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Diversity of cyanobacteria and cyanotoxins in Hartbeespoort Dam, South Africa

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Abstract. The South African Hartbeespoort Dam is known for the occurrence of heavy *Microcystis* blooms. Although a few other cyanobacterial genera have been described, no detailed study on those cyanobacteria and their potential toxin production has been conducted. The diversity of cyanobacterial species and toxins is most probably underestimated. To ascertain the cyanobacterial composition and presence of cyanobacterial toxins in Hartbeespoort Dam, water samples were collected in April 2011. In a polyphasic approach, 27 isolated cyanobacterial strains were classified morphologically and phylogenetically and tested for microcystins (MCs), cylindrospermopsin (CYN), saxitoxins (STXs) and anatoxin-a (ATX) by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and screened for toxin-encoding gene fragments. The isolated strains were identified as *Sphaerospermopsis reniformis*, *Sphaerospermopsis aphanizomenoides*, *Cylindrospermopsis curvispora*, *Raphidiopsis curvata*, *Raphidiopsis mediterrranea* and *Microcystis aeruginosa*. Only one of the *Microcystis* strains (AB2011/53) produced microcystins (35 variants). Forty-one microcystin variants were detected in the environmental sample from Hartbeespoort Dam, suggesting the existence of other microcystin producing strains in Hartbeespoort Dam. All investigated strains tested negative for CYN, STXs and ATX and their encoding genes. The *mcyE* gene of the microcystin gene cluster was found in the microcystin-producing *Microcystis* strain AB2011/53 and in eight non-microcystin-producing *Microcystis* strains, indicating that *mcyE* is not a good surrogate for microcystin production in environmental samples.

Additional keywords: *Cylindrospermopsis*, Hartbeespoort Dam, microcystin, *Microcystis*, *Raphidiopsis, Sphaerospermopsis*.

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Introduction

Periodic cyanobacterial blooms and dominance by cyanobacteria are a common phenomenon in many freshwater ecosystems worldwide and are caused by nutrient over-enrichment because of agricultural, urban and industrial activities (Paerl and Huisman 2009). Cyanobacteria found in such blooms are often able to produce a variety of hepatotoxic and neurotoxic secondary metabolites and are a limiting factor for the utilisation of water from these lakes and reservoirs as drinking water and for irrigation and recreational purposes (Hitzfeld *et al.* 2000; Carmichael 2001; Saqrane and Oudra 2009). Serious chronic human and acute animal health problems, in some cases even mortalities, have been related to the presence of hepatotoxic and neurotoxic metabolites produced by cyanobacteria (Carmichael 2001; Paerl and Huisman 2009).

Since the 1950s, Hartbeespoort Dam has been known for the occurrence of massive blooms of the potentially toxin-producing cyanobacterium *Microcystis aeruginosa* (Kützing) (Allanson and Gieskes 1961; Ashton *et al.* 1985; Zohary and Pais-Madeira 1990; Van Ginkel 2003; Oberholster and Botha 2010; Conradie and Barnard 2012). Occasionally, a few heterocytous cyanobacterial species, e.g. *Anabaena* sp. and *Cylindrospermopsis* sp., and a few non-heterocytous cyanobacterial species e.g. *Oscillatoria maxima*, *Pseudanabaena* sp., *Aphanocapsa* sp., *Planktothrix* sp., have been reported in the phytoplankton community in Hartbeespoort Dam in conjunction with *M. aeruginosa* (Allanson and Gieskes 1961; Zohary 1985; Hambright and Zohary 2000; Van Ginkel 2003; Janse van Vuuren and Kriel 2008; Conradie and Barnard 2012).

In the 1970s, cattle mortalities occurred on the shores of Hartbeespoort Dam and were related to toxins produced by blooms of *M. aeruginosa* (Toerien *et al.* 1976). The livestock mortalities lead to an intensive study of *Microcystis* colonies, toxin production and toxins in Hartbeespoort Dam (Toerien *et al.* 1976). A toxin called D-6 was isolated from a *Microcystis* bloom collected from Hartbeespoort Dam in 1974

(Botes *et al.* 1982*a*). Toxin D-6 was similar to a toxin BE-4 isolated from *Microcystis* strain WR 70 from Witbank Dam in South Africa. Toxin BE-4, now known as microcystin-LA, was the first microcystin to have its structure determined (Botes *et al.* 1982*a*, 1982*b*, 1984). Microcystins are cyclic heptapeptides with the common structure cyclo-(-D-Ala^1-L-X^2-D-isoMeAsp^3-L-Z^4-Adda^5-D-isoGlu^6-Mdha^7). The position of amino acids is indicated by the superscripted number (Diehnelt *et al.* 2006). The most variable L-amino acids are found in the positions 2 and 4 (letters × and Z) in the microcystin molecule (Diehnelt *et al.* 2006). Typical amino acids in position 3 are either D-aspartic acid (Asp) or D-erythro- methylaspartic acid (MAsp). In position 7 either *N*-methyldehydroalanine (Mdha), dehydroalanine (Dha), or 2-amino-2-butenoic acid (Dhb) occur (Diehnelt *et al.* 2006).

Altogether, 10 microcystin (MC) variants have been described from Hartbeespoort Dam in different studies: MC-RR, MC-LR, MC-FR, MC-YR, MC-LA, MC-YA, MC-LAba, MC-WR, MC-(H_4)YR and [Asp^3, Dha^7]MC-RR (Wicks and Thiel 1990; Van Ginkel 2003; Mbukwa *et al.* 2012). However, the number of microcystin variants found in Hartbeespoort Dam is low compared with the more than 100 microcystin variants that have been described worldwide (Neffling 2010). These microcystins are produced by *Microcystis* spp. and members of other cyanobacterial genera e.g. *Planktothrix*, *Anabaena*, and *Nostoc* (Sivonen and Jones 1999).

It is hypothesised that the number of cyanobacterial species and toxins present in Hartbeespoort Dam documented to date is underestimated because most former studies of Hartbeespoort Dam have focussed on *Microcystis* spp. only, and often utilised analytical methods with limited ability to discriminate microcystin analogues and detect other types of cyanobacterial toxins. This study therefore aimed to apply modern analytical methods in a polyphasic approach to elucidate in detail the cyanobacterial composition, phylogeny and toxicity of the cyanobacteria present in Hartbeespoort Dam, and their toxin profiles.

Material and methods

Study area, measurements and sampling

Hartbeespoort Dam is a manmade reservoir located near Pretoria, South Africa. Hartbeespoort Dam was completed in 1923 and filled with water in 1925 (Cochrane 1987). The reservoir has a surface area of around 20 km^2 and a mean depth of 9.6 m (Ashton *et al.* 1985). Hartbeespoort Dam was originally planned as a water supply for Pretoria and Johannesburg but, after completion, was mainly used for irrigation and recreation (Cochrane 1987; Water Research Commission 2008). The initial oligotrophic conditions in Hartbeespoort Dam changed over the next 25 years to eutrophic because of excessive nutrient loading (Allanson and Gieskes 1961). Several studies conducted between 1970 and 2010 have confirmed a further change to hypertrophic conditions in Hartbeespoort Dam (Steyn *et al.* 1975; Ashton *et al.* 1985; Wicks and Thiel 1990; Van Ginkel 2003; Oberholster and Botha 2010).

The sampling point at Hartbeespoort Dam was close to the northern shore (25°44′05.34″S, 27°52′08.64″E). Samples for analysis of phytoplankton composition, cyanobacterial toxins and for the isolation of cyanobacterial strains were taken in April 2011. The growing season for cyanobacteria in Hartbeesport Dam is from January until April according to Conradie and Barnard (2012). For quantitative phytoplankton analysis, a 125 mL subsample was removed from a sample taken from the lake surface, and fixed with Lugol's solution. A 50 mL water sample for isolation of cyanobacteria was taken and kept in a cool shady place and gently shaken twice per day before analysis in Norway.

For cyanotoxin analysis, 10 L of lake water from the surface was sampled in a plastic container, frozen, thawed and then shaken with 30 g of activated HP-20 resin (DIAION, Mitsubishi Chemical Corporation, Tokyo, Japan) overnight to extract microcystins (Miles *et al.* 2012). The sample was filtered through nylon netting (200 µm mesh) and the resin recovered and stored at 4°C until transportation to Norway. The resin was rinsed with distilled water and eluted slowly with methyl alcohol (MeOH) (3 × 50 mL), the eluates were evaporated to dryness *in vacuo* and dissolved in MeOH (5 mL). A specimen was diluted 10-fold for analysis.

Isolation of strains and morphological characterisation

Using a microcapillary, single colonies of *Microcystis* and filaments of *Sphaerospermopsis*, *Cylindrospermopsis* and *Raphidiopsis* were isolated. They were washed five times and placed in wells on microtiter plates containing 300 µL Z8 medium (Kotai 1972). After successful growth, the samples were placed in 50 mL Erlenmeyer flasks containing 20 mL Z8 medium and maintained at 22°C. Strains were classified on the basis of morphological traits according to Komárek and Anagnostidis (1998), Horecká and Komárek (1979), Komárek and Komárkova (2006) and Cronberg and Annadotter (2006). Morphological characterisations were conducted using an Olympus BX50 light microscope with an Olympus Dp72 camera and CellSense Digital Image software (Olympus, Oslo, Norway). The morphological identification was determined on the basis of the following criteria: (i) size of vegetative cells, heterocytes and akinetes and (ii) nature and shape of filaments or colonies. Length and width of 50–250 vegetative cells and of 20–50 heterocytes and akinetes were measured. All strains used in this study are maintained at the Norwegian Institute for Water Research, Oslo, Norway.

Genomic DNA extraction, PCR amplification and sequencing

Fresh culture material of all cyanobacterial strains was frozen and thawed three times and boiled for 5 min to break the cell walls and remove mucilage surrounding the filaments or colonies. After centrifugation (5 min, 16000 *g*) the supernatant was discarded. Autoclaved zirconium beads (0.5 g), 600 µL sodium phosphate buffer (pH 8) and 100 mL 25% sodium dodecylsulfate (SDS) were added to each pellet. After horizontal vortexing for 10 min, the sample was centrifuged (6 min, 14000 *g*). The supernatant was transferred into a new 2 mL Eppendorf tube. The pellet was washed with 500 µL sodium phosphate buffer, mixed thoroughly and centrifuged (6 min, 14000 *g*). The supernatants were combined and 200 µL lysozyme (10 mg/mL in TE buffer (Tris–EDTA)) was added. After incubation at 37°C for 15 min, 150 µL 25% SDS and 10 µL proteinase K (20 mg/mL) were added, followed by incubation at 60°C for

15 min. To separate the DNA from proteins, 600 µL ice-cold 7.5 M ammonium acetate was added and the sample centrifuged for 8 min (14000 *g*). The supernatant was transferred to a new 2 mL Eppendorf tube, and 0.7 volumes of isopropanol was added. After centrifugation at 14000 *g* for 60 min, the pellet was washed twice with 80% ethanol and centrifuged for 5 min (16000 *g*). The pellet containing genomic cyanobacterial DNA was dissolved in 40 µL TE buffer and stored at −20°C.

All PCRs were performed on a Peltier thermal cycler PTC 200 (MJ Research, Inc., San Francisco, CA) using the Taq PCR core kit (Qiagen GmbH, Hilden, Germany). The reaction mixture contained 0.1 µL Taq DNA polymerase (5 U/µL), 0.5 µL deoxynucleoside triphosphate mix (10 mM), 2 µL Qiagen PCR buffer, 1 µL forward and reverse primer (10 µM), and 1 µL genomic DNA (total volume 20 µL). The primers PCβf and PCαr were used to amplify the intergenic spacer and flanking regions of the *cpcB* and *cpcA* genes of the phycocyanin operon (PC-IGS) (Neilan *et al.* 1995). PCR was also used to check whether the isolated strains were potential producers of ATX, CYN, MCs or STXs. A polyketide synthase (PKS) encoding gene (*anaF*) of the anatoxin gene cluster was amplified using the primer atxoaf (Ballot *et al.* 2010*a*) and the newly designed primer atxoar (acctccgactaaagctaggtcg). Amplification of the *cyrJ* gene fragment was conducted using the primers cynsulfF and cylnamR (Mihali *et al.* 2008). The primers sxtaf and sxtar were used to amplify a part of the *sxtA* gene of the saxitoxin gene cluster (Ballot *et al.* 2010*b*). A part of the *mcyE* gene of the microcystin gene cluster was investigated using the primers mcyEF2 and mcyER4 and the PCR program according to Rantala *et al.* (2004). The cycling protocol for the PC-IGS fragment was one cycle of 5 min at 94°C and then 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C with a final elongation step of 72°C for 5 min. PCR products were visualised by 1% agarose gel electrophoresis with GelRed staining and UV illumination.

Amplified PC-IGS and *mcyE* products were purified through Qiaquick PCR purification columns (Qiagen, Hilden, Germany). Sequencing of the purified PC-IGS and *mcyE* products was performed using the same primers as for PCR. For each PCR product, both strands were sequenced on an ABI 3130 XL genetic analyser using the BigDye terminator V.3.1 cycle sequencing kit (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) according to the manufacturer's instructions.

Phylogenetic analysis

Sequences of the PC-IGS locus in all *Sphaerospermopsis*, *Cylindrospermopsis*, *Raphidiopsis* and *Microcystis* strains were analysed using Bioedit (Hall 2007) and Align (version 03/2007) MS Windows-based manual sequence alignment editor (Hepperle 2008) to obtain DNA sequence alignments, which were then corrected manually. Segments with highly variable and ambiguous regions and gaps making proper alignment impossible were excluded from the analyses.

A PC-IGS set containing 443 positions was used in the Nostocales PC-IGS tree. Nostocaceae Cyanobiont (AY181211) was employed as the outgroup and 31 additional Nostocales sequences derived from GenBank were included in the PC-IGS analyses. A set containing 521 positions was used for the *Microcystis* PC-IGS analysis. *Pseudanabaena mucicola* (HQ662535) was employed as the outgroup and 35 additional African *Microcystis* sequences derived from GenBank were included in the PC-IGS analyses. Phylogenetic trees for PC-IGS were constructed using the maximum likelihood (ML) algorithm in PAUP* *v.*10b (Swofford 2002). In the ML analyses, evolutionary substitution models were evaluated using the AIC criterion in jModelTest *v.*0.1.1 (Guindon and Gascuel 2003; Posada 2008). The TIM2+G evolutionary model was found to be the best-fitting evolutionary model for the PC-IGS tree (Nostocales) and TrNef+G for the PC-IGS tree (*Microcystis*). ML analyses of both trees were performed with 1000 bootstrap replicates using PAUP* *v.*10b (Swofford 2002). The sequence data were submitted to the EMBL Nucleotide Sequence Database under the accession numbers listed in Table 1.

Toxin analysis

Fresh culture material of all cyanobacterial strains was frozen and thawed three times, ultrasonicated for 5 min and filtered through Spin-X centrifuge tube filters (Corning Inc., Corning USA), at 10000 *g*. The filtrate was used for analysis of STXs. For analysis of MCs, the filtrate (100 µL) was mixed with MeOH (100 µL) (Miles *et al.* 2012), and for analysis of CYN and ATX the filtrate was mixed with acetonitrile (1: 4).

Table 1. Cyanobacterial strains isolated from Hartbeespoort Dam, strain codes and accession numbers.

Species	Strain	Accession nr. PC-IGS
Sphaerospermopsis		
S. reniformis	AB2011/03	HE979808
S. aphanizomenoides	AB2011/04	HE979809
S. reniformis	AB2011/05	HE979810
S. aphanizomenoides	AB2011/08	HE979811
S. aphanizomenoides	AB2011/24	HE979812
S. aphanizomenoides	AB2011/34	HE979813
S. aphanizomenoides	AB2011/43	HE979814
S. aphanizomenoides	AB2011/48	HE979815
Cylindrospermopsis		
C. curvispora	AB2011/30	HE979816
Raphidiopsis		
R. curvata	AB2011/25	HE979817
R. mediterranea	AB2011/37	HE979818
Microcystis		
M. aeruginosa	AB2011/06	HE979819
M. aeruginosa	AB2011/07	HE979820
M. aeruginosa	AB2011/27	HE979821
M. aeruginosa	AB2011/31	HE979822
M. aeruginosa	AB2011/32	HE979823
M. aeruginosa	AB2011/33	HE979824
M. aeruginosa	AB2011/35	HE979825
M. aeruginosa	AB2011/36	HE979826
M. aeruginosa	AB2011/38	HE979827
M. aeruginosa	AB2011/42	HE979828
M. aeruginosa	AB2011/44	HE979829
M. aeruginosa	AB2011/46	HE979830
M. aeruginosa	AB2011/51	HE979831
M. aeruginosa	AB2011/52	HE979832
M. aeruginosa	AB2011/53	HE979833
M. aeruginosa	AB2011/55	HE979834

Microcystin analysis

Standards

Microcystin (MC-RR, MC-LR, MC-YR, MC-WR, MC-LA, MC-LY, MC-LF, MC-LW) standards were purchased from Alexis Biochemicals (Grünberg, Germany), an NMR-quantitated standard of [Dha^7]MC-LR was obtained from IMB NRC, Halifax, NS, Canada, and MC-RY was isolated from a cyanobacterial bloom (Miles *et al.* 2013*b*). [Asp^3]MC-LY (Miles *et al.* 2012) isolated from *M. aeruginosa* CYA548, and with its structure confirmed by NMR and mass spectral analysis (C. O. Miles, H. E. Nonga, M. Sandvik, S. Chaudhry, A. L. Wilkins, F. Rise and A. Ballot, unpubl. data), was also used as a standard. Standards of MC-WR and MC-LW in 1 : 1 MeOH–water (1 mL) were each treated with 30% H_2O_2 (50 µL) and allowed to stand at room temperature for a week to cause partial oxidation of tryptophan (Puddick *et al.* 2013). The major oxidation product from MC-WR was identical by LC-MS^2 to MC-NfkR identified in a *Microcystis* extract (Puddick 2012; Puddick *et al.* 2013), whilst the major oxidation product from from MC-LW showed LC-MS^2 retention, mass and fragmentation pattern consistent with MC-LNfk.

Freeze-dried culture material of *Nostoc* 152 (containing [ADMAdda^5]MC-LR, [ADMAdda^5]MC-LHar and [Asp^3, ADMAdda^5]MC-LR as the major microcystins (Namikoshi *et al.* 1990)) was obtained from K. Sivonen (Helsinki University, Finland), and a specimen (8 mg) extracted with MeOH–H_2O (1 : 1, 1.5 mL) as for the fresh culture material. Aliquots of the extract were treated with pH 9.7 carbonate buffer (Miles *et al.* 2012) (to produce [DMAdda^5]-microcystins by hydrolysis) at 30°C, and progress of the reaction monitored by LC-MS^2 for 2.5 days. Treatment of hydrolysed and unhydrolysed aliquots (in carbonate buffer) with mercaptoethanol (to derivatise the Mdha^7-group), followed by LC-MS^2 analysis (Miles *et al.* 2012), was used to confirm the identity of the major hydrolysis products ([DMAdda^5]MC-LR, [DMAdda^5]MC-LHar and [Asp^3, DMAdda^5]MC-LR) and the hydrolysed extract was then used as a qualitative standard for these microcystins.

LC-MS^2 analysis

LC-MS^2 analysis with and without mercaptoethanol derivatisation was performed as described by Miles *et al.* (2012). Briefly liquid chromatography was performed on a Symmetry C18 column (3.5 µm, 100 × 2.1 mm; Waters, Milford, MA, USA), using a Surveyor MS Pump Plus and a Surveyor Auto sampler Plus (Finnigan, Thermo Electron Corp., San Jose, CA, USA) eluted (0.3 mL min^{-1}) with a linear gradient (300 µL min^{-1}) of acetonitrile (A) and water (B) each containing 0.1% formic acid. The gradient was from 22.5% to 42.5% A over 4 min, then to 75% A at 10 min, to 95% A at 11 min (1 min hold) followed by a return to 22.5% A with a 3-min hold to equilibrate the column. The HPLC system was coupled to a Finnigan LTQ ion trap mass spectrometer (Finnigan Thermo Electron Corp., San Jose, CA, USA) operated in full-scan positive ion ESI mode (*m*/*z* 500–1600).

Microcystins were analysed by LC-MS^2, and quantitated from their $[M+H]^+$ ions in scan mode relative to the most closely related commercial standard available (e.g. MC-YR-analogues relative to MC-YR etc). Identities were considered confirmed when retention time and fragmentation pattern were identical to commercial standards or to analogues with, or derived from, authenticated structures (MC-RY, [Asp^3]MC-LY, MC-NfkR, [DMAdda^5]MC-LR and [Asp^3, DMAdda^5]MC-LR). Identification was considered tentative if peaks with appropriate retention times yielded appropriate fragmentation patterns (Miles *et al.* 2012). Oxidised MC-WR analogues in the samples were identified by comparison with MS^2 spectra of related compounds (Puddick 2012).

Cylindrospermopsin and anatoxin-a analyses

Liquid chromatography was performed on a SeQuant ZIC-HILIC column (3.5 µm, 150 × 2.1 mm) (Merck, Darmstadt, Germany), using an Accela HPLC module (Thermo Scientific, San Jose, CA, USA). Separation was achieved using step gradient elution at 0.2 mL min^{-1} starting with 20% A (water containing 5 mM ammonium acetate and 0.1% acetic acid) and 80% B (95% MeCN containing 5 mM ammonium acetate and 0.1% acetic acid) for 8 min, then rising to 60% A over 15 min followed by a return to 20% A (8 min hold) before the next injection. The HPLC system was coupled to a TSQ Quantum Access triple-quadrupole mass spectrometer operating with an ESI interface (Thermo Scientific, San Jose, CA, USA). Typical ESI parameters were a spray voltage of 3.5 kV, heated capillary temperature at 250°C and nebulizer gas at 600 L h^{-1} of N_2. The mass spectrometer was operated in MS/MS mode with argon as collision cell gas at 1.4×10^{-3} Torr. Ionisation and MS/MS collision energy settings (typically 25–30 eV) were optimised while continuously infusing (syringe pump) 200 ng/mL of CYN and ATX, at a flow rate of 5 µL min^{-1}. Screening of CYN and ATX were performed with multiple-reaction monitoring (MRM) in positive ionisation mode using the following transitions: *m*/*z* CYN 416.1 → 176.0, 416.1 → 194.0, ATX *m*/*z* 166.1 → 131.1, 166.1 → 149.1. Certified cylindrospermopsin and anatoxin-a (NRC CRM) from National Research Council, Halifax, NS, Canada were used as standards. The detection limit for both toxins was 10 µg L^{-1}.

Saxitoxin analysis

Analysis of STXs was conducted according to the HPLC method of Rourke *et al.* (2008), except that separation was achieved on a Waters T3 Atlantis column and the acetonitrile content of mobile phases A and B were 4% and 16%, respectively.

Results

Phytoplankton community

Cyanobacteria dominated the phytoplankton sample from Hartbeespoort Dam in April 2011 and comprised 96.9% of the total phytoplankton biomass of 27.7 mg L^{-1} (Table 2). The most dominant cyanobacterium was *M. aeruginosa* with a biomass of 26.3 mg L^{-1} wet weight, or 97.9% of the cyanobacterial biomass. Other cyanobacterial species present belonged to the genera *Sphaerospermopsis*, *Cylindrospermopsis*, *Raphidiopsis*, *Pseudanabaena* and *Aphanocapsa* which together comprised a biomass of 0.56 mg L^{-1} wet weight (2.1% of the cyanobacterial biomass). Other phytoplankton groups observed were Bacillariophyceae, Chlorophyceae, Cryptophyceae and Euglenophyceae with a total biomass of 0.85 mg L^{-1} or 3.1% of the total biomass (Table 2).

Morphological and phylogenetic characterisation

Twenty-seven potentially toxin producing cyanobacterial strains were isolated from Hartbeespoort Dam (Table 1).

On the basis of morphological features e.g. presence and form of vegetative cells, heterocytes and akinetes, six of the isolated strains were identified as *Sphaerospermopsis aphanizomenoides* (Forti) Zapomelová, Jezberová, Hrouzek, Hisem, Reháková & Komárková, and two strains as *Sphaerospermopsis reniformis* (Lemmermann) Zapomelová, Jezberová, Hrouzek, Hisem, Reháková & Komárková. The *S. aphanizomenoides* strains were characterised by straight filaments and the *S. reniformis* strains by coiled filaments. The cell size of the vegetative cells varied between 2.2–13.2 × 1.8–6.8 µm in *S. aphanizomenoides* and 2.6–7.6 × 3.0–7.2 µm in *S. reniformis*. Round to ellipsoid heterocytes with a cell size of 3.7–8.2 × 2.8–6.6 µm and 4.4–7.6 × 4.6–7.8 µm were observed in strains of *S. aphanizomenoides* and *S. reniformis*, respectively. Round to slightly ellipsoid akinetes were observed adjacent to heterocytes in four *S. aphanizomenoides* strains and in both *S. reniformis* strains with cell sizes of 6.5–14.2 × 4.9–11.1 µm and 6.9–12.0 × 7.0–11.7 µm, respectively (Fig. 1, Table 3).

One strain was identified as *Cylindrospermopsis curvispora* M. Watanabe. It was characterised by coiled filaments, vegetative cells with a cell size of 2.4–10.4 × 1.9–3.6 µm. ellipsoid heterocytes with a cell size between 2.9–7.4 × 2.0–3.7 µm and kidney shaped akinetes with a cell size of 9.4–19.6 × 3.1–4.7 µm (Fig. 1, Table 3). One strain was determined as *Raphidiopsis curvata* F.E.Fritsch & M.F.Rich and one strain as *Raphidiopsis mediterranea* Skuja (Fig. 1, Table 3). The *R. curvata* strain was characterised by curved filaments and the *R. mediterranea* strain by straight filament. In both strains no heterocytes were observed. The size of the vegetative cell ranged from

Table 2. Biomass of phytoplankton groups in Hartbeespoort Dam in April 2011

Phytoplankton groups	Biomass mg L^{-1} wet weight	Biomass%
Bacillariophyceae	0.043	0.15
Chlorophyceae	0.060	0.22
Cryptophyceae	0.681	2.46
Cyanobacteria	26.860	96.94
Euglenophyceae	0.065	0.23

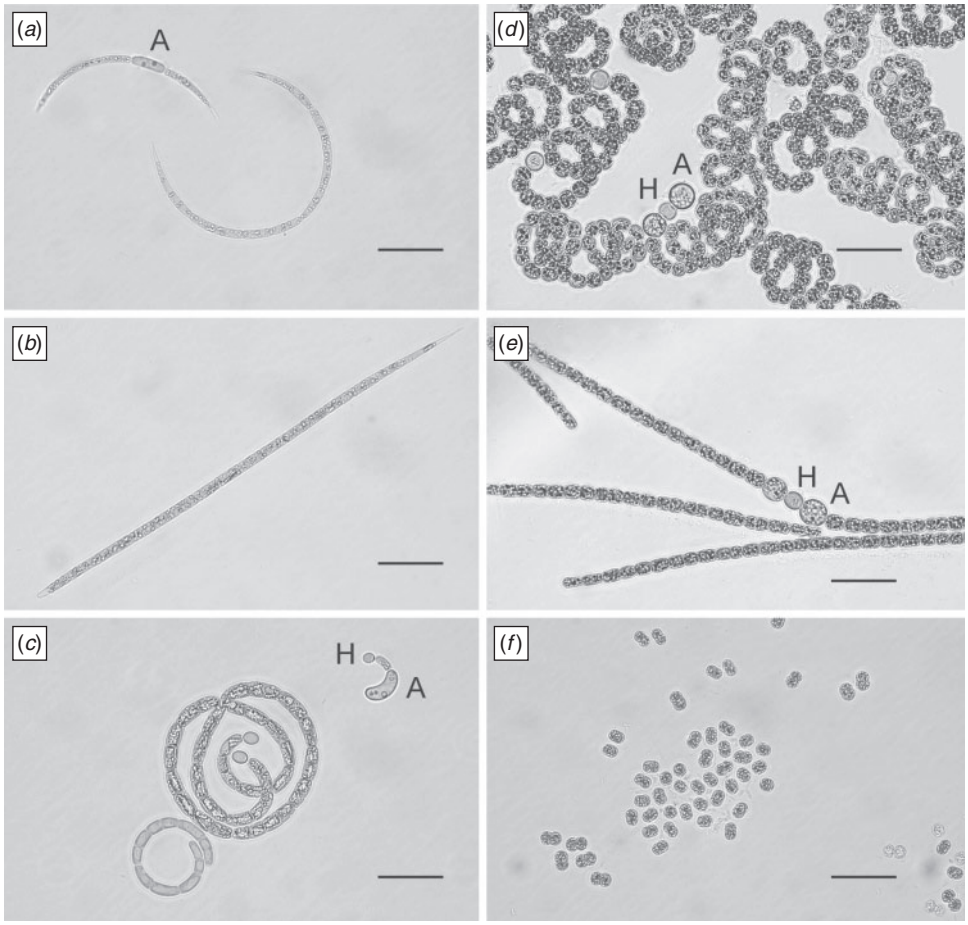

Fig. 1. Micrographs of cyanobacteria investigated in this study. (a) *Raphidiopsis curvata*; (b) *Raphidiopsis mediterranea*; (c) *Cylindrospermopsis curvispora*; (d) *Sphaerospermopsis reniformis*; (e) *Sphaerospermopsis aphanizomenoides*; (f) *Microcystis aeruginosa*. A = akinete, H = heterocyte. Scale bars indicate 25 µm.

Table 3. Morphological characteristics of *Sphaerospermopsis*, *Cylindrospermopsis and Raphidiopsis* strains from Hartbeespoort Dam, South Africa, grown under culture conditions.

Strain	Characteristics						
	shape of trichomes	vegetative cells		heterocytes		akinetes	
		length (μm)*	width (μm)*	length (μm)*	width (μm)*	length (μm)*	width (μm)*
S. aphanizomenoides							
AB2011/04	straight	4.5 (2.8, 6.9)	4.2 (2.6, 6.4)	5.9 (5.2, 6.8)	5.6 (4.2, 6.6)	10.0 (6.1, 12.7)	8.7 (6.2, 11.1)
AB2011/08	straight	5.2 (2.8, 9.9)	2.8 (2.1, 3.8)	6.1 (5.3, 7.4)	5.0 (4.2, 5.1)	n.o.	n.o.
AB2011/24	straight	5.0 (3.0, 11.0)	5.8 (4.7, 8.2)	5.6 (4.7, 8.2)	4.2 (3.3, 5.7)	9.9 (7.8, 11.9)	7.6 (6.6, 9.2)
AB2011/34	straight	5.5 (2.6, 13.2)	2.7 (1.8, 3.5)	5.2 (3.7, 8.2)	3.4 (2.8, 4.3)	8.8 (6.5, 12.3)	6.6 (4.9, 8.4)
AB2011/43	straight	3.7 (2.2, 7.2)	4.8 (2.6, 5.5)	7.2 (6.8, 7.6)	5.4 (5.1, 5.9)	11.3 (9.7, 14.2)	10.0 (9.2, 11.1)
AB2011/48	straight	4.8 (2.7, 9.8)	3.3 (2.3, 4.2)	5.6 (4.4, 8.0)	4.4 (3.6, 5.6)	n.o.	n.o.
S. reniformis							
AB2011/03	coiled	4.5 (3.0, 6.5)	4.5 (3.0, 5.5)	5.3 (4.4, 6.2)	5.5 (4.6, 6.1)	8.4 (6.9, 9.6)	8.6 (7.0, 9.7)
AB2011/05	coiled	5.0 (2.6, 7.6)	5.9 (4.0, 7.2)	6.9 (6.4, 7.6)	7.1 (7.4, 7.8)	11.3 (9.9, 12.0)	10.8 (9.7, 11.7)
C. curvispora							
AB2011/30	coiled	6.7 (2.4, 10.4)	3.0 (1.9, 3.6)	4.8 (2.9,7.4)	2.8 (2.0, 3.7)	13.5 (9.4, 19.6)	3.9 (3.1, 4.7)
R. curvata							
AB2011/25	curved	8.8 (3.8, 17.3)	2.2 (1.4, 2.8)	n.o.	n.o.	9.4 (6.7, 12.5)	3.3 (2.4, 4.0)
R. mediterranea							
AB2011/37	straight	9.9 (5.9, 17.8)	2.2 (1.6, 2.7)	n.o.	n.o.	n.o.	n.o.

3.8–17.3 × 1.4–2.8 μm in *R. curvata* and from 5.9–17.8 × 1.6–2.7 μm in *R. mediterranea*. Akinetes with a size of 6.7–12.5 × 2.4–4.0 μm were observed in *R. curvata* only (Fig. 1, Table 3).

Sixteen strains were identified as *M. aeruginosa* (Fig. 1). The mean cell diameter of the various *Microcystis* strains ranged from 3.2 μm (strain AB2011/53) to 5.4 μm (strain AB2011/42) (data not shown).

The morphological determination of the isolated strains was supported by phylogenetic features (Figs 2, 3, Table 1). Phylogenetic relationships of the investigated strains are presented in the ML tree of the PC-IGS region of Nostocales strains (Fig. 2) and a separate tree of African *Microcystis* strains (Fig. 3). In the ML-tree in Fig. 2 the *Cylindrospermopsis* and *Raphidiopsis* spp. were grouped in a distinct cluster (cluster I) which is supported by a bootstrap value of 100%. *Cylindrospermospis* spp. and *Raphidiopsis* spp. could not be distinguished phylogenetically and formed mixed subclusters. *Cylindrospermopsis curvispora* from Hartbeespoort Dam could not be distinguished from other *C. raciborskii* strains (Fig. 2).

All *S. aphanizomenoides* and *S. reniformis* strains were grouped in a separate cluster (cluster II) supported by a bootstrap value of 95%. They were grouped closer to *Anabaena* and *Aphanizomenon* strains than to *Cylindrospermopsis* and *Raphidiopsis* strains. *Sphaerospermopsis reniformis* formed mixed subclusters with *S. aphanizomenoides* and *A. aphanizomenoides* strains and could not be distinguished phylogenetically (Fig. 2).

The *Microcystis* strains from Hartbeespoort Dam were grouped in 3 clusters which were separated from other African *Microcystis* strains. The exception was cluster III, where a *Microcystis* strain from Lake Victoria, Uganda (AM048621), was included, forming a subcluster (Fig. 3). The microcystin producing strain AB2011/53 was located in cluster IIIb. Its PC-IGS sequence was characterised by a similarity of 100% to those of seven non-microcystin producing strains.

Identification of cyanobacterial toxins and toxin producing strains

As determined by LC-MS^2 analysis, 41 microcystin variants were found in the sample from Hartbeespoort Dam from April 2011 (Table 4). The most abundant variants were MC-RR, MC-LR, MC-YR and MC-(H_4)YR (Fig. 4). The MC-LR concentration was 0.93 μg L^{-1} and the total microcystin concentration was ~3.6 μg L^{-1}. For 23 of the 41 microcystins, the concentrations were below the limit of quantification (0.01 μg L^{-1}). All microcystins in Table 4, with the exception of the [Mser^7]-congeners, reacted with mercaptoethanol in the presence of carbonate buffer, indicating that they contained Mdha or Dha, rather than Mdhb or Dhb, as the amino acid at site-7 (Miles *et al.* 2012; Miles *et al.* 2013*a*).

Fifteen of the 16 *M. aeruginosa* strains isolated from Hartbeespoort Dam did not produce microcystins. However, one strain (AB2011/53) produced 35 microcystins as determined by LC-MS^2 (Table 4), with a total microcystin concentration (extra- and intracellular) of 943 μg g^{-1} wet weight, equivalent to 0.024 pg cell^{-1}.

All 27 cyanobacterial strains investigated in this study tested negative for CYN, ATX and STXs by LC-MS and HPLC analysis.

Amplification of toxin encoding genes

Amplification of the *mcyE* gene was observed in the MC-producing *M. aeruginosa* strain AB2011/53 and in 8 other non-MC producing *Microcystis* strains from Hartbeespoort Dam. None of the 27 strains exhibited amplification of the *sxtA* gene (saxitoxin gene cluster), *cyrJ* gene (cylindrospermopsin gene cluster) and the *anaF* gene (anatoxin-a encoding gene cluster).

Discussion

This study clearly demonstrated the presence of the potentially toxic Nostocales cyanobacteria *C. curvispora*, *R. curvata*,

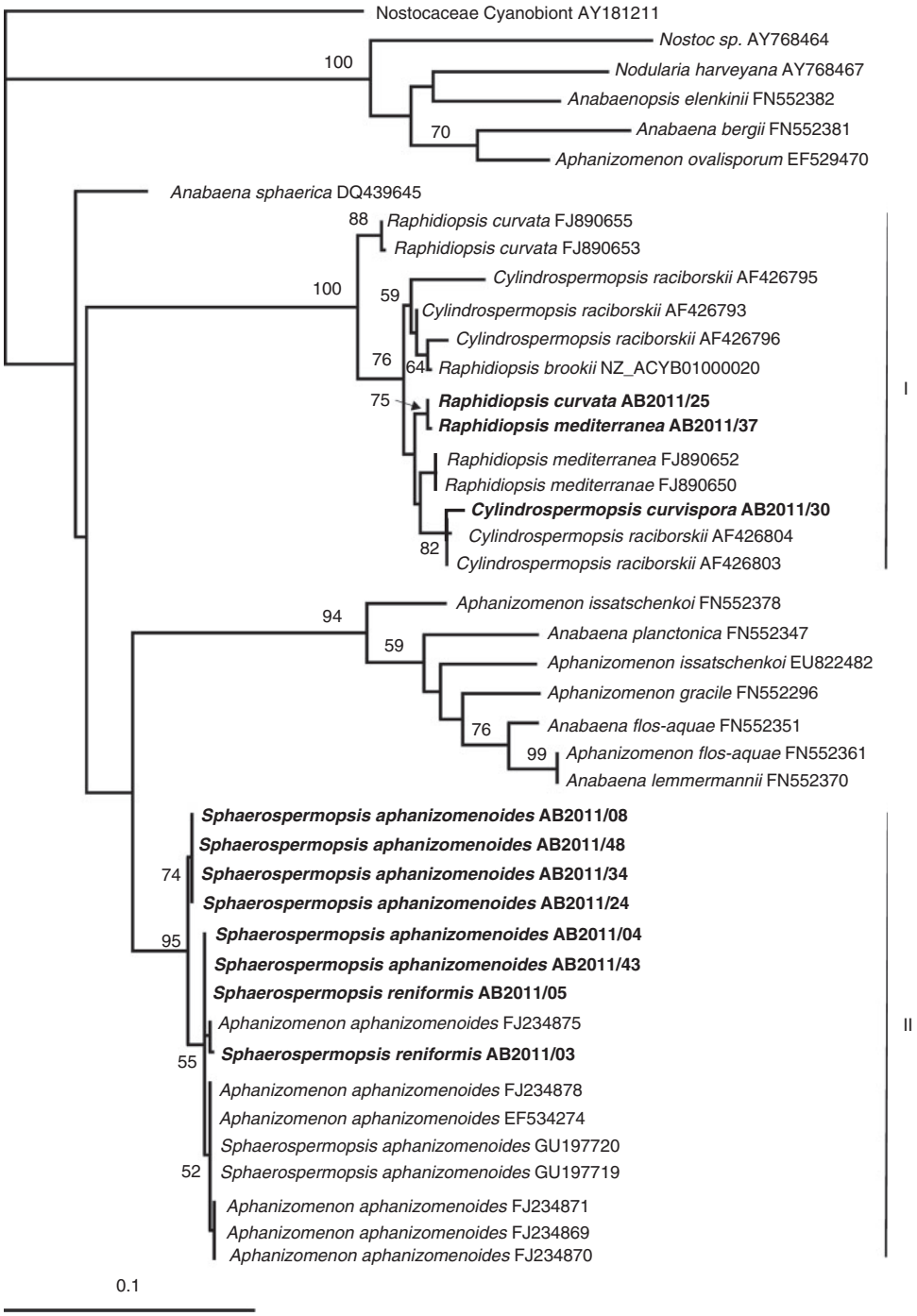

Fig. 2. Maximum likelihood tree determined on the basis of partial PC-IGS gene sequences of 42 Nostocales strains. Outgroup = Nostocaceae Cyanobiont (AY181211). Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 10% sequence divergence.

R. mediterranea, *S. aphanizomenoides* and *S. reniformis* in the phytoplankton community of Hartbeespoort Dam, South Africa. None of these species have previously been detected in Hartbeespoort Dam, but have been reported from tropical and subtropical regions of Africa (Cronberg and Komárek 2004; Cronberg and Annadotter 2006). Van Ginkel (2003) has detected *Cylindrospermopsis* spp. (later described as *C. raciborskii* by Janse van Vuuren and Kriel (2008) for the first time in South Africa in the Orange River in 2000, and later in low numbers in Hartbeespoort Dam. *Cylindrospermopsis curvispora* has been described only from a few countries in the world. It was initially detected in a Japanese reservoir by Watanabe (1995), and was later also found in Sri Lanka, in western Africa in Senegal, and in southern Africa in Zambia and Botswana (Cronberg and

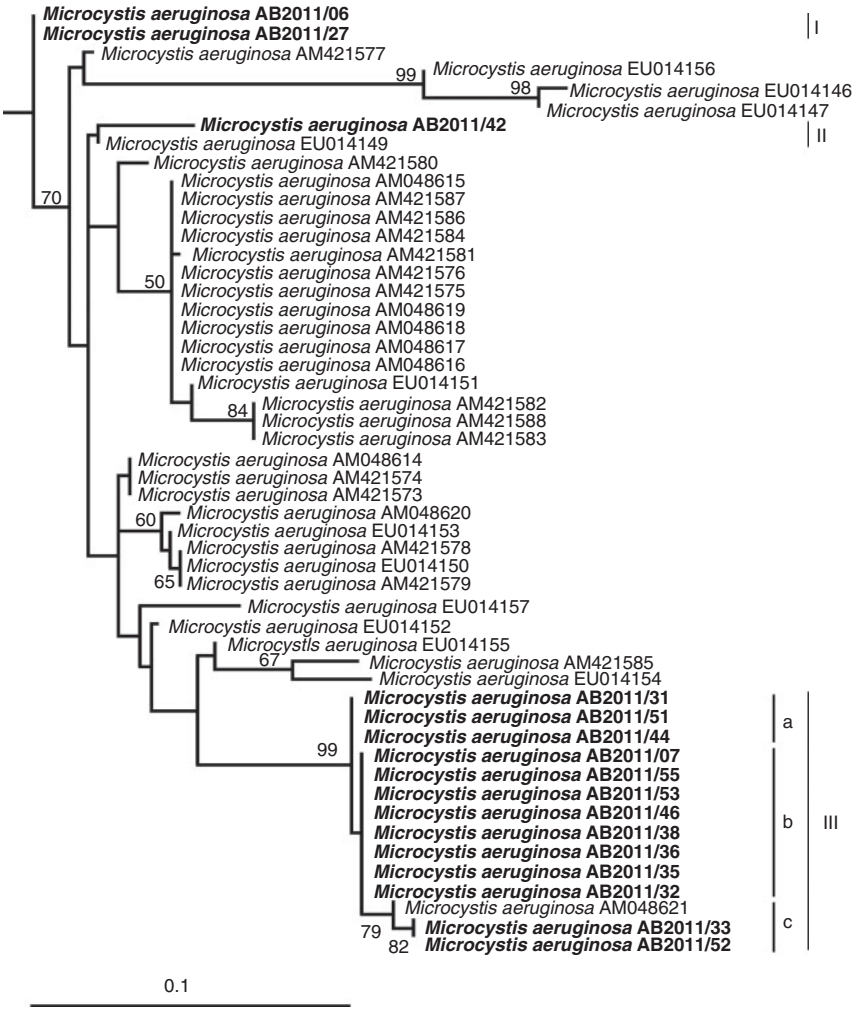

Fig. 3. Maximum likelihood tree determined on the basis of partial PC-IGS gene sequences of 51 African *Microcystis* strains. Outgroup = *Pseudanabaena mucicola* (HQ662535). Strains from this study are marked in bold. Bootstrap values above 50 are included. The scale bar indicates 10% sequence divergence.

Komárek 2004; Thomazeau *et al.* 2010). McGregor and Fabbro (2000) have described coiled morphotypes of Australian *C. raciborskii* with a similar morphology to *C. curvispora* strain AB2011/30. Therefore, it cannot be excluded that *C. curvispora* is actually another morphotype of *C. raciborskii*. This is supported by a study from Thomazeau *et al.* (2010), who concluded that *C. curvispora* cannot be distinguished genetically from *C. raciborskii* using 16S rRNA gene sequences.

Cylindrospermopsis spp. and *Raphidiopsis* spp. are clearly distinguished morphologically by the possession or lack of heterocytes. *Rhaphidiopsis curvata* is characterised by short crescent filaments and *R. mediterranea* by short straight filaments (Cronberg and Annadotter 2006), features which could be clearly seen for filaments of both species in the environmental sample from Hartbeespoort Dam. However, in culture, both isolated *Raphidiopsis* strains AB2011/25 and AB2011/37 grew mostly as long straight, or slightly curved, filaments. Only a small proportion of the *R. curvata* culture AB2011/25 was observed growing as short crescent filaments. Such morphological variations between cyanobacterial strains growing in natural environments or under culture conditions were also reported in other studies (e.g. Ballot *et al.* 2008; Zapomělová *et al.* 2008). This demonstrates that a correct identification, using morphological traits only, in some cases is misleading or not even possible. An intensive study on the cyanobacterial composition should therefore always include a combination of classical methods (e.g. microscopy) and newer genetic methodologies.

Cylindrospermospsis curvispora, *R. curvata* and *R. mediterranea* strains can be clearly distinguished using morphological criteria. However, the mixed cluster (cluster I) of *Cylindrospermopsis* and *Raphidiopsis* sequences from Hartbeestpoort Dam and those derived from GenBank in the phylogentetic tree in (Fig. 2) confirms suggestions by McGregor and Fabbro (2000), Moustaka Gouni *et al.* (2009) and Stucken *et al.* (2010), that *Raphidiopsis* and *Cylindrospermopsis* in fact constitute a single genus. Cluster I in Fig. 2 also clearly indicates that *C. curvispora* from Hartbeespoort Dam is very closely related to other *C. raciborskii* strains and is closer to *R. mediterranea* and

Table 4. Microcystin variants found by LC-MS^2 in a water sample from Hartbeespoort Dam and in *Microcystis* culture AB2011/53 isolated from Hartbeespoort Dam
+ = concentration <0.01 μg/L; X = unidentified amino acid; nd = not detected

m/z	Compound	Status [A]	R_t (min)	AB2011/53 μg g^{-1} [B]	Hartbeespoort Dam μg L^{-1}
1035.8	[DMAdda^5]MC-(H_4)YR	Tentative	2.06	+	+
1024.8	[Asp^3]MC-RR	Tentative	2.12	5	0.02
1038.8	MC-RR	Confirmed	2.13	344	1.28
1031.8	[DMAdda^5]MC-YR	Tentative	2.40	3	0.01
1061.8	[DMAdda^5]MC-Y(OMe)R	Tentative	2.40	+	+
967.8	[Asp^3, DMAdda^5]MC-LR	Confirmed	2.57	+	+
981.8	[DMAdda^5]MC-LR	Confirmed	2.59	1.4	0.01
1054.8	[DMAdda^5]MC-WR	Tentative	3.11	+	+
1035.8	[Asp^3]MC-(H_4)YR	Tentative	3.25	+	+
1029.8	MC-RR analogue	Unidentified	3.37	+	+
1031.8	[Asp^3]MC-YR	Tentative	3.47	+	0.01
1035.8	[Dha^7]MC-(H_4)YR	Tentative	3.52	+	+
1049.8	MC-(H_4)YR	Tentative	3.56	54	0.27
1061.8	[Asp^3]MC-Y(OMe)R	Tentative	3.60	+	+
1013.8	[Mser^7]MC-LR	Tentative	3.65	+	+
1063.8	[Mser^7]MC-YR	Tentative	3.68	+	+
1031.8	[Dha^7]MC-YR	Tentative	3.75	+	+
1013.8	MC-XR	Tentative	3.77	+	+
1045.8	MC-YR	Confirmed	3.84	155	0.43
981.8	[Asp^3]MC-LR	Tentative	3.87	2.6	0.08
1075.8	MC-Y(OMe)R	Tentative	3.87	10	0.03
995.8	MC-LR	Confirmed	3.89	285	0.93
967.8	[Asp^3, Dha^7]MC-LR	Tentative	3.93	+	+
1100.8	MC-NfkR	Tentative	3.99	+	+
981.8	[Dha^7]MC-LR	Confirmed	3.99	15	0.10
1085.8	MC-XR	Tentative	4.01	+	+
1054.8	[Asp^3]MC-WR	Tentative	4.13	+	+
1009.8	MC-HilR	Tentative	4.15	12	0.03
1029.9	MC-FR	Tentative	4.21	19	0.05
1068.8	MC-WR	Confirmed	4.32	26	0.08
1054.8	[Dha^7]MC-WR	Tentative	4.35	+	+
1037.8	MC-AnaR	Tentative	5.05	11	nd
1031.8	[Asp^3]MC-RY	Tentative	5.12	+	+
1045.8	MC-RY	Confirmed	5.31	+	0.01
896.8	[DMAdda^5]MC-LA	Tentative	6.18	nd	+
1029.8	MC-RF	Tentative	6.34	+	+
896.8	[Asp^3]MC-LA	Tentative	7.50	nd	+
960.8	MC-YA	Tentative	8.13	nd	+
988.8	[Asp^3]MC-LY	Confirmed	8.15	nd	0.14
910.8	MC-LA	Confirmed	8.29	nd	0.04
1002.8	MC-LY	Confirmed	8.48	nd	0.04
924.8	MC-LAba	Tentative	9.08	nd	+

[A] 'Unidentified' indicates unknown analogue with characteristic microcystin-like MS^2 fragmentation pattern, 'tentative' indicates microcystin with appropriate R_t, MS and MS^2 fragmentation pattern for the proposed analogue, and 'confirmed' indicates R_t, MS and MS^2 fragmentation pattern were identical to those of an authentic standard (for MS^2 spectra, see Supplementary data).
[B] μg g^{-1} wet weight of algal biomass.

R. curvata from Hartbeespoort Dam than to *Cylindrospermopsis* and *Raphidiopsis* species from other locations. These findings raise the question of whether *C. curvispora*, *R. curvata* and *R. mediterranea* can be regarded as separate species or are most likely just rare morphotypes of *C. raciborskii*.

Strains of *Cylindrospermopsis* and *Raphidiopsis* from Australia, Brazil, China, Japan and Thailand produce CYN, STXs or ATX (Hawkins *et al.* 1997; Saker and Neilan 2001; Li *et al.* 2001; Namikoshi *et al.* 2003; Soto-Liebe *et al.* 2010). However, all the *Cylindrospermopsis* and *Raphidiopsis* strains isolated from Hartbeespoort Dam tested negative for production of cyanotoxins and their encoding genes. Interestingly, no CYN-, STX- or ATX-producing *Cylindrospermopsis* or *Raphidiopsis* strains have been located on the African continent to date although genetic data have suggested the colonisation of Australia by African *Cylindrospermospis* strains (Gugger *et al.* 2005;

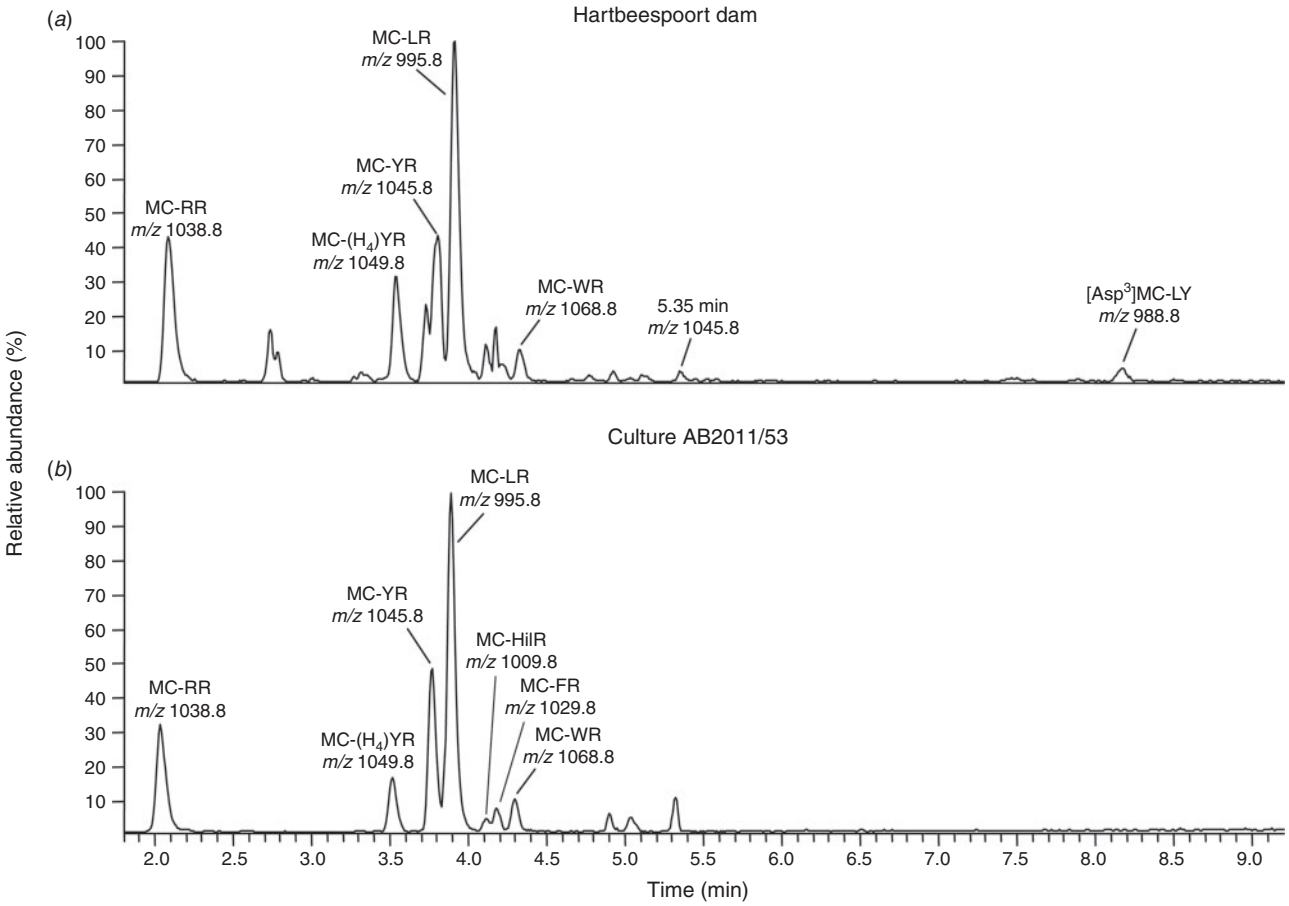

Fig. 4. Liquid chromatography–mass spectrometry (LC-MS) chromatograms (*m/z* 890–1150, 1.8–9.2 min) of extracts from bloom material from Hartbeespoort Dam and from *M. aeruginosa* culture AB2011/53. Chromatograms were produced by subtraction of the corresponding mercaptoethanol-derivatisated chromatograms (Miles *et al.* 2012) to reduce peaks not attributable to microcystins.

Haande *et al.* 2008). The only possible exception is in Egypt, where *C. raciborskii* strains with hepatotoxic effects and *R. mediterranea* strains with neurotoxic effects on mice were detected (Mohamed 2007). However, the findings by Mohamed (2007) were not supported by LC-MS analyses and the supposed toxins were not identified.

Sphaerospermopsis aphanizomenoides and *S. reniformis* have also not been described from Hartbeespoort Dam before. The filaments of both species were clearly visible among the dominant *Microcystis* colonies. Coiled and straight filaments of *Sphaerospermopsis* are readily confused with *Anabaena* spp. if akinetes and heterocytes are lacking. In culture, but not in the environmental sample from Hartbeespoort Dam, some of the *Sphaerospermopsis* filaments possessed heterocytes and akinetes. There is a possibility that *Anabaena* spp. observed in an earlier study by Van Ginkel (2003) were in fact *Sphaerospermopsis* spp. So far, only a few findings of *S. reniformis* or other coiled species with a similar morphology (*S. torques reginae*, *A. eucompacta*, *A. oumiana*) have been reported from water bodies in Africa, Asia, Europe and Central and South America (Li and Watanabe 1999; Cronberg and Annadotter 2006; Zapomělová *et al.* 2009; Werner *et al.* 2012). However, this dearth of reports could be attributed to misidentification of this morphospecies (Cronberg and Annadotter 2006; Werner *et al.* 2012). In the PC-IGS tree, all *Sphaerospermopsis* spp. from Hartbeespoort Dam are grouped together and are separated from other Nostocales cyanobacteria. This supports findings by Zapomělová *et al.* (2009, 2010), who reclassified former *Aphanizomenon aphanizomenoides* and *Anabaena reniformis* into the new genus *Sphaerospermopsis* according to their morphological and phylogenetic characteristics. *Planktothrix* spp. which was described by Conradie and Barnard (2012) as occurring in low numbers in samples preserved with Lugol's solution from Hartbeespoort Dam in 2005, was not observed in samples collected for the current study.

So far worldwide, no *Sphaerospermopsis* strains have been found to possess genes which encode for the biosynthesis of CYN, STXs, ATX, and MCs or producing these toxins, including in our study. However, the existence of toxin producing *Sphaerospermopsis* strains cannot be excluded because in many other Nostocales genera, e.g. *Cylindrospermopsis*, *Aphanizomenon*, *Anabaena*, non-toxin and toxin producing strains have been described (Ballot *et al.* 2010*a*, 2010b; Li *et al.* 2001; Haande *et al.* 2008).

Similar to other studies conducted at Hartbeespoort Dam (e.g. Allanson and Gieskes 1961; Zohary and Pais-Madeira 1990;

Van Ginkel 2003; Conradie and Barnard 2012), the present study confirmed that *M. aeruginosa* is the dominant cyanobacterium. Blooms of *M. aeruginosa* in Hartbeespoort Dam have been recorded since the 1950s, and this species has continued to dominate the phytoplankton community of this reservoir (Allanson and Gieskes 1961; Wicks and Thiel 1990; Conradie and Barnard 2012). Harding *et al.* (2004) and Conradie and Barnard (2012) have described frequent *Microcystis* dominances of up to 100% of the phytoplankton biomass in Hartbeespoort Dam.

The difference between the 41 MC variants found in the water sample from Hartbeespoort Dam and the 35 variants produced by *Microcystis* strain AB2011/53 shows clearly that other MC producing cyanobacteria (most likely other MC producing *Microcystis* strains) must have been present in Hartbeespoort Dam at the time of investigation. The novel variant MC-AnaR (tentatively identified from its MS^2 fragmentation pattern) found in *Microcystis* strain AB2011/53 was not detected in the water sample from Hartbeespoort Dam, probably because its concentration in the water sample was below the detection limit of the LC-MS analysis. The number of microcystins detected in this study is considerably higher than the 10 MC variants (MC-RR, MC-LR, MC-YR, MC-FR, MC-YA, MC-LA, MC-LAib, MC-WR, MC-(H_4)YR, [Asp^3, Dha^7)]MC-RR) described in previous studies of Hartbeespoort Dam using HPLC analysis (Botes *et al.* 1984; Wicks and Thiel 1990; Van Ginkel 2003; Mbukwa *et al.* 2012). This is probably primarily because of the analysis method here. Use of thiol derivatisation permitted subtraction of chromatograms (Fig. 4) to assist in identifying minor components. Thiol reactivity also provided greater certainty in the identification of reacting components as putative microcystins, which could then be evaluated by examination of their MS^2 spectra (Miles *et al.* 2012; Miles *et al.* 2013*b*). In the current investigation, MC-RR, MC-LR, MC-YR were the most prevalent microcystins, whereas Wicks and Thiel (1990) described MC-LR and MC-FR, and Van Ginkel (2003) MC-LA, as the most abundant microcystins in Hartbeespoort Dam. This suggests a varying dominance of different MC producing *Microcystis* strains.

LC-MS^2 analysis revealed production of [$DMAdda^5$]MC-LR, [$DMAdda^5$]MC-LHar and [Asp^3, $DMAdda^5$]MC-LR (pseudo-first order kinetics, $t_{1/2}$ ca 30 h) in carbonate buffer caused by hydrolysis of the acetate group from the major analogues in the *Nostoc* 152 extract ([$ADMAdda^5$]MC-LR, [$ADMAdda^5$]MC-LHar and [Asp^3, $ADMAdda^5$]MC-LR (Namikoshi *et al.* 1990)). Hydrolysed *Nostoc* 152 extract was used as a qualitative LC-MS standard to confirm the identities of [$ADMAdda^5$]-microcystins in the extracts from Hartbeespoort Dam and *M. aeruginosa* culture AB2011/53. [$DMAdda^5$]-analogues of the major microcystins in Hartbeespoort Dam and AB2011/53 extracts, including [$DMAdda^5$]MC-LR and [Asp^3, $DMAdda^5$]MC-LR, were readily identified from their shorter retention times (by ~1.5–2-min) and prominent fragment ions at *m*/*z* 585 (rather than *m*/*z* 599 in their [$Adda^5$]-congeners) and $[MH-120]^+$ (rather than $[MH-134]^+$) in their MS^2 spectra (Supplementary data). [$DMAdda^5$]-microcystins were typically present at ca 1% of the levels of the parent [$Adda^5$]-analogues in the samples from Hartbeespoort Dam, suggesting that they are minor products of normal microcystin biosynthesis. Additionally, MC-NfkR, a tryptophan-oxidised congener of MC-WR, was identified at low levels by LC-MS^2 in the extracts from Hartbeespoort Dam and *M. aeruginosa* culture AB2011/53 and its identity confirmed by oxidation of an authentic specimen of MC-WR using the method of Puddick *et al.* (2013). This appears to be the first report a tryptophan-oxidised microcystin congener in a field sample.

The water sample from Hartbeespoort Dam contained 0.93 $\mu g\,L^{-1}$ of MC-LR, which is slightly below the World Health Organisation's provisional guideline (1 $\mu g\,L^{-1}$ MC-LR) for drinking-water (WHO 1998), although the total MC concentration (3.6 $\mu g\,L^{-1}$) was considerably higher. However, Harding *et al.* (2004) measured a much higher median MC concentration of 580 $\mu g\,L^{-1}$ (between 0 and 28930 $\mu g\,L^{-1}$) during a survey in 2003 and 2004, and Conradie and Barnard (2012) detected microcystin concentrations up to 3200 $\mu g\,L^{-1}$ in Hartbeespoort Dam in 2005. In the studies by Harding *et al.* 2004 and Conradie and Barnard 2012, biomass was measured as chlorophyll-a and no correlation was found between the highest microcystin concentrations and the highest chlorophyll-a concentations. Conradie and Barnard (2012) used an ELISA for the detection of microcystins and could therefore not distinguish the microcystin variants in their study.

The low MC concentrations detected in this study, can be explained by the dominance of non-MC producing *Microcystis* in Hartbeespoort Dam. Of the 16 *Microcystis* strains isolated, only one (AB2011/53) produced microcystins. Interestingly, we identified the *mcyE* gene, a glutamate-activating adenylation domain which is part of the microcystin-encoding gene cluster (Tillett *et al.* 2000), not only in the MC-producing strain AB2011/53, but also in eight non-microcystin-producing strains from Hartbeespoort Dam. The presence of the *mcyE* gene in non-MC-producing cyanobacteria has also been described by Noguchi *et al.* (2009) and this raises a question as to the suitability of the *mcyE* gene to quantify toxin-producing *Microcystis* spp. in quantitative PCR investigations. Other genes of the microcystin encoding gene cluster, e.g. *mcyA*, *mcyB*, and *mcyT*, have been reported in non-MC-producing *Microcystis* and *Planktothrix* strains (Mikalsen *et al.* 2003; Kurmayer *et al.* 2004; Christiansen *et al.* 2008). Genes encoding the biosynthesis of other cyanobacterial toxins, e.g. CYN and STXs, have been detected in several non-toxin-producing cyanobacteria (Wood *et al.* 2007; Rasmussen *et al.* 2008; Ballot *et al.* 2010*b*). Various mechanisms, such as horizontal gene transfer, mutations, insertions and deletions, have been proposed as explanations for non-toxin-producing cyanobacteria possessing parts of toxin-encoding gene clusters (Christiansen *et al.* 2008; Tooming-Klunderud *et al.* 2008; Moustafa *et al.* 2009).

As depicted in the PC-IGS tree in Fig. 3, the toxin-producing *Microcystis* strain AB2011/53 cannot be distinguished from seven non-MC-producing strains which are grouped in subcluster IIIb. *Microcystis* strains with similar PC-IGS sequences are present worldwide in North America, Asia, and Europe when using NCBI Blast (NCBI). However, differences can be seen when comparing the mean cell sizes. Vegetative cells of MC-producing strain AB2011/53 measured only 3.2 µm, which was considerably smaller then the other *Microcystis* strains in subcluster IIIb (mean cell sizes between 3.8 and 5.2 µm). The other eight *Microcystis* strains of this study in cluster I, II, IIIa

and IIIB possess PC-IGS sequences which are unique to Hartbeespoort Dam according to NCBI Blast and are distributed in different clusters. However, the number of PC-IGS sequences in GenBank is relatively low and further studies could reveal a wider distribution of *Microcystis* species with similar PC-IGS sequences.

By investigating more locations in Hartbeespoort Dam over a longer time period we probably could have found a higher cyanobacterial diversity and more diverse cyanotoxin composition. However, the current study shows clearly that a carefully conducted polyphasic approach even of samples taken at one selected date and at one location can result in a detailed overview about the cyanobacterial and cyanotoxin composition in a certain part of a lake. It is obvious that previous studies conducted at Hartbeespoort Dam did not reveal a similar diverse cyanobacterial community and cyanotoxin composition even though those studies were conducted over longer time periods and more locations were sampled. In the current study, the proportion of MC producing *Microcystis* strains was low in Hartbeesport Dam compared with non-MC producing *Microcystis* strains. A shift to the dominance of MC producing *Microcystis* strains could increase the MC concentrations in the water body considerably threatening the use of Hartbeespoort Dam for irrigation, fishing and water sports and increasing the risk to human and animal health during the growth season of cyanobacteria.

Furthermore, the massive *Microcystis* blooms could be curtailed by reducing nutrient loading in Hartbeespoort Dam. However, such measures could then promote the growth of the potential toxin producing *Cylindrospermopsis*, and *Sphaerospermopsis* or other heterocytous cyanobacteria. The ability of those heterocytous cyanobacteria to fix atmospheric nitrogen would be an advantvage and enable them to outcompete *Microcystis* spp. which are dependant on dissolved inorganic nitrogen compounds (Sukenik *et al.* 2012).

In conclusion, this is the first report of *S. aphanizomenoides*, *S. reniformis*, *C. curvispora*, *R. mediterranea* and *R. curvata* in Hartbeespoort Dam. None of the isolates of these species produced cyanobacterial toxins although *Cylindrospermopsis* and *Raphidiopsis* spp. are known toxin producers in Australia, Asia and South America. Forty-one MC variants were present in an environmental sample from Hartbeespoort Dam and 35 MC variants were detected in a *Microcystis* strain isolated from the same water sample. The majority of the isolated *Microcystis* strains did not produce MCs, which can explain the relatively low MC concentrations in the water sample from Hartbeespoort Dam.

Abbreviations Used

Aib, amino isobutyric acid; Adda, (2*S*,3*S*,8*S*,9*S*,4*E*,6*E*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid; Aba, aminobutyric acid (unspecified stereochemistry); ADMAdda, 9-*O*-acetyl-desmethylAdda; Ana, aminononanoic acid (unspecified stereochemistry); Ala, alanine; ATX, anatoxin-a; CYN, cylindrospermopsin; Dha, dehydroalanine; Dhb, dehydrobutyrine; DMAdda, 9-*O*-desmethylAdda; Glu, glutamic acid; (H_4)Y, tetrahydrotyrosine; MC, microcystin; Mdha, *N*-methyldehydroalanine; Mdhb, *N*-methyldehydrobutyrine; ML, maximum likelihood; Nfk, *N*-formylkynurenine; PC-IGS, intergenic spacer and flanking regions of the cpcB and cpcA genes of the phycocyanin operon; SDS, sodium dodecylsulfate; STXs, saxitoxins; TE, tris(hydroxymethyl)aminomethane–EDTA; (H_4)Y, tetrahydrotyrosine; Y(OMe), methoxytyrosine.

Supplementary data

Supplementary data (MS^2 spectra extracted during LC-MS^2 analysis of water from Hartbeespoort Dam, culture of *M. aeruginosa* strain AB2011/53, hydrolysed culture of *Nostoc* sp. strain 152, and microcystin standards) associated with this article can be found in the on-line version at http://dx.doi.org/10.1071/MF13153

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