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Full Length Research Paper

Use of PCR based technologies for risk assessment of a winter cyanobacterial bloom in Lake Midmar, South Africa.

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Toxic freshwater cyanobacterial blooms are potential health hazards in water supply reservoirs and therefore predicting bloom events is an important goal of monitoring fresh water programmes. The recent identification of the *mcy* genes in the production of microcystin synthetase for the first time provides an avenue to study microcystin production at a genetic level. This paper reports analysis of a winter cyanobacterial bloom by use of quantitative real-time PCR, ELISA and PP2A methods for detection of strains present and determination of their toxigenicity in Lake Midmar South Africa. We further investigated the taxonomic composition of phytoplankton at different sampling sites and the physical and chemical changes caused in the surface water of Lake Midmar by waterfowl. Our study clearly demonstrates that the interaction between low surface water temperatures and productivity was overshadowed by the response to nutrients and nutrient availability. We also confirmed the presence of the toxic cyanobacterial strains through the use of molecular markers that detect the presence of some of the *mcy* genes in the *mcy* gene cluster that is able to synthesize microcystin toxins in *Microcystis* spp.

Key words: Winter cyanobacterial bloom, waterfowl, TN:TP ratio, *mcy* gene cluster and quantitative real-time PCR.

INTRODUCTION

Toxic cyanobacteria are a diverse and widely distributed group of organisms that can contaminate natural and man-made bodies of water. Under certain environmental conditions, some species of cyanobacteria (such as *Microcystis aeruginosa* and *Anabaena flos-aquae*) produce toxins that are released in water upon the death of the cells. The most studied class of these toxins, the microcystins, are compose of 7 amino acid cyclic peptide hepatotoxins and so far 65 structural isoforms have been described, each with a unique level of toxicity (Carmichel, 1997, 2001). Hazards to human health may result from chronic exposure via contaminated water supplies. Studies in Europe and North America have demonstrated that 25 - 75% of blooms produced by toxic strains encountered in eutrophic lakes are toxic to humans. In Bahia, Brazil, *Anabaena* and *Microcystis* spp. were responsible for a lethal outbreak attributed to cyanobacterial toxins present in drinking water, which resulted in the death of 88 children from over 2000 cases of gastroenteritis over a period of 42 days (Teixera et al., 1993). Illnesses caused by cyanobacterial toxins to humans fall into three categories: gastroenteritis and related diseases, allergic and irritations reaction, and liver diseases. Microcystins have also been implicated as powerful tumour promoters and inhibitors of protein phosphatase 1 and 2A and they are suspected to be involved in the promotion of primary liver cancer in humans (Codd, 1999; Zegura et al., 2003). Evaluation of the development of toxin concentrations in cyanobacterial populations during bloom events is important for the prediction of potential health hazards. Changing toxin concentrations in cyano-

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bacterial blooms most probably reflect alterations in species and strain composition with various toxins and toxicities, as well as the regulation of toxin biosynthesis in specific strains under certain environmental conditions.

The environmental or abiotic factors, which are known to influence toxic bloom formation, are temperature, pH, light intensity, and nutrient concentration (van der Westhuizen and Eloff, 1985). In an attempt to account for the variation in toxin content that has been observed on both temporal and spatial scales, culture studies have been carried out to investigate the influence of environmental conditions such as illumination (Rapala and Sivonen, 1998), or the concentration of nutrients, such as phosphorus (Oh et al., 2001) and nitrogen (Sivonen, 1990) or temperature (Rapala et al., 1997). Changes in toxin production due to variable laboratory conditions are usually lower than the observed differences in toxin levels between strains of a given species or that observed in natural blooms of *M. aeruginosa* (Sivonen et al., 1999). The recent 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) made it possible for the design and construction of primer sets, which can then be used to identify strains bearing *mcy* genes (Oberholster et al., 2006a). This 55-kb gene cluster consists of six open reading frames (ORFs) with a mixed non-ribosomal peptide synthetase/polyketide synthase nature (*mcyA* to *mcyE* and *mcyG*) and four smaller ORFs with putative precursor and tailoring functions (*mcyF* and *mcyH* to *mcyJ*) (Tillett et al., 2000).

It could be demonstrated that the occurrence of *mcy* genes in cells is correlated with their ability to synthesize microcystin and, *vice versa*, that microcystin-free cells usually do not contain *mcy* genes (Kurmayer et al., 2002). This approach is appealing as an early warning diagnostic and is very sensitive because of the amplification achieved by PCR. The aim of the work described here was to focus on the use of ELISA, PP2A and quantitative real-time PCR as methods for detecting toxic strains of cyanobacteria and the expression levels of the *mcyA-D* genes as representatives of the microcystin peptide synthetase and polyketide synthase genes at a low surface water temperature, as well as possible environmental factors responsible for the development of a cyanobacterial winter bloom in Lake Midmar. Kruger and Eloff (1978) found a correlation between the water temperature and the development of *Microcystis* blooms in eutrophic impoundments in South Africa. They reported that *Microcystis* blooms started to develop in open lake water, once temperatures reach 16 - 17°C. Their results show the effect of temperature on specific growth rate occurs after the upper temperature limit is surpassed. Although prevailing water temperatures in South Africa is generally suitable for cyanobacterial growth during the greater portion of the year, this is to our knowledge the first report of a cyanobacterial winter bloom in South Africa.

MATERIALS AND METHODS

Study site description

The Mgeni river system has particular significance in the Province of KwaZulu Natal, South Africa because it is strategically positioned as the water supply for the Pietermaritzburg-Durban complex. Some 45% of the population of the province is dependent upon it for their water supply and it supports 20% of the industrial output of the whole country. The catchment lies within the Karroo System, the highlands comprising of shales, mudstones and sandstones of the Beaufort Series while the rest of the catchment, the mistbelt and uplands between 915 m and 1372 m consist of erodible soft sandstones and shales of the Ecca Beds. Lake Midmar was built in the Mgeni river catchment between 1962 and 1965, with a surface area of 1560 ha and a maximum depth of 23 m at full supply level (Breen, 1983) (Figure 1). Lake Midmar, which is a prime fishing and recreation spot, support emerged and submerged macrophytes in the littoral zone, but also presented large expanses of open water pelagic habitat which is less suitable for foraging by most aquatic birds. The central portions of the lake are used by only a few, deep-diving species, such as the White-breasted Cormorant (*Phalacrocorax carbo*) and the Reed Cormorant (*Phalacrocorax africanus*) (Elmberg et al., 1994).

Sampling strategy

Phytoplankton samples were collected in the winter months of 2005 at 4 sampling sites in Lake Midmar, using a syringe sampler modified after Baker et al. (1985). Duplicate monitoring samples were taken at the surface and 50 cm depth intervals down to 5 m at each site. On each date, the integrated water samples were transferred from the field to the laboratory in a dark coolbox, within no more than 3 h. Monitoring began in June to the end of August and was performed once every two weeks when the cyanobacterial bloom occurs at sampling sites 1 and 2. The duplicate samples (5 L) were preserved in the field by addition of acid Lugol's solution to a final concentration of 0.7%, followed after one hour by addition of buffered formaldehyde to a final concentration of 2.5%.

Cyanobacterial population growth

The net rate increase of population growth per unit time was estimated from the logarithms of biomass (cell number) during the continuous increase of cyanobacterial cells in June, July as

$$k = (\ln N_2 - \ln N_2)/(t_2 - t_1)$$

where N_1 is the biomass at time t_1 and N_2 is the biomass at time t_2. One day was considered as the unit of time (k = day^{-1}) (O'Sullivan and Reynolds, 2003).

Microscopic analysis

All identifications were made using a compound microscope with 200 - 800x oculars and appropriate keys (Wehr and Sheath, 2003). Field or strip counts were made until at least 100 individuals of each of the dominant phytoplankton species were counted. Colonies of *Microcystis* were disintegrated by ultrasonication prior to counting (40 impulses per s over 4 min for a 10 ml sample) (Kurmayer et al., 2003). All counts were based on numbers of cells observed and the

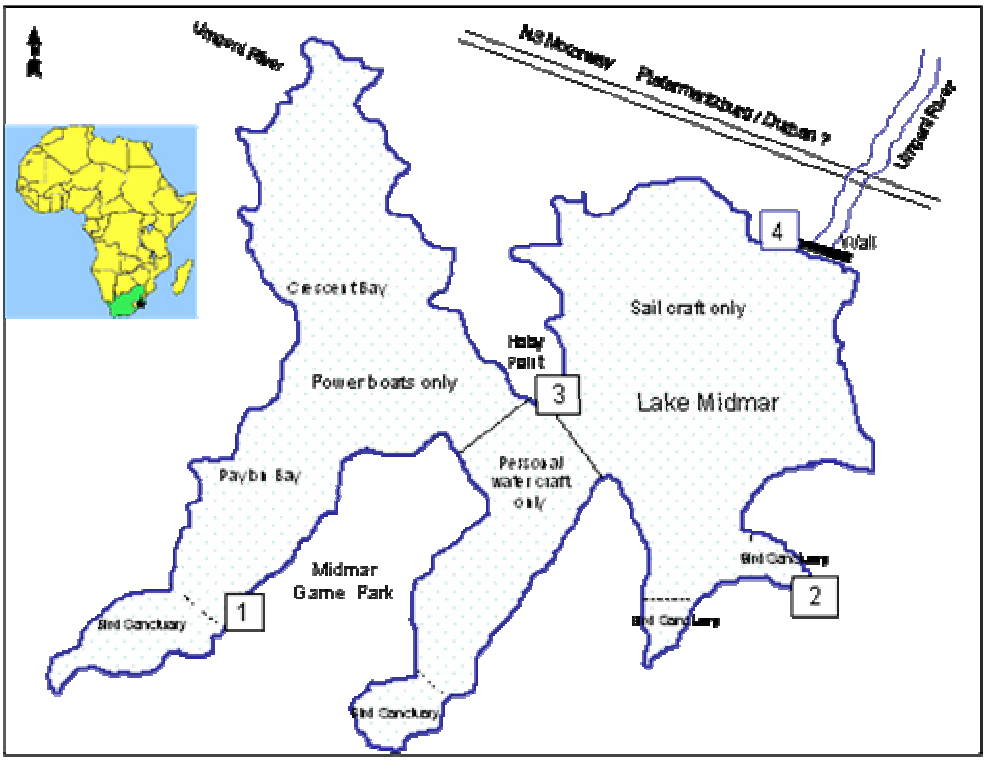

Figure 1. Lake Midmar that is part of the Mgeni river system in the Province of KwaZulu Natal, South Africa.

individual data grouped into major algal classes at each sampling site. Community comparisons were made using percent community similarity (the sum of the minimum relative abundance for all taxa between any two samples) to compare all study sites in each sampling event (Brower et al., 1990).

Chlorophyll and physicochemical measurement

Chlorophyll *a* was extracted from lyophilized GF filters using N, N-dimethylformamide for 2 h at room temperature and measured photospectrometrically at 647 and 664 nm according to the calculations of Porra et al. (1989). Nutrients dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) was analyzed using classical spectophotometric methods (American Public Health Association, American Water Work Association, and Water Pollution Control Federation, 1980). Temperature profiles, pH and conductivity of the water column were measured with a Hach^{TM} sension 156 portable multiparameter (Loveland, CO, USA). Secchi depth (transparency) was measured at all four sampling sites with a 20 cm Secchi disc, while the trophic state of the sampling sites were also characterized by their Secchi disc transparency (OECD, 1982). Wind velocity was measured at each of the sampling sites 1 m above the water surface with a Weather monitor 2 (Hayward, CA 94545 USA). Water column stability was measured using the Brunt-Väsäilä buoyancy frequency squared term (N^2), calculated (Patterson et al., 1984; Viner, 1985) from:

$$N^2 = (-g/\rho)\ (\partial\rho/\partial z)$$

Where g = 9.81 m s^{-2} as acceleration due to gravity, $\partial\rho/\partial z$ the density gradient determined for the entire water column, of the mean density ρ. Values of N^2 reported in this study have the unit 10^{-4} s^{-2}.

Reference cyanobacterial culture

The axenic *M. aeruginosa* strain PCC 7806 were obtained from the Institute Pasteur (PCC; Paris, France). The strain was cultured in liquid MA (Ichimura, 1979) medium at 25 ± 2°C under continuous illumination of 25 µmol photons m^{-2} s^{-1}. At 21 days growth, 2 ml of the culture was transferred to a serum vial and lyophilized for 48 h. The sample was then stored under vacuum until DNA was extract**.**

Pretreatments of environment samples for whole-cell PCR

For whole-cell PCR, cyanobacterial cells were collected from the environmental samples of sites 1 and 2, by placing the samples mixed with distilled water in a glass cylinder under fluorescent light. Under these conditions, the cyanobacterial cells floated to the surface and the lower water layers were siphoned off. Before resuspension in distilled water to define volume, the cells were washed three times with distilled water and subjected to a freeze-thraw treatment for PCR template preparation (Baker et al., 2001). DNA was extracted from the environment samples as well as from the reference culture strains PCC 7806 using DNAzol®-Genomic DNA Isolation reagent following the manufacturer's procedures (Molecular Research Center, Inc., USA). Extracted DNAs were purified once (culture strain) or twice (environmental strains) with a Prep-A-Gene DNA Purification Kit (Bio-Rad) according to the manufacturer's instructions and eluted in 60 µl.

Table 1. Oligonucleotides used for RT-PCR and PCR analysis.

Gene region	Primer set	Primer sequence	T_m (ºC)	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	Grobbelaar et al., 2004
	Tox 1M	5'-TAAGCGGGCAGTTCCTGC-3'	58.2		Oberholster et al., 2006a, b.
	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 ***McyD***	Tox2+	5'-AGGAACAAGTTGCACAGAATCCGCA-3'	50		
	Tox2-	5'-ACTAATCCCTATCTAAACACAGTAACTCA-3'	50	~200 bp	Kaebernick et al., 2002.
	McyDF2	5'-GGTTCGCCTGGTCAAAGTAA-3'	50		
	McyDR2	5'-CCTCGCTAAAGAAGGGTTGA-3'	50	~297 bp	
McyB	FAA	5'-CTATGTTATTTATACATCAGG-3'			
	RAA	5'-CTCAGCTTAACTTGATTATC-3'	40	~580 bp	Neilan et al., 1999.

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 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). The PCR amplification with whole cells started with 6 µl of crude sample, pretreated sub sample with an approximate cell density of 8 x 10^6 cells/ml, or 0.1 µg 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 that 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 amplification conditions were identical to those for the samples described above. An extra ramp rate of 3 s/°C between the denaturing and annealing steps was set 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.

RNA extraction and quantitative PCR

Cells were homogenized using liquid nitrogen and RNA extracted using the Qiagen RNA easy kit (Qiagen Inc., USA) according to the manufacturers' instructions, and using DEPC-treated equipment and solutions.

Quantitative PCR was performed using 5 ng of total RNA per reaction and with 10 µM of each primer (Table 1). Quantitative real-time PCR was performed using the iScript One-Step RT-PCR Kit (Bio-Rad, USA) and analysed using the iCycler iQ Real-Time PCR Detection Instrument (Bio-Rad). The cycling parameters consisted of 1 cycle at 95℃ for 10 min; 40 cycles starting with 1 cycle at 95℃ for 10 s, primer specific annealing T℃ for 5 s, 72℃ for 10 s; followed by the melting curve analysis (95℃ for 0 s, 65℃ for 15 s, 95℃ for 0 s), and cooling (40℃ for 30 s). A minimum of 7 reactions was done for each fragment analyzed, standard curves were generated using dilution series (1:1, 1:10, 1:100, 1:1000) and repeated. After primer design from sequence information using Primer Designer 5 (ver. 5.03, Scientific and Educational Software) purified salt-free primers were synthesized (IDT). In order to calculate relative expression ratios for target genes, these were normalised with the expression of the unregulated 16S rRNA transcript (Pfaffl, 2001).

Protein phosphatase inhibition and ELISA assays

Toxicity was determined by using the same methods as described in Boyer et al. (2004). Briefly, 5 liter samples were collected from sites 1 and 2 where the cyanobacterial bloom occurred during June to August. The water was poured gently through a 934 AH glass fiber filters in the field, frozen on dry ice, and returned to the laboratory in a coolbox for toxin analysis. Filters for toxin analysis were extracted by grinding with 10 ml of 50% methanol containing

1% acetic acid and clarified by centrifugation. This extract was used for analysis of microcystins using the protein phosphatase inhibition assay (PPIA) as described in Carmichael and An (1999).

ELISA assay was conducted with a Quanti^{TM} Kit for microcystins (EnviroLogix, USA) as described by the manufacturers. The concentration of microcystin was measured by reading the optical density (OD) of the EnviroLogix calibrators and negative control, and respective collected samples at an absorbance of 450 nm. A semi-log curve was constructed using the EnviroLogix calibrators and the microcystin concentration calculated. Since the limit of detection (LOD) of the kit is 0.147 ppb, % B_o was also incorporated during the calculations, where % B_o = (OD of sample or calibrator/OD of negative control) x 100. The LOD was determined by interpolation at 81.3% B_o from a standard curve, where 81.3% B_o was determined to be 3 standard deviations from the mean of a population of negative water samples. 100% B_o equals the maximum amount of microcystin-enzyme conjugate that is bound by the antibody in the absence of any microcystin in the sample. The results were obtained by reading the plate on a multiskan ascent (Thermo Labsystems, USA).

Bird counting protocol

Birds were counted from shore during June to August with an x45 spotting scope. A running record of location was kept for all individual birds encountered to decrease the likelihood that individuals were counted more than once. For community analysis, we used only non-passerine birds that feed at or beneath the surface of the water at the 4 sampling sites of 50 m^2 each. Classification of the different bird species was done according to Everyone's guide to South African birds (Johnson, 1981).

RESULTS

Species composition, meteorological events and stability

During the winter month of June 2005 a cyanobacterial bloom started to developed near the shores of sampling sites 1 and 2 with a average cell abundance of 1.21 x 10^8 cells/ml, reaching a peak in July with a maximum abundance of 1.80 x 10^9 cells ml^{-1} and decline at the end of August when wind velocity increase from an average speed of 0.41 m s^{-1} in June to 3.2 m s^{-1} during high-wind and storm events in mid August. The estimated N^2 for Lake Midmar during June, July and August were 3.10, 4.38 and 4.51. The highest chl-*a* (0.092 mg/l) was observed at site 1, while chl-*b* (0.041 mg/l) was the highest at site 3. Identification of individual cyanobacteria colonies collected at sites 1 and 2 revealed the occurrence of two morphospecies: *M. aeruginosa* (Smith, 1950) and *Microcystis wesenbergii* (Teiling, 1941) (Figure 4). The phytoplankton composition from the four sampling sites revealed a dominance of 91% Cyanophyceae at the surface water of sites 1 and 2, while a dominance of 68% Bacillariophyceae (*Melosira granulata*) and 23% Chlorophyceae (*Botryococcus braunii*) were observed at sites 3 and 4. The highest cyanobacterial population growth net rate increase per unit time (k[day^{-1}]) was during the peak period in July and was estimated at 0.33 (Figure 2).

Amplification products obtained from the cyanobacteria spp. sampled in Lake Midmar provided for supporting evidence that the environment strains *M. aeruginosa* and *M. wesenbergii* at sampling sites 1 and 2, contained representative genes within the *mcy* gene cluster present in the *M. aeruginosa* culture strain PCC7806, and that is normally associated with toxin production (Figure 5, Table 2). Low expression levels of the *mcyA-D* genes at sampling sites 1 and 2 were observed after analysis of RNA from the strains isolated from the environmental samples using quantitative real-time PCR (Figure 4). The toxicity of the environmental strains was also determined using ELISA and inhibition of PP2A assays and the toxicity levels were compared to the toxin levels present in the cultured PCC7806 strain. Although we found variations in the toxigenic levels of the cyanobacterial samples, microcystin-LR were detectable in all samples of sites 1 and 2, varying from 0.09 to 0.17 μg L^{-1} on different sampling dates. The levels of microcystin-LR in Lake Midmar never exceeded the World Health Organization (WHO) drinking water threshold fixed at 1 μg L^{-1} during the study period.

Bird composition

The bird densities in Lake Midmar were typically highest in the shallow and highly productive littoral zone. The dominant bird taxa during our survey in the littoral zone of sampling site 1 and 2 included Egyptian Goose (*Alopochen aegyptiacus*), Red-konbbed Coot (*Fulica cristata*) and the Whitefaced Duck (*Dendrocygna viduata*). While in the large stretches of open water the White-breasted Cormorant (*Phalacrocorax carbo*), Reed Cormorant (*Phalacrocorax africanus*) and the Little Grebe (*Tachybaptus ruficollis*) were observed. The highest number of individual birds counted at sampling site 1, was on June 10th with an average of 163 birds per 50 m^2 (Figure 3).

Transparency, pH, temperature and nutrients

The mean Secchi depth transparency at sampling site 3 and 4 was (1.8 + 0.2) and (0.69 + 0.4) at sites 1 and 2 during the cyanobacterial peak exhibited in July. The eutrophic state at sampling sites 1 and 2 can be largely characterized due to phytoplankton rather than inorganic particles or colour. The estimated thickness of the euphotic zone was 1.51 m at sites 1 and 2, compared to 3.96 m at sites 3 and 4. The average water temperature was 11.3°C for June and 10.1°C for July during the peak of the cyanobacterial bloom, while surface pH values were consistently near 7.9 for the same period at the four sampling sites. The high cyanobacterial cell abundance at sampling sites 1 and 2 during the July peak was associated with a low TN:TP ratio (< 10). The average TP for July during the peak of the cyanobacterial bloom at site 1 and 2 were 800 μg L^{-1}, while the average TN was 2300

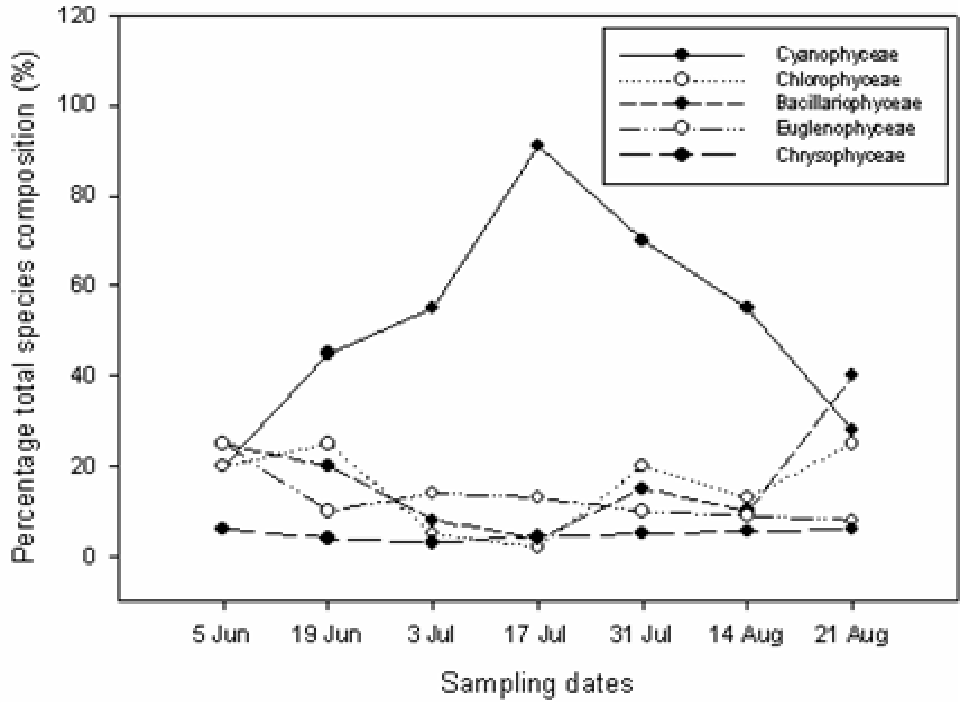

Figure 2. Percentage total species composition at sampling sites 1 and 2 during winter cyanobacterial bloom.

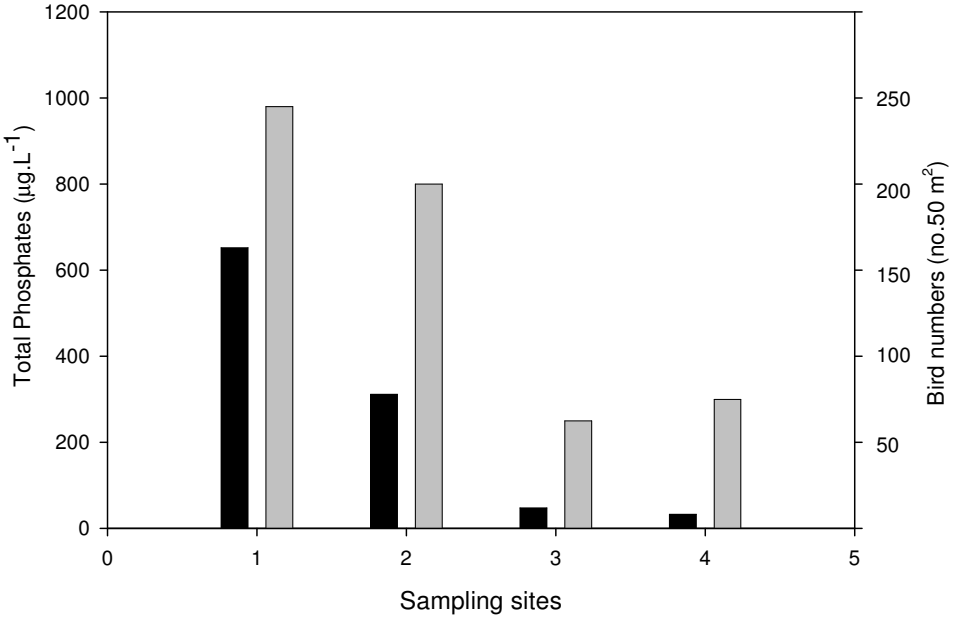

Figure 3. Relationship between bird numbers and total phophate as measured at the different sampling sites in Lake Midmar on the 10th of June.

µg L^{-1} that was in strong contrast with the average TP of 300 µg L^{-1} and TN of 400 µg L^{-1} at sites 3 and 4. There was, however no significant difference in the silica concentration at the four sampling sites with an average of 19,500 µg L^{-1} at sites 1 and 2, and 20,000 µg L^{-1} at sites 3 and 4.

Table 2. 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).

Strain	**Isolation date**	**Geographic origin**	**PCR**										**RT-PCR**[a]	**PP2A**	**ELISA**
Microcystis aeruginosa			*McyB*-Tox3P/2M	*McyB*-Tox1P/1M	*McyB*-Tox7P/3M	*McyB*-Tox10P/4M	*McyB*-Tox2+/-	*McyB2*-FAA/RAA	*McyA*-MSR/MSF	*McyD*-F2/R2	*McyA*-MSI	*Uma1*-UMF/R			
PCC7806 (Cultured strain)	1972	Braakman Reservoir, The Netherlands	+	+	+	+	+	+	+	+	+	+	/	+	+
UP10 (Environment strain)	2004	Roodeplaat Reservoir, ZA	+	-	-	-	-	+	-	+	-	+	+	+	+
UP40 (Environment strain)	2005	Midmar Lake, ZA	-	-	-	-	+	-	+	+	-	+	+	+	+
UPUS1 (Environment strain)	2004	Sheldon Lake Colorado, US	-	+	-	-	-	-	+	+	-	+	+	+	+

[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.

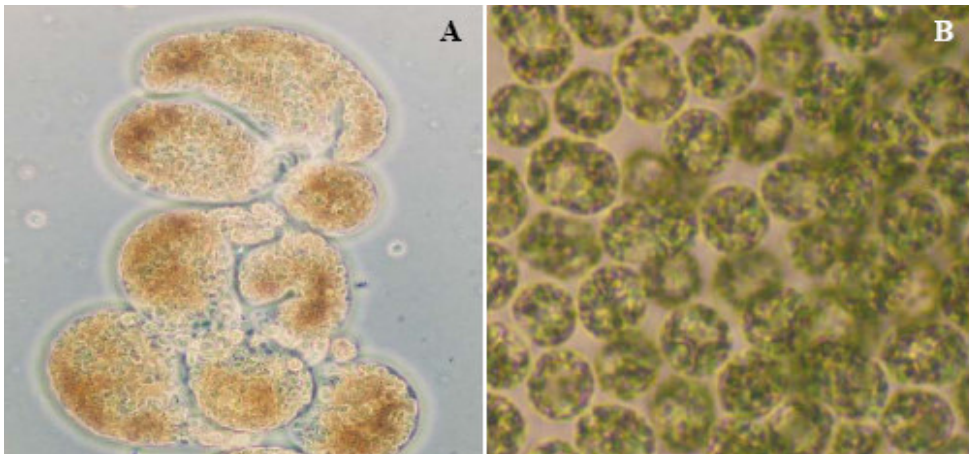

Figure 4. (A) *Microcystis wesenbergii* (after Teiling 1941, Wojciechowski 1971); Unstained, bright-field microscopy, 200 x; (B) *Microcystis aeruginosa* (after Smith 1950); Unstained, bright-field microscopy, 1200 x.

DISCUSSION

In general, the northern lake sites had lower phytoplankton densities than southern lake regions, suggesting conditions are more meso-oligotrophic in the northern lake sites. Cyanobacteria, primarily composed of *Microcystis* spp., were very prevalent in the southern lake regions where shallow water sites contained the highest densities of cyanobacteria with the predominant blue-green taxon *M. aeruginosa* accounting for over 70% of the total cyanobacterial density. For excessive and rapid growth of cyanobacteria to occur certain environmental conditions have to be met. These are eutrophication of water with inorganic nutrients, especially phosphorus and nitrogen, and various combinations of low hydraulic flows, high temperatures, pH and calm weather (Wicks et al., 1990). The N^2 values for stratification in the water column during June, July and August were typically small, indicating that stratification was weak, while wind speed exceeding 3.1 m s^{-1} in August tended to reduce stability causing the collapse of the dominant cyanobacterial bloom at the end of August. Scott et al. (1969) reported that wind speeds greater than 2.4 m s^{-1} are required for vertical mixing of the water column that is comparable with our observations. During the winter months June, July there was a detectable heat loss from the lake gained during the day, due principally to low night temperatures and clear skies. Robarts and Zohary (1987) found that *Microcystis* was severely limited at water temperatures below 15°C and was optimal at temperatures around 25°C.

Despite these previous observations by Robarts and Zohary (1987) a cyanobacterial winter bloom of *Microcystis* was observed at sites 1 and 2 with an average surface water temperature of 10.1°C in July, which appears to be totally antithetical to this paradigm. Generally dominance by diatoms is restricted to periods when the temperature is low (less than 15°C) as in the case of sampling sites 3 and 4 and not cyanobacteria as observed at sites 1 and 2 (Løvstad and Bjørndalen, 1990). Foy and Gibson (1993) have demonstrated in culture experiments on three planktonic diatom species that growth rate show a progressively decreasing response to increasing temperature above 10°C. The only explanation possible is that temperature alone may only in part determine bloom formation of *Microcystis* spp. in Lake Midmar and that a combination of factors are responsible for the bloom development at sites 1 and 2 during the winter months of June and July 2005. In Lake Hartbeespoort (South Africa) *Microcystis* is clearly the dominant autotrophy in summer at temperatures exceeding 20°C, but also in autumn and winter, when the water temperature drops to as low as 12.8°C (Robarts, 1984). Similar data have been reported for an eutrophic Danish lake by Jensen (1985) and a hypertrophic Japanese lake by Imamura (1981). Implying that at these low temperatures growth of *Microcystis* spp. should be close to zero. Yet, pre-existing standing stocks maintain themselves through the winter by successfully remaining in suspension while experiencing low loss rates (Oberholster et al., 2006b). However the growth rate (k[day^{-1}]) of 0.33 observed during the peak of the cyanobacterial bloom in July was near the growth rate of 0.48 observed in colony cultures by Reynolds (1984) and 0.37 in field populations by Padisak (unpublished). Due to the fact that growth conditions in experimental systems are optimized for light, nutrients, temperature, pH and loss is reduced (no grazing or sinking, etc), significantly higher rates are measured than in field populations.

A plausible explanation for the high nutrient values at sites 1 and 2 are, that these sites are part of the Midmar game park bird sanctuary, and waterfowl may be a contributory factor to the high nutrient values in the winter months (Suter, 1994) (Figure 3). Large flocks of Egyptian

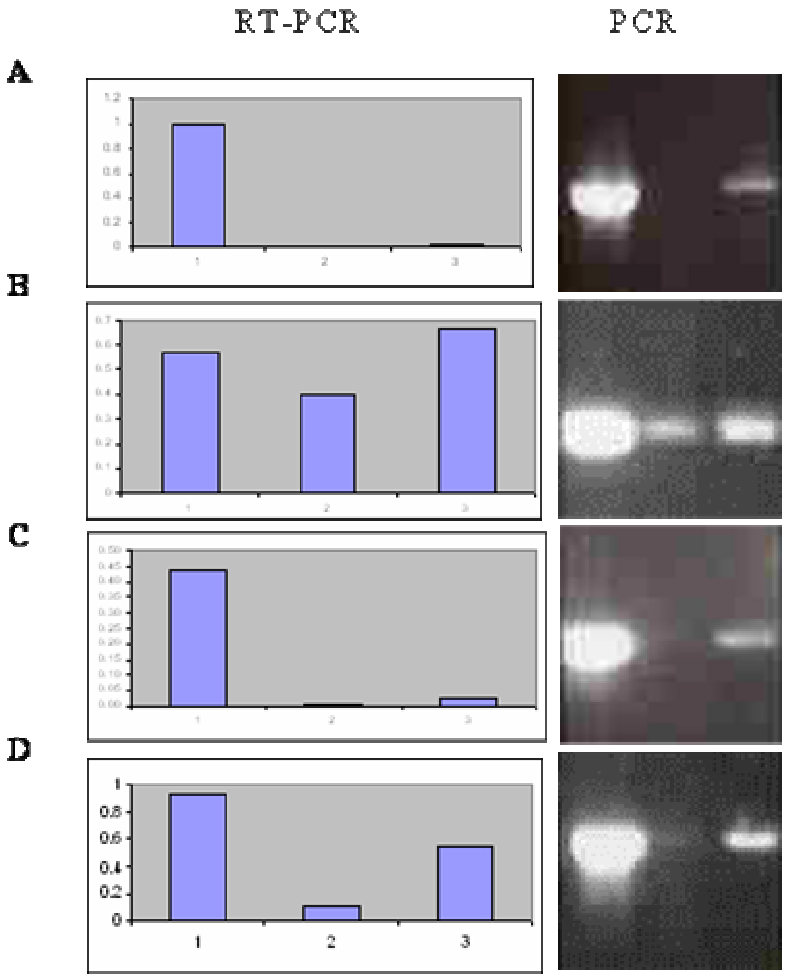

Figure 5. Quantitative PCR of RNA from *Microcystis aeruginosa* strains UPUS1 (1), UP40 (2) and UP10 (3). (A) *McyB* primer set Tox3P/2M; (B) *McyB* primer set McyB2-FAA/FBB; (C) *McyD* primer set McyD-R2/F2; and (D) *Uma1* with primer set Uma1-UMR. 16SRNA was included as standard. Separation of PCR amplicons obtained after PCR of *Microcystis aeruginosa* strain PCC7806 (1); UP40 (2) and UP10 (3) using different primers as in the case of the RT-PCR analysis on a 2% agarose gel. M = Hyperladder^{TM} IV, Bioline, USA.

Goose (*A. aegyptiacus*) and Red-knobbed Coot (*F. cristata*) were observed in the littoral zone during the sampling period at sample sites 1 and 2. Of particular relevance is that the nutrient concentration of these sampling sites was much higher than at the northern sites that are not part of the bird sanctuary, and which are used for recreation purposes i.e. yachting and power-boats. It is generally accepted that the TN:TP ratio is an important determinant of the species composition of natural populations in lakes (Takamura et al., 1992). Studies showed shifts from green algae and diatoms to blue-green algae as the TN:TP ratio in the lakes decrease (Schindler, 1977; Kotak et al., 2000). In Lake Hartbeespoort (South Africa) a somewhat deeper reservoir than Lake Midmar, the absence of *M. aeruginosa* during 1988 and 1989 was ascribed to the low epilimnetic phosphate concentration and the increasing N:P ratios, i.e. from about 4 to 10 (Chutter, 1989). What is evident from these observations is that low TN:TP or inorganic N:P ratios are most probably associated with the stimulation of cyanobacterial growth. In our study the average TN:TP ratio at all four sampling sites were < 10 : 1 by atoms which reflects a nitrogen limitation (Smith, 1982; Kalff, 2002). Literature shows that nitrogen-fixing species such as *Anabaena* should dominate at these low TN:TP ratio range, however the results of this study contradict this relation, since *M. aeruginosa* was the dominance species at sampling sites 1 and 2 with a average TN:TP ratio of 3:1 by atoms. Previous studies by Breen (1983) on Lake Midmar reported the dominance of green algae, diatoms and members of the Cryptophyceae during the winter months, which correspond with our findings of the proportion of phytoplankton at sites 3 and 4 with high silica values and a TN:TP ratio of 1.3:1 by atoms, but is in

contrast with our findings at sites 1 and 2. The only explanation for this compositional difference is that the interaction between temperature and productivity was overshadowed by the response to nutrients and nutrient availability at sites 1 and 2. This is in agreement with the data and conclusions drawn by others (e.g. Konopka and Brock, 1978; Zevenboom and Mur, 1980; Smith, 1986), but in disagreement with Tilman and Kiesling (1984) who reported that temperature was generally the most important variable controlling dominance by major taxonomic groups of algae in chemostat experiments with natural populations. However, in Lake St. George, USA the proportion of cyanobacteria in the plankton was negatively correlated with the ratio NO_3^- N:TP and positively correlated with temperature. However when the water temperature was below 21°C and the ratio NO_3^- N:TP exceeded 5:1, cyanophyte blooms never occurred (McQueen and Lean, 1987).

Toxicity

Van der Westhuizen and Eloff (1985) determined that temperature has a most pronounced effect on toxicity of *M. aeruginosa* in culture studies. The highest growth rate was obtained at 32°C, while the highest toxicity was found at 20°C, but declined at temperatures higher than 28°C. At temperatures of 32°C and 36°C toxicity was 1.6 and 4 times, respectively less than cells cultured at 28°C, suggesting that highest growth rate is not correlated with highest toxicity. They considered the decreased toxin production to be possibly related to decreased stress levels at temperatures above 20°C. Temperature changes were also found to induce variations in both the concentration and peptide composition of the toxin (Yokoyama and Park, 2003). The studies by Wicks and Thiel (1990) on environmental factors that affect the production of microcystins in *M. aeruginosa* scum in Lake Hartbeespoort (South Africa) confirms that microcystins were either not detectable or occurred in very low concentrations during the winter months May to August. This is comparable with results obtained from our study of Lake Midmar where low detectable levels of microcystin-LR varying from 0.09 to 0.17 $\mu g.L^{-1}$ occurred. Many reports have noted the variable toxicity of samples from cyanobacterial water blooms with regard to site, season, week or even day of collection (Codd and Bell, 1985). Kotak et al. (2000) found that the TN:TP ratio explained most of the variation in microcystin concentration ($\mu g\ L^{-1}$). They suggested that a shift in the N:P ratio could increase the incidence of toxic blooms and the production of toxins. Watanabe and Oishi (1985) reported a remarkable decrease in toxicity (microcystin-LR) of *M. aeruginosa* strain M228 when the nitrogen concentration in culture medium was reduced, but only minor changes were observed when the phosphorus concentration was lower. Lee et al. (2000) observed a strong correlation between the TN concentration of culture medium and the microcystin-LR cellular content of *M. aeruginosa*. In reply to our study, we measured low concentrations of microcystin-LR between 0.09 to 0.17 $\mu g\ L^{-1}$ at nitrogen-limited conditions (TN:TP, 3:1), with a low temperature range of 10.1°C in the environmental strains of *Microcystis* spp. collected at sampling sites 1 and 2 in July, using ELISA and verified by comparison with a protein phosphatase inhibition assay. The ELISA assay was used due to the fact that the presence of the genetic markers of microcystin synthetase does not always guarantee that a strain will be competent for microcystin production (Tillett et al., 2001). We further observed low levels of expression of the selected genes *mcyA-D* of the *mcy* gene cluster after quantitative real-time PCR of RNA isolated from the *Microcystis* strains collected at sites 1 and 2 with an average surface water temperature of 10.1°C in July. These findings are contradictory to findings of Sivonen (1990) who found that toxin production in culture strains of *Oscillatoria* sp. responded positively to increasing phosphorus levels between 0.1 and 0.4 mg phosphorus L^{-1} and Hee-Mock et al. (2000) who showed that phosphorus was an important factor in the control of both the production of microcystin and the type of microcystin produced and that the reduction of phosphorus in eutrophic waters may lower the growth and microcystin producing rate of *M. aeruginosa*, resulting in reduction of toxic bloom formation. In a recent study of 22 lakes in southern Quebec, Canada, Giani et al. (2005) observed stronger responses of toxin concentration to nitrogen content than to TP, which is in concurent to the results of this study.

Conclusion

Due to the fact that drinking water treatment processes in South Africa are very basic and conventional, comprising mainly of alum flocculation, sedimentation, rapid sand filtration and chlorination, the removal of cyanotoxins by such treatment processes are inadequate (Lawton and Robertson, 1999). The detection of toxic cyanobacteria and their cyanotoxins is therefore fundamental for sound water management. The use of PCR-based methods are fast and sensitive tools and allows, in a single PCR step, to determine whether a body of water sample bears microcystin-producing cyanobacteria (Oberholster et al., 2004; 2005). We further observed that the determining factor for winter bloom of toxic *Microcystis* spp. in Lake Midmar to be a combination of biotic and abiotic factors and not temperature alone, but that nitrogen plays a large role in changes in the phytoplankton community composition of the different sites in the lake and possible alteration of cell-specific toxicity levels.

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