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Iron Dependence of *Lyngbya majuscula*

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ABSTRACT

The filamentous alga (Cyanobacterium) *Lyngbya majuscula* which is a nuisance aquatic plant in the southeastern United States, shows iron dependence in Gorham's medium in laboratory conditions, using iron (III) EDTA, NaFe (EDTA), as the iron source. The addition of iron stimulated oxygen production as determined volumetrically with a Warburg apparatus to a concentration of 1.4 ppm Fe as Fe $(EDTA)^-$. An optimum range of 1.4 to 6.1 ppm added iron was observed, and concentrations above 6.4 ppm added iron were inhibitory. The growth of this cyanobacterium thus could be reduced by treatments that would affect the availability of iron, provided the cyanobacterium does not synthesize an iron chelator or siderophore. Thorough, repetitious, and diagnostic studies indicated that no detectable levels of siderophore were produced. The lack of siderophores and the dependence of lyngbya on iron for growth suggests that lowering iron concentrations in the water might reduce photosynthesis and growth. However, if lyngbya is capable of generating siderophores, management of the plant by controlling iron would not be feasible, so the absence of siderophore generation is significant.

Key words: siderophores, cyanobacteria, chelators, iron dependence, filamentous algae, geosmin.

INTRODUCTION

Lyngbya, a benthic, filamentous blue-green alga (cyanobacterium) causes significant problems in natural waters of the southeast United States (Beer et al. 1986, Lembi 1986, Speziale et al. 1988, 1991; Speziale and Dyck, 1992). In Florida, aquatic plant surveys indicate filamentous algae was the sixteenth most abundant aquatic plant in 1988 (Schardt 1989), and *L. majuscula* was one of seven algae in this group. Control of this species is difficult and relatively high concentrations of copper are required for its management. According to Schardt (pers. comm. 1990), no algicide licensed in Florida effectively controlled lyngbya.

The control of lyngbya is desired for several reasons, including maintaining the favorable state of a lake. Lyngbya can form dense mats contributing to the eutrophication of freshwater lakes (Canfield and Hoyer 1988, Canfield et al. 1989) and contributing to the buildup of plant detritus that can fill lakes and choke streams (Schardt 1989). Excessive amounts of cyanobacteria in a lake can favor survival of rough fish over sport fish, decreasing the ecological and recreational value of the lake (Williams et al. 1985).

In addition, control of lyngbya is desirable as a means of controlling the chemicals that it produces. *Lyngbya spp.* produce geosmin (1,10-trans-dimethyl-trans-9-decanol), an earthy, moldy smelling compound first isolated from a culture of Streptomycetes (Gerber and Lechevallier 1965). Geosmin and methylisoborneol can be responsible for the muddy taste in surface water supplies (Gosselin et al. 1989) and off-flavor in catfish (Persson 1984). Finally, during periods of abundant growth, geosmin, elaborated by floating mats of Lyngbya can be released into the air causing irritation of human respiratory membranes (Persson 1984).

Lyngbya spp. also produce skin irritants that cause dermatitis or swimmers itch (Moikeha et al. 1971a,b). A causative agent, Lyngbyatoxin A, was isolated from *Lyngbya majuscula* growing at Kahala Beach, Oahu, Hawaii (Cardellina et al. 1979). Lyngbyatoxin A is also a potent tumor-promoting agent (Fujiki et al. 1984). Other tumor promoters, aplysiatoxin and debromoaplysiatoxin, were isolated by Kato and Scheuer (1974, 1976).

There may be useful products that can be isolated from *L. majuscul*a, but the present concern is focused on gaining information leading to potential control. One possibility is to limit the plant growth by controlling the availability of an essential element. We examined the iron dependence of the plant under controlled laboratory conditions. In addition, we also considered the possibility that *L. majuscula* is capable of synthesizing siderophores (potent iron-chelating agents); the availability of these agents would probably preclude control of the plant by manipulation of iron levels.

MATERIALS AND METHODS

Samples of lyngbya were obtained from Horseshoe Lake in Lakeland, FL. and were identified as *L. majuscula* by Clinton P. Dawes (Martin and Martin 1987, Johnson and Martin 1988). Extraneous material was removed, and the alga (cyanobacterium) was washed several times in 5 L of distilled water, agitating, draining until the water was clear and all observable trash was removed. A stock culture was maintained in well water from the Floridian aquifer in a 200 liter, open, plastic drum on the roof of the Science Center at the University of South Florida.

Effect of added iron on oxygen production by *L. majuscula* in modified Gorham's medium (Bold and Wynne 1985) was measured (using 0.2 g, fresh weight of lyngbya) with a Warburg apparatus as previously described (Martin and Martin 1987, Dyer et al. 1992). We used a constant temperature bath at 30C. The Warburg flask was illuminated with cool-white fluorescent lamps arranged vertically around the bath (100 uE/m^2/sec PAR, as measured by a LiCor radiometer/pho-

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tometer). After a 30-minute incubation period, manometer readings were recorded every ten minutes for about an hour. When constant rate of oxygen production was obtained, the contents of the side-arm flask containing a known amount of NaFeEDTA in Gorham's medium was added, and the new rate was measured.

Lyngbya cultures were grown under various conditions in an effort to induce the cyanobacterium to produce a siderophore. Typically the cyanobacterium was washed in distilled water for a minimum of ten times and maintained in deionized-distilled water for at least 12 hours before inoculation. In searching for siderophores, media were inoculated with 12 to 384g of lyngbya. Culturing periods in iron-free media ranged from 4 hours to 104 days. Iron-deficient medium (Simpson and Neilands 1976) was used with three different modifications: (1) 2 mM NH_4Cl was used instead of 0.9 mM; (2) $Na_2CO_3.H_2O$ (0.234g /L of medium) was added; and (3) thiamine hydrochloride (0.0020 g/L) was added. Cultures were maintained at 24 ± 2C and at 30 ± 2C.

Two methods were used to extract siderophores. The Armstrong-Van Baalen method (1979) involved a chloroform extraction to isolate an iron siderophore. A second method involved chloroform-phenol (1:1) extraction to isolate the siderophore (hydroxamic acid) (Mullis et al. 1971).

Rhodotorulic acid, obtained from Sigma Chemical Co., was used as a standard dihydroxamate to confirm the reliability of extraction methods and our technique.

Three assays were used to determine the presence of a hydroxamate. An iron(III) chloride assay consisted in adding 0.1 mL of partially purified chromatography fraction or 0.5mL of supernatant medium to 3 mL of $FeCl_3$ reagent (2%v/v iron chloride in 5 mM HCl), then measuring the absorbance (400-500 nm) using a 2-cm quartz cell (Gibson and Magrath 1969). A second assay consisted in adding 2.5 mL iron perchlorate solution (5 mM in 0.1 M $HCl0_4$) to the test sample as in the previous assay, and measuring the absorbance as before (Atkin et al. 1970). The third assay was the Csa'ky test (Csa'ky 1948) using modifications suggested by Gillam and co-workers (1981).

A fourth assay, the universal chemical assay for siderophores (Schwyn and Neilands 1987) was applied as a last check. Media from several sources were screened using the ternary complex—chrome azurol S/iron (III)/hexadecyltrimethylammonium bromide. When a siderophore removes iron from the intensely colored ternary complex, the color of the solution changes from blue to orange. Equilibria with pure hydroxamates was known to be slow, so 5-sulfosalicylic acid was used as a "shuttle", i.e., to chelate with the iron rapidly, then give up the iron to the more stable siderophore. The ternary complex solution was prepared: 6 mL of 10 mM aqueous hexadecyltrimethylammonium bromide was placed in a 100-mL volumetric flask, treated with 1 mL of iron(III) chloride solution (1 mM $FeCl_3.6H_20$ in 10 mM HCl) and 7.5 mL of 2 mM aqueous chrome azurol S (CAS) solution. Then 4.307 g of piperazine and 6.25 mL of 12 M hydrochloric acid was added, and the entire mixture was diluted to 100 mL. Shuttle solution was prepared by treating the ternary complex solution with 5-sulfosalicylic acid at a concentration of 4 mM. The solutions were stored in the dark. Presence of a siderophore was determined by adding 2.0 mL of solution to be tested to 2.0 mL of shuttle solution. After two hours, the absorbance was measured at 630 nm using a uv-vis spectrometer.

RESULTS

There is a problem with nomenclature for cyanobacteria. Our samples were identified as *Lyngbya majuscula* by Clinton Dawes (Martin and Martin 1987, Johnson and Martin 1988). Speziale (1990) identified samples from the same source as *L. wollei*. Speziale and Dyck (1992) preferred to assign the species name *wollei* to indicate separation in having the name reflect the source (freshwater versus marine). The cyanobacterium *L. majuscula* is a marine species that is able to adapt to freshwater and vice versa (Shannon et al. 1992). Cowell (pers. comm. 1991) reported that this adaptive process occurs naturally in such estuaries as Crystal River, Florida, where *L. majuscula* is found from the fairly saline environment of King's Bay (Romie) to freshwater up-river locations.

For the Warburg runs, the change in manometer reading (in cm) was plotted as a function of time (min) and fitted to a straight line by regression analysis. Quality of fit was indicated by statistically significant linear correlation coefficients (0.94 or better) and consistency for the control run, then the same procedure was applied to the rate when the iron concentration (as $Fe(EDTA)^-$) was increased. [Raw data are presented elsewhere (Gross 1994).]

Under the conditions used, the rate constant increased as a function of added iron (Figure 1) until a maximum rate (k = 0.027 cm/min) was attained at 1.4 ppm added iron. The maximum (or optimum) rate continued until the apparent inhibitory concentration of 6.4 ppm added iron, and the rate constant decreased to a minimum (k = 0.0011 cm/min) and remained until the highest concentration of iron studied (10.4 ppm).

The validity of the rate plot, rate constant as a function of added iron (Figure 1), was checked by estimating the concentration of iron in Gorham's medium (known to be 0.8

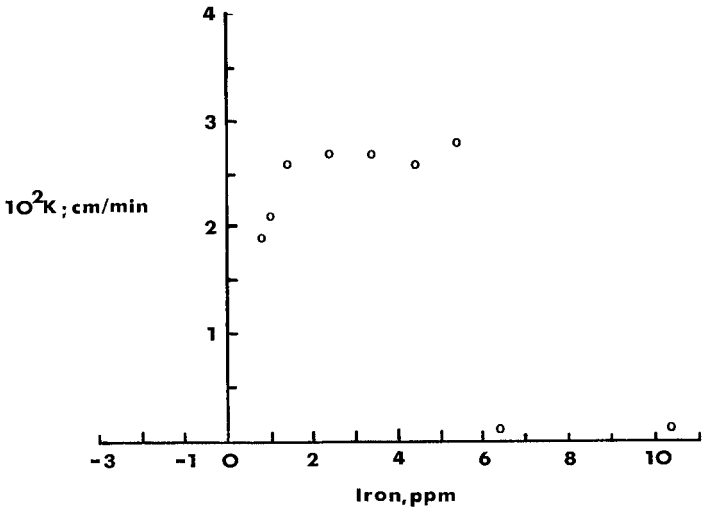

Figure. 1. Growth rate, change in manometer reading, cm/min, as a function of concentration (ppm) of iron added (as FeEDTA-) added to 0.2 g of lyngbya in Gorham's medium in the Warburg apparatus. Each point is the slope of the line passing through a minimum of five measurements.

ppm). The points occurring in the rate-increase phase, were extrapolated to the abscissa by means of a least-squares treatment (the so-called "standard addition" technique). The abscissa intercept was -0.8 ppm, indicating the initial concentration of iron in Gorham's solution was 0.8 ppm, which was in good agreement with the amount that was added to the medium.

Two methods were used to extract any siderophores that may have been produced. The Armstrong-Van Baalen (1979) method used chloroform and extracted the siderophore as the iron-siderophore complex. The second method (Mullis et al. 1971) used chloroform:phenol (1:1 w/w) as the extract and removed the siderophore as the free hydroxamic acid. Neither method produced a detectable product. Rhodotorulic acid, a known hydroxamate, was used as a standard to test the efficacy of the method and technique. The first procedure was unsuccessful, but the second was successful, and about 80% of the material was extracted. Over 40 experiments were run under varying conditions (for details, see Gross, 1994) without success.

We used the universal chemical assay to test our ability to detect a siderophore. Desferal was used as the standard. Results from Desferal standards and on media from several sources are listed in Table 1. Had a siderophore been present, it would have removed the iron from the ternary complex causing a decrease in absorption relative to the reference, such as was seen with Desferal solutions with concentrations greater than 0.5 ppm or greater. In short, we were able to detect concentrations of a siderophore greater than 9×10^{-7} M.

DISCUSSION

Studies of the iron dependence of *Lyngbya majuscula* under controlled laboratory conditions indicate that the plant shows a distinct iron dependence. The data are best demonstrated for the production of oxygen using a Warburg apparatus. Under these conditions, the method of standard addition allows extrapolation to calculate the amount of chelated iron that was in the original medium, and the agreement between calculated and known iron concentrations was good. The data indicate a significant range for the optimum concentration (Figure 1), and a toxic level was indicated.

Table 1. Absorbances at 630 nm for desferal standards and lyngbya media for the ternary complex.

Sample	Absorbance at 630 nm	Positive result?
Water with CAS[1] in reference cell		
Desferal, 500 ppm (8.92×10^{-4}M)	-0.993	Yes
Desferal, 5 ppm	-0.297	Yes
Desferal, 0.5 ppm	0.007	Barely
Desferal, 0.05 ppm	0.028	No
Desferal, 0.005 ppm	0.026	No
Gorham's medium with CAS[1] in reference cell		
Medium, iron-deficient lyngbya, after 2weeks growth:		
a. Trial 1	0.110	No
b. Trial 2	0.136	No
c. Trial 3	0.132	No
d. Trial 4	0.126	No
Supernatant water from lyngbya in deionized water, after 1 week growth:		
a. Trial 1	0.136	No
b. Trial 2	0.160	No

[1]CAS, complex chrome azurol S solution

The dependence of plant growth, as measured by oxygen production, on iron concentration has obvious application to the concept of management of the plant by adjusting the iron concentration. Though toxic levels of iron were observed, no one would suggest iron enrichment to those levels for several reasons, including the effect of ultimate dilution to an optimum level.

On the other hand, it is conceivable that iron levels could be controlled by pH adjustment, much as has been done already for Alabama ponds in efforts to control blue-green algae (Tucker and Boyd 1985) to avoid contamination of catfish by geosmin and related materials. If iron concentration is determined by the solubility of iron(III) hydroxide, then given the solubility product constant of 1×10^{-39}, at pH 7 or above the calculated iron concentration is 10^{-18} M, or about 6×10^{-14} ppm. Typically, most media contain 0.5-1.0 ppm iron.

Many cyanobacteria synthesize siderophores under iron poor conditions (Atkin et al. 1970). If this were the case with *L majuscula*, then control of iron by pH adjustment would be futile because the siderophore would solubilize sufficient iron.

The present study indicated that under the conditions tested, no siderophore was detected. In addition, we demonstrated our ability to isolate a known siderophore. Had rhodotorulic acid or a hydroxamate of similar molecular weight and properties been present, it could have been extracted at a concentration of 1.5×10^{-5} M. Three different assays, used routinely in about fifty separate experiments failed to detect a siderophore. In addition, a more sensitive screening assay was used as a check. We estimated that siderophore concentrations of 9×10^{-7} M or greater would have been detected.

The failure to detect siderophores under the conditions studied leads to one of two conclusions. First it is possible that our methods were not sufficiently sensitive. While that is plausible, the concentration of chelated iron at the siderophore levels we could detect would not have been especially stimulatory. The second conclusion is simply that *L. majuscula* in iron-deficient media did not produce siderophores. If that conclusion, which seems a reasonable one, may be extrapolated to the environment, the failure to find a siderophore, while not as exciting a result, is surely the more encouraging one.

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LITERATURE CITED

Armstrong, J. E. and C. Van Baalen. 1979. Iron transport in microalgae: the isolation and biological activity of a hydroxamate siderophore from the blue-green alga *Agmenellum quadruplicatum*. J. Gen. Microbiol. 111: 253-262.

Atkin, C. L., J. B. Neilands, and H. J. Phaff. 1970. Rhodotorulic acid from species of *Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. J. Bacteriol. 103(3): 722-733.

Beer, S., W. Spencer, and G. Bowes. 1986. Photosynthesis and growth of the filamentous blue-green alga *Lyngbya birgei* in relation to its environment. J. Aquat. Plant Manage. 24: 61-65.

Bold, H. C. and M. J. Wynne. 1985. Introduction to the Algae: Structure and Reproduction. 2nd ed., Prentice-Hall, Englewood Cliffs, NJ. 720 pp.

Canfield, Jr., D. E. and M. V. Hoyer. 1988. The eutrophication of Lake Okeechobee. Lake and Reservoir Manage. 4(2): 91-99.

Canfield, Jr., D. E., E. Phlips, and C. M. Duarte. 1989. Factors influencing the abundance of blue-green algae in Florida lakes. Can. J. Fish. Aquat. Sci. 46: 1232-1237.

Cardellina II, J. H., F. Marner, and R. E. Moore. 1979. Seaweed dermatitis: structure of lyngbyatoxin A. Science. 204: 193-195.

Csa'ky, T. Z. 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. 2: 450-454.

Dyer, J. R., D. Forgie, B. B. Martin, and D. F. Martin. 1992. Effects of selected copper(II)-chelate compounds on the rates of production of oxygen by filamentous algae. Biomedical Lett. 47: 363-369.

Fujiki, H., M. Suganuma, H. Hakii, G. Bartolini, R. E. Moore, S. Takayama, and T. Sugimura. 1984. A Two-stage mouse skin carcinogenesis study of lyngbyatoxin A. J. Cancer Rsch. and Clin. Oncol. 108(1): 174-176.

Gerber, N. N. and H. A. Lechevallier. 1965. Geosmin, an earthy-smelling substance isolated from actinomycetes. Appl. Microbiol. 13: 935.

Gibson, F. and D. I. Magrath. 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-I. Biochim. Biophys. Acta 192: 165-174.

Gillam, A. H., A. G. Lewis, and R. J. Andersen. 1981. Quantitative determination of hydroxamic acids. Anal. Chem. 53: 841-844.

Gosselin, P., D. Joulain, P. Laurin, and F. Rouessac. 1989. Synthesis of earthy-moldy smelling compounds-I. stereoselective synthesis of (±)-geosmin. Tetrahedron Lett. 30 (21): 2775-2778.

Gross, E. D. 1994. Studies of the effects of chelated iron(III) and photodynamic action on the growth rate of *Lyngbya majuscula*. Dissertation, University of South Florida Tampa, FL. 190 pp.

Gross, E. D., D. F. Martin, and W. C. Sexton. 1991. A convenient method for measuring fresh weight of filamentous algae. Biomedical Lett. 46: 35-37.

Johnson, K. B. and D. F. Martin. 1988. Effect of fluorescein family dyes on the growth of the filamentous alga, *Lyngbya majuscula*. Microbios Lett. 38: 21-26.

Kato, Y. and P. J. Scheuer. 1974. The aplysiatoxins. Pure Appl. Chem. 41: 1-14.

Kato, Y. and P. J. Scheuer. 1976. The aplysiatoxins: reactions with acid and oxidants. Pure and Appl. Chem. 48: 29-33.

Lembi, C. A. 1986. Algal identification. Aquatics 8(2): 12-16.

Martin, B. B and D. F. Martin. 1987. Effects of four dyes on rates of oxygen production by three filamentous algae. Microbios Lett. 35: 151-154.

Martin, B. B. and D. F. Martin. 1988. Effect of fluorescein-family and other dyes on the growth of the red tide organism *Ptychodiscus brevis*. J. Environ. Sci. Health A23(8): 757-764.

Moikeha, S. N. and G. W. Chu. 1971. Dermatitis-producing alga *Lyngbya majuscula* Gomont in Hawaii. II. Biological properties of the toxic factor. J. Phycol. 7: 8-13.

Moikeha, S. N., G. W. Chu, and L. R. Berger. 1971. Dermatitis-producing alga *Lyngbya majuscula* Gomont in Hawaii. I. Isolation and chemical characterization of the toxic factor. J. Phycol. 7: 4-8.

Mullis, K. B., J. R. Pollack, and J. B. Neilands. 1971. Structure of schizokinen, an iron-transport compound from *Bacillus megaterium*. Biochem. 10(26): 4894-4898.

Persson, P. E. 1984. Uptake and release of environmentally occurring odorous compounds by fish. Water Res. 18(10): 1263-1271.

Schardt, J. D. 1989. 1988 Florida aquatic plant survey. Florida Department of Natural Resources, Tallahassee, Florida. 118 pp.

Schwyn, B. and J. B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160: 47-56.

Shannon, K., E. D. Gross, and D. F. Martin. 1992. Variation of growth of *Lyngbya majuscula* as a function of salinity. Biomed. Letters 47: 29-33.

Simpson, F. B. and J. B. Nielands. 1976. Siderochromes in cyanophyceae: isolation and characterization of schizokinen from *Anabaena* sp. J. Phycol. 12: 44-48.

Speziale, B. J. 1990. Department of Biological Sciences, Clemson University, Clemson, South Carolina. Personal communication.

Speziale, B. J. and L. A. Dyck. 1992. Lyngbya infestations: comparative taxonomy of *Lyngbya wollei* comb. nov. (cyanobacteria). J. Phycol. 28: 693-706.

Speziale, B.J., E. G. Turner, and L. A. Dyck. 1991. Physiological characteristics of vertically-stratified *Lyngbya wollei* mats. Lake and Reserv. Manage. 7(1): 107-114.

Speziale, B. J., E. G. Turner, and L. A. Dyck. 1988. "Giant" Lyngbya. Aquatics 10(2): 4-11.

Tucker, C. S. and C. E. Boyd. 1985. *In:* Channel Catfish Culture. Elsevier Science Publishers B.V., Amsterdam.

Williams, V. P., D. E. Canfield, Jr., M. M. Hale, W. E. Johnson, R. S. Kautz, J. T. Krummrich, F. H. Langford, K. Langeland, S. P. McKinney, D. M. Powell, and P. L. Shafland. 1985. Lake habitat and fishery resources of Florida. *In:* W. Seaman, Jr., Florida Aquatic Habitat and Fishery Resources, Florida Chapter, American Fisheries Association, Kissimmee, FL., pp. 43-119.