

MOLECULAR INVESTIGATIONS OF CYLINDROSPERMOPSIN PRODUCTION IN
FLORIDA LAKES

By

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To my parents, who supported me in every stage of my PhD.

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Abstract of Dissertation Presented to the Graduate School
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MOLECULAR INVESTIGATIONS OF CYLINDROSPERMOPSIN PRODUCTION IN
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Large numbers of toxic cyanobacterial blooms have been reported for lakes, reservoirs, and rivers around the world. The presence of toxic species in surface waters that are used for drinking water and recreation have received special attention due to health concerns and effects on ecosystems. Cylindrospermopsin (CYN), a cyanobacterial toxin, shows hepatotoxic, cytotoxic and genotoxic activities. In an attempt to identify CYN-producers in Florida, USA, different species and strains of filamentous cyanobacteria were isolated from lakes in the state. *Cylindrospermopsis raciborskii* is generally assumed to be the source of CYN in lakes and rivers in Florida. However, none of the eight Florida isolates of this species that were tested contained the genetic determinants involved in toxin production, nor did they produce CYN. I show for the first time that *Aphanizomenon ovalisporum* isolated from a pond in this state has the genes associated with CYN production. Analysis by liquid chromatography with mass spectrometric detection (LC/MS) revealed that it produced CYN in the range of 7.39- 9.33 $\mu\text{g mg}^{-1}$ freeze-dried cells. 16S rDNA sequences of this strain showed 99.6 % and 99.9 % identity to published *A. ovalisporum* and *Anabaena bergii* 16S sequences, respectively. To determine if CYN genes were present in the lakes examined and to reveal the variety of CYN producers, water samples were collected from different freshwater lakes in Florida. However, due to inhibitors co-purified

with genomic DNA, it was not possible to amplify CYN genes by using these templates. Humic substances are believed to be the most common inhibitors of enzymes used in molecular biology, such as Taq polymerase in PCR. A new approach to genomic DNA isolation from environmental cyanobacteria samples was developed. The genomic DNA obtained by the new method was of high quality, had a high molecular weight, was amplified in the Polymerase Chain Reaction (PCR), and was amenable to restriction nucleases. These methods, to my knowledge, are the first to describe the removal of PCR inhibitors from lake samples during cyanobacterial DNA isolation. CYN genes, polyketide synthase (PKS) and peptide synthetase (PS) were detected in three samples from Crescent Lake, Buffalo Bluff, and Lake George collected in May 2007. All sites had high numbers of *C. raciborskii* and low numbers of *A. ovalisporum*. Restriction Fragment Length Polymorphism (RFLP) analysis of PS genes suggested *A. ovalisporum* as the likely source of CYN in these systems.

CHAPTER 1 INTRODUCTION

Bacteria, cyanobacteria and fungi produce chemically and functionally different types of peptides and polyketides, which are collectively called secondary metabolites. Some of these secondary metabolites act as proteinase inhibitors, anti-algal and anti-fungal agents, immunosuppressors, and promoters of cell differentiation. Cyanobacterial toxins, such as microcystins and cylindrospermopsin, are examples of such secondary metabolites (Dittmann et al. 2001)

A large number of toxic cyanobacterial blooms have been reported for lakes, reservoirs, and rivers around the world (Paerl et al. 2001; Philips 2002). The presence of toxic species in surface waters represents a potential threat to the integrity and sustainability of aquatic ecosystems. Due to use of some affected systems as sources of drinking water and recreation, they are receiving special attention as a threat to human and animal health (Hitzfeld et al. 2000). In 1996 more than 50 dialysis patients died as a result of cyanobacterial toxins in a water supply in Brazil, and in 1989, a *Microcystis* bloom in a water storage reservoir in England killed 20 lambs and 15 dogs (Haider et al. 2003).

Classification of cyanobacterial toxins is generally based on two features: Chemical structure and mode of action (Table 1-1). Cylindrospermopsin is an unusual alkaloid (Figure 1-1) having both a cyclic guanidine group and a sulphate group that make it zwitterionic and highly water soluble, with a molecular weight of 415 (Norris et al. 2002; Griffiths and Saker 2003). It is stable in aqueous solutions of different pH and light conditions (Chiswell et al. 1999). The pyrimidine ring is essential for toxicity (Griffiths and Saker 2003)

There are three different chemical forms of cylindrospermopsin: Cylindrospermopsin, 7-epicylindrospermopsin, and deoxycylindrospermopsin. 24 hour LD₅₀ of cylindrospermopsin in a mouse bioassay is 2 mg kg⁻¹, whereas 5 day LD₅₀ of this toxin is 0.2 mg kg⁻¹. In the mouse

bioassay, cylindrospermopsin and 7-epicylindrospermopsin have the same toxicity.

Deoxycylindrospermopsin shows greatly reduced toxicity (Metcalf et al. 2002).

In Florida, USA, two species of cyanobacteria have been singled out as the most important sources of toxins in freshwater ecosystems, *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* (Chapman and Schelske, 1997; Philips, 2002). The focus of this dissertation was *C. raciborskii*, which is one of the most widespread and prolific bloom-forming species of algae in Florida.

Cylindrospermopsin has been reported to be produced by *Cylindrospermopsis raciborskii* isolated from various regions globally (Griffiths and Saker 2003). In 1979, *C. raciborskii* caused 148 residents of Palm Island, Australia to be hospitalized with hepatitis-like symptoms. In addition, deaths of cattle have been reported from different regions of Australia where *C. raciborskii* blooms occurred (Griffiths and Saker 2003). Symptoms of cylindrospermopsin damage in liver are distinguishable from damages caused by other hepatotoxins such as microcystins (Saker et al. 2003). There are no pathological differences of cylindrospermopsin toxicity in mice when the toxin is administered either as a suspension of freeze dried culture or purified compound, and there are no differences if it is administered orally or intraperitoneally (Norris et al. 2002).

The initial objectives of this dissertation were to define 1) The relationship between *Cylindrospermopsis* blooms and the presence of the toxin cylindrospermopsin (CYN), and 2) the environmental and genetic factors that may regulate the level of toxin production. *Cylindrospermopsis raciborskii* is generally assumed to be the source of CYN in lakes and rivers in Florida (Chapman and Schelske 1997). Early studies of CYN concentrations in Florida lakes have shown a poor relationship between the amount of toxin present and the size of

Cylindrospermopsis blooms (Aubel et al., 2006). Originally it was hypothesized that this observation was based on environmental regulation of toxin production. In the early phases of the current research effort, strains of *C. raciborskii* from a number of lakes and rivers in Florida were isolated and tested for both presence of the toxin and the genetic determinants for CYN production. Surprisingly, none of the *Cylindrospermopsis* isolates contained the genes for the production of the toxin. This led to a more intensive search for the organisms that may be responsible for the toxin production, which led to the discovery of a species of *Aphanizomenon* with the genetic determinants. This search forms the basis of Chapter 2 of this dissertation.

Building upon the unexpected results of the first phase of research, the search for CYN genes was extended to lake and river water samples in an effort to establish the scope of influence of *A. ovalisporum* as a producer of CYN. Cyanobacteria were harvested by filtration and used in whole community genomic DNA extraction. The presence of inhibitors in natural water samples co-purified with genomic DNA, however, made it impossible to amplify CYN genes. Humic substances are believed to be the most common inhibitors of enzymes used in molecular biology, such as Taq polymerase in PCR (Pan et al. 2002).

The third chapter of the dissertation describes the development of a new approach to cyanobacterial DNA isolation from lake samples. This method resolves problems associated with inhibition of PCR reactions. The genomic DNA obtained using the improved method was of high quality, had a high molecular weight, and was digested with restriction nucleases. This method, to my knowledge, is the first work to describe the removal of PCR inhibitors from lake samples during cyanobacterial DNA isolation.

The fourth chapter describes screening of lake samples from Hillsborough County, the Harris Chain of Lakes, and the St. Johns River system for CYN biosynthesis genes using the

improved DNA isolation method from environmental cyanobacteria. Amplification products from lakes were subjected to Restriction Fragment Length Polymorphism (RFLP) analyses and compared with fragments from *C. raciborskii* and *A. ovalisporum*. Results indicated that *A. ovalisporum* is the likely CYN producer in samples containing the CYN genes.

Table 1-1. Classification of cyanotoxins.

Type of toxin	Toxin	
Chemical structure	Cyclic peptides	microcystin, nodularin
	Alkaloids	anatoxin-a, anatoxin-a(s), cylindrospermopsin
Mode of action	Hepatotoxins	microcystin, cylindrospermopsin, nodularin
	Neurotoxins	anatoxin-a, anatoxin-a(s), saxitoxin
	Cytotoxins	cylindrospermopsin

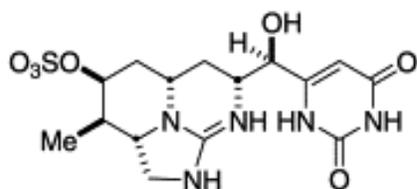


Figure 1-1. Structure of cylindrospermopsin.

CHAPTER 2
A COMPARATIVE STUDY OF FLORIDA STRAINS OF *Cylindrospermopsis* AND
Aphanizomenon FOR CYLINDROSPERMOPSIN PRODUCTION

Introduction

Cyanobacteria are prominent features of many freshwater and brackish systems around the world. Under suitable conditions they can reach high biomass levels and form blooms (Paerl et al., 2001; Philips, 2002). One of the major concerns with blooms is that certain cyanobacteria have the ability to produce cyanotoxins as a secondary metabolite. Cyanotoxins not only pose a risk to the integrity of aquatic ecosystems, but also to human health (Chorus and Bartram, 1999). One of the cyanotoxins of concern is cylindrospermopsin (CYN). First identified from *Cylindrospermopsis raciborskii*, CYN is a tricyclic guanidine alkaloid associated with production of harmful metabolites and the inhibition of protein synthesis (Froscio et al., 2003). It has been linked to gastrointestinal distress, liver damage, and, to a lesser degree, damage to other organs in a variety of animals (Falconer et al., 1999; Seawright et al., 1999; Shaw et al., 2000). CYN is genotoxic, causing DNA strand breakage (Humpage et al., 2000; Shen et al., 2002). The human health risk associated with *C. raciborskii* was highlighted by a poisoning incident on Palm Island, Australia in 1979, in which 148 people were hospitalized with hepatitis-like symptoms after exposure to CYN-contaminated water (Bourke et al., 1983; Hawkins et al., 1985).

Initially described as a tropical and sub-tropical species, *Cylindrospermopsis* has also been found in temperate lakes across Asia, Europe, North and South America, which demonstrates its wide physiological tolerance (Padisák, 1997; Briand et al., 2004). Although found worldwide, only the Australian and some Asian strains of *Cylindrospermopsis* have been shown to produce CYN (Hawkins et al., 1985; Li et al., 2001a). Some Brazilian strains have been shown to produce saxitoxin (Lagos et al., 1999), and some European strains of *C. raciborskii* have shown

varying degrees of toxicity to mice, rat hepatocytes and human cell lines, but without the demonstrated ability to produce CYN or saxitoxin (Fastner et al., 2003; Saker et al., 2003). Over the past decade, species of cyanobacteria other than *C. raciborski* have been shown to produce CYN, including *Umezakia natans* from Japan (Harada et al., 1994), *Raphidiopsis curvata* from China (Li et al., 2001b), *Anabaena bergii* from Australia (Schembri et al., 2001), *Aphanizomenon ovalisporum* from Israel (Banker et al., 1997), *Aphanizomenon flos-aquae* from Germany (Preussel et al., 2006), *Anabaena lapponica* from Finland (Spoof et al., 2006) and *Lyngbya wollei* from Australia (Seifert et al., 2007).

The recent observation that CYN is generated by a range of cyanobacteria species has initiated efforts to define the spatial distribution and sources of CYN. The goal of this study was to explore the capacity for CYN production among strains of *Cylindrospermopsis* encountered in Florida, USA, as well as other potential toxin-producing genera. *Cylindrospermopsis* was first reported in Florida in 1986 (Hodgson et al., 1986). Subsequent research has shown that *Cylindrospermopsis* is a major bloom-forming species in this state (Cichra et al., 1995; Chapman and Schelske, 1997; Philips et al., 2003). Although CYN has been detected in Florida lakes (Aubel et al., 2006), none of the isolated strains of this genus produce CYN or contain the genetic determinants involved in toxin production (Neilan et al., 2003; Kellmann et al., 2006). Schembri et al. (2001), by utilizing degenerate primers, determined for the first time that genes encoding polyketide synthase (PKS) and peptide synthetase (PS) enzymes are found in cylindrospermopsin-producing *C. raciborskii* and *Anabaena bergii* (*var. limnetica*), but are absent in strains that do not produce the toxin. Later work by Shalev-Alon et al. (2002) identified three genes putatively involved in cylindrospermopsin biosynthesis in *Aphanizomenon ovalisporum*. These genes, *aoaA* encoding an amidinotransferase, *aoaB* encoding a hybrid non-

ribosomal peptide synthetase/polyketide synthase, and *aoaC* encoding a polyketide synthase, were located in the same genomic region, suggesting their putative involvement in CYN biosynthesis. In the current study, individual strains of cyanobacteria were isolated from environmental samples collected from various sites in Florida and were screened for the presence of the two genes putatively associated with the production of CYN, PKS and PS. Representative isolates were tested for CYN production. Genetic relationships between isolates were also examined based on the gene sequence analyses of 16s rRNA. While none of the *Cylindrospermopsis* isolates produced CYN, I report for the first time the isolation of a CYN-producing *Aphanizomenon ovalisporum* from Florida.

Materials and Methods

Isolation, Culture and Morphometric Measurements of the Cyanobacteria

Water samples collected from lakes and rivers in Florida (USA) were enriched in screw cap tubes with BG-11 medium (Stanier et al., 1971) excluding a nitrogen source. After growth of cultures, individual filaments were transferred into 5 ml of fresh medium by micro-pipette. Isolated species were grown at 25 °C having a 12:12-h light-dark photoperiod under 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ supplied with cool white fluorescent lamps. They were screened for PKS and PS determinants after reaching sufficient density for DNA extraction.

Two *C. raciborskii* strains (QHSS/NR/Cyl/03 and FAS-C1) and an *A. ovalisporum* strain (FAS-AP1) were grown in 4-L flasks containing 2-L of medium in a manner similar to that described above. The remaining strains were kept in culture tubes and used in the Polymerase Chain Reaction (PCR) and Enzyme-Linked Immuno-Sorbent Assay (ELISA). A *C. raciborskii* strain QHSS/NR/Cyl/03 obtained from Queensland Health Scientific Services (Queensland, Australia) was used as a positive control for CYN production and genetic determinants. The list of strains used in this study and their sources are provided in Table 2-1.

Key morphological characteristics of cultured *A. ovalisporum* and *C. raciborskii*, preserved with Lugol's were determined microscopically using a Leica DM IL phase contrast inverted microscope (Leitz, Wetzlar Germany). Measurements of cells were at 400X and 1000X.

Genomic DNA isolation, Amplification and Sequencing

Cyanobacterial cells were harvested by centrifugation at 13000 rpm for 5 min. Genomic DNA isolation was performed using the Wizard Genomic DNA Purification Kit (Promega, WI, USA) following the method for gram-positive bacteria in accordance with the manufacturer's instructions. Amplification of PKS and PS gene fragments were performed on a Biometra thermal cycler (Biometra GmbH i. L., Goettingen, Germany) according to Schembri et al. (2001). Each PCR reaction contained 10-20 ng of genomic DNA, 20 pmol of each primer (MWG-Biotech Inc., NC, USA), 200 μ M of each deoxynucleoside triphosphates (Fisher Scientific Company L.L.C, PA., USA), 1.5 mM MgCl₂, 10 μ l of 5X green buffer, and 1.5 units of GoTaq DNA polymerase (Promega, WI, USA) in a total volume of 50 μ l. For amplification of partial PKS gene (624 bp), the PKS-specific primer pair M4 and M5 was used. PS-specific primers M13 and M14 were used to amplify a region of the PS gene (597 bp).

Approximately the 1470 bp region of the 16s rDNA was amplified with the primer pair 27F1 (Neilan et al. 1997) and 1516R (5'-ATCCAGCCACACCTTCCGG), the latter designed in this study. Each PCR reaction contained approximately 10-50 ng of genomic DNA, 20 pmol of each primer (MWG-Biotech Inc., NC, USA), 200 μ M of each deoxynucleoside triphosphates (Fisher Scientific Company L.L.C, PA., USA), 2.5 mM MgCl₂, 10 μ l of 5X green buffer, and 1.25 units of GoTaq DNA polymerase (Promega, WI, USA) in a total volume of 50 μ l. Amplification conditions for all were 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. To determine sizes, 100 bp DNA

ladder (Invitrogen, CA, USA) was run with PCR products on a 1.5% agarose gel. Gels were visualized under UV transillumination after staining with ethidium-bromide.

PCR generated fragments of PKS, PS and 16S rDNA were used for sequencing. Fragments were purified from 1.5% agarose gels using a Qiaquick MinElute gel extraction kit (Qiagen, CA, USA). For PKS and PS, the same primers used in PCR amplification were used for sequencing of both strands. 16S rDNA fragments were sequenced by using the newly designed sequencing primers 379F (5'-GAATTTTCCGCAATGGGC), 1118F (5'-AACGAGCGCAACCCTCG), 522R (5'-CCGTATTACCGCGGCTG), and 1180R (5'-CTTGACGTCATCCCCACCTT) along with PCR primers to obtain sequences on both strands. This was accomplished with a Perkin Elmer 377 automated DNA sequencer (Perkin Elmer, MA, USA), using the fluorescent dideoxy terminator method of cycle sequencing (Smith et al., 1986). Sequences were corrected manually using Vector NTI software (Invitrogen, CA, USA). Published sequences were obtained from the National Center for Biotechnology Information (NCBI) databases (<http://www.ncbi.nlm.nih.gov/>). DNA sequence alignments were performed by using Vector NTI software. GenBank accession numbers of the sequences obtained in this work are: EU076457 to EU076459 (16S rRNA gene); EU076460 and EU076462 (*aoaC*); EU076461 and EU076463 (*aoaB*).

Cylindrospermopsin Analysis

Two *C. raciborskii* strains (QHSS/NR/Cyl/03 and FAS-C1) and one *A. ovalisporum* strain (FAS-AP1) were analyzed for CYN by LC/MS. Cultures in the late exponential phase were harvested by filtration and freeze-dried. Two sets of cultures, 30-day old and 40-day old, were analyzed for each strain. FAS-C1 was the only Florida strain of *C. raciborskii* that reached sufficient density to harvest and freeze-dry at the time of analysis. The remaining Florida *C. raciborskii* isolates were tested with a CYN ELISA kit (Abraxis LLC, PA, USA).

For LC/MS analysis, CYN was isolated from freeze-dried tissues by extraction into a 1.5% solution of formic acid using a method based on the work by Törökne et al. (2004). Resulting extracts were analyzed using an HP1100 high-performance liquid chromatography system (Hewlett-Packard Company, Wilmington, DE) with UV detection in series with an LCQ MS (Thermo Electron, Cincinnati, OH). The analytes were separated across an Adsorbosphere HS C18 column (150 x 4.6 mm, 5 μ m; Alltech Chromotography, Deerfield, IL) under isocratic conditions at a flow rate of 0.6 ml/min in a mobile phase consisting of 5:95 methanol: water with 0.1% formic acid. All samples were monitored first at 262 nm before being introduced into the mass spectrometer via ESI probe in positive mode, where an m/z range of 300-2000 was evaluated for characteristic CYN fragmentation pattern. Based on the M + H⁺ ion (m/z 416), quantitation of CYN was determined against standard calibration curves ($R^2 > 0.995$) having at least five points. The limit of detection was $< 0.01 \mu\text{g mg}^{-1}$ freeze-dried cells. Both freeze-dried samples of 40-day old cultures and the remainder of the strains were also analyzed by an ELISA kit for CYN. This is a direct competitive ELISA kit utilizing polyclonal antibodies. Exponential phase cultures of each strain were harvested by centrifugation and were washed once with distilled water. Approximately 12 mg wet weight from each strain was extracted for CYN with glass beads in distilled water. After centrifugation, supernatants were used in the ELISA. Freeze-dried cultures were extracted in distilled water by glass-beads. After centrifugation, supernatants were used in the ELISA according to manufacturer's instructions.

Results

Morphology of *A. ovalisporum* and *C. raciborskii* Strains

Strain FAS-AP1 was identified as *Aphanizomenon ovalisporum* in accordance with morphological features designated by Komárek and Kováčik (1989) and Pollingher et al. (1998) (Figure 2-1). Morphological measurements (n= 30) of this strain are given in Table 2-2.

Terminal cells were elongated and blunt. Among the 100 trichomes observed, 48 had terminal cells that were completely hyaline, 31 were partially hyaline and 21 were not hyaline.

Heterocysts were roughly spherical. Terminal heterocysts were identified in 13% of trichomes (n=30) observed in the morphological analysis. Heterocysts were also located at various locations throughout the trichomes. Akinetes were either spherical or broadly oval.

Cylindrospermopsis raciborskii strains were identified according to the morphological features outlined by Komárek and Kling (1991). Morphological characteristics of *C. raciborskii* (n=30) revealed solitary, straight or slightly curved trichomes that had a length range of 41µm-182µm, with a mean of 89µm. The width of trichomes varied from 1.9µm to 2.7µm, with a mean of 2.4µm. Heterocysts were ovoid in shape and always terminal, with lengths from 6.0µm to 13µm and a mean of 8.2µm. The width at the base of the heterocysts ranged from 1.9µm to 2.7µm with a mean of 2.4µm. There were either one or two terminal heterocysts on each trichome.

Comparison of 16S rRNA Gene Sequences

16S rDNA sequence for *Aphanizomenon ovalisporum* strain FAS-AP1 (1431bp) showed 99.6 % identity to a previously published *A. ovalisporum* 16S rDNA sequence in the NCBI databases (1131/1136 identity with accession number AY335547). Similarly, the same sequence showed 99.9% identity to a published 16S rDNA sequence for *Anabaena bergii* (1398/1399 identity with accession number AF160256). *C. raciborskii* Florida strain FAS-C1 and the Australian strain of *C. raciborskii* QHSS/NR/Cyl/03 were also very similar in terms of their 16 S rDNA sequences with 99.7% (1428/1432) identity. Identity of 16S rDNA sequence for strain *A. ovalisporum* FAS-AP1 to sequences for the Florida strain of *C. raciborskii*, FAS-C1 and Australian strain QHSS/NR/Cyl/03 were lower, 93.1 % (1336/1435) and 93.2 % (1338/1435), respectively.

Amplification and Sequences of Partial PKS and PS Genes

Nine strains of *C. raciborskii* were included in this study, eight from Florida waters. Three of these strains (FL-D, FL-F, FL-I) were negative for PKS and PS determinants in previous studies (Neilan et al., 2003; Kellmann et al., 2006). The five remaining Florida isolates (FL-E, FL-L, FAS-C1, FAS-C2, FAS-C3) also tested negative for PKS and PS genes (Fig. 2-2). The known CYN-producing strain of *C. raciborskii* from Australia (QHSS/NR/Cyl/03) and the newly isolated and identified cyanobacterium *Aphanizomenon ovalisporum* (FAS-AP1) were the only strains giving positive amplification products for both of the genes (Fig.2-2).

Partial PKS sequence (583 bp) from *A. ovalisporum* strain FAS-AP1 shared 99.8% (539/540) and 100% (583/583) identity with published PKS sequences for *A. bergii* (accession number AF170844) and *A. ovalisporum* (accession number AF395828), respectively, in the NCBI databases. Partial PKS sequence (583 bp) from *C. raciborskii* strain QHSS/NR/Cyl/03 showed 99% to 100% identity to other published PKS sequences from *C. raciborskii*. Identity between strains FAS-AP1 and QHSS/NR/Cyl/03 was 99.3% (579/583) for the PKS sequences.

Partial PS sequence (552 bp) of *A.ovalisporum* strain FAS-AP1 shared 99.4% (531/534) and 100% (552/552) identity with sequences from *A. bergii* (accession number AF170843) and *A. ovalisporum* (accession number AF395828). Similarity of PS sequences between the *C. raciborskii* strain QHSS/NR/Cyl/03 (552bp) and other published *C. raciborskii* PS sequences ranged from 99% to 100%. PS sequence of QHSS/NR/Cyl/03 showed 96.4% (532/552) identity to PS sequence for FAS-AP1.

Toxin Content of Florida and Australian Isolates

CYN was not detected in the 30- or 40-day old cultures of *C. raciborskii* strain FAS-C1 by either the LC/MS (Figure 2-3) or ELISA methods. CYN was also not detected in the remaining seven Florida strains of *C. raciborskii* using ELISA. Concentrations of CYN obtained by

LC/MS and ELISA for strains QHSS/NR/CYL/03 and FAS-AP1 are shown in Table 2-3. In general, 40-day old cultures contained higher levels of CYN than the 30-day old cultures for these strains. As evaluated by LC/MS, CYN levels for FAS-AP1 were consistently higher than for QHSS/NR/CYL/03. Although the same freeze-dried materials used for LC/MS evaluation were extracted and tested independently with different lots of the CYN ELISA kit, the ELISA results were never in agreement with the LC/MS results for FAS-AP1; in each case the ELISA results were about half the level determined by LC-MS. In contrast, CYN concentrations, determined by LC/MS and ELISA for strain QHSS/NR/CYL/03, were in excellent agreement. The difference in performance between the LC/MS and ELISA methods for FAS-AP1, as compared to QHSS/NR/CYL/03, may be due to the presence of an unidentified CYN isomer or congener produced by one of the strains. While a full comparative method evaluation was beyond the scope of this study, I intend to investigate the cause(s) of these differences in future work.

Discussion

The results of this study demonstrate that the ability to produce certain toxins can vary considerably among strains of cyanobacterial species that are largely indistinguishable from a morphological perspective. This is a significant observation considering the fact that cultural eutrophication of surface waters has increased the world-wide occurrences of putatively toxic algal blooms (Chorus and Bartram, 1999; Philips, 2002). In many freshwater ecosystems, intense algal blooms are dominated by cyanobacteria (Canfield et al., 1989). Florida contains over 7,000 lakes, many of which are eutrophic, and the toxin-producing ability of ubiquitous species like *C. raciborski* is a critical water management issue. *C. raciborskii* blooms have been observed in numerous lakes and rivers in this state (Cichra et al., 1995; Chapman and Schelske, 1997; Philips et al., 2003). Owing to the fact that Australian and Brazilian strains of *C. raciborskii* produce the

toxic compounds, CYN and saxitoxins, widespread occurrences of *C. raciborskii* blooms have raised serious concerns about risks to the integrity of affected ecosystems, as well as human health. To my knowledge, however, none of the isolated strains of this genus from Florida produce CYN or carry the genetic determinants involved in CYN production (Neilan et al., 2003; Kellmann et al., 2006). The eight Florida strains of *C. raciborskii* examined in this study also did not produce PCR bands corresponding to PKS and PS gene fragments. These conclusions are further supported by the results of CYN analysis of unialgal cultures of Florida isolates of *C. raciborski* using LC/MS and ELISA, which did not yield measurable quantities of CYN, in contrast to significant quantities present in cultures of *C. raciborskii* from Australia (QHSS/NR/Cyl/03). While these data do not rule out the possibility of a CYN-producing *C. raciborskii* in Florida, it adds weight to the hypothesis that most Florida strains do not produce CYN. These results also help explain the general lack of a defined relationship between abundance of *C. raciborskii* in freshwater ecosystems of Florida and observed concentrations of CYN (Aubel et al., 2006). The latter observation raises the possibility that previous reports of CYN may have been caused by coincidental, but unrecorded presence of another CYN-producing species.

In an effort to identify species of cyanobacteria that may be responsible for CYN production, a number of lake and river water samples were screened in this study for genes putatively associated with CYN production. A sample from Duval County, Florida gave positive bands for PKS and PS determinants (data not shown). This sample contained both *C. raciborskii* and an unidentified filamentous cyanobacterium. Isolation and testing of both species revealed that the genetic determinants for PKS and PS were present only in the unidentified cyanobacterium. Morphological observations of the latter isolate (FAS-AP1) indicate that it is

most closely allied with *Aphanizomenon ovalisporum*. Intracellular concentrations of CYN associated with *A. ovalisporum* strain FAS-AP1 (i.e. 7.39 $\mu\text{g mg}^{-1}$ freeze-dried cells) fall within the higher range of reported CYN concentrations in all species tested (Fastner et al., 2003; Törökne et al., 2004; Preussel et al., 2006). To my knowledge, this is the first report of a CYN-producing cyanobacterium in the USA.

Morphological observations and measurements of *A. ovalisporum* FAS-AP1 overlap with shapes and sizes of vegetative cells, heterocysts, and akinetes of strains previously identified as *A. ovalisporum*, *Anabaena bergii* and *A. minderi* (*A. bergii* var. *limnetica*) (Pollingher et al., 1998; Shaw et al., 1999; Hindák, 2000). Komárek and Kováčik (1989) suggest that all three species should be placed in the genus *Aphanizomenon*. However, the debate as to whether these species belong to *Anabaena* or *Aphanizomenon*, or whether they should be in a different genus, continues (Komárek and Kováčik, 1989; Hindák, 2000; Gkelis et al., 2005). According to our observations and measurements, strain FAS-AP1 is most similar to *A. ovalisporum* and *A. bergii*. The akinetes of strain FAS-AP1 are either spherical or broadly oval, contrary to long elliptical akinetes in *A. minderi* (*A. bergii* var. *limnetica*) (Hindák, 2000). It should also be noted that different culture conditions may affect shapes and sizes of cells, heterocysts and akinetes (Pollingher et al., 1998), complicating morphological comparisons.

The results of sequence analyses of 16S rDNA of the Florida strain of *A. ovalisporum* show 99.6 % and 99.9% identity to published 16S rDNA sequences for *A. ovalisporum* and *Anabaena bergii* (var. *limnetica*), respectively. Although FAS-AP1 is more similar to *A. bergii* (var. *limnetica*) in terms of their 16S sequences, based on the morphological criteria discussed above, I identify FAS-AP1 as *Aphanizomenon ovalisporum*. In addition, there are no available morphological descriptions for *A. bergii* (var. *limnetica*) for which the 16S rDNA sequence is

available. It has been suggested that these two species are morphological variants of the same species (Schembri et al., 2001). In a phylogenetic study based on 16S rRNA gene sequences, *A. bergii* (var. *limnetica*) clustered closely with some *Nodularia*, *Cyanospira*, and *Anabaenopsis* strains (Rajaniemi et al., 2005). Another phylogenetic study also clustered *A. ovalisporum*, *A. bergii* (var. *limnetica*), a *Umazekia*, an *Anabaenopsis* and a *Cyanospira* strain together. Based on similarity of 16S rDNA sequences, Kellmann et al. (2006) suggested that these species can be included in a revised genus. My results further support the need for a taxonomic revision of *Aphanizomenon ovalisporum*, *Anabaena bergii* and *Anabaena minderi*.

In the case of *Cylindrospermopsis*, comparisons of 16S rDNA sequences between the Australian strain of *C. raciborskii* QHSS/NR/Cyl/03 and the Florida strain FAS-C1 reveal high sequence similarity between these two strains (99.7 % identity). This is in good agreement with previous reports of *Cylindrospermopsis* strains from different geographic regions having high sequence similarities and forming a monophyletic group. However, slightly lower similarities are found between FAS-C1 and some of the previously published 16S rDNA sequences for Florida strains, ~ 99.1% as already reported by Neilan et al. (2003) for other Florida strains.

PKS sequences for *A. ovalisporum*, *A. bergii* and *C. raciborskii* share high sequence identity (99 - 100%). Although PS sequence identities within *A. ovalisporum*, *A. bergii* and *C. raciborskii* strains are very high (99-100%), they are lower when compared with each other (95-96%). Kellmann et al. (2006) suggest that a horizontal gene transfer event may have occurred between these species.

Considering the variety of CYN producers observed around the world, *A. ovalisporum* may be one of several species of cyanobacteria that produce CYN in freshwater ecosystems of

Florida. More species should be isolated and tested from these systems. Furthermore, this study demonstrates that *C. raciborskii* is probably not a major CYN producer in Florida.

Table 2-1. List and origins of the strains used in the study.

Species	Strain	Origin
<i>C. raciborskii</i>	FL-D	St. Johns River, Florida
	FL-E	St. Johns River, Florida
	FL-F	St. Johns River, Florida
	FL-I	St. Johns River, Florida
	FL-L	St. Johns River, Florida
	FAS-C1	Lake Griffin, Florida
	FAS-C2	Fishpond, Jacksonville, Florida
	FAS-C3	Lake Brant, Florida
	QHSS/NR/Cyl/03	Australia
<i>A. ovalisporum</i>	FAS-AP1	Fishpond, Jacksonville, Florida

Table 2-2. Morphometric measurements (μm) of *Aphanizomenon ovalisporum* strain FAS-AP1.

		Mean	\pm SD (n=30)	Minimum	Maximum
Trichome	Length	243.9	127.1	34.0	628.0
Vegetative cell	Length	4.0	0.9	2.5	5.5
	Width	4.5	0.9	2.0	5.0
Terminal vegetative cell	Length	4.9	1.0	3.0	7.9
	Width	3.8	0.9	3.0	6.0
Heterocyst	Length	6.4	1.1	4.8	8.2
	Width	7.1	1.1	5.8	8.0
Akinete	Length (n=11)	10.7	1.8	9.0	15.0
	Width (n=11)	7.6	1.8	6.0	12.0

Table 2-3. Concentrations of CYN in *C. raciborskii* strains and *A. ovalisporum* as determined by LC/MS and CYN ELISA.

Strain	CYN (LC/MS) ($\mu\text{g mg}^{-1}$ freeze-dried cells)		CYN (ELISA) ($\mu\text{g mg}^{-1}$ freeze-dried cells)
<i>C. raciborskii</i> QHSS/NR/Cyl/03	3.44 ^a	6.73 ^b	6.61 \pm 0.20 ^b (n=3) ^c
<i>C. raciborskii</i> FL-D	— ^e	—	ND ^d
<i>C. raciborskii</i> FL-E	—	—	ND
<i>C. raciborskii</i> FL-F	—	—	ND
<i>C. raciborskii</i> FL-I	—	—	ND
<i>C. raciborskii</i> FL-L	—	—	ND
<i>C. raciborskii</i> FAS-C1	ND ^a	ND ^b	ND ^b
<i>C. raciborskii</i> FAS-C2	—	—	ND
<i>C. raciborskii</i> FAS-C3	—	—	ND
<i>A. ovalisporum</i> FAS-AP1	7.39 ^a	9.33 ^b	5.03 \pm 0.36 ^b (n=3) ^c

^a30-day old culture; ^b40-day old culture; ^cSamples for ELISA were run in triplicate, ^dNot detected, ^eNot analyzed.

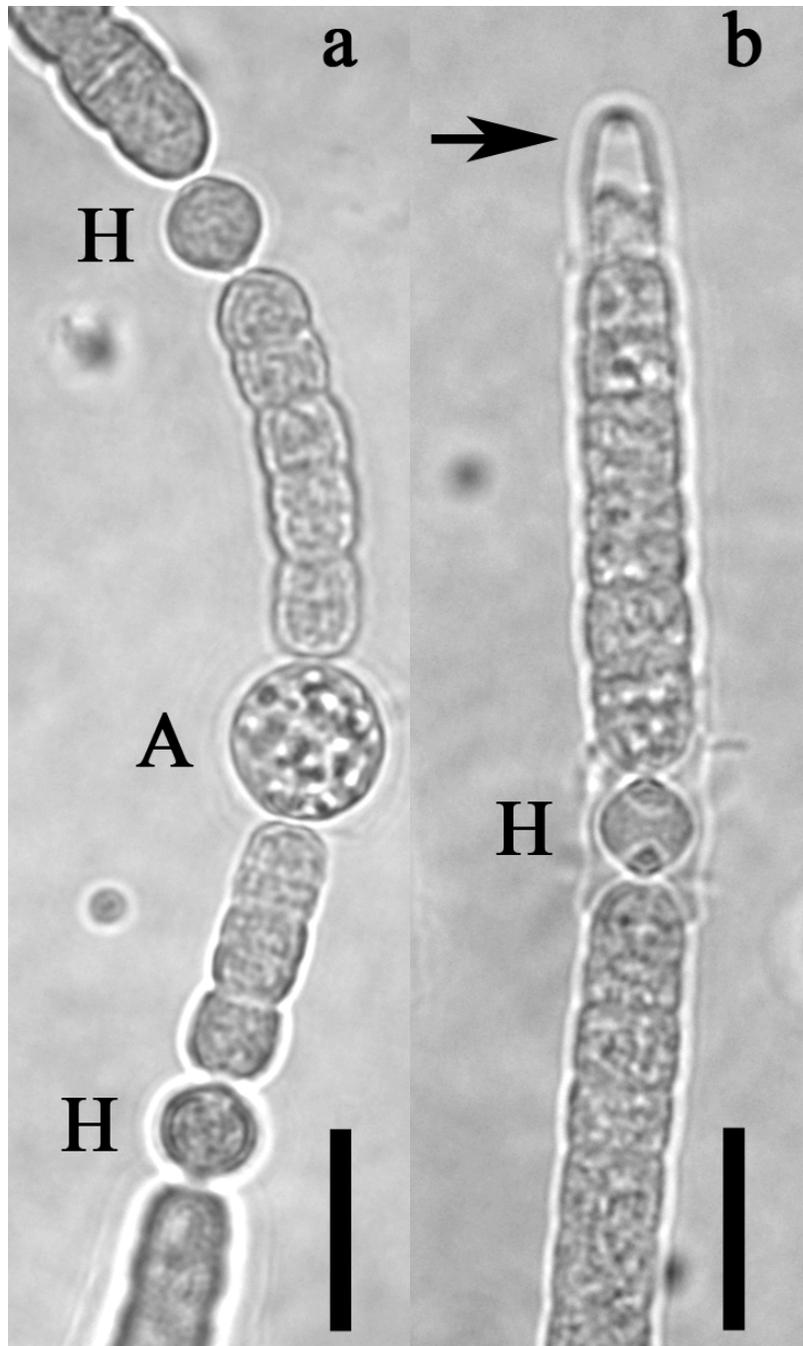


Figure 2-1. Trichomes (a, b) of isolated *A.ovalisporum* strain FAS-AP1 showing heterocysts (H), akinete (A), and terminal hyaline cell (arrow).Bar 10 μ m.

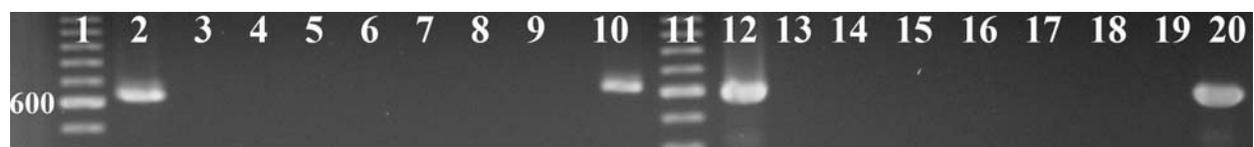


Figure 2-2. Lanes 1 and 11, size marker; Lanes 2-10, PCR products for partial PKS genes: Lanes 2) QHSS/NR/CYL/03; 3) FAS-C1; 4) FAS-C2; 5) FAS-C3; 6) FL-D; 7) FL-E; 8) FL-F; 9) FL-L; 10) FAS-AP1. Lanes 12-20, PCR products for partial PS genes: 12) QHSS/NR/CYL/03; 13) FAS-C1; 14) FAS-C2; 15) FAS-C3; 16) FL-D; 17) FL-E; 18) FL-F; 19) FL-L; 20) FAS-AP1. 600 denotes the 600 bp band in the size marker.

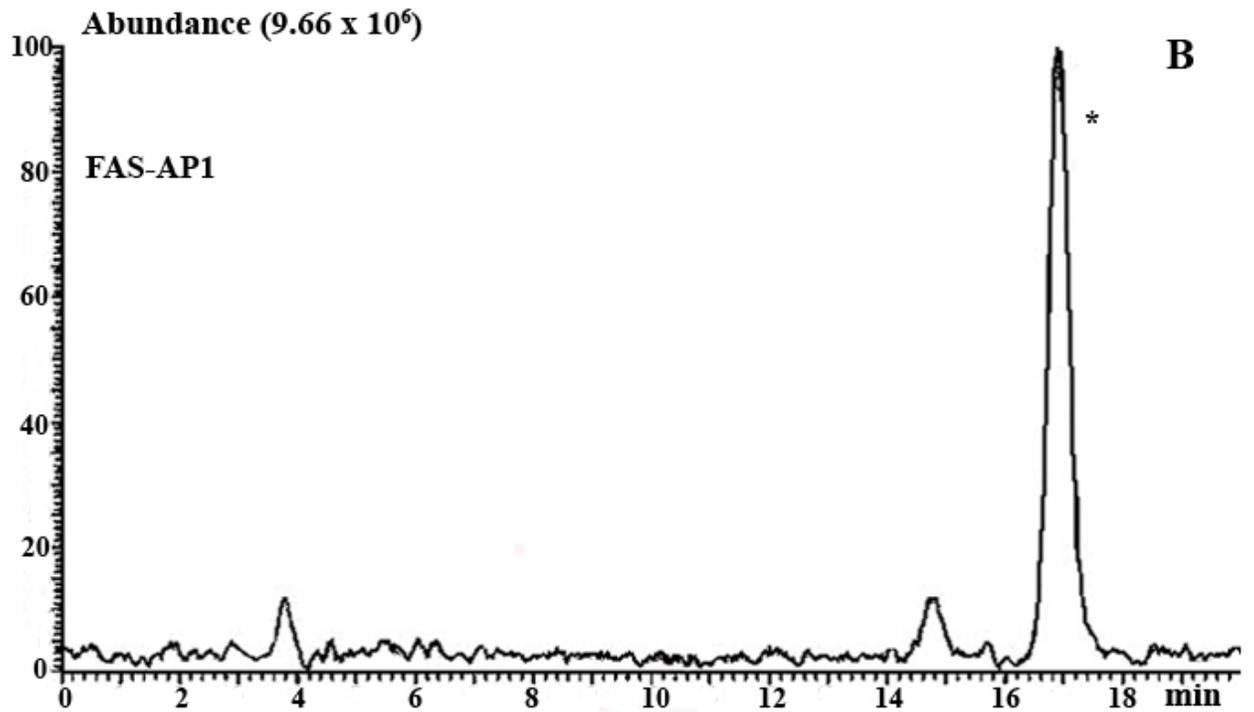
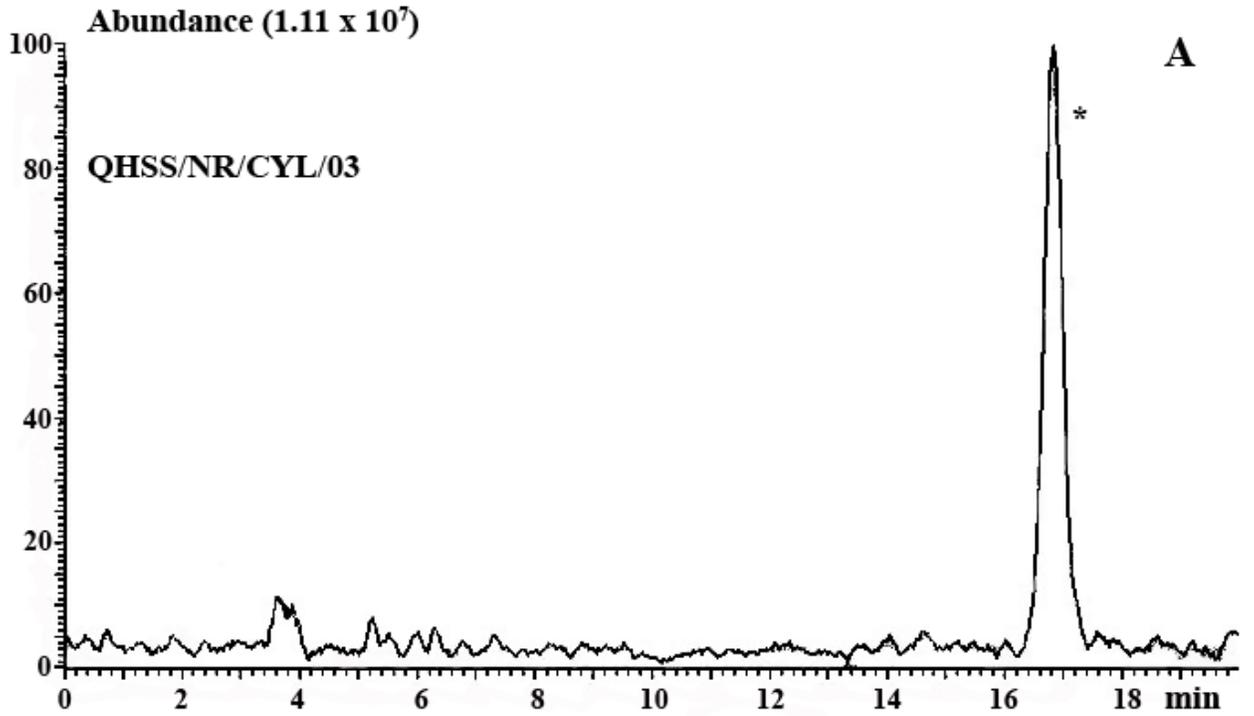


Figure 2-3. Selected ion chromatograms (m/z 416) for QHSS/NR/CYL/03 (A), FAS-AP1 (B), and FAS-C1 (C). The characteristic peak for CYN (*) appears at a retention time of 16.8 min for the two strains shown to produce the toxin.

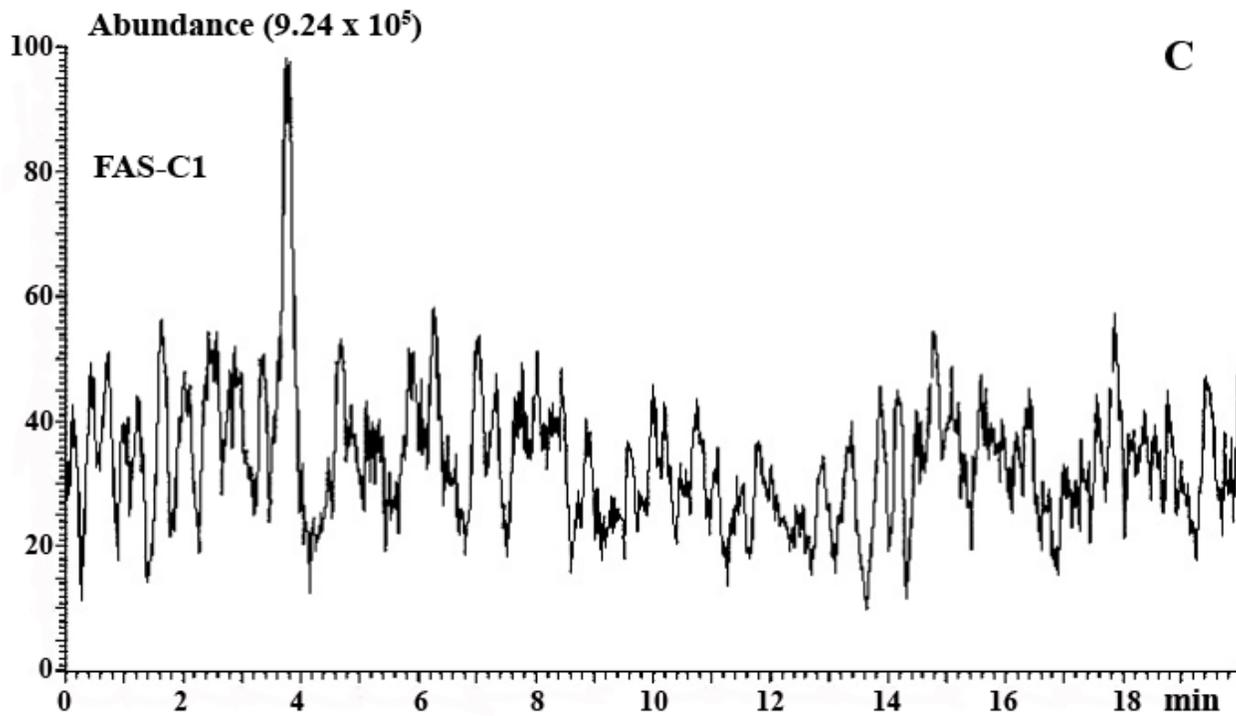


Figure 2-3. Continued.

CHAPTER 3
AN IMPROVED METHOD FOR THE ISOLATION OF INHIBITOR-FREE
CYANOBACTERIAL DNA FROM ENVIRONMENTAL SAMPLES

Introduction

Molecular approaches have greatly expanded understanding of the diversity of microorganisms in freshwater and marine environments (Beja 2004; Tringe and Rubin 2005). Given that the majority of these microorganisms cannot be cultured in the laboratory, environmental DNA isolation and Polymerase Chain Reaction (PCR) of samples from the natural environment, along with cloning and gene sequencing, provide a powerful tool for identifying novel microorganisms and their metabolites (Amann et al. 1995).

Cyanobacteria are a large, diverse and important group of organisms in freshwater environments. They commonly reach very high cell densities and form blooms in many freshwater ecosystems (Phlips 2002). Species within the group have various competitive advantages over other algae, such as the ability to fix atmospheric nitrogen, the ability to regulate buoyancy in the water column, and production of toxins. The ability to produce toxins also make certain species of cyanobacteria a concern for human and animal welfare (Haider et al. 2003).

Isolation of individual species of cyanobacteria has been an effective method for the identification of toxins produced by certain key species (Sivonen et al. 1990; Harada et al. 1994). Cultures of isolated cyanobacteria have also been used to identify genes responsible for the biosynthesis of toxins (Tillett et al. 2000; Schembri et al. 2001). Degenerate or specific primers designed from these gene sequences have been used to search for toxin genes in environmental samples (Schembri et al. 2001; Hisbergues et al. 2003; Yilmaz et al. 2007). This approach has been used to determine the species responsible for toxin production in natural water samples, either by cloning, sequencing strategies or RFLP methods (Hisbergues et al. 2003). All these methods require so-called “inhibitor-free” DNA, because these molecular methods are

easily inhibited by environmental contaminants (Pan et al. 2002). The presence of contaminants also poses serious barriers to the quantification of environmental DNA (Zipper et al. 2003).

Cyanobacterial DNA exists free in the cellular cytoplasm surrounded by a multilayered envelope, demonstrating both Gram-positive and Gram-negative features. Some filamentous cyanobacteria possess S-layers, fibrillar structures and sheaths outside of the outer membrane, making it difficult to lyse cells for extraction of DNA (Hoiczky and Baumeister 1995).

Methods exist for DNA isolation from cultured cyanobacteria. Not every method for DNA extraction performs equally well for different cyanobacterial strains due to variations in cell wall characteristics, cell size, and the presence or absence of sheaths. Modifications of basic methods, however, can be applied to yield good quality genomic DNA from cyanobacterial isolates cultured under laboratory conditions (Mak and Ho 1992; Fiore et al. 2000; Tillett and Neilan 2000). Problems, however, can arise when cyanobacteria are harvested directly from the natural environment. Contaminants such as humic (HA) and fulvic acids are often co-purified along with DNA from environmental samples. These substances inhibit PCR and restriction digestion of the DNA (Zhou et al. 1996; Jackson et al. 1997; Pan et al. 2002). Current methods of cyanobacterial DNA isolation do not effectively eliminate these inhibitory substances. Bovine serum albumin or skim milk has been used to circumvent the effects of inhibitors (Pan et al. 2002). Chelex-based kits have also been applied successfully in some DNA isolations (Wilson et al. 2000). Both approaches, however, have yielded only partial success.

Similar inhibitors of PCR and restriction digestion of DNA are commonly encountered in DNA isolation from bacteria in soil samples. Soils rich in organic content often contain high levels of humic and fulvic acids. Many methods exist for eliminating inhibitors from soil DNA, however, no single method is used universally (Schneegurt et al. 2003). In most cases, after

isolation, DNA is purified using chromatography columns, agarose gel electrophoresis, or glass milk to remove humics and other inhibitors, thereby increasing the cost and time needed to obtain good quality DNA and reducing ultimate DNA yields (Miller et al. 1999; England et al. 2001).

Humic substances are also very abundant in the freshwater and marine environments, constituting a significant portion of the colored dissolved organic matter (CDOM) in these systems. In freshwater systems, humic substances originate from decay of algae, bacteria and plants, including soil leaching and surface runoff from terrestrial environments (Anesio et al. 2005). During a study on the distribution of toxic cyanobacteria in lakes in Florida (USA), I encountered problems in isolating inhibitor-free DNA from these freshwater ecosystems. Several approaches including commercial kits and published methods did not yield DNA suitable for PCR. Building upon published methods on soil DNA extraction, and modifying the xanthogenate nucleic acid isolation method of Tillett and Neilan (2000), I developed an improved method for removing inhibitory substances from DNA of environmental cyanobacteria samples collected from natural environments characterized by high amounts of colored dissolved organic matter (CDOM). Because a large number of lakes, rivers and estuaries throughout the world contain high levels of CDOM, this method will provide a valuable new tool for expanded application of molecular genetics to limnological and oceanographic research.

Materials and Methods

Cyanobacterial Strains Used and Collection of Lake Samples

Microcystis aeruginosa PCC 7806 and *Aphanizomenon ovalisporum* FAS-AP1 were grown in BG-11 medium (Stanier et al. 1971) at 25 °C. Light was provided on a 12:12hr light:dark photoperiod at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ from cool white fluorescent lamps. Cells in

exponential growth phase were harvested by centrifugation at 13,000 rpm for 10 minutes. Wet-weight of cells was recorded, and tubes were kept frozen at -20 °C until genomic DNA isolation.

Natural water samples were collected from a suite of Florida lakes (Table 3-1). Water samples were collected using a vertical sampling pole and filtered onto 5 or 10 µm nylon filters depending on the phytoplankton species present and algal cell density of the sample. Microorganisms were scraped off filters and suspended in distilled water by pipetting. Equal amounts of cells were distributed in pre-weighed microcentrifuge tubes, and tubes were centrifuged for 5-7 minutes at 13,000 rpm. Supernatants were removed, tubes were weighed and samples were kept frozen at -20°C until DNA isolation. In the case of cells with gas vacuoles, these were disrupted by pressure in a syringe before centrifugation.

Genomic DNA Isolations

Genomic DNA was isolated from laboratory cultures and lake samples using the xanthogenate nucleic acid isolation method (XS-isop method) (Tillett and Neilan 2000) with modifications introduced in this study. In addition, Instagene matrix (Bio-Rad, CA, USA) was tested for its ability to remove PCR inhibitors. Instagene matrix isolations were performed according to Neilan (2002). Approximately 10-20 mg wet-weight of cells was used for each DNA isolation.

In the standard XS-isop method, cells were suspended in 50 µl TE buffer at pH 7.4 (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8). Then, 750 µl of XS buffer (1% potassium ethyl xanthogenate; 100 mM Tris-HCl, pH 7.4; 20 mM EDTA, pH 8; 800 mM ammonium acetate; and 1% SDS) were added to the suspension. Tubes were incubated for 2 h at 70 °C. After incubation, the tubes were vortexed for 30 s and kept on ice for 30 min. Following centrifugation for 10 min at 13,000 rpm, supernatants were transferred to clean micro-centrifuge tubes, and 0.7 volumes of isopropanol were added. Tubes were then incubated at room temperature for 10 min. After

centrifugation at 13,000 rpm for 10 min, pellets were washed in 70% ethanol, air dried and suspended in double distilled and autoclaved water.

New modifications to the aforementioned method were: (1) DNA was precipitated with 7% final concentration of polyethylene glycol 8000 (PEG 8000) with 10mM MgCl₂ (XS-PEG) instead of 0.7 volumes of isopropanol. (2) 3% polyvinylpolypyrrolidone (PVPP) was included in the XS buffer, and DNA was precipitated with either 0.7 volumes of isopropanol (XS-3%PVPP-isop) or 7% PEG 8000 with 10 mM MgCl₂ (XS-3%PVPP-PEG). (3) Cells were suspended in 50 µl of TE buffer at pH 7.4. Before addition of XS buffer with 3% PVPP, cells were prewashed with 1 ml of TE buffer pH 7.4 containing 3% PVPP by vortexing at high speed for 30 s. Two µl of 3 M CaCl₂ was added to precipitate PVPP, followed by another 30 s vortexing. After three minutes on the bench, supernatants were transferred to clean micro-centrifuge tubes and cells were pelleted by centrifugation at 13,000 rpm for 5 minutes (Wechter et al. 2003). Cells were re-suspended in 50 µl of TE buffer at pH 7.4, and 750 µl of XS buffer with 3% PVPP was added. DNA was precipitated with either 0.7 volumes of isopropanol (prewashed-isop) or 7% PEG 8000 with 10 mM MgCl₂ (prewashed-PEG). For all methods, DNA pellets were washed with 70% ethanol (twice if PEG was used), air-dried, and suspended in double distilled and autoclaved water.

DNA Quantification

DNA was quantified by three methods: (1) Spectrophotometric measurements were performed on 2 µl aliquots of processed samples using a Nanodrop ND-1000. When calculating DNA concentrations, 340 nm absorbance values were used to normalize 260 nm readings with this instrument. In trials, isopropanol and PEG methods resulted in significant differences in 340-nm values. Absorbance values around 340 nm can be a consequence of humic substances in the samples. For this reason I used 260-nm readings without 340-nm normalization to calculate

DNA concentrations. (2) Fluorometric measurements were performed using 5-10 μ l aliquots of processed genomic DNA solution with a Quant-iT dsDNA HS assay kit and a Qubit fluorometer (Invitrogen, CA, USA), in accordance with the manufacturer's instructions. (3) DNA concentrations were also determined using electrophoretic methods. Ten μ l aliquots of genomic DNA samples were run on 0.8% agarose gels along with 0.5 μ g λ -HindIII DNA fragments (Invitrogen, CA, USA). After ethidium bromide staining, gels were visualized under UV-transillumination. Band intensities of genomic DNAs were compared to those of the λ -HindIII fragments using the ImageJ software (<http://rsb.info.nih.gov/ij/>). Samples from Lakes Dora (06/26/2007) and Griffin (05/30/2007) and the *A. ovalisporum* FAS-AP1 culture were used for DNA yield comparisons. Duncan's multiple range test ($p=0.05$) was used to compare mean values obtained using different methods.

Polymerase Chain Reaction

To test effectiveness of the new DNA isolation methods in removing PCR inhibitors, genomic DNAs isolated with different methods were amplified for a 1470 bp region of 16S rDNA, 685 bp region of the phycocyanin intergenic spacer region (PC-IGS), and approximately 300 bp of one of the condensation domains of the *mcyA* gene within the microcystin synthetase operon. Primers used in the study are listed in Table 3-2. 16S and PC-IGS reactions contained 2 μ l of genomic DNA, 20 pmol of each primer (MWG-Biotech Inc., NC, USA), 200 μ M of each deoxynucleoside triphosphates (Fisher Scientific Company L.L.C, PA, USA), 2.5 mM $MgCl_2$, 10 μ l of 5X green buffer, and 2 units of GoTaq DNA polymerase (Promega, WI, USA) in a total volume of 50 μ l. Reaction components for *mcyA* PCR were similar except that 300 μ M of each deoxynucleoside triphosphate was added, and the $MgCl_2$ concentration was increased to 4.5 mM (Hisbergues et al. 2003). Amplification for PC-IGS and *mcyA* started with denaturation of the genomic DNA at 95 $^{\circ}C$ for 3 min, followed by 30 cycles of 95 $^{\circ}C$ for 30 s, annealing at 55 $^{\circ}C$ for

30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Amplification conditions for 16S rDNA were similar except that annealing temperature was increased to 60 °C. PCR products were pooled, normalized and run on a 2% agarose gel in TBE buffer (89 mM Tris, 2 mM EDTA, 89 mM Boric Acid), stained with ethidium bromide and visualized with UV transillumination.

Restriction Endonuclease Digestion of Genomic DNA

Genomic DNAs from *Microcystis aeruginosa* PCC 7806 laboratory cultures and lake samples were isolated using XS-isop, XS-PEG, or XS-3%PVPP-PEG methods. Each restriction reaction contained 1 U of the restriction endonuclease BmrI (New England Biolabs, Inc), 5 µl of buffer, and 40 µl of genomic DNA solution (80-400 ng DNA), in a total volume of 50 µl. Samples were incubated overnight at 37 °C. Negative controls used the same amount of template without the enzyme BmrI. Digests were run on a 0.8% agarose gel in TBE buffer, stained with ethidium bromide and visualized with UV transillumination.

Results

DNA Yield with Different Measurement Methods

For almost all cases, the spectrophotometric (Nanodrop) quantification method yielded more genomic DNA than fluourometric (Qubit) or gel densitometry (Image J) methods. In ten of twelve comparisons, fluourometry and gel densitometry values were not significantly different from each other (Table 3-3).

For lake samples, in general, there was no significant difference between XS-isop and XS-PEG methods in terms of DNA yield obtained using the fluourometric and gel densitometry quantification methods. Only the Lake Griffin sample showed a statistically significant decrease in fluourometry between the mentioned isolation methods. There were significant differences between the two isolation methods when the spectrophotometric method was used.

DNA yield was similar between the XS-isop and XS-3%PVPP-isop methods for the lake samples, except for the Lake Griffin sample with fluorometry and Lake Dora sample with spectrophotometry. Only the Lake Griffin sample with the spectrophotometric method, and the Lake Dora sample with the fluorometric method yielded significantly different DNA concentrations when XS-PEG and XS-3%PVPP-isop methods were compared.

In general, the XS-3%PVPP-PEG method yielded the lowest DNA concentrations. Concentrations with this method were significantly lower when compared to those of the XS-isop method. The XS-3%PVPP-PEG method yielded a similar concentration only in the gel densitometry for the Lake Dora sample. For this sample, there were no significant differences in gel densitometry concentrations regardless of the isolation methods used. XS-3%PVPP-PEG values were significantly different from XS-PEG and XS-3%PVPP-isop methods in seven of twelve comparisons.

The situation was different with the *A. ovalisporum* FAS-AP1 culture. All isolation methods yielded significantly different concentrations when spectrophotometry was used. With fluorometry and gel densitometry, XS-isop and XS-PEG yields were significantly different most of the time, while there was no significant difference between XS-isop and XS-3%PVPP-isop. XS-PEG and XS-3%PVPP-isop methods yielded significantly different concentrations for all measurement methods. For fluorometry, the yield was significantly different between XS-PEG and XS-3%PVPP-PEG, while no difference was observed when yield was analyzed with gel densitometry. XS-3%PVPP-PEG concentrations were significantly different from XS-PEG and XS-3%PVPP-isop in five of six comparisons.

Isopropanol versus PEG Precipitation

Cells collected from Lake George (07/10/2007) were used in DNA isolations to determine effects of isopropanol versus PEG 8000 precipitation of genomic DNA in obtaining PCR

amplifiable templates,. The samples from Lake George were characterized by high CDOM levels (>50 platinum cobalt units). Amplification of 16S rDNA, PC-IGS and *mcyA* genes was inhibited when genomic DNA was precipitated with isopropanol whether in regular XS buffer or XS buffer with 3% PVPP (Figure 3-1). Isopropanol precipitation was only successful when cells were prewashed with PVPP and DNA isolated in XS buffer with 3% PVPP (data not shown). PEG 8000 precipitated genomic DNA was amplified for all three genes. Instagene matrix was not effective in removing PCR inhibitors from this sample. *Aphanizomenon ovalisporum* FAS-AP1 and *Microcystis aeruginosa* PCC 7806 were used as positive controls for each DNA isolation method. Expected amplifications from each strain were obtained from all isolation methods. *A. ovalisporum* did not have any *mcyA* bands, in agreement with microcystin ELISA results that were negative for this strain (data not shown). Instead, this strain produced cylindrospermopsin.

The effect of the XS-PEG method in removing PCR inhibitors was also tested for six other lake samples for the *mcyA* gene (Figure 3-2). Lake samples used in these analyses exhibited high CDOM levels (i.e. 50 platinum cobalt units). While all XS-isop preparations were inhibited, the XS-PEG method provided DNA that was amplified for all lakes.

Restriction Digestion of Genomic DNA

Samples from Lake Griffin (06/26/2007), Crescent Lake (07/10/2007), Buffalo Bluff (07/10/2007), and Lake George (08/09/2007) were used in genomic DNA isolations with XS-isop, XS-PEG and XS-3%PVPP-PEG methods. *Microcystis aeruginosa* PCC 7806 genomic DNA was used as the positive control, and its DNA was cut with BmrI for all isolation methods. The restriction enzyme was inhibited with XS-isop preparations for all lake samples, whereas all samples from XS-PEG and XS-3%PVPP-PEG preparations were digested with the enzyme used.

A gel sample is shown in Figure 3-3 for Crescent Lake, Buffalo Bluff and *M. aeruginosa* PCC 7806 comparing activity of the restriction enzyme on XS-isop and XS-PEG isolations.

Discussion

The proposed new methods provided DNA that is easily amplified in PCR, and cut with restriction enzymes. Additional PCR additives such as BSA were not necessary. Obtained DNA was of high molecular weight, and yield was similar to that of the original protocol (XS-isop). XS-isop protocol was previously shown to yield high quality genomic DNA not only from cyanobacteria, but from Gram-positive, Gram-negative bacteria and the Archea. The new improved methods should be applicable for a wide range of microorganisms in aquatic environments, facilitating removal of inhibitors. This has important implications in an era when non-culture methods of microbial community analyses have become very important.

My initial intention was to incorporate PVPP in the extraction solution. PVPP has been used to remove humics from soil DNA isolations either as part of the extraction buffer or as part of a purification step after DNA isolation (Zhou et al. 1996; Arbeli and Fuentes 2007). PVPP did not cause any negative effects on the XS buffer, and a range of PVPP concentrations in the buffer was tested in terms of recovery of DNA and prevention of inhibition of PCR (data not shown). Inclusion of PVPP alone (1-5%) did not remove PCR inhibitors from lake samples. Wechter et al. (2003) reported pre-washing of soil samples in potassium phosphate buffer containing PVPP, followed by another PVPP addition before the protein precipitation step. When I incorporated pre-washing of cells in TE buffer containing 3% PVPP, followed by isolation of DNA in XS buffer with 3% PVPP and isopropanol precipitation, I was able to obtain PCR amplifiable templates from all tested lake samples. DNA yield with pre-washing was comparable to that from XS-3%PVPP-isop for cultures. There was, however, a significant loss

in yield when lake samples were used. This was probably due to freezing of cells before isolation, and some cells and DNA were lost during pre-washing (data not shown).

Polyethylene glycol (PEG) precipitation of DNA has been reported in the literature mainly for size fractionation of DNA. Size of DNA precipitated by PEG is dependent on concentration of PEG used, and higher molecular weight DNA is recovered by lower PEG concentrations (< 10% PEG) (Lis and Schleif 1975; Paithankar and Prasad 1991). Lis and Schleif (1975) reported that 15% PEG 6000 precipitated λ DNA fragments down to 100 bp, while fragments smaller than 700 bp were not precipitated when 7% final concentration of PEG was used. More recently, use of PEG has appeared in several papers as an approach to remove humic contaminants from soil DNA crude extracts. A four fold reduction in humic substances is observed when compost DNA is precipitated with 10% PEG 8000 and 1.1 M NaCl, compared with isopropanol precipitation (LaMontagne et al. 2002). Arbeli and Fuentes (2007) obtained 48.5-136.8-fold lower humic contaminants compared to isopropanol precipitation when genomic DNA was precipitated with 5% PEG 8000 in the presence of 0.6 M NaCl with no difference in DNA yield. In my case, replacement of isopropanol precipitation of DNA with PEG 8000 in the XS buffer (XS-PEG), resulted in PCR amplifiable template, without need for addition of PVPP or prewashing. These DNA preparations were also free of DNases and were cut with the restriction enzyme BmrI. Initially I used 5% PEG in the XS buffer, however this resulted in significant loss in DNA yield. With 7% PEG, yield of DNA was similar to isopropanol precipitation (Table 3-3). If the inhibitors in my samples are humic substances, I may have prevented the precipitation of lower molecular weight humics by using 7% PEG in the extraction buffer. In general, aquatic humic substances are smaller than terrestrial humic substances in terms of molecular weight (MW). An MW range of 0.8-1.5 kDa has been reported for Suwannee stream humics (Beckett et al. 1987).

For two rivers in Florida, dominant molecular masses of humics were determined to be around 2 Kda, < 4.5% being in the higher molecular weight fraction (13 Kda) (Zanardi-Lamardo et al. 2002). The MW distribution of humic substances, if any, in my lake samples is not known. However the color of the DNA pellets after XS-isop isolation from all lake samples was brownish suggesting presence of humics. This color decreased gradually in the order XS-PEG, XS-3%PVPP-isop, XS-3%PVPP-PEG, prewashed-isop and pre-washed-PEG. The effect of PEG compared to isopropanol precipitation can also be seen in Figure 3-3, where fragments smaller than 564 bp are absent in XS-PEG DNA templates.

Among the three quantification methods tested on lake samples and *A. ovalisporum* culture, spectrophotometric measurements yielded greater DNA values than flourometric and gel analyses measurements. Spectrophotometry overestimates DNA concentrations, especially from environmental samples. Zipper et al.,(2003) reported 10 to 30 times higher DNA concentrations compared to flourometric methods, when HA is present in samples. HA also absorbs in the UV range, thereby complicating DNA quantification. Different HA preparations differ in their absorbances in the UV region, and it is not possible to correct for HA absorbance to quantify DNA with UV-VIS spectrophotometry (Zipper et al. 2003). For this reason, flourometry and gel densitometry are commonly preferred over spectrophotometry in soil DNA isolations (Zhou et al. 1996; LaMontagne et al. 2002; Howeler et al. 2003). Although flourometry or gel densitometry is more reliable than spectrophotometry, binding of humic substances to fluorescent dyes has also been reported (Zipper et al. 2003). I have obtained values 10 times higher with spectrophotometry than flourometry and gel analyses. In general, the latter two methods agreed well for DNA values, and I suggest using either of them for both environmental samples and cyanobacteria cultures. I did not attempt to quantify humic acids in my samples

simply by using specific wavelengths for the reasons mentioned above. Instead, inhibition was tested by PCR and restriction assays.

For environmental samples, DNA yields using XS-isop and XS-PEG were similar and the improved method can be used safely for these samples without loss in DNA yield. It is likely that others may encounter more difficult samples in terms of preventing the inhibition of PCR. If the XS-PEG method is not sufficient, I suggest pre-washing the cells with 3%PVPP and precipitation with PEG if DNA yield is not the major concern. It is advisable to wash cells just after harvest and before freezing so that loss of cells and DNA is minimized.

Table 3-1. Lake samples used, collection dates and locations.

Source		Collection date	Location
Harris Chain of Lakes	Lake Dora	05/30/2007	Lake County, Florida
		06/26/2007	
	Lake Griffin	05/30/2007	Lake County, Florida
		06/26/2007	
St. Johns River System	Buffalo Bluff	07/10/2007	Palatka, Florida
	Crescent Lake	07/10/2007	Putnam County, Florida
		08/09/2007	
	Lake George	07/10/2007	Volusia County, Florida
		08/09/2007	

Table 3-2. Oligonucleotide primers used in the study.

Name	Sequence (5'-3')	Reference
16S rRNA		
27F1	AGAGTTTGATCCTGGCTCAG	(Neilan et al. 1997)
1516R	ATCCAGCCACACCTTCCGG	(Yilmaz et al. 2007)
PC-IGS		
PC β F	GGCTGCTTGTTTACGCGACA	(Neilan et al. 1995)
PC α R	CCAGTACCACCAGCAACTAA	(Neilan et al. 1995)
mcyA		
mcyA-Cd1F	AAAATTAAAAGCCGTATCAAA	(Hisbergues et al. 2003)
mcyA-Cd1R	AAAAGTGTTTTATTAGCGGCTCAT	(Hisbergues et al. 2003)

Table 3-3. Total genomic DNA yield (μg) from two lake samples and *A. ovalisporum* FAS-AP1 culture with different methods of isolation and DNA quantification*.

Sample Source	Quantification Method	DNA		Isolation	Methods
		XS-isop	XS-PEG	XS-3%PVPP-isop	XS-3%PVPP-PEG
Lake Griffin					
	Nanodrop	5.65 \pm 1.65 (A) (a)	1.72 \pm 0.20 (B) (a)	4.30 \pm 1.02 (A) (a)	2.31 \pm 0.18 (B) (a)
	Qubit	0.63 \pm 0.02 (A) (b)	0.50 \pm 0.06 (B) (b)	0.53 \pm 0.03 (B) (b)	0.33 \pm 0.01 (C) (b)
	Image J	0.82 \pm 0.10 (A) (b)	0.86 \pm 0.14 (A) (c)	0.86 \pm 0.07 (A) (b)	0.36 \pm 0.11 (B) (b)
Lake Dora					
	Nanodrop	2.55 \pm 0.54 (A) (a)	1.27 \pm 0.65 (BC) (a)	1.82 \pm 0.32 (B) (a)	0.89 \pm 0.04 (C) (a)
	Qubit	0.26 \pm 0.07 (AB) (b)	0.20 \pm 0.02 (B) (b)	0.28 \pm 0.04 (A) (b)	0.19 \pm 0.02 (B) (b)
	Image J	0.23 \pm 0.08 (A) (b)	0.26 \pm 0.03 (A) (b)	0.25 \pm 0.11 (A) (b)	0.22 \pm 0.02 (A) (b)
<i>A.ovalisporum</i>					
	Nanodrop	5.00 \pm 0.82 (A) (a)	0.93 \pm 0.12 (B) (a)	3.13 \pm 2.36 (C) (a)	2.22 \pm 0.02 (D) (a)
	Qubit	1.17 \pm 0.07 (A) (b)	0.36 \pm 0.01 (B) (b)	1.06 \pm 0.06 (A) (b)	0.53 \pm 0.12 (C) (b)
	Image J	0.84 \pm 0.07 (A) (b)	0.56 \pm 0.03 (B) (c)	0.87 \pm 0.02 (A) (b)	0.56 \pm 0.02 (B) (b)

*Each extraction was performed in triplicate. Each measurement was repeated twice, except for Qubit, which was conducted once. Sample means for each lake and culture were compared to other values using Duncan's multiple range test. Means with the same letter are not significantly different from each other. Upper case letters are for comparisons among the four DNA isolation methods for each quantification method (rows). Lower case letters are for comparisons among the three different quantification methods for each DNA isolation method (columns).

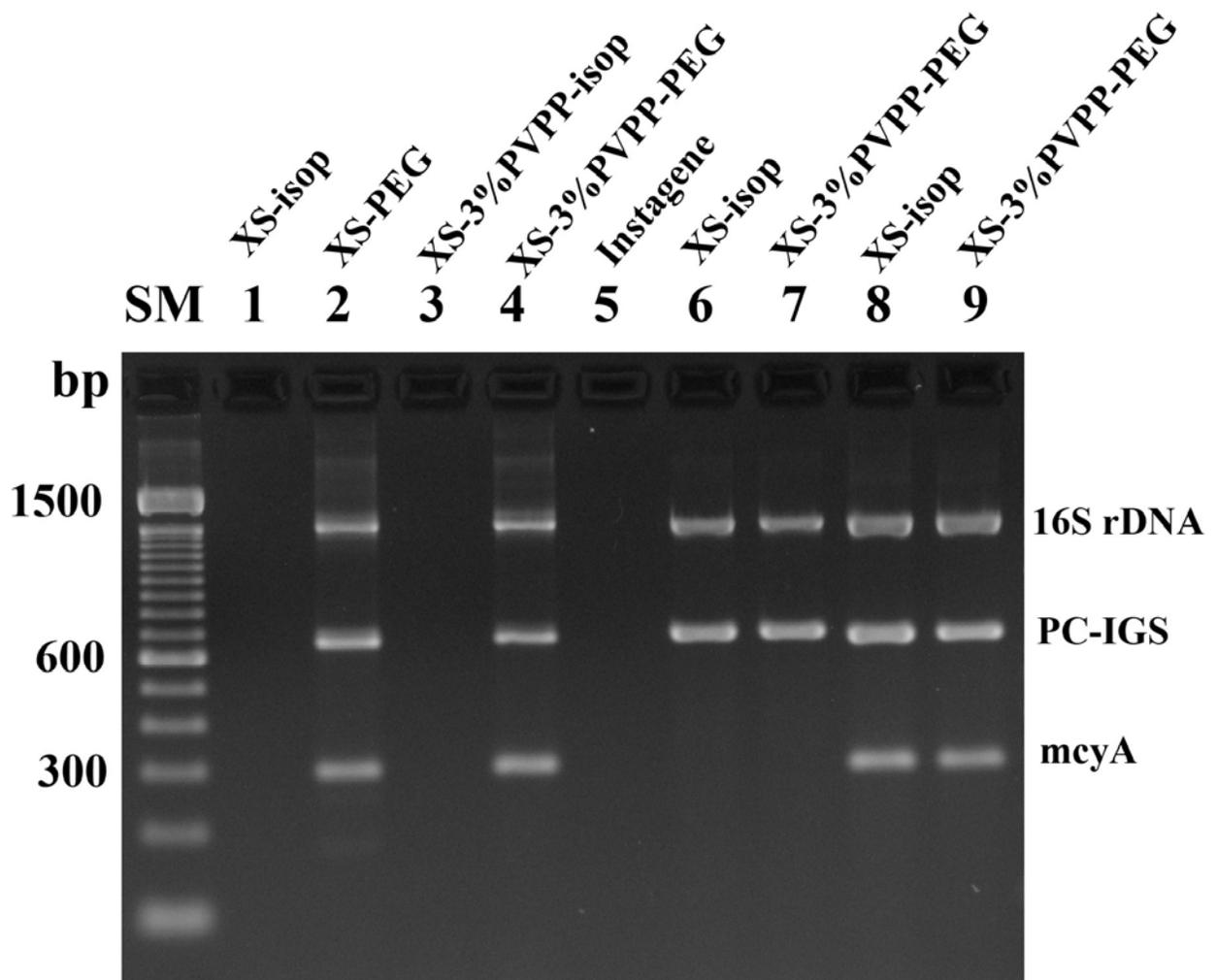


Figure 3-1. PCR results for the 16S rDNA (upper bands), PC-IGS (middle bands) and *mcyA* (lower bands) with genomic DNA from the Lake George sample (lanes 1-5) collected on 07/10/2007. *A. ovalisporum* FAS-AP1 (lanes 6-7) and *M. aeruginosa* PCC 7806 (lanes 8-9) were used as positive controls. Size marker (SM) is the 100 bp DNA ladder.

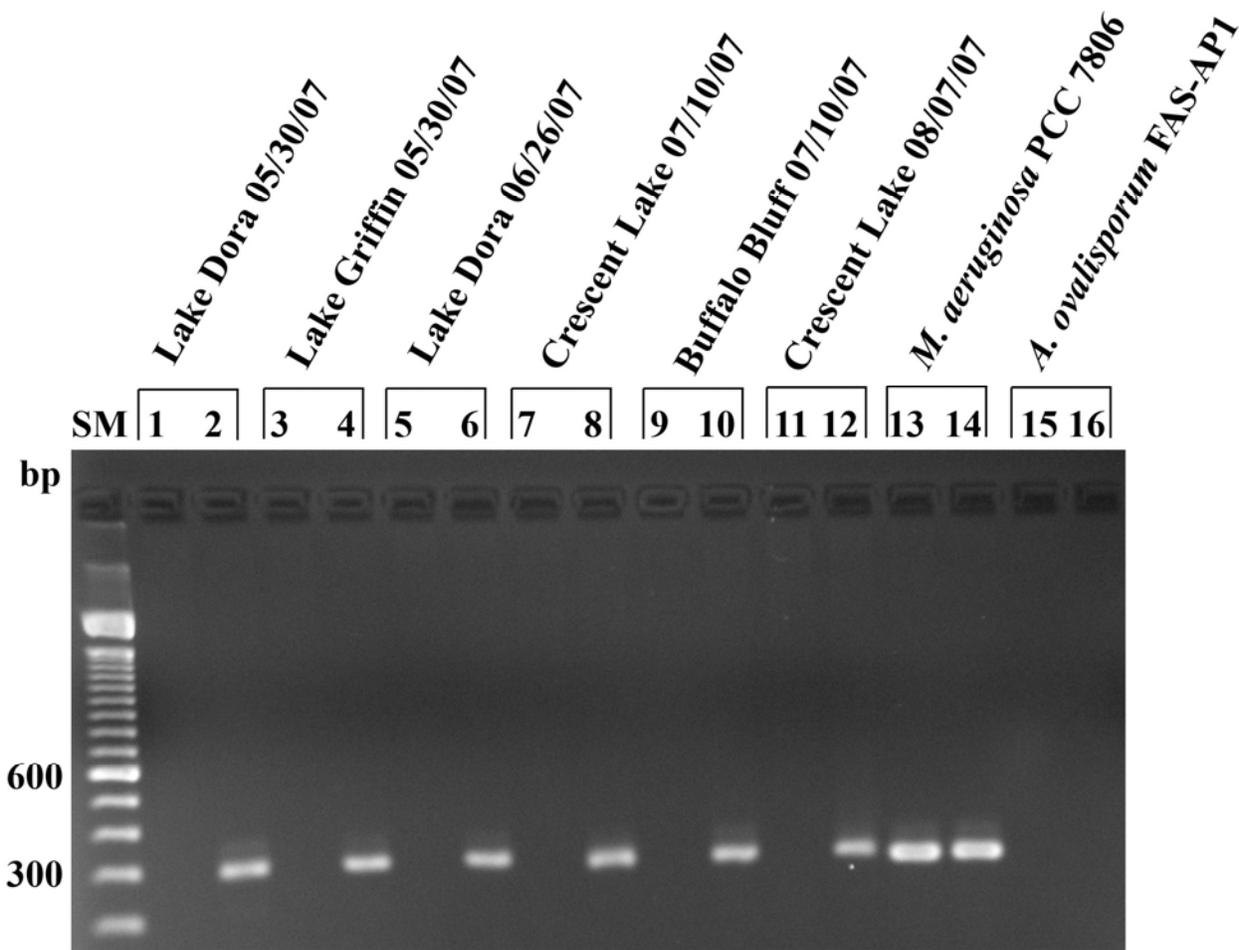


Figure 3-2. *mcyA* PCR results for lake samples and two cultures isolated either with XS-isop (odd lanes) or XS-PEG (even lanes). *M. aeruginosa* PCC 7806 was the positive control, and *A. ovalisporum* FAS-API was the negative control for the *mcyA* gene. Size marker (SM) is the 100 bp DNA ladder.

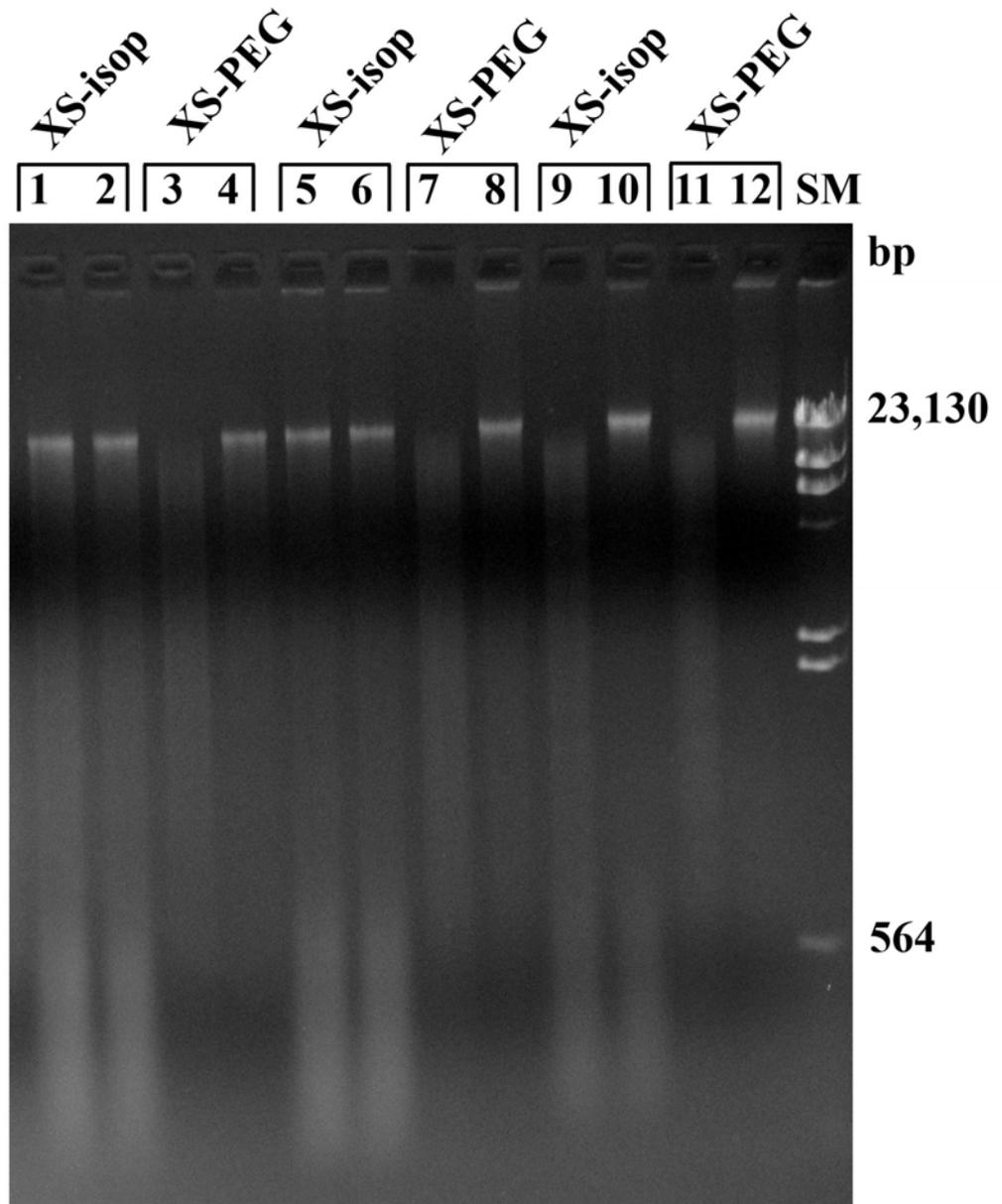


Figure 3-3. Restriction digestion of genomic DNA with BmrI, isolated either with XS-isop or XS- PEG. Odd lanes are samples incubated with the enzyme. Even lanes are samples incubated without the enzyme. Lanes (1-4), Crescent Lake (07/10/2007); lanes (5-8) Buffalo Bluff (07/10/2007); lanes (9-12), *Microcystis aeruginosa* PCC 7806 as the positive control. Size marker (SM) is 0.5 μ g λ DNA Hind-III fragment.

CHAPTER 4
RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSES OF
CYLINDROSPERMOPSIN (CYN) BIOSYNTHESIS GENES IN FLORIDA LAKES

Introduction

Cyanobacteria are a morphologically diverse group of microorganisms capable of plant-like photosynthesis. They contain chlorophyll-a in addition to other accessory pigments such as phycocyanin and phycoerythrin (Bryant and Frigaard 2006). They are considered one of the oldest life forms on earth, and have adaptations to survive in a variety of environments from lakes to oceans, and from deserts to polar regions (Eldridge and Greene 1994; Paerl et al. 2000).

Under appropriate environmental conditions, cyanobacteria can reach very high cell densities in lakes and oceans (Phlips 2002). They are a concern in aquatic ecosystems around the world due to the ability of some bloom-forming species to produce toxic secondary metabolites (Haider et al. 2003). Although the function of these toxins is a matter of debate, they can have adverse effects on the function of the ecosystem, including human resources (Kaebernick and Neilan 2001). Concerns regarding the effect on humans are related to the drinking water supply, recreational activities, and food supply (Haider et al. 2003).

Many cyanobacterial toxins are either hepatotoxic (e.g., microcystins, cylindrospermopsin) or neurotoxic (e.g., saxitoxins, anatoxin-a) (Kaebernick and Neilan 2001). Some toxins are produced by a variety of cyanobacteria species, and some species can produce several types of toxins. The ability to produce toxins depends on the presence of toxin biosynthesis genes, most commonly polyketide synthases, peptide synthetases or a combination of both (Tillett et al. 2000; Schembri et al. 2001). For some species such as *Microcystis aeruginosa*, the gene clusters responsible for the biosynthesis of microcystins (MC) have been identified and sequenced (Dittmann et al. 2001). In the case of cylindrospermopsin (CYN), biosynthesis genes from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* have been identified and

partially sequenced (Schembri et al. 2001; Shalev-Alon et al. 2002). The availability of genetic information has opened new areas of research, including: (1) regulation of toxin biosynthesis (Kaebernick et al. 2000; Dittmann et al. 2001; Kaebernick et al. 2002), (2) determination of toxic species from isolated cultures (Li et al. 2001), (3) rapid screening of lakes for toxic cyanobacteria (Pan et al. 2002), and (4) determination of species responsible for toxin production in mixed phytoplankton populations (Hisbergues et al. 2003).

As described in Chapter 2, I have identified a CYN-producing *Aphanizomenon ovalisporum* from a freshwater pond in Florida using Polymerase Chain Reaction (PCR) for the putative CYN biosynthesis genes, namely polyketide synthases (*pk*s) and peptide synthetases (*ps*) (Schembri et al. 2001; Kellmann et al. 2006). *A. ovalisporum* FAS-AP1 PKS sequences were 99.8% and 100% similar to previously published sequences from *A. bergii* and *A. ovalisporum*. Similarity to PKS sequences from *C. raciborskii* was also high (i.e., over 99%). Identities of PS sequences from *A. ovalisporum* FAS-AP1 to PS sequences of *A. bergii* and *A. ovalisporum* were 99.4% and 100%, respectively. Contrary to PKS sequences, PS sequence identity between *A. ovalisporum* FAS-AP1 and *C. raciborskii* strains was lower (~ 96.4%).

Amplification of toxin genes from environmental samples has previously been performed for MC genes. These studies formed a model to screen lakes for CYN biosynthesis genes, followed by RFLP analyses to determine the producer organisms. MC can be produced by several different cyanobacteria including *Microcystis*, *Anabaena* and *Planktothrix* (Haider et al. 2003). Measuring MC concentrations with a biochemical method alone, however, does not give any information regarding the producer(s) of the toxin. One way to find the producers is to amplify the toxin genes by PCR from an environmental sample, purify the fragments and clone them, followed by sequencing of the fragments (Tringe and Rubin 2005). Obtained sequences

will reveal the variety of toxin producers, or, if there is sufficient sequence information from isolated cultures, will identify the toxin-producer organisms in that sample. The other method to differentiate sequences of toxin-producer organisms again uses purified toxin gene fragments. These are then restricted with a variety of restriction enzymes. Different sequences are cut with different enzymes or from different sites. The resulting so-called “restricted” fragments are visualized on an agarose gel and compared with fragments from known toxin-producer isolates (Hisbergues et al. 2003). This method is called Restriction Fragment Length Polymorphism (RFLP). Gene sequence information of toxin genes was used previously to detect MC genes from lakes in Germany and China (Pan et al. 2002; Hisbergues et al. 2003). By taking advantage of differences in sequences of MC genes from different cyanobacteria, Hisbergues et al. (2003) purified amplified MC fragments from lake samples and digested them with different restriction enzymes. In this way, they were able to identify the source of MC in the investigated lakes.

In this chapter I use sequence information from *A. ovalisporum* FAS-AP1, along with previously published sequences from other CYN producing cyanobacteria, to identify CYN producers in Florida lakes.

C. raciborskii is a major bloom-forming species in Florida and has long been assumed to be the source of CYN in lakes (Chapman and Schelske 1997). In Chapter 2, I demonstrated that none of the eight Florida isolates of this species obtained in this study produced this toxin. Using the newly established DNA isolation method from environmental samples described in chapter 3, I undertook screening of lake samples in Florida for CYN biosynthesis genes to determine producer species by RFLP methods.

Materials and Methods

Control Cyanobacterial Strains

Cyanobacterial strains *A. ovalisporum* FAS-AP1, *C. raciborskii* strains QHSS/NR/CYL03 and FAS-C1 were grown in BG-11 medium (Stanier et al. 1971) at 25 °C. Light was provided on a 12:12hr light:dark photoperiod, at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ from cool white fluorescent lamps. Cells in exponential phase of growth were collected by centrifugation at 13,000 rpm for 5 minutes and frozen at -20 °C until DNA isolation.

Lake Samples

Lake samples were collected using a vertical sampling pole. After transport to the laboratory, they were filtered onto 5-10 μm nylon filters, scraped off the filters and suspended in double distilled water in 1.5 ml centrifuge tubes. Tubes were centrifuged for 5-7 minutes at 13,000 rpm. Supernatants were removed, and cells were frozen at -20 °C until DNA isolation. A list of lake samples used in this study is provided in Table 4.1. CYN concentrations were determined using a CYN ELISA kit (Abraxis, LLC, PA, USA) according to manufacturer's instructions.

Genomic DNA Isolation

Genomic DNA from lake water samples was isolated according to the XS-PEG method described in chapter 3. Approximately 10-20 mg wet weight of cells was suspended in 50 μl of TE buffer, to which 750 μl of freshly prepared XS buffer was added. Tubes were incubated at 70 °C for 2 h. Following incubation on ice for 30 min to precipitate proteins, tubes were centrifuged for 10 min at 13,000 rpm. 750 μl of the supernatants were transferred to clean microcentrifuge tubes and 187.5 μl of PEG stock solution (35% PEG with 50 mM MgCl_2) was added to each tube. Tubes were mixed by inverting several times and incubated at room temperature for 10 min, followed by 10 min centrifugation at 13,000 rpm. Supernatants were removed and the DNA

pellet washed twice with 70% Ethanol. Washed DNA was pelleted by centrifugation for 5 min at 13,000 rpm and air dried. DNA was dissolved either in double distilled autoclaved water or TE buffer.

Polymerase Chain Reaction (PCR) and Internal Control Fragment (ICF) Design

A 624 bp region of the PKS gene and 597 bp region of the PS gene were amplified from lake samples and isolated cultures on a Biometra thermal cycler (Biometra GmbH i. L., Goettingen, Germany) according to Schembri et al. (2001). Each PCR reaction contained 10-20 ng of genomic DNA, 20 pmol of each primer (M4-5 and M13-14) (MWG-Biotech Inc., NC, USA), 200 μ M of each deoxynucleoside triphosphates (Fisher Scientific Company L.L.C, PA, USA), 1.5 mM MgCl₂, 10 μ l of 5X green buffer and 1.5 units of GoTaq DNA polymerase (Promega, WI, USA) in a total volume of 50 μ l. Amplification conditions for both included denaturation of the template DNA at 95 °C for 3 min, 30 cycles of 95 °C for 30 s, annealing of the primers at 60 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. PCR products were run on a 1.5% agarose gel along with a 100 bp DNA ladder, stained with ethidium bromide and visualized under UV transillumination.

Internal Control Fragments for PKS and PS genes were designed as described in Wilson et al. (2000) for the DNA-dependent RNA polymerase gene. They were designed so that the same primers used in toxin gene amplification were used to amplify ICFs. For the construction of PKS-ICF, a 33 bp primer PKS-INT (5'-TGGAATCCGGTAATTGGTCTCAGTTCATCAAGC) was designed. A 20 bp fragment at the 3' end of PKS-INT matched exactly to the sequence 220 bp downstream of the primer M4. The 13 bp overhang at the 5' end of PKS-INT matched exactly with the fragment at the 3' end of M4. PKS-INT and M5 were used in the first PCR and the resulting product was used in the second PCR with the primers M4 and M5 to give an ICF of 404 bp. ICF for PS was constructed in a similar fashion. A 33 bp primer PS-INT (5'-

TGATAGCCACGAGCTTTTCTGAAAGAGGTTGGC) was designed to match a contiguous 19 bp fragment 206 bp downstream of primer M13. The 14 bp overhang at the 5' end of PS-INT matched exactly with a fragment at the 3' end of M13. PS-INT and M14 were used in the first PCR and their product was used as a template in the second PCR with M13 and M14 to give an ICF of 391 bp. PCR conditions were the same as described for the PKS and PS amplifications described above. ICF fragments were added in PCR reactions during amplification of CYN genes to visualize the success of the PCR. PKS, PS and ICF fragments were purified from agarose gels with the Qiaquick MinElute gel extraction kit (Qiagen, CA, USA), according to manufacturer's instructions.

Restriction Digestion of PS Fragments

Approximately 150 ng of purified PS PCR products were used in each restriction assay with 1.5 U of the enzymes BmgBI or NciI (New England Biolabs) in a total volume of 50 μ l with the buffers supplied with the enzymes. They were incubated overnight at 37 °C.

Phylogenetic Analysis

Partial 16S rDNA sequences were aligned using Clustal W within the Mega 4 package (Tamura et al. 2007). Sequence alignments were checked manually. Phylogenetic trees were constructed from pairwise distance matrix using neighbor-joining. Alignments were bootstrapped with 1000 resamplings.

Results

Determination of Optimum ICF Concentration

Three different ICF concentrations (10, 20, 30 fg) were tested in PCR reactions for PKS-ICF and PS-ICF, with the same amount of template DNA from *A. ovalisporum* FAS-AP1. Both the gene fragment, and the ICF were amplified in all cases, with the expected length fragments (Figure 4.1). Ten fg was used as the amount of ICF in subsequent PCR reactions.

Screening of Lake Samples for CYN Biosynthesis Genes

Fourteen samples obtained from five lakes in Hillsborough County were chosen for genetic screening based on the high abundances of *C. raciborskii*. Genomic DNAs obtained from these sites were subjected to amplification for the PKS and PS genes along with the corresponding ICFs. ICFs were used to confirm the success of PCR reactions. As described in chapter 3, environmental DNA samples usually contain PCR inhibitors, but the inhibition was removed using the newly developed method of isolation. None of the 14 samples contained CYN biosynthesis genes. Results for the PKS gene are shown in Figure 4-2.

Seven other samples from Lakes Dora, Griffin, and the St. Johns River system collected in April and May of 2007 were also screened for CYN biosynthesis genes, i.e. PKS and PS. All sites had high densities of *C. raciborskii* (~ 100,000 cells/ml) as observed by microscopy (Mary Cichra, personal communication). Detection of CYN genes was initially tested with ICFs prior to final PCR for purification. CYN genes were absent in samples from Lakes Dora and Griffin. However both PKS and PS genes were amplified in samples from the St. Johns River System (Figure 4-3). Despite presence of CYN genes, ELISA analysis did not detect measurable quantities of CYN. Samples from the St. Johns River System also had *A. ovalisporum* in low numbers. Low concentrations of CYN have been reported for lakes in Florida (Aubel et al. 2006). CYN concentrations in St. Johns River samples were below detection limits, in agreement with the low numbers of *A. ovalisporum* present in the samples.

Purification and RFLP Analyses of PS Fragments from the St. Johns River System Samples

High sequence similarity between PKS sequences of *A. ovalisporum* and *C. raciborskii* Australia strain QHSS/NR/CYL/03 (> 99%) did not permit the use of restriction enzymes to differentiate between the two species. However, PS sequence similarity between the two species

was lower (96.4%). Two restriction enzymes, namely BmgBI and NciI, yielded different length fragments for the PS fragments from *C. raciborskii* and *A. ovalisporum*. The *C. raciborskii* PS fragment was not cut with BmgBI, whereas this enzyme yielded two fragments 310 bp and 287 bp, in *A. ovalisporum* FAS-AP1. PS fragments from both species were cut with the enzyme NciI. *C. raciborskii* yielded three fragments, 329 bp, 234 bp, and 51 bp, whereas *A. ovalisporum* yielded two fragments, 319 bp and 278 bp. These fragment sizes obtained from the sequence information for the two species were in agreement with the fragments visualized after gel electrophoresis (Figure 4-4). Amplified PS fragments from Crescent Lake, Buffalo Bluff, and Lake George, in the St. Johns River ecosystem, collected in May of 2007, were purified from agarose gels and restricted with the mentioned enzymes. The fragment patterns of all three sites for both enzymes were the same as patterns obtained from *A. ovalisporum*, but differed from *C. raciborskii*. There are faint bands at 600 bp (Figure 4-4), which was not cut by the enzyme BmgBI. This is probably due to incomplete digestion by this enzyme. Cyanobacterial DNA is not cut by all restriction enzymes, and can sometimes be incompletely digested. It may, however, represent amplifications from a *C. raciborskii* PS fragment. This is unlikely since the same fragments were cut with the enzyme NciI, giving the same length fragments as *A. ovalisporum*.

Phylogenetic Relationship of *A. ovalisporum* to Other Cyanobacteria

Sequence information and genetic relatedness of *A. ovalisporum* to other strains of *A. ovalisporum* and *A. bergii* were described in Chapter 2. To illustrate its relationship in other cyanobacteria, partial 16S rDNA sequences obtained in this work and from published sequences have been used to construct the tree presented in Figure 4-5. The *A. ovalisporum* FAS-AP1 strain clustered together with other *A. ovalisporum* and *A. bergii* strains from around the world. Discrepancies in identification and naming of these two species were discussed in Chapter 2. Interestingly, both species were separated from other *Anabaena* and *Aphanizomenon* strains. *C.*

raciborskii Florida strain FAS-C1 and Australian strain QHSS/NR/CYL/03 clustered with other *C. raciborskii* strains from Florida and around the world forming a monophyletic group.

Discussion

C. raciborskii is one of the most common bloom-forming species of cyanobacteria in Florida lakes. Year-round blooms have been observed in some lakes (Chapman and Schelske, 1997; Phlips et al, 2003). It has been considered a threat to the health of the ecosystems in Florida due to its putative ability to produce CYN, as reported in other countries (Chorus and Bartram, 1999). There has, however, been no confirmation of CYN production from isolated species of this genus in Florida (Kellmann et al. 2006). I showed in Chapter 2 that none of the eight Florida isolates of *C. raciborskii* produce this toxin. Instead, *A. ovalisporum* isolated from a pond in Duval County, Florida contained CYN biosynthesis genes and produced the toxin. To further investigate CYN producers in different lakes, I used PCR for the putative CYN biosynthesis genes on environmental samples. The results provided the first example of screening of lake water samples in Florida by molecular methods for the production of CYN.

All of the Hillsborough County lakes, as well as Lakes Dora and Griffin, were negative for the presence of CYN genes despite high *C. raciborskii* biomass. However, CYN genes were detected in samples from the St. Johns River System, the first reported observation of these genes in the US. It was possible to discriminate between PS fragments in *C. raciborskii* and *A. ovalisporum* using two different restriction enzymes. Following purification of these fragments from lake samples and cultures, they were subjected to RFLP analysis. Patterns obtained from lakes were the same as those from *A. ovalisporum*, but different from *C. raciborskii*. These results suggest that PS genes involved in CYN biosynthesis in Crescent Lake, Buffalo Bluff and Lake George are from *A. ovalisporum*. There is a possibility that a CYN-producing strain of *C. raciborskii* exists in lakes not included in this study. It is advisable to test with more restriction

enzymes, preferably after a nested PCR. Cloning and sequencing of these PS fragments would also reveal the variety of PS sequences in these samples, and such a test should be performed before arriving at definitive conclusions.

The phylogenetic tree illustrated in Figure 4-5 shows the clustering of *A. ovalisporum* and *A. bergii* strains. As they are separated from other *Anabaena* and *Aphanizomenon* strains, a taxonomic revision of these two species is needed as discussed in Chapter 2.

Along with the newly developed DNA isolation methods from lake water samples, a variety of sites can be screened, and the organisms responsible for toxin production can be identified, which is not possible by chemical methods or microscopy alone. This study provides the first example of such research in Florida lakes.

Table 4-1. Lake samples used in PCR amplifications for CYN genes.

Source	Collection Date	Location
Lake Brant	07/27/2005	Hillsborough County, Florida
	10/13/2005	
	07/18/2006	
	08/15/2006	
Lake Carroll	09/28/2005	Hillsborough County, Florida
	07/18/2006	
Egypt Lake	07/27/2005	Hillsborough County, Florida
Little Lake Wilson (LLW)	07/26/2005	Hillsborough County, Florida
	07/17/2006	
	08/14/2006	
Lake Wilson	07/26/2005	Hillsborough County, Florida
	09/27/2005	
	07/17/2006	
	08/14/2006	
Lake Dora	04/15/2007	Lake County, Florida
	05/30/2007	
Lake Griffin	04/15/2007	Lake County, Florida
	05/30/2007	
Buffalo Bluff	05/20/2007	Palatka, Florida
Crescent Lake	05/20/2007	Putnam County, Florida
Lake George	05/20/2007	Volusia County, Florida

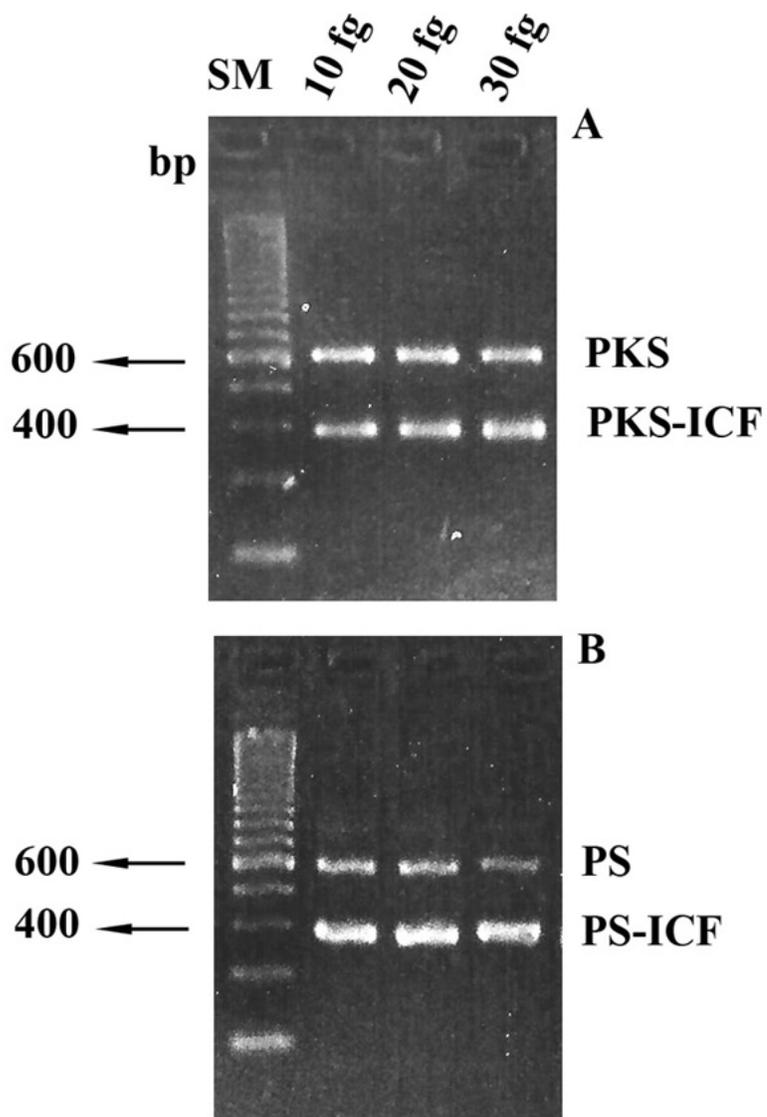


Figure 4-1. PCR results with different concentrations of ICF (10, 20, 30 fg) for PKS (A) and PS (B) genes with template from *A. ovalisporum*. Size marker (SM) is the 100 bp DNA ladder.

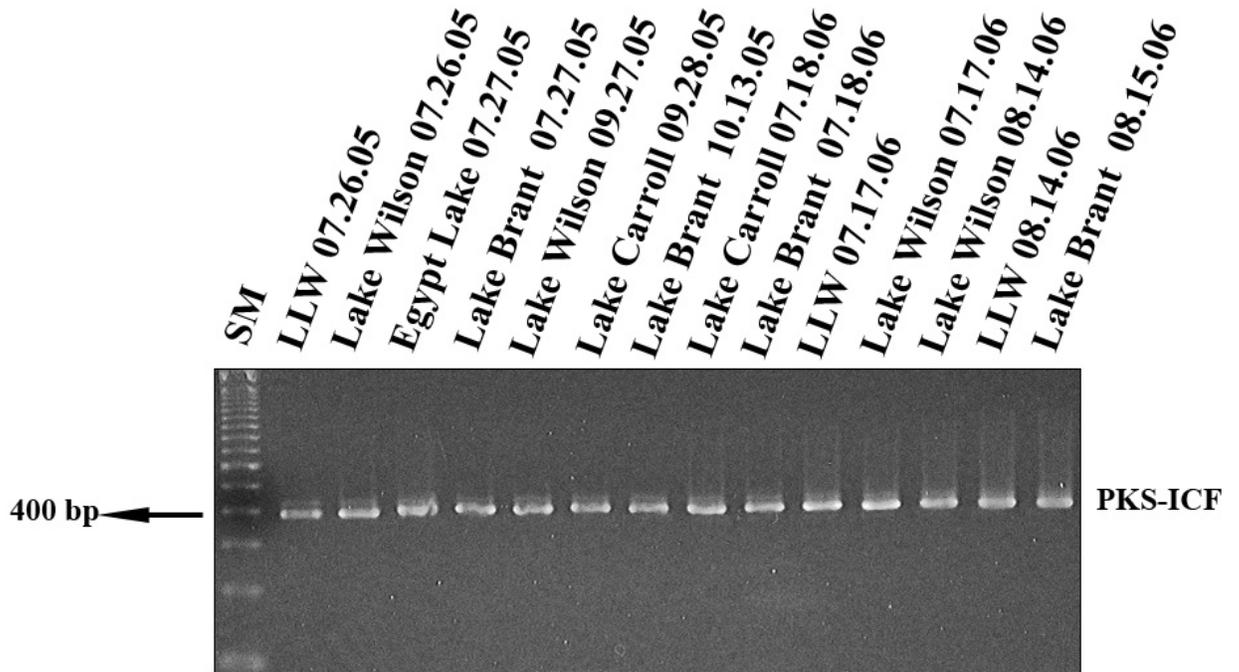


Figure 4-2. PCR results for the amplification of PKS genes from Hillsborough County lake samples. None of the sites had PKS genes. 400 bp fragment is the ICF added into the PCR reactions for conformation of proper PCR function. Size marker (SM) is the 100 bp DNA ladder. See table 4-1 for lake locations and abbreviations.

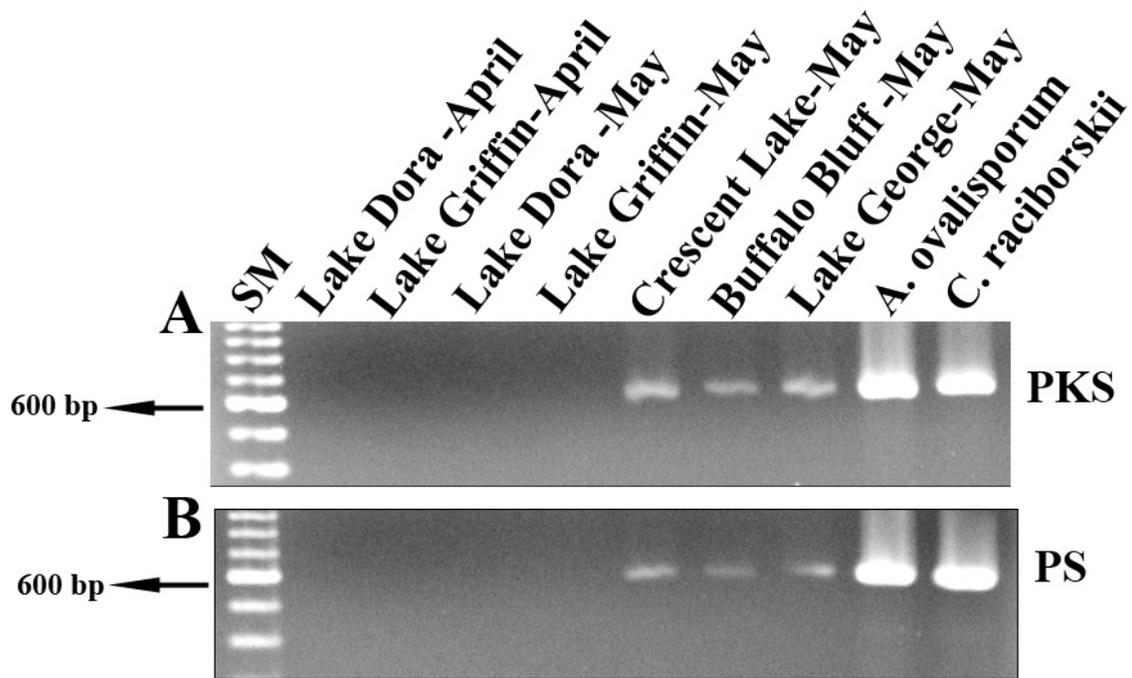


Figure 4-3. PCR results for the PKS (A) and PS (B) genes. Amplifications were obtained only from the St. Johns River Samples. *A. ovalisporum* and *C. raciborskii* were the positive controls. SM is the 100 bp DNA ladder.

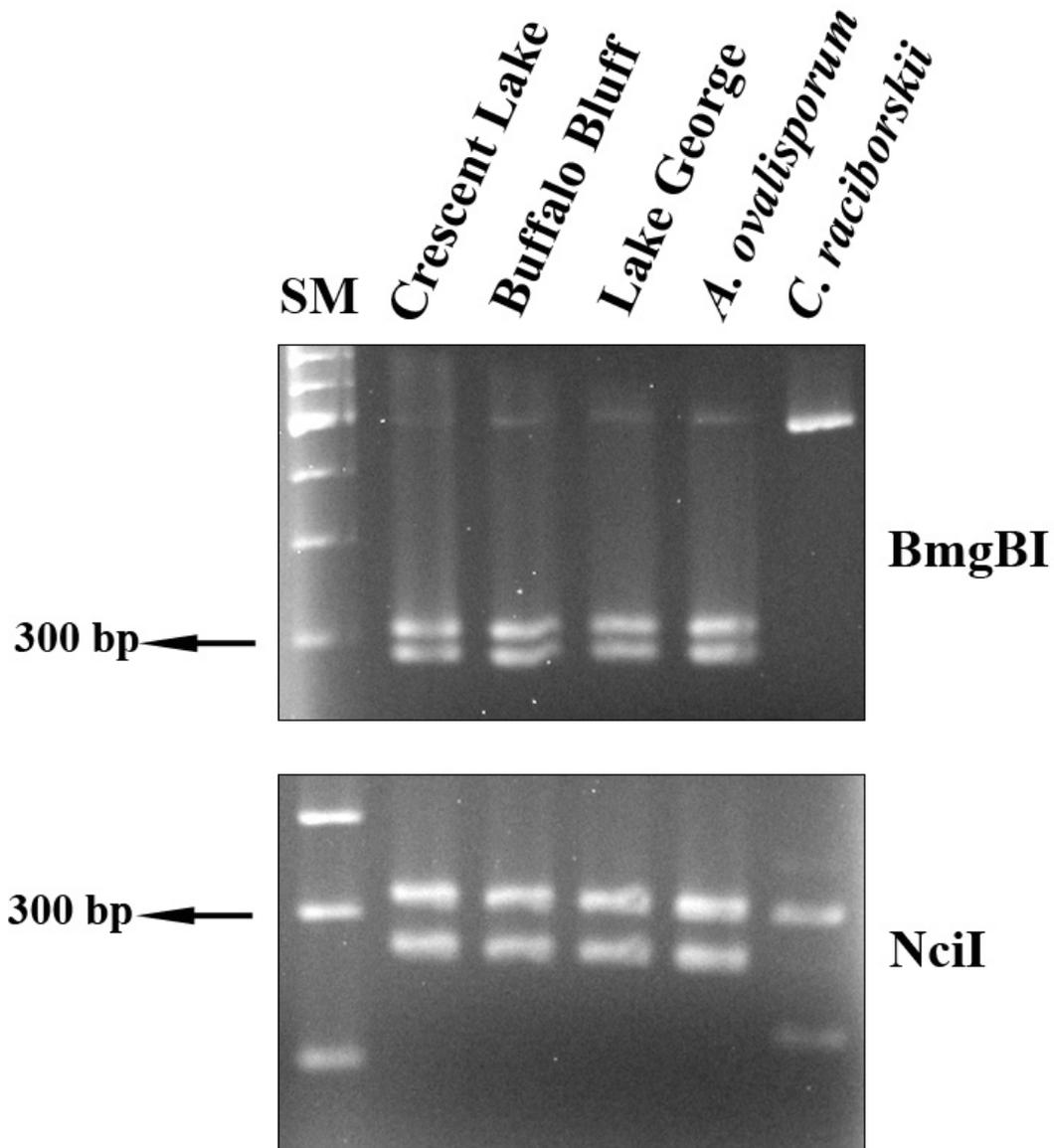


Figure 4-4. RFLP analyses on the PS fragments obtained from the St. Johns River Samples, *A. ovalisporum* and *C. raciborskii*. SM is the 100 bp DNA ladder. 51 bp fragment from the NciI treated *C. raciborskii* PS fragment was not visible in the gel due to its low concentration.

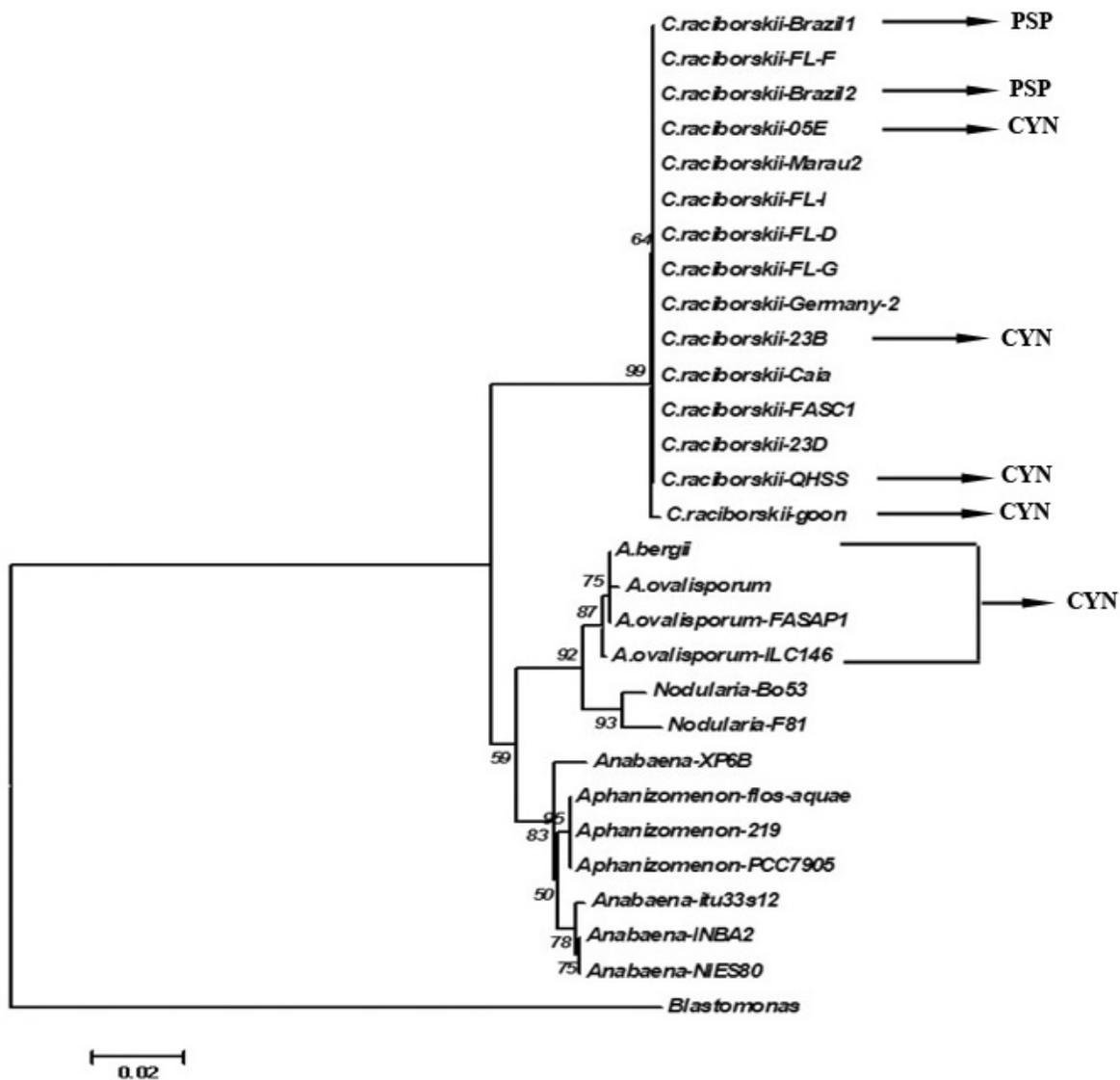


Figure 4-5. Neighbor-joining tree of selected cyanobacteria based on partial 16S rDNA sequences. Bootstrap values above 50% are shown. PSP, paralytic shellfish poisons, and CYN, cylindrospermopsin.

CHAPTER 5 CONCLUSIONS

A significant percentage of lakes in Florida, USA are eutrophic and subject to blooms of planktonic algae, including potentially toxic species. The threat that toxic algae pose for the integrity of aquatic ecosystems and human health in Florida has become a major issue for water managers. Although there has been considerable research on phytoplankton in many eutrophic lakes by universities and government agencies, data on toxic algae have only recently been forthcoming (Phlips, 2003; Phlips et al. unpublished, Yılmaz et al. unpublished). Two species of cyanobacteria have been identified as the major toxic algae threats in Florida, *M. aeruginosa* and *C. raciborskii* (Phlips, 2002; Phlips et al. 2003). The link between *M. aeruginosa* and the hepatotoxin microcystin has been clearly established. For example, a *M. aeruginosa* bloom that covered many square kilometers in the St. Lucie Estuary was tested for the presence of microcystins, revealing up to 800 µg/L microcystin-LR equivalents (Phlips et al. unpublished), 800 times higher than the World Health Organization's provisional guideline value of 1 µg/L in drinking water (Chorus and Bartram, 1999). A two year investigation of different systems resulted in a wide range of MC concentrations, generally lower than 20 µg/L (Bigham et al. unpublished; Yılmaz et al. unpublished).

Establishing the link between *C. raciborskii* and the toxin CYN has been less clear cut despite the widespread distribution and intensity of blooms around Florida. This study helped to explain the basis for this confusion. The fact that all isolates of *C. raciborskii* from lakes and rivers in Florida lacked genes associated with CYN production suggests that the CYN observed in Florida lakes is produced by other species of cyanobacteria.

Here, I reported on the isolation of a CYN-producing *A. ovalisporum* for the first time in the USA. The fact that *A. ovalisporum* is not a common bloom-forming species in Florida helps

explain why very high concentrations of CYN have not been observed in Florida (Aubel et al., 2006) despite the common occurrence of intense *C. raciborskii* blooms. In order to identify the variety of CYN producers in the freshwater systems of Florida by molecular methods, lake samples were collected during a period of two years from lakes in Hillsborough County, the Harris Chain of Lakes, and the St. Johns River System. Whole community genomic DNA isolated from these samples proved to be unsuitable for molecular analyses. The improved methods described in chapter 3 yielded DNA suitable for many molecular biology applications. To my knowledge, this is the first report on removal of inhibitors from cyanobacterial DNA isolations in freshwater systems. These new methods should have broad application in aquatic molecular ecology because the modified DNA isolation method was previously shown to yield high quality DNA from cyanobacteria, other Gram-negative and Gram-positive bacteria, as well as the Archea (Tillet and Neilan, 2000).

Chapter 4 of this dissertation illustrates the usefulness of the established DNA isolation methods. As explained in that chapter, lakes with high *C. raciborskii* biomass were screened for the putative CYN biosynthesis genes, and only three contained organisms harboring these genes. RFLP analyses on the amplified gene fragments suggested the newly isolated *A. ovalisporum* was the source of CYN genes in these systems. Cloning and sequencing of these gene fragments, however, are needed to ascertain the variety of CYN genes in the positive samples. In addition, isolation and screening of more cyanobacteria species are needed to understand the variety of toxin producers fully.

This dissertation supports the usefulness of molecular methods in ecological research and describes novel methods that can be used to answer a variety of questions in aquatic molecular ecology.

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BIOGRAPHICAL SKETCH

Mete Yilmaz was born in Ankara, Turkey in 1976. He started his undergraduate degree at Ege University in 1995, majoring in aquaculture. Upon graduation in 1999, he started his M.Sc. in aquaculture there and completed the program in 2001. He worked on mass cultivation of a cyanobacterium, *Spirulina platensis*, to be used in human consumption, and optimized conditions for the production of a pigment (phycocyanin) produced by the algae. He became interested in pigment biosynthesis and went to Brown University in 2002 to work on this topic with Sam Beale. He came to the University of Florida in 2003 and started his PhD under the supervision of Edward J. Philips. He has been working on the molecular ecology of toxic cyanobacteria since then.