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Title: Effects of Light and Nutrients on Intraspecific Secondary Metabolite Variation In the Benthic Cyanobacterium *Lyngbya majuscula*.

Approved: Valerie J. Paul
Dr. Valerie J. Paul, Chairperson, Thesis Committee

Intraspecific chemical variation in secondary metabolite production has been noted for a variety of plants in marine and terrestrial environments. Cyanobacteria, blue-green algae, are primary producers that can respond to physical environmental factors such as light and nutrients in ways similar to plants. In this study, the predictions of the carbon/nutrient balance hypothesis were tested in the laboratory by manipulating the light and nutrient resources available to the benthic marine cyanobacterium *Lyngbya majuscula*, which is known to produce differing amounts and types of secondary metabolites. Light significantly affected the growth and the concentration of organic extract and the major secondary metabolite pitipeptolide A. Conversely, enhanced nitrogen and phosphorus did not influence growth or secondary metabolite production. These results suggest that light may be the major factor influencing secondary metabolite formation for *L. majuscula*.

TO THE OFFICE OF THE GRADUATE SCHOOL AND RESEARCH

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EFFECTS OF LIGHT AND NUTRIENTS ON INTRASPECIFIC SECONDARY METABOLITE VARIATION IN THE BENTHIC CYANOBACTERIUM *LYNGBYA MAJUSCULA*

BY

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INTRODUCTION

Compounds from marine algae may function in a variety of ecological roles, including the deterrence of herbivores (Hay, Fenical and Gustafson, 1987; Hay, 1996; Thacker, Nagle and Paul, 1997; Nagle and Paul, 1998), allelopathy (Hay, 1996), and antifouling (Schmitt, Hay and Lindquist, 1995; Hay, 1996). Due to the nature of earlier biomedical goals, natural products chemists have generally not been interested in the ecological functions of the chemicals, ignoring potential variation within or between individuals (Paul, 1992). Only recently has chemical variability been studied for marine algae (Paul, 1992; Hay, 1996).

Researchers working with terrestrial plants have focused on the importance of understanding the trade-offs in allocation of nutrient resources towards primary and secondary metabolites (Tuomi, 1992). Various hypotheses developed assume that variation may be due to plants allocating their resources to maximize their fitness, with a cost accrued in the production and storage of these secondary metabolites (Tuomi, 1992; Cronin and Hay, 1996a). Some of these hypotheses include the growth-differentiation balance hypothesis, which predicts that rapidly dividing cells are less likely to produce secondary metabolites; and the optimal defense theory, which predicts that plants allocate their defenses to tissue most vulnerable to herbivory (Tuomi, 1992). This research focused on one hypothesis, which has been studied mostly in terrestrial plants, the carbon/nutrient-balance (CNB) hypothesis. This hypothesis suggests that the allocation of resources to the production of secondary metabolites in a plant depends primarily on environmental resources (Bryant, Chapin and Klein, 1983; Bryant, 1987; Ralphs, Manners and Gardner, 1998; Crone and Jones, 1999). According to the CNB hypothesis,

resource availability affects the phenotypic expression of chemical defenses. Allocation of resources to chemical defenses can change as environmental conditions such as light or nutrient availability change. The predictions of the model, therefore, depend on the relative availability of carbon and nitrogen to the plants (Bryant et al., 1983; Bryant, 1987).

The CNB hypothesis predicts that for plants that produce carbon-based secondary metabolites (such as terpenes or polyphenolics), conditions that increase the carbon: nitrogen (C:N) ratio (e.g. high light, low nitrogen) should cause plants to use excess photosynthate as substrate for C-based secondary metabolites increasing the concentration of secondary metabolites and decreasing growth rates. Conditions that decrease the C:N ratio (e.g. low light, high nitrogen) should cause plants to allocate most of their photosynthate to growth accompanied by a decrease in the concentration of secondary metabolites. For plants that produce nitrogen-based secondary metabolites, conditions that increase the C:N ratio should result in a decrease in the concentration of secondary metabolites while more resources are used for growth. Conditions that decrease the C:N ratio should result in an increase in the concentration of secondary metabolites at the expense of growth.

In the marine environment, considerable intraspecific variation of secondary metabolite production in algae has been demonstrated in the few studies that have looked for it (Arnold, Tanner and Hatch, 1995; Peckol, Krane and Yates, 1996; Puglisi and Paul, 1997). Although research in terrestrial plants has produced important hypotheses to explain chemical variation, not until recently has research looked at the effects of nutrient availability on the production of secondary metabolites in marine algae (Yates and

Peckol, 1993; Arnold et al., 1995; Cronin and Hay, 1996b; Peckol et al., 1996; Puglisi and Paul, 1997).

Tropical marine waters around coral reefs can be considered to be nutrient limited in which nitrogen, phosphate, or other trace elements limit plant growth (Lapointe, 1987; Atkinson, 1988). One study done by Larned (1998) assessed the importance of nitrogen or phosphorus on growth in 9 species of algae. Nitrogen limited growth rates for 8 species and phosphorus limited growth rates for 1 species studied.

One group of marine algae, which has been shown to produce bioactive secondary metabolites, is the blue-green algae or cyanobacteria (Gerwick, Roberts, Proteau and Chen, 1994; Patterson, Larsen and Moore, 1994; Faulkner, 1995; Moore, 1996). Unlike marine macroalgae, secondary metabolites from cyanobacteria usually contain nitrogen, probably due to their ability to fix atmospheric nitrogen (Paerl, 1990). The majority of natural products from cyanobacteria are cyclic peptides and depsipeptides (Moore, 1996). Benthic cyanobacteria are a common component of coral reefs and may form tufts similar to other macroalgae (Pennings, Pablo and Paul, 1997).

Cyanobacteria, often described as "pond scum," have been implicated in animal deaths and have raised public health concerns, such as the development of cancer (Carmichael, 1994). Studies on planktonic forms of freshwater species have demonstrated differences in toxicity in samples from the same species (Utkilen and Gjolme, 1992). The mechanisms involved in this apparent chemical variation are unclear and must be understood in order to predict toxin production (Sivonen, 1990).

For cyanobacteria, which produce nitrogen-based secondary metabolites, the CNB hypothesis predicts that conditions that decrease the C:N ratio (e.g. high nitrogen, low

light) should result in an increase in the concentration of secondary metabolites as less resources are used for growth, while conditions that increase the C:N ratio should result in a decrease in the concentration of secondary metabolites and an increase in growth.

Although research on planktonic forms of cyanobacteria has increased over the past two decades, ecological research on natural products from benthic marine cyanobacteria is limited (Nagle and Paul, 1998, 1999). Many important bioactive natural products have been isolated from filamentous benthic cyanobacteria, such as *Lyngbya majuscula* (Moore 1982, 1996; Gerwick et al., 1994). This alga is an ideal model alga to test current hypotheses about the factors influencing secondary metabolite production because it is known that concentrations of the secondary metabolites of this alga can vary considerably among different collections of the same species. Compounds from this species have been shown to deter feeding by fish (Thacker, Nagle and Paul, 1997). Mesograzers, which are usually found living on the cyanobacterium, do not seem to be affected at low concentrations of these secondary metabolites, but are usually deterred at higher concentrations (Pennings and Paul, 1993; Nagle, Camacho and Paul, 1998).

My experiments were designed to investigate the physical environmental factors influencing the variability in secondary metabolite production by *Lyngbya majuscula*, a common inhabitant of tropical reef communities (Thacker et al., 1997; Nagle and Paul, 1998). The main objective of my work was to manipulate nutrients (nitrogen, phosphorus) and light in laboratory experiments to determine their effects on secondary metabolite production by the filamentous cyanobacterium *Lyngbya majuscula*.

My research assessed how well the CNB hypothesis predicted growth and secondary metabolite formation for *Lyngbya majuscula*. The CNB hypothesis would

predict that nitrogen enrichment would lead to increased levels of secondary metabolites in *L. majuscula*. It would also predict that a decrease in light would lead to an increase in levels of secondary metabolites and a decrease in growth. Phosphorus is not addressed in the CNB hypothesis, but from previous studies, phosphorus has been shown to be limiting to growth for macroalgae (Larned, 1998) and may increase levels of secondary metabolite formation in cyanobacteria (Sivonen, 1990).

METHODS

Collection and Maintenance of Cyanobacteria

In the first collection, separate clumps of the cyanobacterium *Lyngbya majuscula* (N = 10) were collected at Piti Bomb Holes on the western side of Guam in January 1999. They were brought back to the Marine Laboratory and placed in buckets filled with 2L of seawater. Algae were then allowed to grow for one week and cleaned of any visible epiphytes and invertebrates. A second collection was made in February 2000 and handled in the same way as the first collection.

Fertilization Experiments

The first laboratory fertilization experiment was conducted from January 14, 1999 to February 4, 1999. Each clump (N=10) was divided into 4 pieces, spun (20 times) in a salad spinner and weighed (1.1 g each, wet mass), and cultured without aeration in 250mL cups. Four treatments were used: control (unenriched seawater), nitrate (10μM) enriched seawater, phosphate (1μm) enriched seawater, and nitrate + phosphate enriched seawater. Nutrients were added to the seawater drawn from Pago Bay, nitrogen in the form of $NaNO_3$ and phosphate in the form of NaH_2PO_4. The seawater and treatments in each cup were changed daily. After three weeks I ended the nutrient experiment, removing each clump from the cups. Excess water from each sample was removed by spinning 20 times in a salad spinner, and then the alga was weighed. The clumps were then freeze-dried, weighed, and extracted as described below.

The second laboratory fertilization experiment was conducted from March 1, 2000 to March 28, 2000. Ten large clumps of *L. majuscula* were divided into 3.0 gram pieces after spinning 20 times in a salad spinner. The 10 large clumps were each divided

into 8 treatments and placed into 80 separate 750 mL bowls, which were set in a factorial design with two levels each of light, phosphate, and nitrate. This experiment was comprised of 10 replicates of eight treatments: control, nitrate enrichment, phosphate enrichment, nitrate + phosphate enrichment, low light, low light + nitrate enrichment, low light + phosphate enrichment, and low light + nitrate + phosphate enrichment. Nutrients were added to the seawater drawn from Pago Bay, nitrogen (20μM) in the form of $NaNO_3$ and phosphate (1μM) in the form of NaH_2PO_4. Mesh screens were placed over the cups to simulate low light conditions. The differences in irradiance between light treatments were measured using an IL 1700 Research Radiometer (International Light) with a SUD 033 probe for photosynthetic active radiation (PAR). The light intensity measured midday on a clear day for the higher light treatment ranged from 135 to 169 μmol photon m^{-2} s^{-1} and for the lower light treatment it ranged from 78 to 84 μmol photon m^{-2} s^{-1}, a 50% decrease. Air was supplied to each cup by air stones. Seawater in each treatment was changed daily. After 4 weeks I ended the nutrient experiment, removing each clump from the cups. Excess water from each sample was removed by spinning 20 times in a salad spinner, and then the alga was weighed. The clumps were then freeze-dried, weighed, extracted and major metabolites quantified as described below. Growth was measured as final weight minus initial weight. The growth rate was analyzed by Three-Way ANOVA (Sokal and Rohlf, 1981) for the three factors: shade, nitrate, phosphate. StatView 5.0 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Extraction and Characterization of Major Metabolites

One bulk sample of the cyanobacterium was freeze-dried and weighed followed by extraction (3x) with 1:1 ethyl acetate (EtOAc): methanol (MeOH) over a 72-hr period. This solution was dried down by rotary evaporation. The dried extract was dissolved in EtOAc and then partitioned between EtOAc and water to remove most of the salts. The EtOAc layer was dried and the extract weighed. The organic extract of the bulk collections was partitioned on a Bond Elut (Varian) silica column in a 0 – 75% EtOAc / 100 – 25% hexanes gradient. Qualitative separation was achieved by preparative normal phase high performance liquid chromatography (HPLC). The HPLC system used was a Waters 501 HPLC pump and a R401 differential refractometer. The column was an Alltech Econosil 10U silica column (25 cm x 10 mm). Two major compounds were separated in 70% EtOAc/ 30% hexanes solvent system.

Quantification of the major metabolites in the extracts from the individual clumps (80 total) from the second fertilization experiment was carried out on a Beckman high performance liquid chromatograph (HPLC) with integrator. The HPLC used included a Beckman model 110B solvent pump, model 156 refractive index detector and model 427 integrator. The analytical column used was an Alltech Econosil 5U column (25 cm x 5 mm). The solvent system used was 65% EtOAc/ 35% hexanes as the mobile phase. The extracts were completely dissolved in 400 μL of 65% EtOAc / 35% hexanes of which 15 μL was injected on the column through a 20 μL injection loop. A calibration curve based on pure compounds was established for the two major metabolites.

Data Analyses

The quantity of secondary metabolites in each sample was calculated using the relationship between areas of peaks of the major compounds and the standard curves generated by HPLC with integrator. The concentrations of metabolites were calculated as percentages of the cyanobacterial dry weight. The percent yields of the major metabolites were statistically analyzed by Three-Way ANOVA (Sokal and Rohlf, 1981): the three factors were shade, nitrate, and phosphate.

RESULTS

Growth

In the first fertilization experiment, *Lyngbya majuscula* grew by about 50% of its original weight over the three weeks (Figure 1A). There were no significant effects of the enrichment treatments on growth (Figure 1A). In the second fertilization experiment, *L. majuscula* grew twice as much over the 4 week experiment for the higher light treatments as compared to the lower light treatments (Figure 2 and Table 1). The effect of decreasing the light intensity on the growth of the cyanobacterium *L. majuscula* grown in the laboratory revealed that light was the limiting factor in this experiment. Higher concentrations of neither nitrate nor phosphate significantly affected growth; therefore neither nutrient was limiting growth (Figure 2 and Table 1).

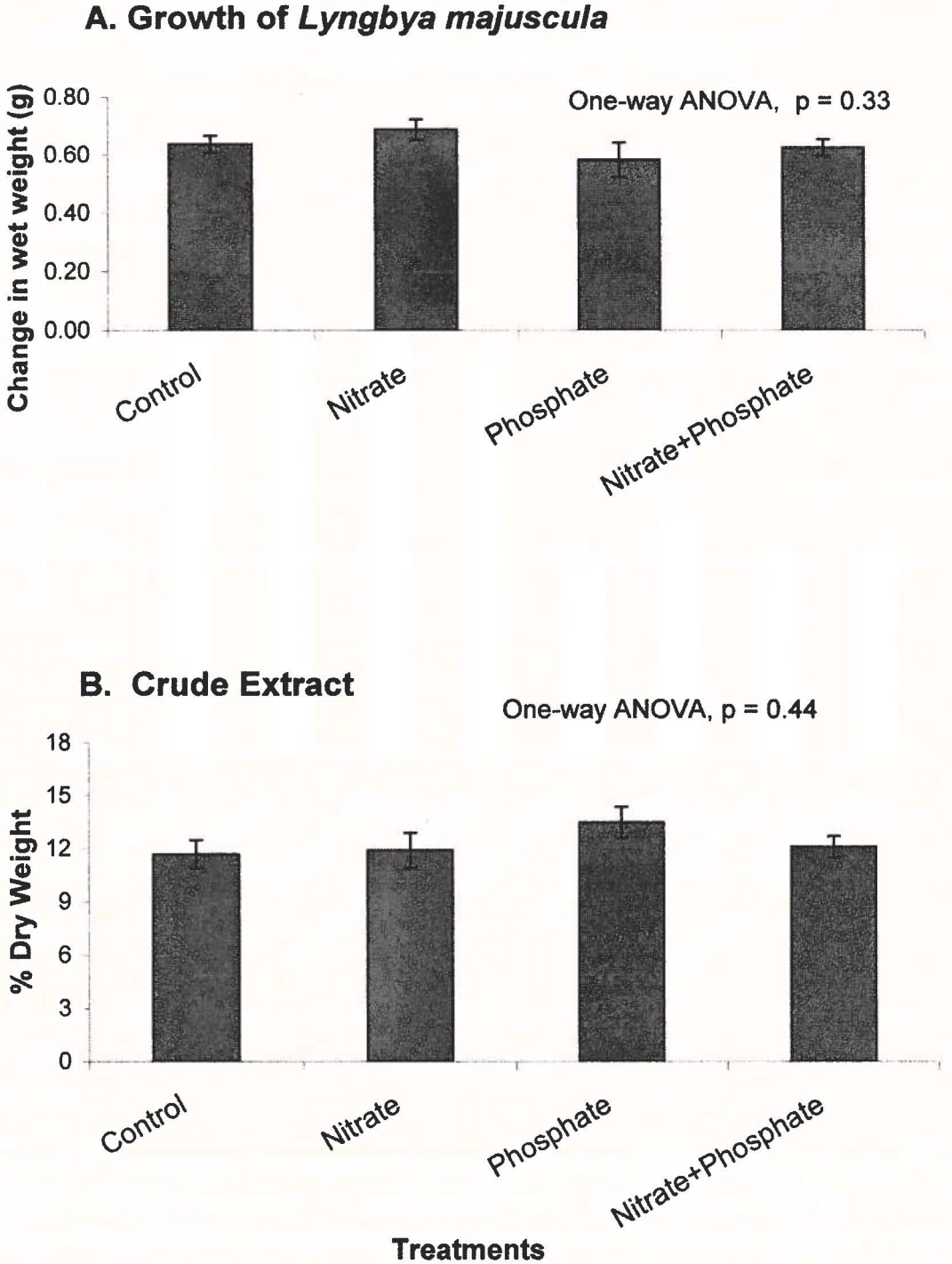


Figure 1. Histogram bars represent A) mean growth and B) crude extract yield for control and treated *Lyngbya majuscula* +/- one standard error. Data were analyzed by One-Way ANOVA to compare means. Neither growth nor % crude extract was significantly affected by the treatments in the first fertilization experiment.

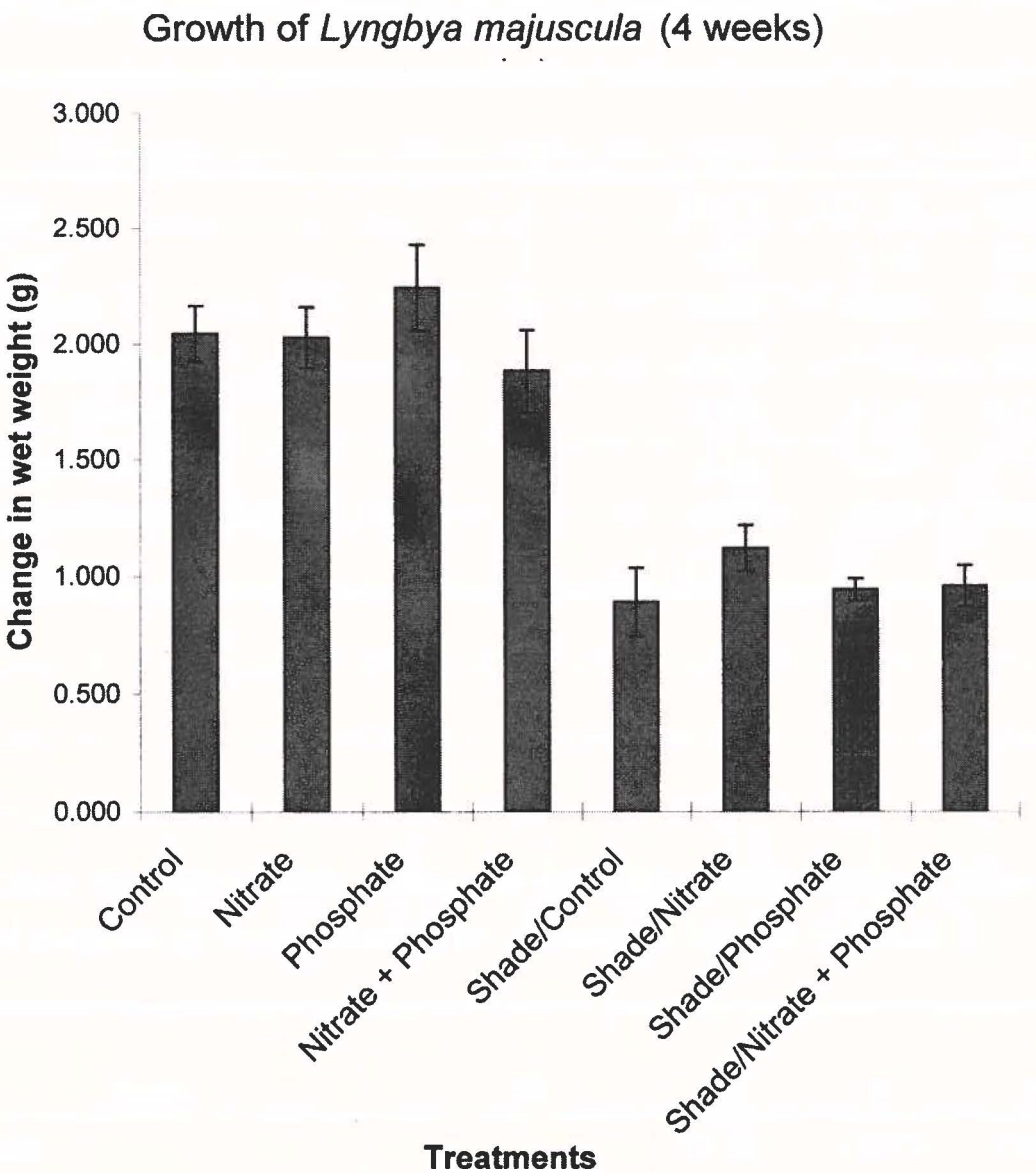


Figure 2. Histogram bars represent mean algal growth in wet weight for control and treated *Lyngbya majuscula* +/- one standard error. Data were analyzed by Three-Way ANOVA to compare means. Growth was significantly affected by the shaded treatment; but, there was no difference observed in growth among the other treatments.

Table 1. Summary of results of three-way ANOVA for the effects of light, nitrate, and phosphate on growth (change in wet weight) in *Lyngbya majuscula.*

Factor	df	SS	F	P
Light	1	23.130	132.124	0.0001
Nitrate	1	0.027	0.156	0.6941
Phosphate	1	0.006	0.033	0.8563
Light x Nitrate	1	0.515	2.941	0.0906
Light x Phosphate	1	0.025	0.144	0.7055
Nitrate x Phosphate	1	0.360	2.059	0.1556
Light x Nitrate x Phosphate	1	0.016	0.089	0.7668
Residual	72	12.604		

Table 2. Summary of results of three-way ANOVA for the effects of light, nitrate, and phosphate on % of crude extract per dry weight in *Lyngbya majuscula.*

Factor	df	SS	F	P
Light	1	17.955	12.723	0.0006
Nitrate	1	1.081	0.766	0.3843
Phosphate	1	0.253	0.179	0.6732
Light x Nitrate	1	0.210	0.149	0.7007
Light x Phosphate	1	0.006	0.004	0.9477
Nitrate x Phosphate	1	1.770	1.254	0.2665
Light x Nitrate x Phosphate	1	0.990	0.702	0.4050
Residual	72	101.609		

Secondary Metabolite Production

Crude extract did not differ significantly between the different nutrient treatments in the first fertilization experiment (Figure 1B). In the second fertilization experiment, light influenced amount of crude extract and the production of the major secondary metabolite (Figures 3 and 4; Tables 2 and 3). Two major peaks were observed in the analytical HPLC traces of all *Lyngbya majuscula* extracts analyzed (Figure 6 and 7). At lowered light intensity, pitipeptolide A concentration was significantly lower than at higher light intensity (Figure 4; Table 3); but pitipeptolide B concentration was not significantly different between the treatments (Figure 5; Table 4). In contrast, the yields of both crude extracts and the secondary metabolites were not significantly different between the controls and the nitrogen and phosphorus enriched treatments (Figures 3-5; Tables 2-4). No interactive effects of light intensity and nutrient availability on growth or secondary metabolite concentrations were detected (Tables 1-4).

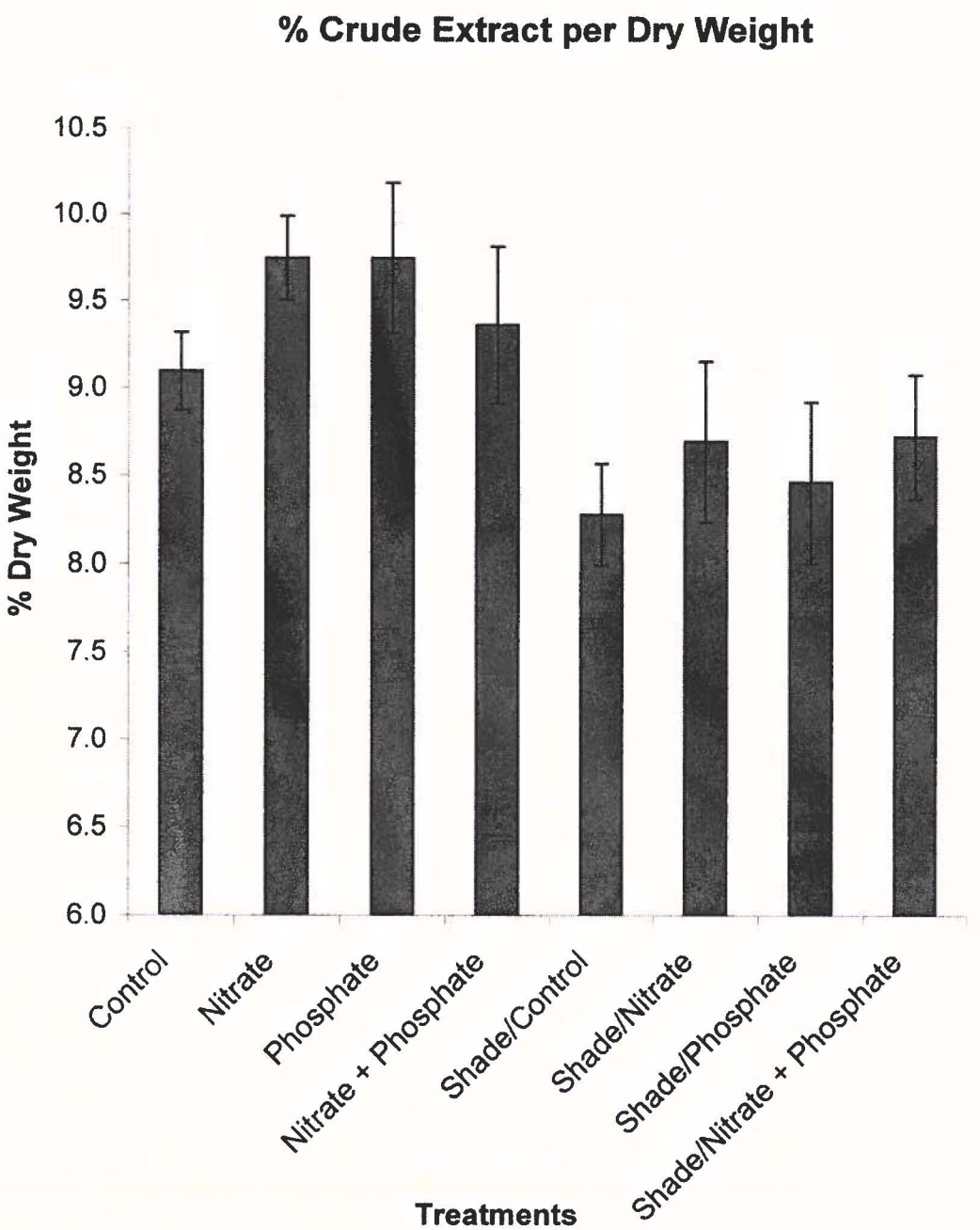


Figure 3. Histogram bars represent mean % crude extract per dry weight +/- one standard error. Amounts of extract were affected by shade but not by the other treatments.

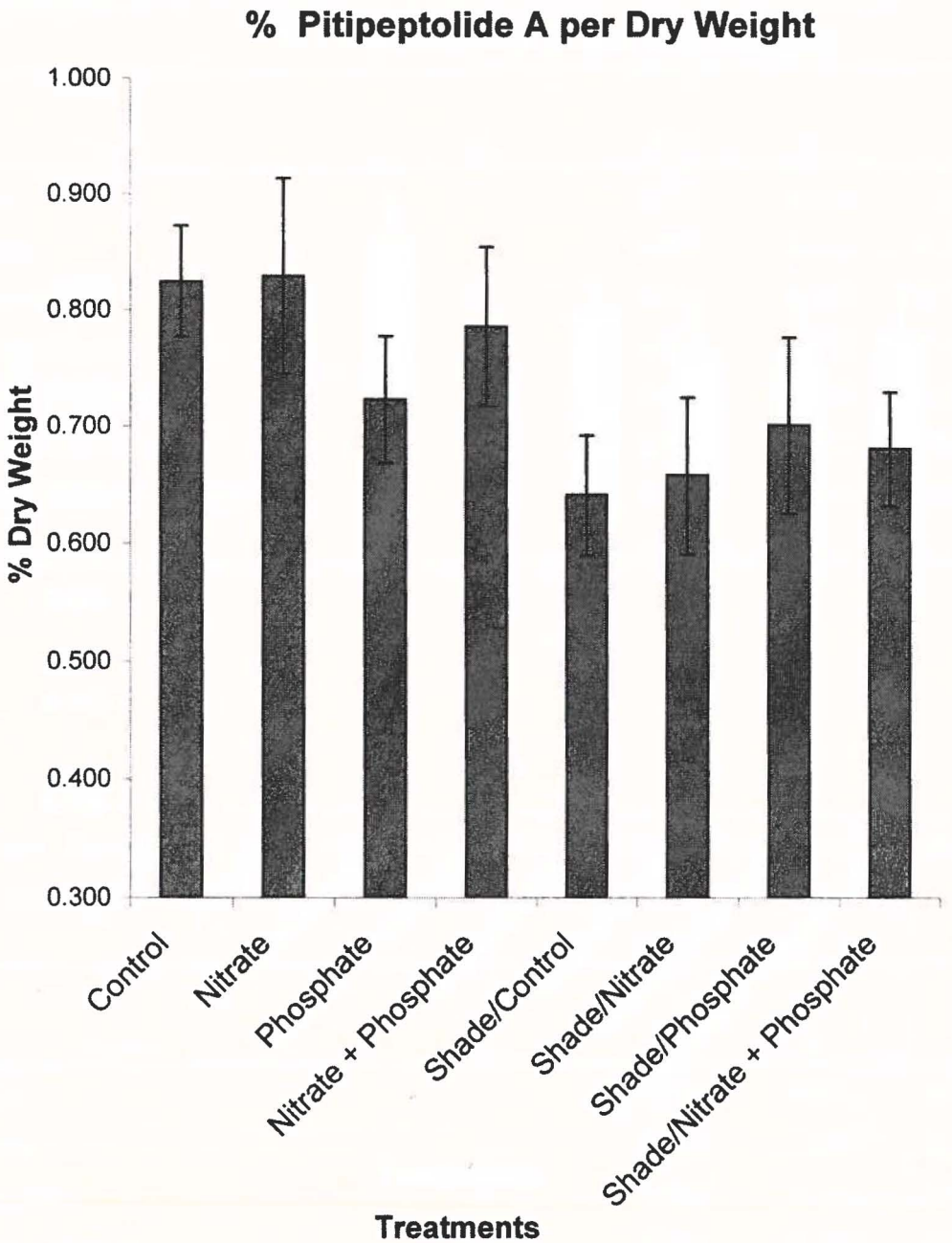


Figure 4. Histogram bars represent mean % major metabolite (pitipeptolide A) per dry weight +/- one standard error. Concentrations of the major secondary metabolite were affected by shade but not by the other treatments.

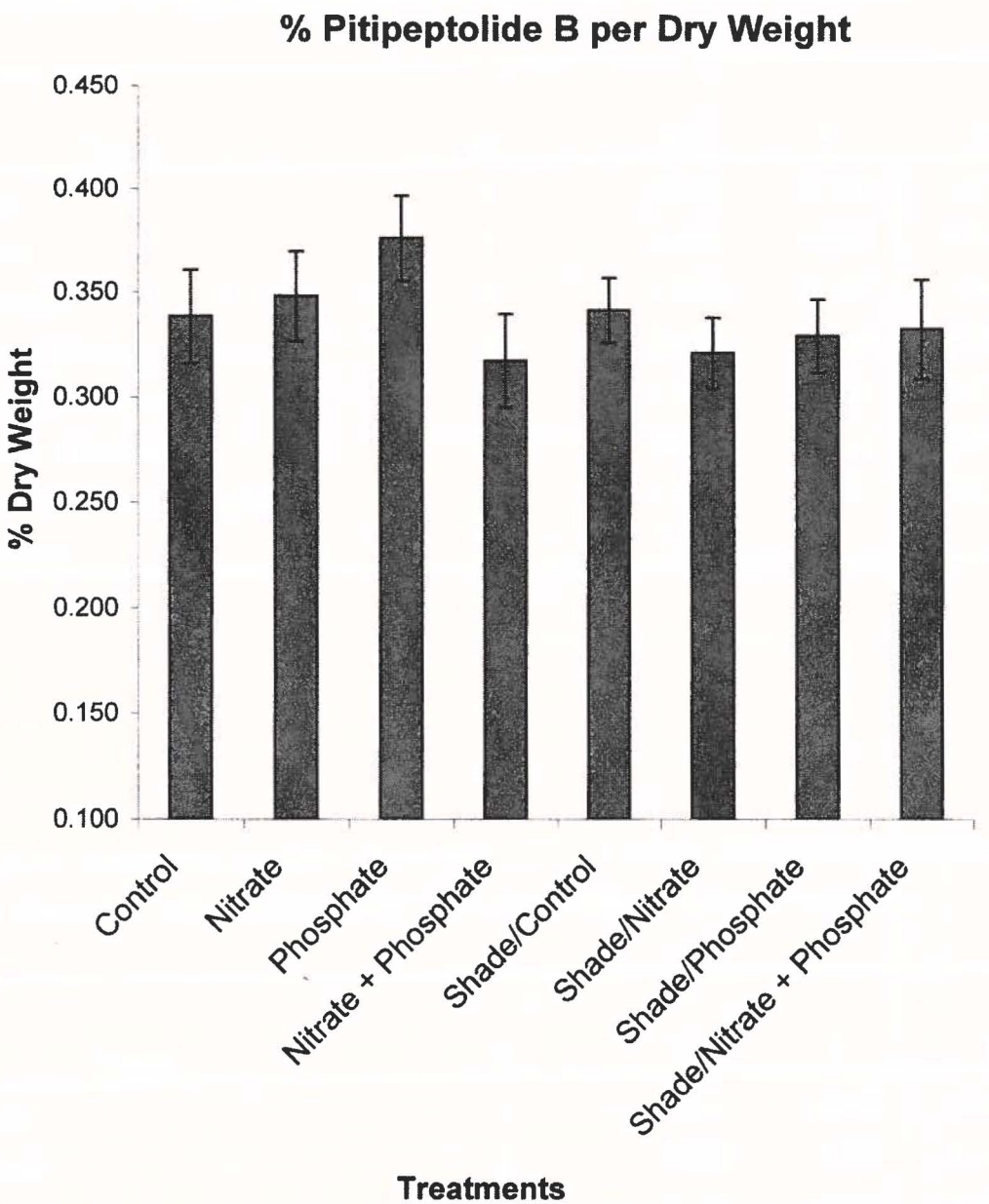


Figure 5. Histogram bars represent mean % pitipeptolide B per dry weight +/- one standard error. Concentration of pitipeptolide B was not significantly different among treatments.

Table 3. Summary of results of three-way ANOVA for the effects of light, nitrate, and phosphate on % of pitipeptolide A per dry weight in *Lyngbya majuscula.*

Factor	df	SS	F	P
Light	1	0.292	6.506	0.0129
Nitrate	1	0.005	0.112	0.7384
Phosphate	1	0.005	0.107	0.7440
Light x Nitrate	1	0.006	0.140	0.7093
Light x Phosphate	1	0.065	1.453	0.2320
Nitrate x Phosphate	1	0.001	0.012	0.9141
Light x Nitrate x Phosphate	1	0.011	0.254	0.6157
Residual	72	3.229		

Table 4. Summary of results of three-way ANOVA for the effects of light, nitrate, and phosphate on % of pitipeptolide B per dry weight in *Lyngbya majuscula.*

Factor	df	SS	F	P
Light	1	0.004	0.852	0.3591
Nitrate	1	0.005	1.207	0.2756
Phosphate	1	0.001	0.010	0.9198
Light x Nitrate	1	0.001	0.286	0.5941
Light x Phosphate	1	0.001	0.015	0.9041
Nitrate x Phosphate	1	0.002	0.549	0.4613
Light x Nitrate x Phosphate	1	0.011	2.339	0.1305
Residual	72	0.328		

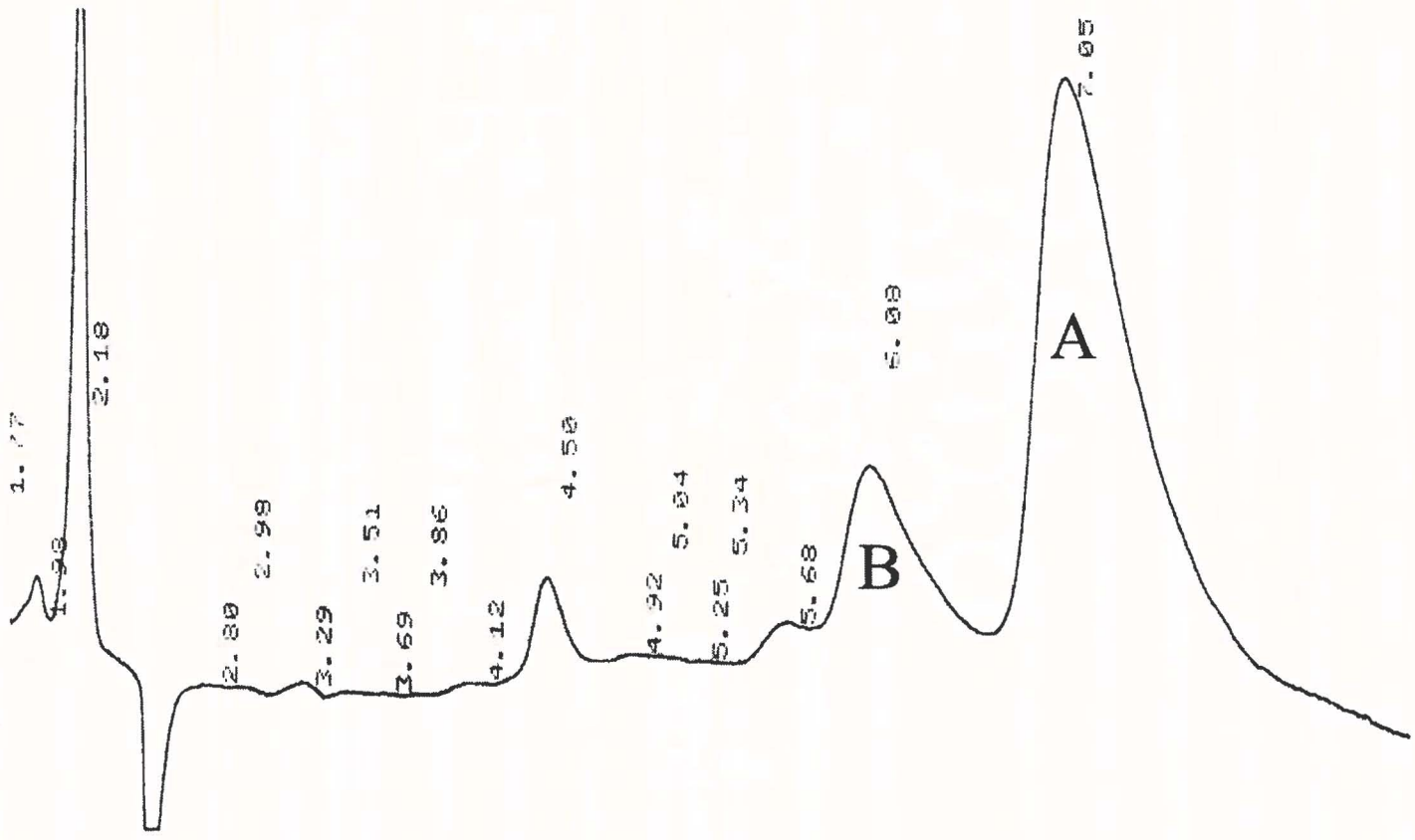
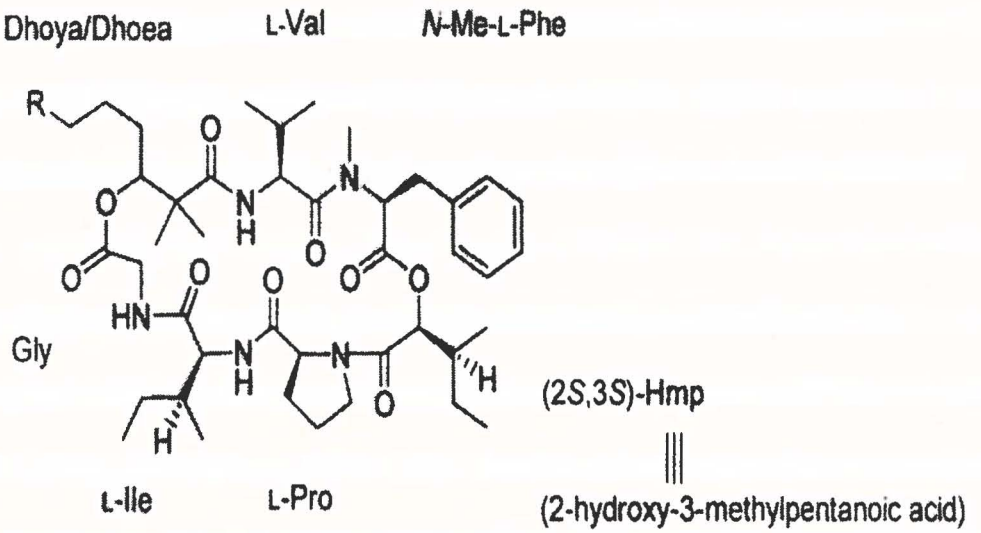


Figure 6. HPLC trace showing peaks of the two secondary metabolites studied: A) pitipeptolide A, B) pitipeptolide B.



R= Pitipeptolide A (major)

R= Pitipeptolide B (minor)

Figure 7. Molecular structures of the major and minor metabolites from the cyanobacterium *Lyngbya majuscula*

DISCUSSION

The carbon/nutrient balance (CNB) hypothesis as introduced by Bryant and coworkers (1983) suggests that the allocation of resources to the production of secondary metabolites in a plant depends primarily on the resource regime available. Studies of the carbon/nutrient-balance hypothesis in the marine environment have focused on macroalgae, which primarily produce carbon-based metabolites. In this study the cyanobacterium *Lyngbya majuscula*, which produces nitrogen-based metabolites, was examined.

The effects of nutrients on the production of secondary metabolites in the freshwater cyanobacterium *Microcystis aeruginosa* have been examined (Watanabe and Oishi, 1985; Utkilen and Gjolme, 1992, 1995). Results of these experiments showed an increase in toxin production due to light and no effects due to nitrogen, contradicting the predictions made based on the CNB hypothesis. In contrast, Sivonen (1990), showed different effects of varying light intensity and nitrogen concentrations on toxin production in the freshwater planktonic cyanobacterium *Oscillatoria agardhii*. Results were in accordance with the CNB hypothesis, where toxin production decreased with increasing light intensity and increased with increasing nitrogen concentration.

In this study, the concentration of the major secondary metabolite, pitipeptolide A, was significantly decreased in lowered light intensity samples, which contradicts predictions made by the CNB hypothesis. Similarly, Watanabe and Oishi (1985) found 3.7-fold-higher toxicity levels, as measured by mouse bioassay, for the cyanobacterium *Microcystis aeruginosa* at 75 than at 7.5 microeinsteins/m^2 s^{-1}. Utkilen and Gjolme

(1992) also found that an increase in light intensity from 20 to 75 μmol photon m^{-2} s^{-1} resulted in an increase in toxin production. Results obtained from *L. majuscula* used in this study and for other cyanobacteria, such as *Anabaena* and *Aphanizomenon* spp. (Rapala et al., 1993) and *Oscillatoria* sp. (Sivonen, 1990) clearly demonstrate that light intensity is a controlling factor for toxicity in many cyanobacteria.

For terrestrial plants, light has been shown to be an important factor affecting both plant nitrogen and allelochemical content (Hagele and Rahier, 1999). It was shown that shaded plants contained higher amounts of nitrogen and an increase in nitrogen-based defenses (i.e., alkaloids) and a decrease in carbon based defenses (i.e., sesquiterpenes) as predicted by CNB hypothesis (Hagele and Rahier, 1999). In contrast, a study on alkaloid concentration in larkspur by Ralphs et al. (1998) showed that restriction of photosynthesis (carbon limitation) did not result in an increase in absolute amounts of alkaloids, which contradicts the CNB hypothesis.

In this study neither growth nor secondary metabolite production was affected by enrichment with nitrate or phosphate in the filamentous cyanobacterium *L. majuscula*. There were also no significant interactive effects of nitrate, phosphate, and light in this study. Secondary metabolite production in the cyanobacterium *Microcystis aeruginosa* was shown not to depend on nitrate (Utkilen and Gjolme, 1995). Other studies with cyanobacteria have found effects with differing nitrogen concentrations (Sivonen, 1990; Rapala, Sivonen, Lyra, and Niemela, 1997). Sivonen (1990) found higher concentrations of secondary metabolites in *Oscillatoria agardhii* with increased nitrogen concentrations. Since light was the limiting factor in my study, results of the enrichment treatments may be different under higher light conditions. In a study of toxin production by *M.*

aeruginosa, it was demonstrated that toxin production increased with increasing light intensity until it reached a maximum, after which it started to decrease with increasing light intensity (Utkilen and Gjolme, 1992).

Phosphorus enrichment studies with cyanobacteria have produced contradicting results. In a study by Rapala et al. (1997), both growth and secondary metabolite production increased in *Anabaena* spp. with increased phosphorus concentrations. Similar studies have shown that secondary metabolite production by some cyanobacteria increased with increasing phosphorus concentration at levels that were growth limiting (Sivonen, 1990; Lehtimaki et al. 1994). Other studies have shown no effect of phosphorus on secondary metabolite production (Rapala, Sivonen, Luukkainen and Niemela, 1993; Utkilen and Gjolme, 1995).

For macroalgae, nutrient and light manipulation experiments and their effects on secondary metabolite production have provided contradicting results. Some results agree with the predictions of CNB hypothesis (Ilvessalo and Tuomi 1989; Yates and Peckol 1993; Arnold et al., 1995). Early studies of the CNB hypothesis in the marine environment focused on the temperate brown alga *Fucus vesiculosus*. The polyphenolics produced by this alga were reported to accumulate under nitrogen deficiency as predicted by the CNB hypothesis, while carbon content did not seem to influence secondary metabolite production, suggesting that external resource availability could modify the accumulation of polyphenolic compounds (Ilvessalo and Tuomi 1989). More direct tests of the effects of nutrient availability with *F. vesiculosus* (Yates and Peckol 1993) and *Lobophora variegata* (Arnold et al., 1995) have also agreed with the CNB hypothesis. In a study done by Arnold et al. (1995) the effects of different concentrations of nitrogen

on polyphenolic content of the tropical brown alga *Lobophora variegata* was examined. Results of this study demonstrated that the polyphenolic concentrations were inversely proportional to nitrogen availability and directly correlated with tissue C:N ratios, in accordance with the CNB hypothesis.

In contrast, several studies with algae that produce terpenoid compounds do not support the CNB hypothesis. A study done by Puglisi and Paul (1997) demonstrated that the manipulation of nitrogen and phosphorus did not lead to a significant change in the monoterpene concentrations in the red alga *Portieria hornemanni*. Because monoterpene concentrations were different between the field and the lab experiments, they suggested the possible importance of light in influencing monoterpene biosynthesis. In another study by Cronin and Hay (1996b) on two brown seaweeds, *Dictyota ciliolata* and *Sargassum filipendula*, light and nutrients were manipulated to determine their effects on carbon-based secondary metabolites and susceptibility to herbivory. Results showed that terpene production was not affected by nutrient addition and was inversely correlated with light intensity, contradicting predictions of the CNB hypothesis. Results that have not been consistent with the CNB hypothesis have hinted at other factors such as UV radiation (Pavia, Cervin, Lindgren and Aberg, 1997) and induction by herbivore damage (Peckol et al., 1996; Hammerstrom, Dethier and Duggins, 1998) as important considerations.

Other studies with cyanobacteria have suggested that other factors may be limiting growth and affecting secondary metabolite production. Utkilen and Gjolme (1995) demonstrated that iron-limited conditions influenced toxin production by *M. aeruginosa* and that iron uptake was light dependent. This leads to the possibility that

light may have an indirect effect on secondary metabolite production. Studies with other cyanobacteria have shown that temperature is an important factor affecting both growth and secondary metabolite accumulation (Van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985; Sivonen, 1990; Rapala et al., 1993, 1997; Lehtimaki, Sivonen, Luukkainen and Niemela, 1994). A study by Watanabe and Oishi (1985), which looked at the effects of temperature on the growth of the cyanobacterium *M. aeruginosa*, demonstrated that temperature may have an effect on secondary metabolite production. Therefore, temperature may have interactive effects with nutrients and/or light.

Although my study seems to contradict the CNB hypothesis, this might be caused by classifying these metabolites as nitrogen-based when they, in fact, contain a great deal of carbon in their molecular skeletons. Therefore, carbon as opposed to nitrogen may be more costly to this organism, which is also able to fix atmospheric nitrogen (Paerl, 1990).

Since generalist consumers do not consume chemically rich cyanobacteria, secondary metabolites may facilitate the formation and persistence of benthic cyanobacterial blooms in coral reef habitats (Thacker et al., 1997; Nagle and Paul, 1998, 1999). In addition, toxic cyanobacterial blooms have been linked to massive fish kills and human health concerns (Hallegraeff, 1993; Carmichael, 1994). My results strongly suggest that light intensity is an important factor in the control of secondary metabolite production in *Lyngbya majuscula*. The insights gained from the laboratory experiments should be tested under field conditions before further conclusions are made. My study does show that light, as opposed to nutrients, is more important in determining the phenotypic response of *L. majuscula*, which suggests that secondary metabolite variation may be due to microhabitat and/or temporal variation.

REFERENCES

Arnold, T. M., C. E. Tanner and W. I. Hatch. 1995. Phenotypic variation in polyphenolic content of the tropical brown alga *Lobophora variegata* as a function of nitrogen availability. Mar. Ecol. Prog. Ser. 123:177-183

Atkinson, M. J. 1988. Are coral reefs nutrient-limited? Proceedings of the 6th International Coral Reef Symposium 1:157-166

Bryant, J. P., F. S. Chapin, III and D. R. Klein. 1983. Carbon/nutrient balance in boreal plants in relation to vertebrate herbivory. Oikos 40:357-368

Bryant, J. P. 1987. Feltleaf willow-snowshoe hare interactions: plant carbon/nutrient balance and floodplain succession. Ecology 68:1319-1327

Carmichael, W. W. 1994. The toxins of cyanobacteria. Scientific American. January 78-86

Crone, E. E. and C. G. Jones. 1999. The dynamics of carbon-nutrient balance: effects of cottonwood acclimation to short- and long-term shade on beetle feeding preferences. J. Chem. Ecol. 25:635-656

Cronin, G. and M. E. Hay. 1996a. Within-plant variation in seaweed chemical defenses: Optimal defense theory versus the growth-differentiation balance hypothesis. Oecologia 105: 361-368

Cronin, G. and M. E. Hay. 1996b. Effects of light and nutrient availability on the growth, secondary chemistry, and resistance to herbivory of two brown seaweeds. Oikos 77:93-106

Faulkner, D. J. 1995. Marine natural products. Nat. Prod. Rep. 12:223-269

Gerwick, W. H., M. A. Roberts, P. J. Proteau and J. L. Chen. 1994. Screening cultured marine microalgae for anticancer-type activity. J. Appl. Phycol. 6:143-149

Hagele, B. F. and M. R. Rahier. 1999. Genetic and environmental-based variability in secondary metabolite leaf content of *Adenostyles alliariae* and *A. alpina* (Asteraceae). A test of the resource availability hypothesis. Oikos 85:234-246

Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32:79-99

Hammerstrom, K., M. N. Dethier and D. O. Duggins. 1998. Rapid phlorotannin induction and relaxation in five Washington kelps. Mar. Ecol. Prog. Ser. 165:293-305

Hay, M. E. 1996. Marine chemical ecology; what's known and what's next? J. Exp. Mar Biol. Ecol. 200:103-134

Hay, M. E., W. Fenical and K. Gustafson. 1987. Chemical defense against diverse coral reef herbivores. Ecology 75:1714-1726.

Ilvessalo, H. and J. Tuomi. 1989. Nutrient availability and accumulation of phenolic compounds in the brown alga *Fucus vesiculosus*. Mar. Biol. 101:115-119

Lapointe, B. E. 1987. Phosphorus- and nitrogen-limited photosynthesis and growth of *Gracilaria tikvahiae* (Rhodophyceae) in the Florida Keys: an experimental field study. Mar. Biol. 93:561-568

Larned, S. T. 1998. Nitrogen- versus phosphorus-limited growth and sources of nutrients for coral reef macroalgae. Mar. Biol. 132:409-421

Lehtimaki, J., K. Sivonen, R. Luukkainen and S. I. Niemela. 1994. The effects of incubation time, temperature, light, salinity, and phosphorus on growth and hepatoxin production by *Nodularia* strains. Arch. Hydrobiol. 130:269-282

Moore, R. E. 1982. Toxins, anticancer agents, and tumor promoters from marine prokaryotes. Pure Appl. Chem. 54:1919-1934

Moore, R. E. 1996. Cyclic peptides and depsipeptides from cyanobacteria: a review. J. Ind. Microbiol. 16:134-143.

Nagle, D. G., F. T. Camacho and V. J. Paul. 1998. Dietary preferences of the opistobranch mollusc *Stylocheilus longicauda* for secondary metabolites produced by the tropical cyanobacterium *Lyngbya majuscula*. Mar Biol. 132:267-273.

Nagle, D. G. and V. J. Paul. 1998. Chemical defense of a marine cyanobacterial bloom. J. Exp. Mar. Biol. and Ecol. 225:29-38

Nagle, D. G. and V. J. Paul. 1999. Production of secondary metabolites by filamentous tropical marine cyanobacteria: ecological functions of the compounds. J. Phycol. 35:1412-1421.

Paerl, H. W. 1990. Physiological Ecology and Regulation of N2 Fixation in Natural Waters. Adv. Microb. Ecol. 11:305-344

Patterson, G. M. L., L. K. Larsen and R. E. Moore. 1994. Bioactive natural products from blue-green algae. J. Appl. Phycol. 6:151-157

Paul, V. J. (editor) 1992. Ecological roles of marine natural products. Cornell University Press, Invited contribution for Cornell Chemical Ecology Series, Ithica, NY

Pavia, H., G. Cervin, A. Lindgren and P. Aberg. 1997. Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum*. Mar. Ecol. Prog. Ser. 157:139-146.

Peckol, P., J. M. Krane and J. L. Yates. 1996. Interactive effects of inducible defense and resource availability on phlorotannins in the North Atlantic brown alga *Fucus vesiculosus*. Mar. Ecol. Prog. Ser. 138:209-217

Pennings, S. C., S. R. Pablo and V. J. Paul. 1997. Chemical defenses of the tropical, benthic cyanobacterium *Hormothamnion entermorphoides*: Diverse consumers and synergisms. Limnol. Oceanogr. 42:911-917

Pennings, S. C. and V. J. Paul. 1993. Sequestration of secondary metabolites by three species of sea hares: location, specificity, and dynamics. Mar. Biol. 117:535-546

Puglisi, M. P. and V. J. Paul. 1997. Intraspecific variation in the red alga *Portieria hornemannii*: monoterpene concentrations are not influenced by nitrogen or phosphorus enrichment. Mar. Biol. 128:161-170

Ralphs, M. H., G. D. Manners and D. R. Gardner. 1998. Influence of light and photosynthesis on alkaloid concentration in larkspur. J. Chem. Ecol. 24: 167-182

Rapala, J., K. Sivonen, R. Luukkainen and S. I. Niemela. 1993. Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and non-toxic *Anabaena*-strains—a laboratory study. J. Appl. Phycol. 5:581-591

Rapala, J., K. Sivonen, C. Lyra and S. I. Niemela. 1997. Variation of microcystins, cyanobacterial hepatoxins, in *Anabaena* spp. as a function of growth stimuli. Appl. Environ. Microbiol. 63:2206-2212.

Schmitt, T. M., M. E. Hay and N. Lindquist. 1995. Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. Ecology 76: 107-123

Sivonen, K. 1990. Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl. Environ. Microbiol. 56:2658-2666

Sokal, R. R. and F. J. Rohlf. 1981. Biometry. The principles and practice of statistics in biological research. Second edition. W. H. Freeman and Company, New York.

Thacker, R. W., D. G. Nagle and V. J. Paul. 1997. Effects of repeated exposures to marine cyanobacterial secondary metabolites on feeding by juvenile rabbitfish and parrotfish. Mar. Ecol. Prog. Ser. 147:21-29

Tuomi, J. 1992. Toward integration of plant defense theories. Trends Ecol. Evol. 7: 365-367

Utkilen, H. and N. Gjolme. 1992. Toxin production by *Microcystis aeruginosa* as a function of light in continuous cultures and its ecological significance. Appl. Environ. Microbiol. 58:1321-1325.

Utkilen, H. and N. Gjolme. 1995. Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl. Environ. Microbiol. 61:797-800

Van Alstyne, K. L., J. J. McCarthy, III, C. L. Hustead and D. O. Duggins. 1999. Geographic variation in polyphenolic levels of Northeastern Pacific kelps and rockweeds. Mar. Biol. 133:371-379

Van der Westhuizen, A. J. and J. N. Eloff. 1985. Effect of temperature and light on the toxicity and growth of the blue-green alga Microcystis aeruginosa (UV-006)*. Planta 163:55-59

Watanabe, M. F. and S. Oishi. 1985. Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. Appl. Environ. Microbiol. 49:1342-1344.

Yates, J. L. and P. Peckol. 1993. Effects of nutrient availability and herbivory on polyphenolics in the seaweed *Fucus vesiculosus*. Ecology 74:1757-1766