CHAPTER 5

Toxic cyanobacterial blooms in a shallow artificially mixed urban lake Colorado

The contents of this chapter have been submitted for publication to *Lakes & Reserviors: Research and Management*.
(Manususcript submission number: 2005/10; Date of acceptance: February 13, 2006)

Abstract

One of the most severe problems related to urban freshwater ecosystem eutrophication is the occurrence of increasingly frequent blooms of toxic cyanobacteria. Cyanotoxins may accumulate in the trophic web and produce diverse intoxication symptoms and chronic effects that are difficult to diagnose and prevent. High mortality of domestic animals and fish has been reported previously under these prevailing conditions. In this study we investigated the taxonomic composition of phytoplankton assemblages during the summer of 2004, a year after restoration of Sheldon Lake. We also analyzed the physical and chemical changes caused by urban runoff, artificial mixing, as well as the usefulness of microcystin molecular markers derived from the *mcy* gene cluster for the detection of toxic cyanobacterial strains in environmental samples of Sheldon Lake. Our study clearly demonstrates that the artificial mixing rate alone is insufficient to cause a transition to a well-mixed system, and that cyanobacteria remained dominant all throughout the summer months. We confirmed the presence of the toxic cyanobacterial strains through the use of molecular markers that detect the presence of the *mcy* gene cluster that is responsible for the production of toxin by *Microcystis* spp. This may have great use-potential in routine analysis of urban aquatic ecosystems. Thus, it may make water monitoring more feasible and allow for the early application of corrective actions especially in cases like Sheldon Lake that is a public recreational focal point.

Keywords: urban runoff, artificial mixing, eutrophication, *Microcystis* spp., molecular markers

5.1 Introduction

Some of the most serious effects of urban runoff are on the aquatic habitat of the receiving waters. Water quality within large spectrum of urban water bodies like reservoirs for flood control or drinking water, stormwater management ponds, or recreational-park ponds is often under great pressure, owing to impacts such as stormwater nutrient and contaminant loading. Urban runoff may carry large amounts of sediment into a receiving waterbody, reducing water clarity and modifying the benthic substrate. Unfortunately sediment is not the only objectionable substance that is found in urban runoff. Urban runoff may also contain high concentrations of nutrients, oxygen-consuming wastes, pathogens, and toxic substances such as pesticides, heavy metals and oil (Peterson *et al.* 1985). Heaney *et al.* (1980) reviewed fish-kill information reported to government agencies from 1970 to 1979 in the U.S. and found that less than 3% of the reported 10 000 fish kills were identified as having been caused by urban runoff. Especially prevalent within urban water bodies are concerns about the amount and distribution of nuisance-forming cyanobacteria. Cyanobacterial blooms may cause a variety of water quality problems, including dissolved oxygen depletion and subsequent fish kills, aesthetic nuisances (e.g., odours, scum fish tainting, unsightliness) and unpalatable and possibly unsafe drinking water (Carmichael 2001). Such problems can severely limit aquatic habitat, recreation activities, fisheries, and use of a water body as a potable water resource.

Cyanobacteria are known to produce a variety of toxins that can be lethal to livestock, pets, wildlife and humans following ingestion of water contaminated with toxic cells

or toxins released from decaying cells (Codd 1999; Azevedo *et al*. 2002). Certain species of *Anabaena* and *Aphanizomenon*, for example, synthesize neurotoxic alkaloids, whereas species of *Anabaena, Microcystis*, *Nodularia* and *Planktothrix* mostly generate hepatotoxic peptides (Carmichael *et al*. 1990). The latter secondary metabolites are contained within the cells and are usually only released upon lyses or when changes in cell wall permeability occur. One of the most common genera in cyanobacteria blooms, *Microcystis*, produces the hepatotoxin microcystins which are rarely reported in high concentrations of open lake water, but concentrations of some hundred µg/L are occasionally encountered at near-shore sites where cyanobacteria accumulate. Children are particularly at risk of ingesting major amounts of cyanobacterial cells when playing in the shallow zones along lakeshores where toxic cyanobacterial scum accumulates. Further support for this statement comes from a Swedish investigation in which the levels of toxicity at different depths of a *Microcystis* bloom were determined (Annadottér *et al*. 1991). Annadottér *et al*. (1991) found that the toxicity was highest at the surface water near shore and decreased with depth during periods of calm weather. Thus, recreational exposure to cyanobacteria should be assessed with respect to the possibility of health hazards both through dermal exposure to cyanobacterial cells and their poorly understood irritable and/or allergenic components, as well as through systemic exposure due to accidental ingestion and aspiration of cell material (Chorus & Mur 1999).

Mammals and birds in general appear to be more susceptible to these toxins than are aquatic invertebrates and fish. Death of fish during cyanobacterial blooms has been reported (Davidson 1959; Koon 1960; Ochumba 1990; Sevrin-Reyssac & Pletikosic 1990) although the evidence linking their deaths to toxins is uncertain.

Histopathological investigations of fish deaths during cyanobacterial blooms in the UK indicated that the cause of death was due to damage to the gills, digestive tract and liver (Rodger *et al*. 1994). However, not all species of cyanobacteria synthesize toxins and the potency of blooms can substantially vary between sites, seasons, weeks, or even days. This variation could be due to changes in species composition, production of different toxins with varying toxicity by one clone, and other reasons influenced by environmental factors (Benndorf & Henning 1989). Since the discovery of the *mcy* gene cluster responsible for microcystin biosynthesis in *Microcystis* it was possible to develop DNA primers for PCR assays, which were then used to discriminate between toxic (i.e., microcystin-producing) and non-toxic (non-microcystin-producing) genotypes (Dittmann *et al*. 1997; Nishizawa *et al*. 1999, 2000; Tillett *et al*. 2000, 2001; Kurmayer *et al*. 2002). The aims of the present study were to use the *mcy* gene cluster to detect the toxicity status of *Microcystis* populations in Sheldon Lake. By doing so it will provide us with more information on the microcystin producing genotypes that form the epilimnetic population and help predicting the formation of toxic blooms, as well as monitoring their development in urban lakes. Also, the study aimed to assess the effect of urban runoff on the 'health' of Sheldon Lake, as well as artificial mixing as a management tool to prevent the formation of blooms.

5.2 Study site description and background

Since the early 1900's, City Park has been a favorite recreational spot for Fort Collins residents in Colorado. The 6,07 hectare Sheldon Lake was excavated in 1874, and is a focal point of the 34,39 hectare park (Fig. 5.1). The area around City Park, the

adjacent cemetery and nine-hole golf course, is highly developed with arterial streets, schools, businesses, residential houses and apartments. In the early years, little thought was given to storm drainage as these areas developed. In 1997, 20.32 cm of rain caused a 5 m high railroad bed, acting as a dam, to break, flooding the city. The flood caused five deaths and millions of dollars in damage (Endres 2004). In December 2002, the Sheldon Lake Drainage Improvement Project started by removing over a 100 years of accumulated sediment at the bottom of the lake to increased the water detention with an additional 30 837.225 m^3. The Sheldon Lake Drainage project was completed in June 2003 at a total cost of 6 million dollars. During this time 746.76 m of storm sewer pipe, 457.2 m of box culvert, 274.32 m of water line and nine stormwater inlets were installed, 30 837.225 m^3 of lake bottom sediment were removed and approximately 21 000 fish were relocated. After completion of the project in June 2003 the lake was restock with largemouth bass (*Micropterus salmoides*); bluegill sunfish (*Lepomis macrochirus*); crappie (*Pomoxis annularus*); channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*). To regulate lake depth variation during summer, water is piped from Pleasant Valley, Cache la Poudre River and the Colorado-Big Thompson watersheds (Sheldon Lake Drainage Improvement Project 2002, 2003a,b).

To enhance water quality in Sheldon Lake, artificial mixing was introduce to manipulate the physical environment so that it becomes less favorable for cyanobacteria and more favorable for less problematic species. The lake was mixed artificially with aerators that were placed on the bottom. Compressed air bubbles were passed from the aerators into the water and were visible as surface boils. Two air compressors connected to a network of tubes provide air. Because of the relatively

high floating velocity of the cyanobacterial colonies, the equipment for aeration was designed in such a way that the vertical mixing velocity would be sufficient to keep

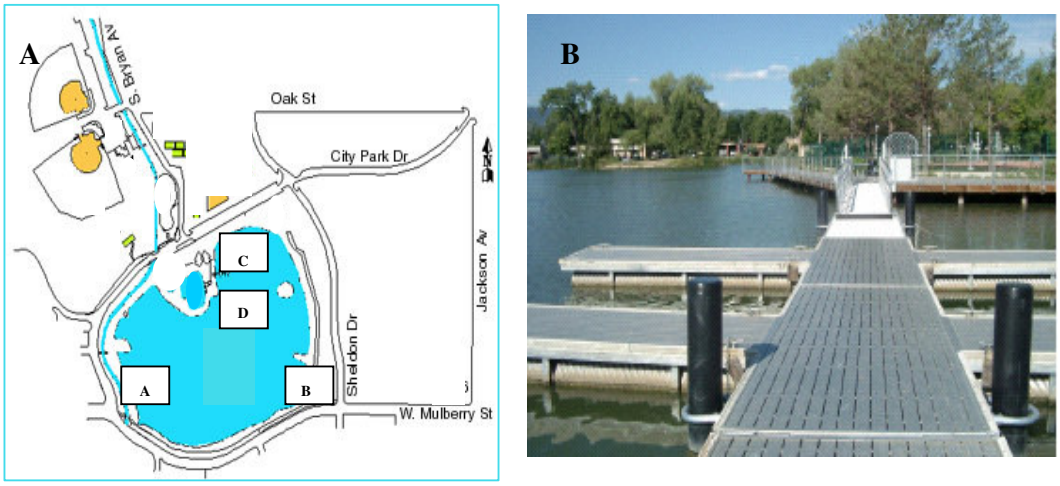

Figure 5.1 (A) Map of Sheldon Lake, Colorado (Sheldon Lake Drainage Improvement Project 2002) (Scale 10 m = 4 mm). (B) Sampling site D.

cyanobacteria entrained in the turbulent flow. In the short term, artificial mixing could prevent cyanobacterial bloom forming since this organism benefits from a stable, stratified water column (Kohler 1992). Gas vesicles provide the cyanobacterial colonies with buoyancy, which enables them to concentrate their biomass in the upper mixed layer and to maximize light interception. In this way, cyanobacteria increases its total daily light dose during periods of calm weather (Ibelings *et al*. 1991; Kohler 1992) while non-buoyant phytoplankton suffers from increased sedimentation losses in a stable lake. In July 2004, a year after the restoration of Sheldon Lake a bloom of *Microcystis wesenbergii* (Teiling 1941; Wojciechowski 1971) started to developed near the shores. This was succeeded by a bloom of *Woronichinia naegeliana* (Smith 1950) in early August, which was precede by a bloom of *Microcystis aeruginosa* (Smith 1950) during the middle of August. The *Microcystis aeruginosa* presented an exponential growth phase that progressed until the middle of September. This phase

was immediately succeeded by a fast declining phase. Beyond this decline *Microcystis aeruginosa* persisted in low numbers and disappeared from the water column at the end of October.

5.3 Material and methods

5.3.1 Bloom Sampling

We sampled the lake two weekly from July to the end of September 2004. The sampling was carried out in the morning (8.00-9.00) and afternoon (16.00-17.00). Four sampling stations were selected. The water column depths of the sampling stations varied from 0.6 to 0.8 m (station A), 0.5 to 0.65 m (station B), 0.4 to 0.5 m (station C) and 0.6 to 0.75 m (station D) due to natural fluctuating intra-and inter-annually depending largely on regional climatic conditions and urban runoff within the sampling area. Water was sampled from the top 0.35 m of the water column, using a Van Dorn bottle. In the laboratory, natural bloom sample water was divided into three sets, the first set were decanted in glass cylinders and placed under fluorescent lights. Under these conditions, the cyanobacteria floated to the surface. The lower water layers were siphoned off, and the cyanobacterial cells were collected, identified and stored at 4 ºC for no more than 1 week, whilst culture was purified for DNA/RNA extrations (Kurmayer *et al*. 2002). Glycerol stocks were also prepared and stored at -80 ºC until further use. Material used for determination of toxicity was processed immediately and frozen at -20 ºC until assayed. The second set of samples was used to determine species composition and community structure. Composition and density were assessed from 1 mL aliquots sampled from 100 mL vertical water samples, then

fixed with buffered 5% (v/v) formaldehyde. Handling, identification and counting of phytoplankton followed the procedures of Lund *et al*. (1958), Brower & Zar (1977), and Padisak (1993). The third set of samples were filtered onto filters with a pore size of 1.0 µm and stored at –20 ºC until chlorophyll*a* (Chl*a*) extraction. For the characterization of water quality during the cyanobacteria bloom period, some physico-chemical and biological variables (temperature, pH, Chl*a*, total nitrogen and phosphate, silica and ammonia, secchi depth, rainfall, windspeed and stormwater runoff) were determined. The entrainment of cyanobacterial colonies in the turbulent flow was investigated by determination of the buoyancy state of the vertical distribution of *Microcystis* colonies, which was measured in the morning and afternoon of 22 August by sampling at intervals of 0.25 m at the different stations. The samples were fixed with 5% (v/v) Lugol's and were counted in a Sedgewick-Rafter chamber.

Chlorophyll*a* (Chl*a*) was measured by the extracting of Chl*a* from lyophilized filters using N,N-dimethylformamide for 2 h at room temperature. Chl*a* was measured spectrophotometrically at 647 and 664 nm and calculated according to Porra *et al*. (1989). Temperature profiles and pH of the water column were measured with an YSI model 2100 thermometer and a 211 microprocessor pH meter. Data of wind speed, direction and rainfall was measured at the meteorological station of the Colorado State University, 2 km away from the lake. Nutrients dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) was analyzed using classical spectrophotometric methods (American Public Health Association, American Water Work Association, and Water Pollution Control Federation 1980).

5.3.2 Bloom toxicity confirmation

5.3.2.1 Pretreatments of environmental samples for whole-cell PCR

For whole-cell PCR cyanobacterial cells were collected from the environmental samples by placing the water sample under fluorescent light in a glass cylinder. The cyanobacterial cells floated under these conditions to the surface and the lower water layers were siphoned off. Before resuspension in distilled water to define volume, the cells were washed one to three times with distilled water and purified by picking colonies (Kurmayer *et al*. 2002). DNA was extracted from an axenic PCC7806 culture strain using DNAzol®-Genomic DNA Isolation reagent following the manufacturers' procedures (Molecular Research Center, Inc., USA).

5.3.2.2 PCR amplification

PCR was performed in a GeneAmp2400 thermocycler (Perkin-Elmer Cetus, Emeryville, Calif., USA). The thermal cycling protocol included an initial denaturation at 94 °C for 2 min, followed by 35 cycles. Each cycle began with 10 s at 93 °C followed by 20 s at the annealing temperature at T_m°C for the specific primer pairs (Table 5.1), and ended with 1 min at 72 °C. When extracted DNA was used, the amplification reactions contained a 10x amplification buffer with 1.5 mm $MgCl_2$, 0.2 mm dNTPs, 20 pmol of each primer and 1 U Taq DNA polymerase, and 3-5 ng purified DNA in a final volume of 50 µL (Dittmann *et al*. 1999, Oberholster 2004, Grobbelaar *et al.* 2004, Grobbellaar 2005). The PCR amplification with whole cells started with 6 µL of crude sample, pretreated subsample with an approximate cell

density of 8 x 10^6 cells/ml, or 0.1µg lyophilized cyanobacterial cells. The sample was added directly to a 20-µL-reaction solution containing bovine serum albumin (0.1 mg/mL) or skim milk (0.1-100 mg/mL, w/v), and a 10x amplification buffer, which contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer, and 0.5 U Taq DNA polymerase (Howitt 1996). The PCR amplifications conditions were identical to those for the samples described above. An extra ramp rate of 3 s/°C was set between the denaturing and annealing steps, when a GeneAmp9600 cycler, instead of GeneAmp2400 was used for PCR amplification. The dosage for the skim milk ranging from 1 to 100 mg/mL was determined to be appropriate based on the results of PCR.

Table 5.1 Oligonucleotides used for RT-PCR and PCR analysis.

Gene region and primer	Sequence	T_m °C)	Fragment size	Authors
McyA NMT				
MSF	5'-ATCCAGCAGTTGAGCAAGC-3'	59	~1.3 Kb	Tillett *et al*. 2001
MSR	5'-TGCAGATAACTCCGCAGTTG-3'	60		
MSI	5'-GAGAATTAGGGACACCTAT-3'	48		
uma1				
UMF	5'-CCTATCGTCGTATTTGGAGT-3'	54	867 bp	
UMR	5'-AAGGAATGGACACGATAGGC-3'	59		
McyB				
Tox 1P	5'-CGATTGTTACTGATACTCGCC-3'	57.9	~350 bp	Oberholster 2004
Tox 1M	5'-TAAGCGGGCAGTTCCTGC-3'	58.2		Grobbelaar et al. 2004
				Grobbelaar 2005
Tox 3P	5'-GGAGAATCTTTCATGGCAGAC-3'	62.4	~350 bp	
Tox 2M	5'-CCAATCCCTATCTAACACAGTACCTCGG-3'	65.1		
Tox 7P	5'-CCTCAGACAATCAACGGTTAG-3'	53.7	~350 bp	
Tox 3M	5'-CGTGGATAATAGTACGGGTTTC-3'	58.4		
Tox 10Pf	5'-GCCTAATATAGAGCCATTGCC-3'	59.8	~350 bp	
Tox 4Mr	5'-CCAGTGGGTTAATTGAGTCAG-3'	57.9		
McyB				
Tox2+	5'-AGGAACAAGTTGCACAGAATCCGCA-3'	50	~200 bp	Kaebernick *et al*. 2000.
Tox2-	5'-ACTAATCCCTATCTAAACACAGTAACTCA-3'	50		
McyD				
McyDF2	5'-GGTTCGCCTGGTCAAAGTAA-3'	50	~297 bp	
McyDR2	5'-CCTCGCTAAAGAAGGGTTGA-3'	50		
McyB				
FAA	5'-CTATGTTATTTATACATCAGG-3'	40	~580 bp	Neilan *et al*. 1999
RAA	5'-CTCAGCTTAACTTGATTATC-3'			

5.3.2.3 RNA extraction and RT-PCR

Picked colonies were homogenized using a mortar and pestle and liquid nitrogen. RNA extracted using the Qiagen RNAeasy kit (Qiagen Inc., USA) according to the manufacturers' instructions. All equiptment, material and solutions were pretreated with Diethyl pyrocarbonate (DEPC) to ensure that it is RNase free. First strand cDNA was synthesised from RNA using the cDNA synthesis System according to the manufacturers' instructions (Roche Molecular Biochemicals, Germany).

Real-time PCR was performed using first strand cDNA (70 ng) from selected total RNA as required. The 20 µL reaction contained 10 pmol forward and reverse primers (Table 5.1), 3 mM $MgCl_2$ and the LightCycler–FastStart DNA Master SYBR Green 1 Mix (Roche Diagnostics Corporation, Germany) as according to manufacturer's procedures (LightCycler–FastStart DNA Master SYBR Green 1 Manual, Roche Applied Science, Germany). Amplification of products consisted of 1 cycle at 95 °C for 10 min; therafter 40 cycles were conducted starting with 1 cycle at 95 °C for 10s, primer specific annealing T°C for 5s, 72 °C for 10s; followed by the melting curve analysis (95 °C for 0s, 65 °C for 15s, 95 °C for 0s), and cooling (40 °C for 30s). A minimum of 7 reactions was done for each fragment analyzed, standard curves were generated using dilution series (1:1, 1:10, 1:100, 1:1 000) and repeated. Results obtained were analyzed using LightCycler Software version 3.5 (Roche Applied Science, Germany) and were expressed as fold change.

5.3.2.4 Fish bioassay

Hepatocytes were isolated from rainbow trout (*Oncorhynchus mykiss*) by a two-step collagenase perfusion of the liver according to Seglen (1976). During the perfusion, buffers were supplemented with 0.2-1% bovime serum albumin. All cells were carried out at 2 x 10^6 cells/ml in a buffer containing 30 mM Hepes, 30 mM TES, 30 mM Tricine, pH 7.4, 68 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1.1 mM KH_2PO_4, 0.7 mM Na_2SO_4 and glucose.

5.3.2.5 Light microscopy

Hepatocytes were fixed for light microscopy in 3% (m/v) paraformaldehyde in phosphate buffer saline (pH 7.4) and mounted on a glass cover slip before viewing using a Zeiss microscope using interference contrast optics.

5.3.2.6 Protein Phosphatase Inhibition and ELISA Assays

Intracellular microcystin content was measured using the colorimetric PP2A inhibition assay (An & Carmichael 1994). The recombinant catalytic subunit of protein phosphatase 1 as expressed in *Escherichia coli* was used as the source of PP enzyme (Zhang *et al.* 1992). This assay has a 50% inhibitory concentration of 6.72 µg/L for the microcystin-LR standard, using 0.5 mg of PP2A per mL, and a limit of detection of 0.033 µg/L or 33 Pm. Prior to the assay, 1 mL of cell suspension in water (supernatant removed) was freeze-thawed three times and diluted 1: 100 to 1: 8 000. Samples were measured in duplicate in the same assay and also in repeated assays. After adjusting each assay to percentages of protein phosphatase activities, the concentration of microcystin was calculated from an average standard curve of all

assays. The PP2A inhibitor in this case microcystin content was calculated for cells per milliliter of sample, resulting in values reported as picomoles of microcystin toxin per cell.

The ELISA assay was conducted with a Quanti TM Kit for microcystins (EnviroLogix, USA). The microcystin concentration was determined from the standard curve of microcystin-LR (Ueno *et al*., 1996). The results were obtained by reading the plate on a multiskan ascent (Thermo Labsystems).

5.3.2.7 Data analyses

The results were recorded on standard Excel spreadsheats for data processing, and statistical analysis was performed using SYSTAT® 7.0.1 (1997).

5.4 Results

5.4.1 Species composition and Physical/chemical measurements

During the study period June to October 2004, cyanobacterial colonies were found on each sampling date, with maximum abundance on 22 August (1.66 x 10^9 cells/ml) and minimum abundance on 30 June (1.30 x 10^8 cells/ml) (Fig. 5.2).

Identification of individual cyanobacteria colonies from June to September revealed a complex pattern of cyanobacterial species succession. The occurrence of three

morphospecies: *Microcystis aeruginosa*; *Woronichinia naegeliana* and *Microcystis wessenbergii* were observed during this time. The phytoplankton composition from

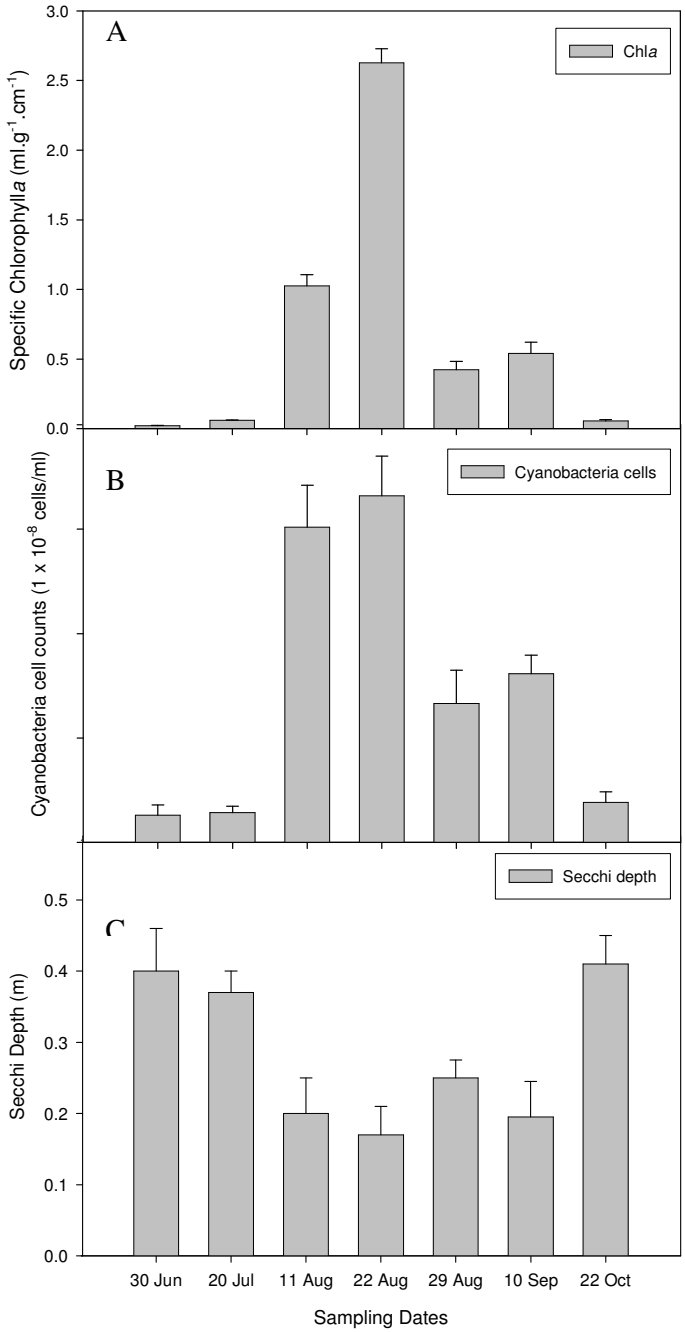

Figure 5.2 Average (a) specfic Chl*a*, (b) cell count and (c) transparency (Secchi depth) during surface blooms in the summer 2004 with artificial mixing, Sheldon Lake, Colorado. Error bars represent standard deviation from the mean value.

live samples taken on three sampling dates in August showed a dominance of Cyanophyceae (*Microcystis aeruginosa*, *Woronichinia naegeliana*) (Fig. 5.3) followed by less dominance of Chlorophyceae (*Cosmarium montrealense*, *Closterium aciculare*); Bacillariophyceae (*Synedra rumpens var fusa*, *Asterionella formosa, Melosira varians*); Euglenophyceae (*Euglena caudate*, *Phacus pleuronectes*) and Chrysophyceae (*Chrysococcus minutus*) (Fig. 5.4).

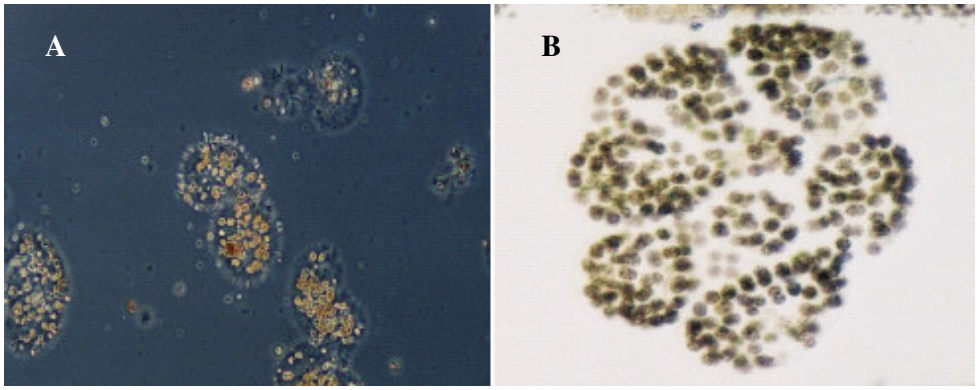

Figure 5.3 (A) *Woronichinia naegeliana* (after Smith 1950); (B) *Microcystis aeruginosa* (after Smith 1950); Unstained, bright-field microscopy, 200 x.

The Secchi depths during the three sampling dates in August were generally restricted to the upper 25 cm of the water column, while a chlorophyll increase together with a 50% reduction in light penetration (as Secchi depth) was observed on 22nd of August 2004 (Fig. 5.2).

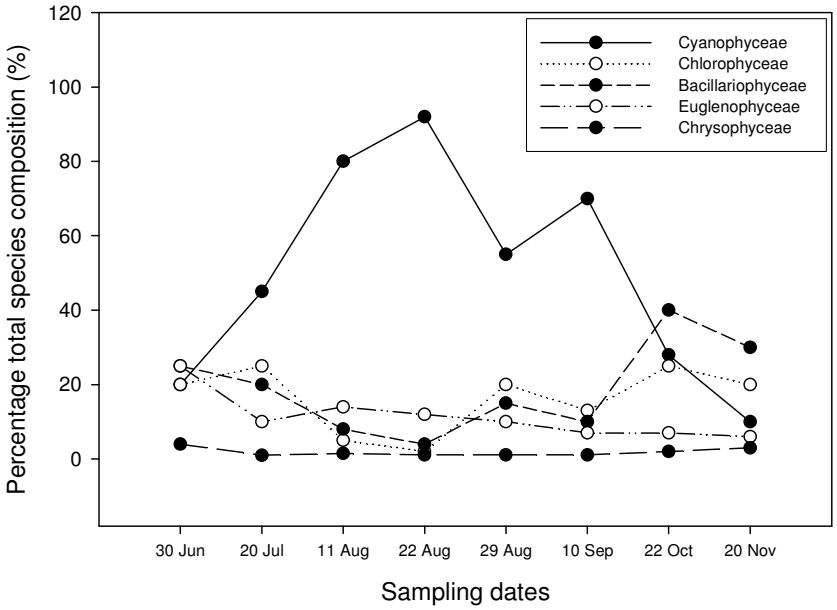

Figure 5.4 Seasonal variations in the relative abundance of five phytoplankton groups with artificial mixing during the summer 2004, Sheldon Lake, Colorado.

Following the Carlson's trophic state index and its associated parameters (Carlson 1977; Kratzer & Brezonik 1981) we presently considered Sheldon Lake eutrophic a year after restoration, due to the summer results of total Secchi disc depth (0.25 m), specific Chl*a* (0.693 mg/L) and Phosphates concentration which ranging from 0.2 to nearly 0.3 mg/L while total Nitrogen value ranging from 0.2 to 0.4 mg/L at the different sampling locations (Fig. 5.2). The concentrations of dissolved inorganic Nitrogen and Phosphates were low while total Ammonia concentration was 0.467 mg/L; Silica 6.3 mg/L and pH 8.9 (also see Chapter 4, Table 4.3).

5.4.2 Artificial destratification

In the morning when samples were taken before artificial mixing of the lake started, an overnight cyanobacteria surface bloom formation was frequently observed, near the shores (Fig. 5.5).

Figure 5.5 Cyanobacterial bloom still visible in the morning on the surface water during artificial mixing at sampling site B.

The average measured wind velocity for most of September was 1.07 ± 0.6 m/s, while the wind speeds increase to an average speed of 10.2 ± 3 m/s during the high-wind and storm events of 4, 21, 25 and 27th September 2004 (Table 5.2).

Table 5.2 Average wind velocity (m/s).

	Year	
Month	**2001**	**2004-2005**
April	0.98	1.02
May	1.04	1.01
June	1.01	1.04
July	1.09	1.07
August	1.13	1.20
September	1.35	1.98
October	1.23	1.34

However during the afternoon when the second set of samples was taken the *Microcystis* bloom next to the shores of the lake had disappeared and a low

concentration of *Microcystis* colonies was found on the surfacewater. We also observed a difference in the number of cyanobacterial colonies with artifical mixing in the morning and afternoon surfacewater at the four sampling sites (Fig. 5.6).

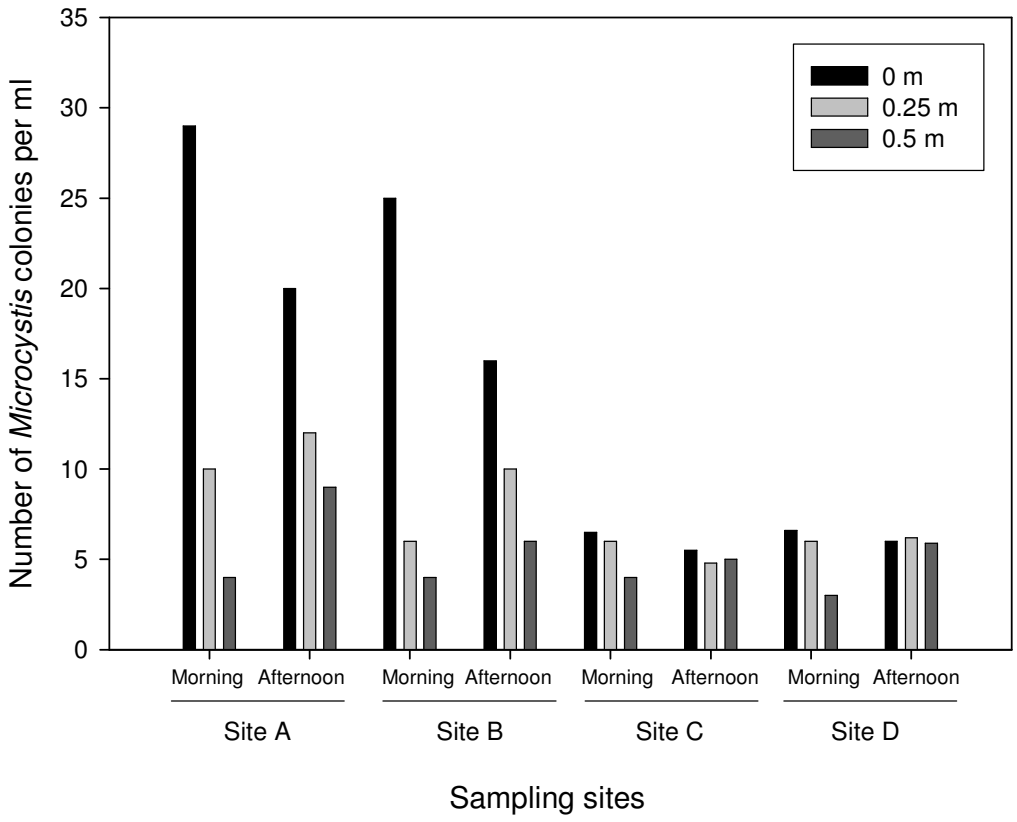

Figure 5.6 The number of *Microcystis* colonies/ml of samples taken at 0.25-m depth intervals at different locations in the lake during artificial mixing, taken on the morning and afternoon of August 22, 2004.

5.4.3 Detection of toxicity

The toxicity of the environment strains were also determined using ELISA and through inhibition of PPA2, and the toxicity levels were comparable to the toxin levels present in the cultured PCC7806 strain (Table 5.3).

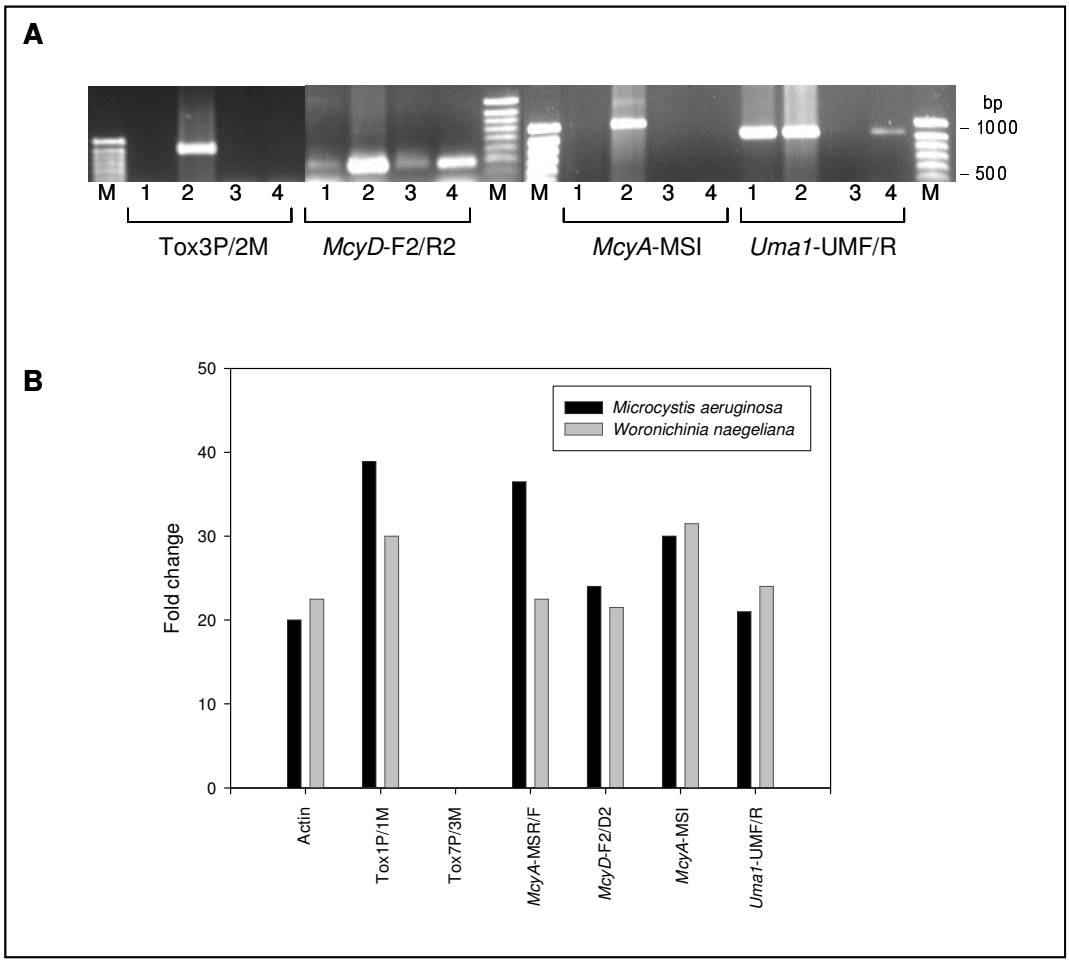

Figure 5.7 (A) Separation of PCR amplicons obtained after PCR of *Microcystis aeruginosa* strain UPUS1 (1); PCC7806 (2); UP37 (3) and *Woronichinia naegeliana* strain UPUS2 (4) using different primers on a 2% agarose gel. M = Hyperladder^{TM} IV, Bioline, USA. (B) Quantitative PCR of RNA from *Microcystis aeruginosa* strains UPUS1 and *Woronichinia naegeliana* strain UPUS2 with selected primers. Actin was included as standard.

Amplification products obtained from the cyanobacteria spp. sampled in Sheldon Lake provided for supporting evidence that the environment *Microcystis aeruginosa* (UP37, UPUS1) and *Woronichinia naegeliana* (UPUS2) strains contained the *mcy* genes present in the culture *M. aeruginosa* strain PCC7806, and that is normally associated with toxin production (Fig. 5.7, Table 5.3). These included amplification products after amplification with TOX1P/1M (*mcyB*) (~350 bp; UP37, UPUS1 and

UPUS2), TOX7P/3M (*mcyB*) (~350 bp; UP37); *mcyA*-MSR/MSF (~1.3 Kb; UPUS1 and UPUS2), *mcyD*-F2/R2 (~297 bp; UP37, UPUS1 and UPUS2), and *Uma1*-UMF/R (~867 bp; UPUS1 and UPUS2) (Fig. 5.7A).

The data was further supported by the expression of selected genes in the *mcy* cluster (i.e., TOX1P/1M, *mcyA*-MSR/MSF, *mcyD*-F2/R2, and *Uma1*-UMF/R) using quantitative PCR on RNA isolated from the environment strains *Microcystis aeruginosa* (UPUS1) and *Woronichinia naegeliana* (UPUS2) (Fig. 5.7B). Similar to the PCR analysis, no expression was obtained after RT-PCR of RNA from the environment strains *Microcystis aeruginosa* (UPUS1) and *Woronichinia naegeliana* (UPUS2) using primer set TOX7P/3M (Fig. 5.7B).

Examination of the rainbow trouts revealed damage to the fins and outer surfaces of the body (Fig. 5.8). Damage to the gills of some of the fish was also observed. Microscopic assessment of the fish hepatocytes revealed clustered blebs and deformation of the cell structure (data not shown).

Figure 5.8 Histopathological investigation of *Oncorhynchus mykiss* revealed damage to gills and fins during cyanobacterial surface blooms during August, 2004.

Table 5.3 Comparison of PCR with different primers, quantitative PCR, ELISA and Protein Phosphatase inhibition (PP2A) assay as determinants of toxicity in strains from different geographical regions. (+ = positive/product; - = negative/no product; / not assayed).

Organism	Isolation date	Geographic origin	PCR										RT-PCR [a]	PP2A* (%)	ELISA (µg/L)
			Tox 3P/2M	Tox1 P/1M	Tox7 P/3M	Tox1 0P/4M	*McyB* Tox2 +/-	*McyB 2*-FAA/RAA	*McyA* -MSR/MSF	*Mcy D*-F2/R2	*McyA* -MSI	*Uma1* -UMF/R			
Microcystis aeruginosa:															
PCC7806 (Cultured strain)	1972	Braakman Reservoir, The Netherlands	+	+	+	+	+	+	+	+	+	+	/	100	44.1 ± 31
UP37 (Environment strain)	2004	Krugersdrift Reservoir, ZA		+	+					+			/	100	61.2± 54
UPUS1 (Environment strain)	2004	Sheldon Lake Colorado, US		+					+	+		+	+	100	32.8 ± 15
Woronichinia naegeliana:															
UPUS2 (Environment strain)	2004	Sheldon Lake Colorado, US		+					+	+		+	+	45	5.6 ± 1.6

***** % of inhibition effect of mycrocystin-LR on protein phosphatase activity. Inhibition was calculated from the activity of PP2A on a colorimetric substrate. The control used to plot a standard curve was MCYST-LR and activity values between 0% and 100% correspond to the log of inhibitor concentrations of 0-3000 nM (assuming the sole PP2A inhibitors in *M.aeruginosa* to be microcystins and the molecular mass of microcystin-LR is 995.2).

[a] Results obtained with primers used for RT-PCR correspond in all experiments to that obtained with PCR, results were comparable with regard to presence of amplicon.

5.5 Discussion

The phenomenon that *Microcystis* form overnight surface blooms are probably due to the fact that under low light conditions (early morning) cyanobacteria increase their buoyancy or at night, cyanobacteria do not actively regulate their buoyancy (Pearson 1990). Thus, if the artificial mixing stopped overnight they may float to the surface, but because it is dark they cannot reverse their buoyancy. Typically, with an underwater circulating aerator there is considerable turbulence close to the surface boil, but further away it decreases. The consequence of this phenomenon is that the *Microcystis* colonies are entrained close to the surface boil but remained disentrained away from the surface boil. This was demonstrated by Visser *et al*. (1996) where *Microcystis aeruginosa* colonies close to the surface boil of the underwater circulating aerator remained buoyant as they experienced a low mean photon density due to regular mixing, and consequently accumulated little carbohydrate. By contrast, *Microcystis* colonies away from the surface boil of the underwater circulating aerator were not entrained, remained close to the water surface and experienced a higher photon density that caused the cells to accumulate carbohydrate, increase their net cell density and lose buoyancy and sink (Ibelings *et al*. 1991). This phenomenon which indicates differences in buoyancy between locations where horizontal mixing of water masses were restricted to an extent that allowed differences in vertical mixing was confirmed by analyzing the distribution of *Microcystis* colonies with depth during sampling in the morning and afternoon of 22 August 2004. Our data clearly highlighted that sampling sites A and B which were a larger distance from the aerators, had a higher number of sinking *Microcystis* colonies per mL in the afternoon. While sampling sites C and D which are near two surface boils of

underwater aerators show a very low percentage of sinking colonies although site C was much shallower than all the other sampling sites (Fig. 5.6). Out of our data we determined that artificial mixing does not prevent bloom forming of cyanobacteria at sampling sites A and B and that the mixing in those areas of the lake was insufficient at an average wind speed of 1.2 m/s.

Kortmann *et al.* (1994) reported that aeration is not always successful in reducing the high relative abundance of the large scum-forming and odour-producing cyanobacterial species or concentrations of available phosphorus. In fact, transparency declined following artificial circulation in over 50% of reported case studies.

5.5.1 Incomplete mixing

In our field studies we observed that the artificial mixing rates in Sheldon Lake alone are insufficient to cause a transition to a well-mixed system and therefore the same species cyanobacteria (i.e., *Microcystis aeruginosa*) remained dominant throughout summer (Fig 5.6). During the end of September major changes in species composition happened when there was a true transition from an incomplete to a complete mixing due to high-wind and storm events the 4, 21, 25 and 27th of September. The average recorded rainfall during the month of September was 7.42 cm. The average windspeed measured for September was 1.07 ± 0.6 m/s, while the wind speeds increase to an average speed of 10.2 ± 3 m/s during the above high-wind and storm events (Table 5.2). Since Sheldon Lake is also a recreational focus-point in Ft. Collins, the lake is surrounded by many large tree species, i.e., poplar, and coniferous species, sheltering the lake against prevailing winds. General ecological theory predicts that incomplete

mixing should promote species coexistence (Levin 1974; Atkinson & Shorrocks 1981; Powell & Richerson 1985; Hsu & Waltman 1993; Tilman 1994).

Field studies of Jacobsen and Simon (1993) on a shallow eutrophic lake in Denmark support these predictions. They observed a massive summer bloom of the cyanobacterium *Aphanizomenon* flos-*aquae*, which at the end of July, during a period of high winds and rainfall collapsed, from a biomass of 140 mm^3/L in July to 6.2 mm^3/L in early August. The lake was still stratified, though, and *Aphanizomenon* continued to dominate, although at much lower biomass. In late August, new storms destroyed the stratification, and *Aphanizomenon* was replaced by cryptomonad species.

The increase in diatom standing stocks in the surface waters (0.25 m) of Sheldon Lake during September was associated with a higher average daily wind speed. Their inoculation into the water column under periods of high wind can lead to significant changes in the phytoplankton composition (Carrick *et al*. 1993). Based on the observations of Smetacek (1985) sedimentation of diatoms can be an integral part of their life cycle and enhance survivorship by incidental predator avoidance or redistribution to more nutrient-rich waters. Padisak *et al*. (1988) stated that as wind turbulence increased, the structure of the surface-water community is altered by the increase in algal biomass due to the results from resuspension of diatoms. Because these alterations occur within brief time frames (hours to days) depending of the meteorological activities (wind velocity and rain), we observe that these processes can account for some of the temporal variation in phytoplankton in Sheldon Lake and thus enhances temporal heterogeneity during October.

5.5.2 Toxicity

The morphological discrimination between toxic and nontoxic cyanobacterial strains are not possible as some cyanobacteria are known to be toxic, some may be genetically capable of producing toxins but do not under all conditions, and some do not produce toxins at all. Since the recently discovery of the *mcy* genes coding for subunits of the microcystin synthetase in *Microcystis* (Dittmann *et al*. 1997; Nishizawa *et al*. 1999, 2000; Tillett *et al*. 2000) it is possible for the first time to develop DNA primers for simple PCR assays, which were then used to identify strains bearing *mcy* genes. By using the PCR assays it could be demonstrated that the occurrence of *mcy* genes in cells is correlated with their ability to synthesize microcystin and vice versa (Kurmayer *et al*. 2002).

In most environmental samples that was analyzed the relative intensity of *mcyB* and *mcyD* bands are not approximately equal. The differential amplification of environmental samples may be indicative of the presence of both nontoxic and toxic *Microcystis* (Ouellette & Wilhelm 2003). In our study, we measured toxicity in the cultured strain *Microcystis aeruginosa* (PCC7806) and environmental strains of *M. aeruginosa* (UP37, UPUS1), as well as in an environmental strain of *Woronichinia naegeliana* (UPUS2) using protein inhibition and ELISA assay. We further observed the expression of selected genes in the *mcy* gene cluster after quantitative PCR of RNA isolated from *M. aeruginosa* (UPUS1) and *Woronichinia naegeliana* (UPUS2), although to our knowledge the latter was not reported previously as toxic. The only cyanobacterial colonies observed by microscopic observation during the surface

bloom of 11th August 2004 was *Woronichinia naegeliana*, while the bloom of 22nd August was dominated by *M. aeruginosa* (Fig. 5.3).

In urban eutrophic aquatic systems like Sheldon Lake, fish stand at the top of the aquatic food chain, and are possibly affected by exposure to toxic cyanobacteria. Cyanotoxins may accumulate in the trophic web (Amorim & Vasconcelos 1999; Saker & Eaglesham 1999) and produce diverse intoxication symptoms and chronic effects that are difficult to diagnose and prevent (Falconer 2001). Many experimental studies have been conducted to document the toxicity of microcystin exposure though gastrointestinal or blood systems on fish such as common carp (Råberg *et al*. 1991; Fischer *et al*. 2000; Li *et al*. 2001; Wu *et al*. 2002), rainbow trout (Sahin *et al*. 1996; Bury *et al*. 1996, 1997; Tencala & Dietrich 1997; Fischer *et al*. 2000) and channel catfish (Zimba *et al*. 2001). However, all these studies were limited to acute toxic experiments, and they were based on either oral gavaging, or intraperitoneal injection, or administration via the dorsal aorta of the toxins, which cannot reflect the uptake route under natural conditions.

Toxins used in these experiments were dried cyanobacteria containing microcystin or purified microcystin, which do not evaluated long-term exposure in natural environments. Histopathological investigation done by us on some rainbow trout deaths during cyanobacteria blooms in Sheldon Lake indicated that the cause of death was likely to the result of damage to the gills and fins (Fig. 5.8). Gill damage by dissolved microcystin-LR has been shown experimentally in tilapia and trout (Garcia 1989; Gaete *et al*. 1994; Bury *et al*. 1996). However, gill damage may also be caused by the high pH values associated with cyanobacterial photosynthesis and the high

ammonia concentrations arising from the decomposition of cyanobacterial cells. Irrespective of this, gill damage almost certainly enhances microcystin uptake leading to liver necrosis. Nevertheless it should be pointed out that histopathological investigation was done on already dead rainbow trout, collected near the shores of Sheldon lake, and that there was no control of the same age to compare our results with.

5.6 Conclusion

This study has shown that Sheldon Lake, during the summer of 2004 a year after restoration, contained toxigenic strains of cyanobacteria and are subject to increasingly negative impacts such as stormwater nutrients and contaminant loading which lead to eutrophication. Especially prevalent within Sheldon Lake, which is a prime fishing spot for residents of Fort Collins, is the concern of health hazard for people and domestic animals from cyanotoxins. Although intoxication has not yet been clearly demonstrated, bioaccumulation of four different cyanotoxins in the trophic chain was observed (Falconer *et al*. 1992; Lindholm *et al*. 1989; Saker *et al*. 1999). However due to the fact that research did not yet clarify long-term effects of cyanotoxins, people should limit or avoid eating fish caught there. Furthermore, we find the PCR assays that were applied during the study of cyanobacterial strains, sensitive in providing a useful indicator of toxicity, although it does not provide a direct measure of the toxin present, it detect toxigenic cells rather than toxins and require little sample preparation and modest capital cost.

Acknowledgements

The authors would like to express their sincere gratitude the National Foundation of Research, and the Water Research Commission, South Africa for provision of funding.

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