Values are means, n = 4 (unless noted).

Species Site Date	Treatment	(By Weight) % C	% N	% P	C:N:P ratio
L. majuscula	C	31.89	2.99	.082	389:36:1
Piti Bomb Holes	N	31.65	2.95	.074	428:40:1
1/21/02 – 1/29/02	P	31.55	2.91	.323	98:9:1
	N + P	31.71	3.18	.314	101:10:1
L. majuscula	C	28.48	2.74	.092	310:30:1
Cocos Lagoon	N	31.09	2.88	.084	370:34:1
2/18/02 – 2/26/02	P	30.28	2.94	.402	76:7:1
	N + P	31.23	3.22	.347	91:9:1
L. bouillonii	C	30.03	1.95	.136	222:14:1
Fingers Reef	N	30.12	3.74	.131	229:29:1
3/4/02 – 3/12/02	P	28.90	2.58	.259	113:10:1
	N + P	29.65	3.85	.325	92:12:1
L. majuscula	C (n=2)	28.32	3.21	.114	249:28:1
Cocos Lagoon	P (n=2)	27.39	2.80	.596	47:5:1
Field Study	N + P (n=3)	25.18	2.61	.176	144:15:1

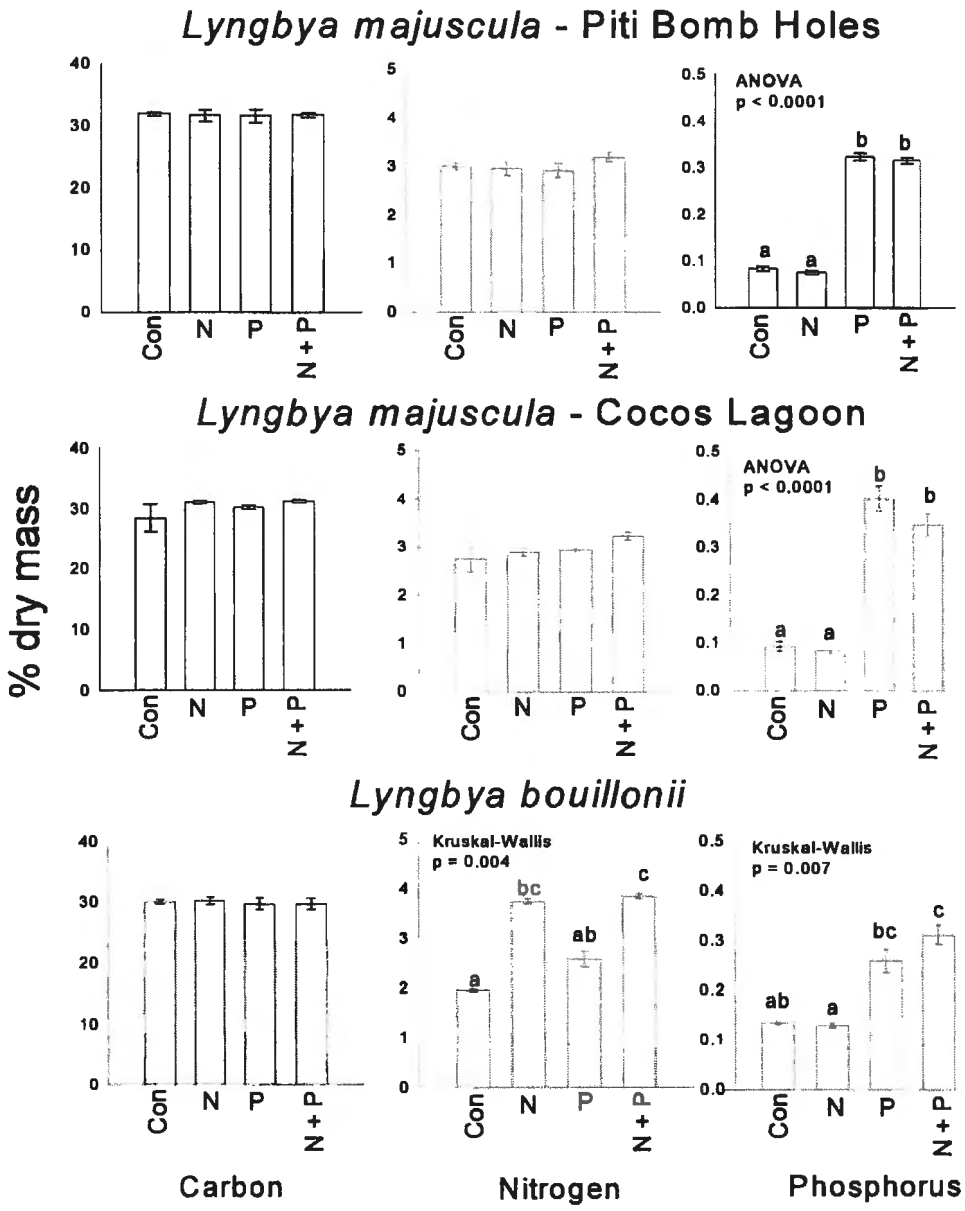

Figure 9. Carbon, nitrogen and phosphorus content in cyanobacteria under different treatment regimes. Control (Con) = DI H_2O, Nitrate (N) = 20 µM, Phosphate (P) = 1 µM, Nitrate + Phosphate (N+P) = 20 µM N + 1 µM P. Error bars represent standard error, n = 4.

***In vitro* solid tumor selective assays**

Both anti-proliferative response and differential activity were the end-points of the *in vitro* solid tumor selective assays. There were four possible positive outcomes: 1.) murine solid tumor selectivity relative to normal cells, 2.) human solid tumor selectivity relative to normal cells, 3.) murine solid tumor selectivity relative to murine leukemia or 4.) human solid tumor selectivity relative to human leukemia.

In the zone assay methodology, the diameter of the filter disk, 6.5 mm, was arbitrarily designated as 200 units. A zone of less than 300 units indicated that the extract was of insufficient activity to be of further interest. A difference in zones between solid tumor cells and either normal or leukemia cells of 250 units defined solid tumor selective compounds.

In this study, a total of 116 extracts (60 lipid and 56 aqueous) were tested for solid tumor selectivity. Of those 116, 20 extracts met the criteria for murine solid tumor selectivity to its respective leukemia cell line (L1210 to Colon 38). Of the twenty that met the criteria, 14 were lipid extracts and 6 were aqueous extracts. Of the twenty that showed selectivity, eighteen were from the cyanobacteria *L. bouillonii*, consisting of 12 of 16 lipid extracts tested and 6 of 16 aqueous extracts. Furthermore, two of those extracts, also met the criteria for murine solid tumor selectivity relative to normal cells (Colon 38 to CFU-GM). The remaining 96 extracts tested did not meet any of the four criteria to be considered a tumor selective compound.

Both lipid and aqueous extracts of *L. bouillonii* had the highest cytotoxic effects on each of the four tumor cells lines (Figures 10 and 11). When treated with nitrate,

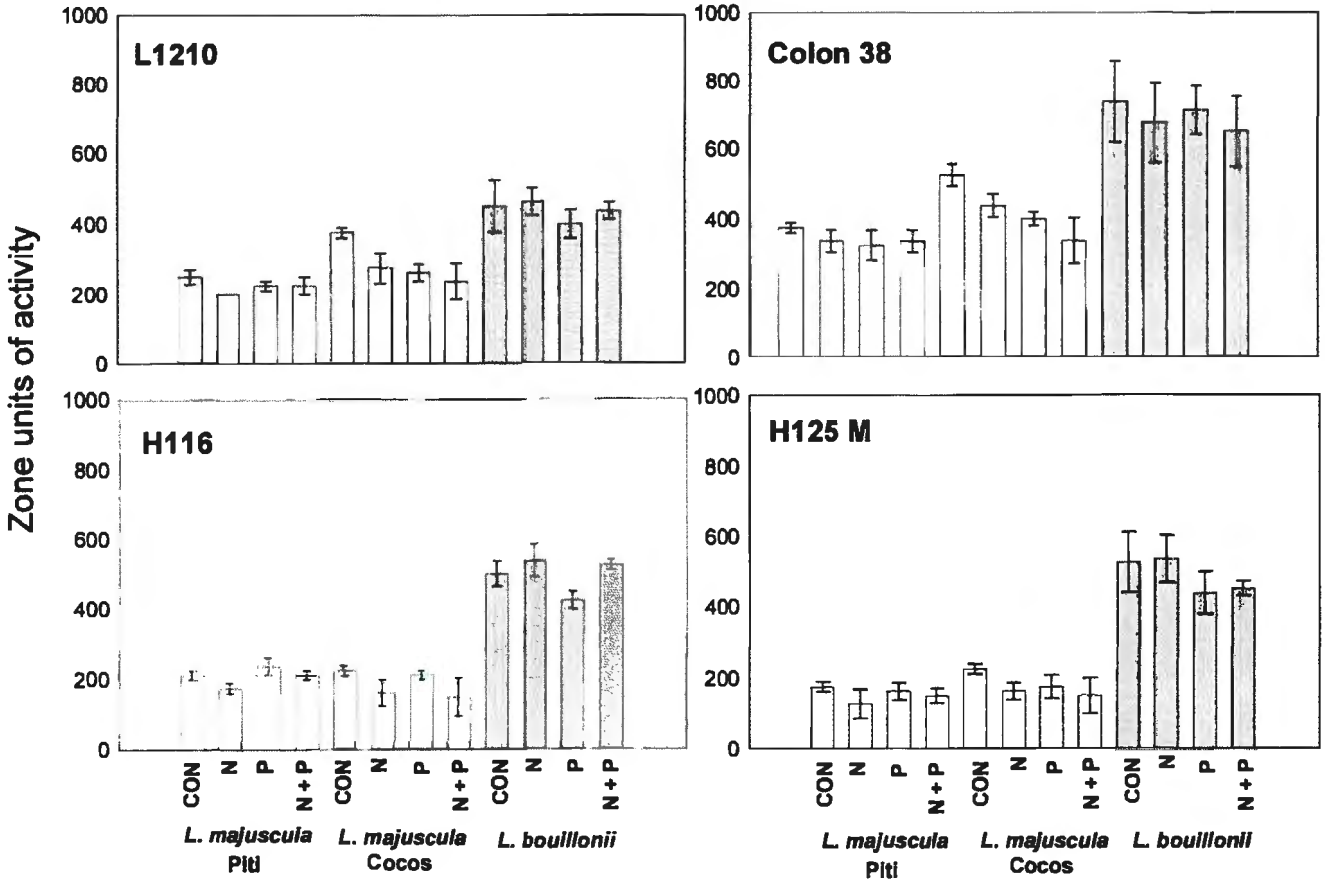

Figure 10. Cytotoxic effects of lipid extracts on four tumor cell lines. Error bars represent standard error, n = 4.

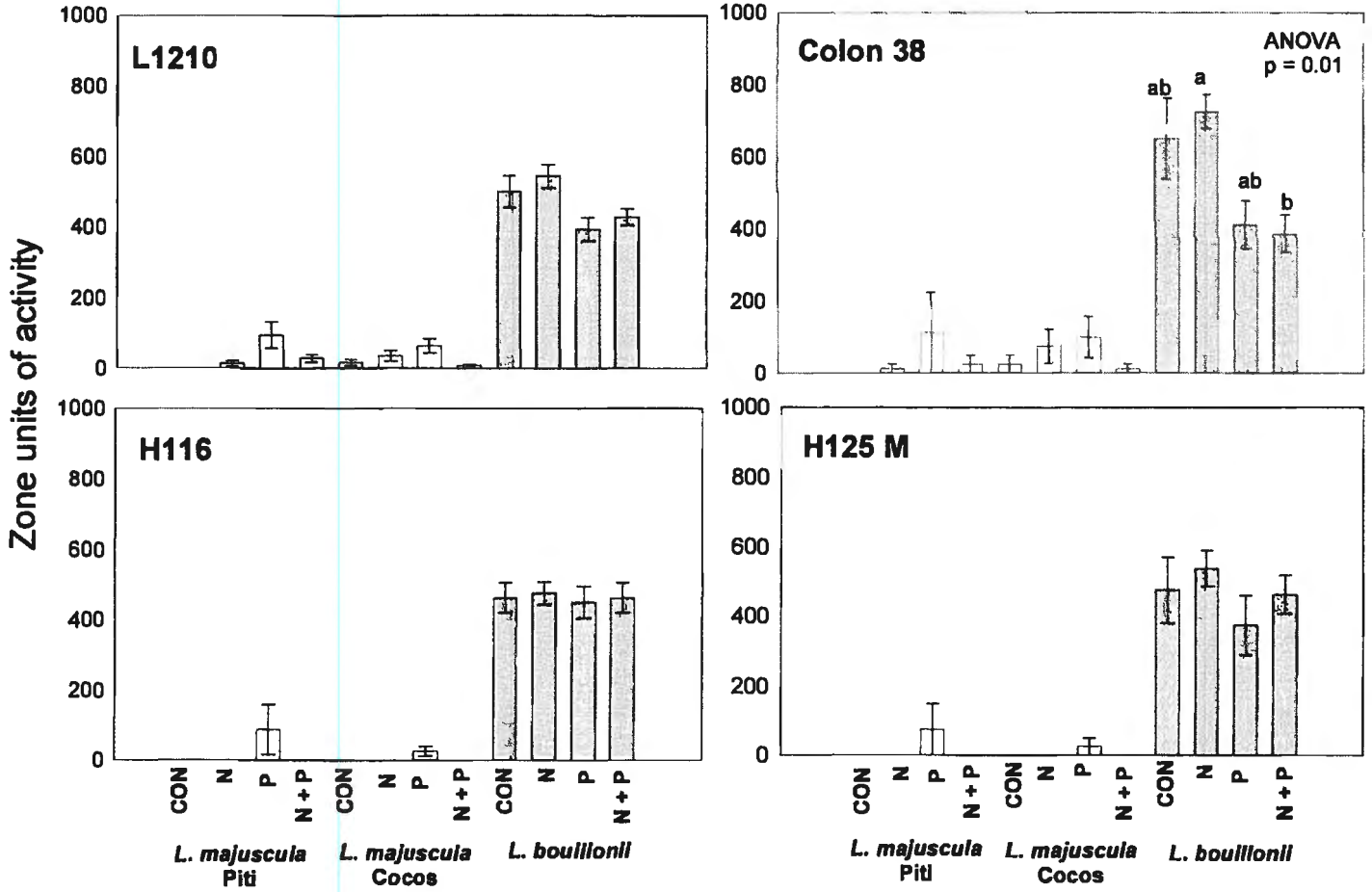

Figure 11. Cytotoxic effects of aqueous extracts on four tumor cell lines. Error bars represent standard error, n = 4.

L. bouillonii had significantly higher cytotoxic effects against Colon 38 cells compared to N+P-treated (ANOVA $p = 0.01$) but not compared to control or P-treated (Figure 11).

Discussion

Growth

On cellular and ecosystem scales, both nitrogen and phosphorus are usually in short supply relative to plant growth (Paerl et al., 2001). In the marine environment, the availability of nitrogen is thought to be the key factor limiting growth and productivity (Paerl, 2000). However, phosphorus has been implicated as a limiting factor for growth as well. Many things, in fact, control growth of cyanobacteria and bloom formation, but nutrient levels and ratios of nitrogen to phosphorus in the water column are probably good indicators of whether conditions for bloom formation are ideal. According to Paerl (2000), water column N:P ratios (by weight) below 20 are conducive to the development and persistence of N_2-fixing genera. Consequently, *Lyngbya majuscula* and *Lyngbya bouillonii*, two nonheterocystous diazotrophic cyanobacteria, have a competitive advantage over other genera in N-deficient waters (Paerl, 2000).

In studies conducted on cyanobacteria around Guam, significant increases in growth took place only when *L. majuscula* from Cocos Lagoon and Piti Bomb Holes were treated with very high concentrations of phosphorus. Studies conducted by Kuffner (2001) also demonstrated that growth of *L. majuscula* was enhanced with phosphate enrichment. However, this increase in wet weight of cyanobacteria may not only be due to "growth" of cyanobacteria, but to an increase in bacterial and epiphytic organisms living in association with cyanobacteria. Because there was no method for removing bacteria and epiphytes from the cyanobacteria, it is not certain how much of the increase in weight was due to cyanobacterial growth alone.

Other factors that influence growth of cyanobacteria may have contributed to the results of these studies as well. The length of study may have been an important factor because cyanobacteria are generally difficult to culture in the laboratory. Some studies were ended earlier than originally planned due to cyanobacteria bleaching or beginning to show signs of stress in the microcosm environment. The study that produced significant increases in growth lasted 15 days.

To solve the problem of culturing cyanobacteria in the laboratory, I attempted to measure growth in the field environment. However, the samples were not caged due to the assumption that *L. majuscula* is chemically defended and generally not a preferred food choice of most fish. Grazing by herbivores was observed at the time of retrieval, so those data were not used to calculate growth.

Light levels were kept constant for each study by covering the tank with mesh screens. However, *L. majuscula* from Piti Bomb Holes and Cocos Lagoon and *L. bouillonii* from Fingers Reef all thrive at different depths, so optimum light levels are different for each species and site. This variation may have influenced growth as well. Studies by Pangilinan (2000) showed that light significantly affected growth of *L. majuscula* from Piti Bomb Holes.

Iron, molybdenum and other trace elements were not addressed in these studies. However, both iron and molybdenum are important for nitrogen fixation; iron is an important enzyme cofactor for numerous biochemical pathways including electron transport, energy transfer, N- assimilation and N_2-fixation. Molybdenum is a cofactor of the N_2-fixing enzyme complex nitrogenase. While trace metal limitation may not be the

most prolific modulator of bloom formation, trace metals do play important synergistic roles with major nutrients (Paerl et al., 2001).

Herbivory is a crucial top-down control of algal and cyanobacterial growth in the marine environment. In the field study conducted at Cocos Lagoon, I chose not to cage the samples after securing them to the reef because of the assumption that this particular species of cyanobacteria was chemically defended against generalist herbivores. However, when food is in short supply, herbivores will sometimes eat less-preferred foods. On the contrary, one specialized marine organism, the sea hare *Stylocheilus longicauda*, actually selects *L. majuscula* in its diet (Paul *et al.*, 2001). Attempts to minimize their effect on growth and natural products chemistry were made by removing any visible sea hares from the samples before the start of the study. In some cases, sea hares that were too small to be detected at the start were observed in the microcosms after several days of feeding. Those samples were excluded from data sets.

Because most of the studies for this research were conducted in the laboratory, some physical factors that may affect growth of cyanobacteria and bloom formation in the field were not considered. *L. majuscula* and *L. bouillonii* are found loosely attached to the substrate or growing at the base of soft corals. Growth and proliferation may be greatly affected by wave action and turbulence. If wave action is fast enough to detach cyanobacteria from the substrate, bloom formation may be altered. This should be taken into consideration during future studies conducted in the field environment.

Uptake of nutrients

Cyanobacteria acquire nutrients from the water column. The rate at which they take them up, however, is dependent on how much nutrient is available and whether the

conditions are ideal for uptake. The cyanobacteria *L. majuscula* and *L. bouillonii* are nonheterocystous, and therefore, must fix nitrogen either spatially or temporally. Light-dark cycles probably play an important role in nitrogen fixation. Circadian rhythms have been shown to control many aspects of cyanobacterial metabolism (Paerl, 2000). These studies did not attempt to separate nutrient uptake temporally. Time course studies were conducted during the daylight hours, so it is not clear if the uptake of nutrients would be different during the night. Uptake of nitrate from the water column in these studies was relatively steady and almost complete at the end of 90 minutes.

Phosphorus uptake has not been positively linked to circadian rhythms, although it is possible that there may be an indirect effect by virtue of nitrogen and phosphorus uptake being linked. In the uptake studies for phosphorus, there was a slight increase in water column phosphate in the N+P-treated samples after one hour. A rather large increase occurred after one hour and twenty minutes. This increase in phosphate in the water column at these times may be the result of release of phosphate back into the water column by the cyanobacteria. Phosphorus is a highly dynamic, biologically active element (Correll, 1998). It is taken up, converted to various forms and released again in relatively short time spans. The lack of release of phosphate back into the water column for the P-treated sample may be an indication that N and P uptake are coupled.

Natural products chemistry

Cyanobacteria, including strains of *L. majuscula*, produce a wide variety of natural products (Paul et al, 2001). These compounds were termed secondary metabolites or natural products because they did not resemble the more classical primary metabolites. Because their functions in the organism were not immediately apparent or found in

cellular processes, they were initially thought to be by-products of detoxification (Fenical, 1982). Many of these unique, and often very complex compounds, have now been isolated and characterized, leading to advances in the pharmaceutical and agricultural industries (Fenical, 1982; Liles, 1996). According to David Newman, a National Cancer Institute chemist, approximately 45% of anticancer drugs approved for commercial use are either natural products or synthetic analogs of natural products (Liles, 1996).

Important functions of secondary metabolites in ecological roles are also more widely understood (Fenical, 1982; Paul et al, 2001). Secondary metabolites have been shown to have feeding deterrent effects against some marine herbivores (Paul et al, 2001). The sea hares *Steilocheilus longicauda* and *Dolabella auricularia* sequester compounds from cyanobacteria to afford protection from fish and other predators. *S. longicauda* specializes on *L. majuscula* (Paul et al, 2001).

The ability of cyanobacteria to produce secondary metabolites is related to the uptake of nutrients from the water column. Because many of the compounds isolated from cyanobacteria are nitrogen-containing secondary metabolites, this research focused on uptake of nitrate and phosphate, two of the most important nutrients in the marine environment. The ability of cyanobacteria to fix nitrogen may explain the presence of nitrogen in these compounds (Paul et al., 2001), many of which are toxic to marine organisms or humans (Paerl et al, 2001) or are pharmacologically bioactive (Luesch et al, 2001).

The range of bioactivity of these natural products is broad. Some of the secondary metabolites isolated from *L. majuscula* include Lyngbyatoxin A, a cyclic

depsipeptide that is a potent phorbol-ester-type tumor promoter; malyngolide, a lipid metabolite with antibiotic activity; curacin A, an antimitotic agent; malyngamides H, I, J, K, and L, all of which are toxic to brine shrimp and goldfish (Paul et al., 2001); lyngbyabellin A, a cytotoxic compound (Luesch et al., 2000a); lyngbyabellin B, an analogue of lyngbyabellin A (Luesch et al., 2000); and apratoxin A, a potent cytotoxin (Luesch et al., 2001).

In these studies, the lipid and aqueous extracts of treated cyanobacteria were tested for cytotoxic effects on four tumor cell lines. The goal was to see if any of the treatments affected the ability of *L. majuscula* or *L. bouillonii* to produce anti-tumor compounds. Although some of the samples showed solid tumor selectivity, only aqueous extracts of *L. bouillonii* differed significantly among on treatments. Those extracts that initially showed selectivity may be more stringently tested as anti-cancer compounds.

From this research, it is apparent that growth and natural products chemistry of the cyanobacteria *L. majuscula* and *L. bouillonii* are dependent on many factors. While one factor, such as nutrient enrichment, may seem to enhance growth under certain conditions, that same factor may not show the same result under different conditions for the same species or under similar conditions for different species. It is important to understand the intricacies of the physical, biological and chemical processes that make up the ecosystem of interest. Predicting the conditions that favor cyanobacterial growth and bloom formation is a daunting task. Extensive research should be undertaken to look at those conditions and the effects of the interactions of multiple conditions on the ecosystem.

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