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Article

The Presence of Toxic and Non-toxic Cyanobacteria in the Sediments of the Limpopo River Basin: Implications for Human Health

Murendeni Magonono[1], Paul Johan Oberholster[2] Addmore Shonhai[3], Stanley Makumire[4] and Jabulani Ray Gumbo[5,*]

[1] Department of Hydrology and Water Resources, School of Environmental Sciences, University of Venda, Thohoyandou, South Africa; murendy22@gmail.com
[2] Council for Scientific and Industrial Research, Natural Resources and the Environment, Stellenbosch, South Africa; poberholster@csir.co.za
[3] Department of Biochemistry, School of Mathematical and Natural Sciences, University of Venda, Thohoyandou, South Africa; addmore.shonhai@univen.ac.za
[4] Department of Biochemistry, School of Mathematical and Natural Sciences, University of Venda, Thohoyandou, South Africa; stanmakster@gmail.com
[5] Department of Hydrology and Water Resources, School of Environmental Sciences, University of Venda, Thohoyandou, South Africa; jabulani.gumbo@univen.ac.za

* Correspondence: jabulani.gumbo@univen.ac.za; Tel.: +27-15-962-8563

Abstract: The presence of harmful algal blooms (HABs) and cyanotoxins in drinking water sources poses a great threat to human health. The current study employed molecular techniques to determine the occurrence of non-toxic and toxic cyanobacteria species in the Limpopo River basin based on the phylogenetic analyses of 16S rRNA gene. The bottom sediments samples were collected from selected rivers: Limpopo, Crocodile, Mokolo, Mogalakwena, Nzhelele, Lephalale, Sand Rivers (South Africa); Notwane (Botswana), Shashe River and Mzingwane River (Zimbabwe). The physical-chemical analysis of the bottom sediments showed the availability of nutrients, nitrates and phosphates, in excess of 0.5 mg/l for most of river sediments, alkaline pH and salinity in excess of 500 mg/l. The FlowCam showed the dominant cyanobacteria species identified from the samples were *Microcystis* species, followed by *Cylindrospermopsis raciborskii, Phormidium and Planktothrix* species and this was confirmed by molecular techniques. Nevertheless, two samples showed the amplification of cylindrospermopsin polyketide synthetase gene (S3 and S9) while two samples showed amplification for microcystin/nodularin synthetase gene (S8 and S13). Thus these findings may imply the presence of toxic cyanobacteria species in the river sediments. The presence of cyanobacteria may be hazardous to human because rural communities and farmers who abstract water from Limpopo river catchment for human consumption, livestock and wildlife watering and irrigation.

Keywords: cyanobacteria; cyanotoxins; nutrient enrichment; akinetes; harmful algal blooms; PCR; phylogenetic analyses

Key Contribution: Presence of viable cyanobacteria akinetes and cysts in river sediments, a source of inoculum of cyanobacteria growth in Limpopo river basin. Some of the cyanobacteria species are toxic.

1. Introduction

Toxic and non-toxic cyanobacteria species are on the increase worldwide including in South Africa. The emergence and resurgence of harmful algal blooms (HABS) is due to eutrophication. The toxic cyanobacteria are known to carry genes that produce cyanotoxins which are lethal to humans. However the toxic and non-toxic cyanobacteria species merely differ in the *mcy* gene content, the peptide synthetase producing microcystin [1]. This may explain the observation of non-detectable microsystin toxin despite the presence of

mcy gene [2]. A study by Frazao et al. [3] using PCR method to determine molecular analysis of genes involved in the production of known cyanotoxins, microcystins, nodularins and cylindrospermopsin. The cyanobacteria genera, *Leptolyngbya, Oscillatoria, Microcystis, Planktothrix and Anabaena*, the toxic strains are known to have in the common the *mcy* (A-E, G, J) genes that are involved in the biosynthesis of microcystin [1,3]. The nodularin cyanotoxin is linked to the *nda* synthetase gene, a polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) and biosynthesized by *Nodularia spumigena* NSOR10 cyanobacteria [4]. The review study of Pearson et al.[4] and Sinha [5] showed that cyanotoxin cylindrospermopsin, is linked to genes, *aoa* or *cyr* (A-O) and now known to be biosynthesized by a number of cyanobacteria genera, *Cylindrospermopsis, Umezakia natans* in Japan, *Aphanizomenon ovalisporum* (Israel, Australia, USA, Spain); *Anabaena bergii* (Australia); *C raciborskii* (Thailand, China, Australia); *Raphidiopsis curvata* (China); *Aphanizomenon flos-aquae* (Germany); *Anabaena lapponica* (Finland); *Lyngbya wollei* (Australia); *Aphanizomenon gracile* (Germany); *Oscillatoria sp* (USA); *Aphanizomenon sp* (Germany); *Raphidiopsis mediterranea*; *Dolichospermum mendotae and Chrysosporum ovalisporum* (Turkey).

The emergence of toxic cyanobacteria species during a bloom period are not linked to any environmental factor such as light, nutrient enrichment and or nutrient depletion and or the presence of predators [4]. Eutrophication is a build-up of organic matter produced by phototrophs, such as cyanobacteria [6-7], this build-up is often seen as algal blooms and is driven by inputs of nitrogen and phosphorus. The cyanobacteria blooms are a major concern worldwide due to the production of cyanotoxins which are harmful to humans [8]. Cyanobacteria tend to dominate during the summer when concentrations of total phosphorus fall between 100-1000 µg/l [9]. A variety of hypotheses exist to explain why cyanobacteria blooms are becoming increasingly prevalent [10-12]. The most common hypotheses focus on nutrient conditions [10-11,13-17] and nutrient cycling [18] within a water body, as well as aspects of cyanobacteria cell physiology, such as their ability to migrate vertically within the water column, fix atmospheric nitrogen and produce cyanotoxins [19-22].

Cyanobacterial blooms are often associated with eutrophic conditions [23-25] so many studies have documented the relationship between nitrogen and phosphorus concentrations, speciation and stoichiometry, and cyanobacteria occurrence [10,13]. Recently it was reported that *Microcystis* growth responds increase to nitrogen over phosphorus [26]. The same study [26] also reported that the growth response of toxic *Microcystis* to nitrogen was greater than non-toxic strains. Some species of cyanobacteria are known for their ability to fix nitrogen giving them high chances for producing cyanotoxins [26]. Other studies have shown that microcystin toxicity is also influenced by change in pH, temperatures and light intensity [27-29]. A study by Beversdorf et al. [30] indicated that some of the non-nitrogen fixing cyanobacteria may produce toxins because of nitrogen stress events.

However there are limited studies on occurrence of toxic and non-toxic cyanobacteria species in the river basin sediments in Africa [31]. Thus the main objectives of the study was to assess the physical-chemical characteristics of river sediments and how these contribute to the resurgence and growth of cyanobacteria species should ideal conditions return especial the river flows and to use Flowcam and molecular techniques to identify toxic and non-toxic cyanobacteria genes in the river sediments and to use 16S rRNA in identifying the cyanobacteria species and explore relationships among the cyanobacteria species in the river sediments.

2. Results

2.1. The physical-chemical characteristics of the river sediments

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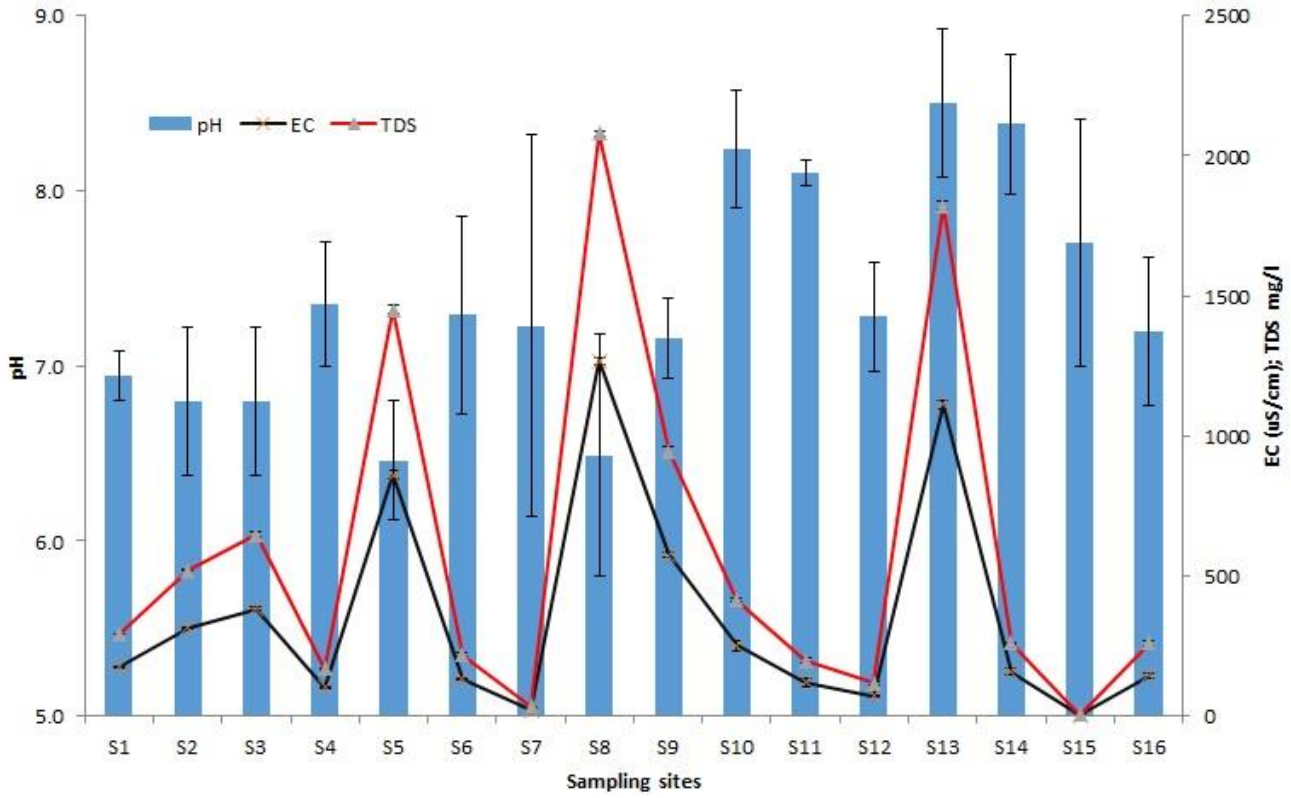

Figure 1. Average values of physical characteristics of the river sediments of the 18 sampling sites. Whiskers reflect standard error.

2.2. The abundance of nutrients in the river sediments

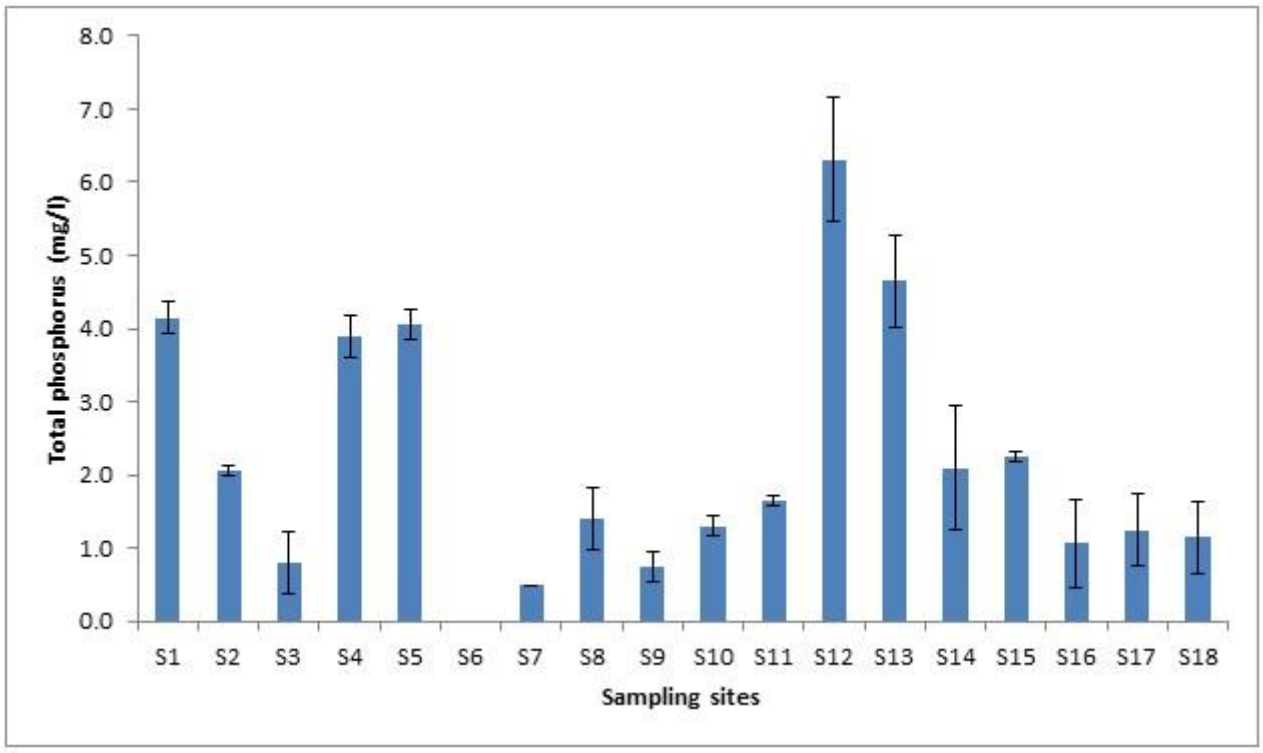

Figure 2. Average values of total phosphorus in the river sediments of the 18 sampling sites. Whiskers reflect standard error.

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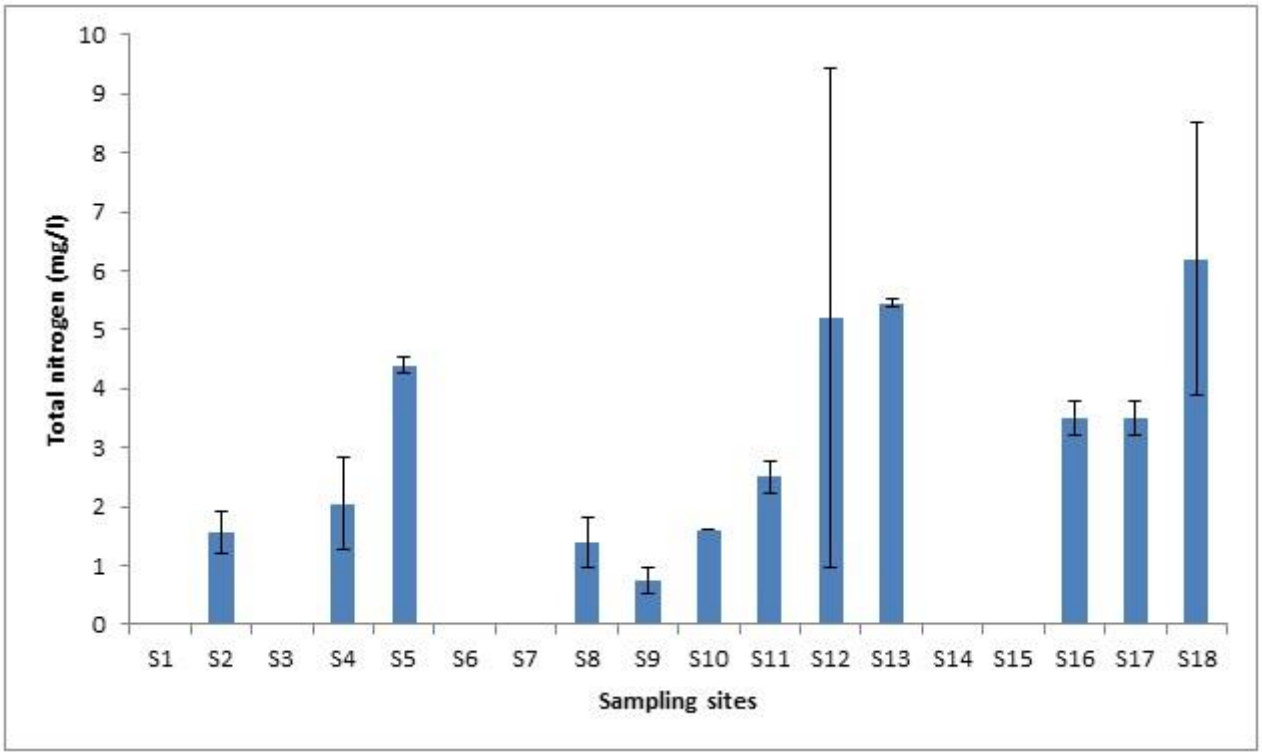

Figure 3. Average values of Total Nitrogen in the river sediments. Whiskers reflect standard error.

2.3. The presence of cyanobacteria in the river sediments

Table 1.Summary of toxic and non-toxic cyanobacteria species in the Limpopo river basin

Cyanobacteria species/sample sites	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18
Aphanizomenon sp.			+*															
Raphidiopsis curvata			+*															
Microcystis aeruginosa	+		+	+	+			++*		+	+	+	++*	+	+		+	
Microcystis panniformis								+*										
Synechocystis PCC 6803			+*				+		+									
Cylindrospermopsis sp.			+*						+*									
Lyngbya sp.	+																	
Leptolyngbya sp.		+														+		
Leptolyngbya boryana								+						+				
Calothrix sp.		+						+	++*				+	+				
Oscillatoria sp.		+	+						++*									
Phormidium sp.		+			+					+					+			
Phormidium uncinatum			+															
Nostoc sp.			+*		+								+*					
Anabaena circinalis					+													
Anabaena oscillarioides					+													
Chroococcus						+												
Anabaechopsis circularis						+												
Spirulina laxissima SAG 256.80										+								
Planktothrix rubescens								+*					+*					+
Alkalinema pantanalense																		+
Gloeocapsa sp.									+*		+							
Arthrospira sp. str *PCC8005*			+*															

Notes: +Flowcam analysis and +Molecular techniques with toxic genes* expression

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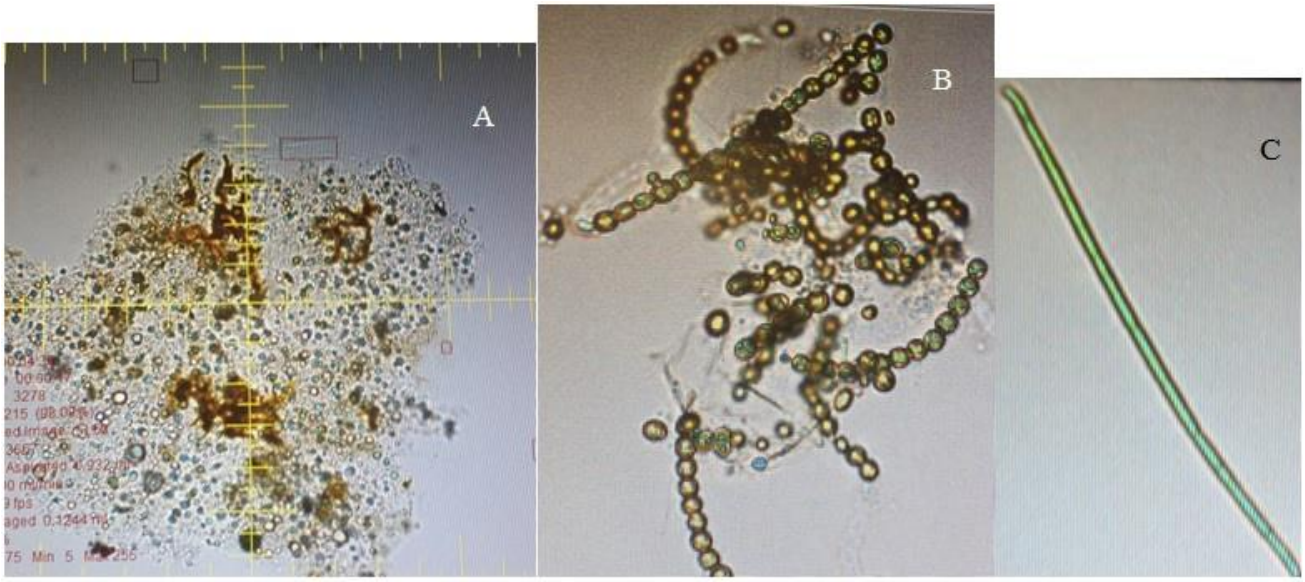

Figure 4. The (A) *Microcystis, (B) Anabaena and (C) Oscillatoria* species in the river sediments

2.4 Polymerase chain reaction amplification of 16S rRNA gene

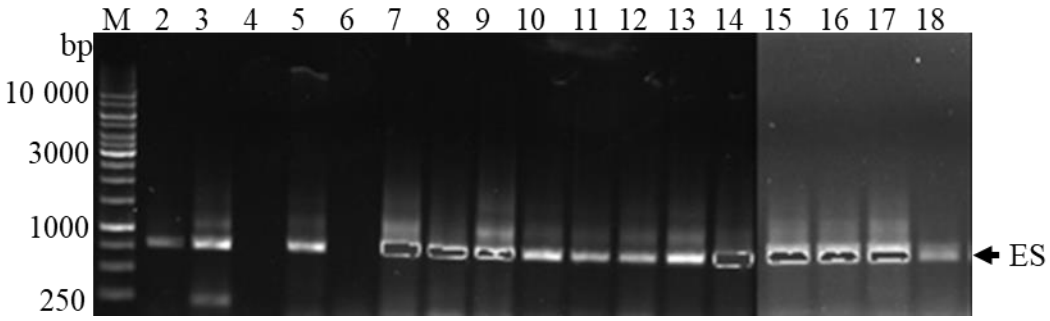

Figure 5. PCR amplification using 27F and 740R primer pair for 16S rRNA gene. ES (estimated fragments); M (Standard Marker), 2-18 Sample numbers. Lane 2=Notwane River; 3=Sand River upstream; 4=Mogalakwena River; 5=Mawoni River; 6=Lephalale River; 7=Mokolo River; 8=Crocodile River downstream of Hartbeespoort Dam; 9=Nzhelele River downstream; 10=Sand River downstream;11=Crocodile River downstream (near bridge on road D1235); 12=Nzhelele River upstream; 13=Mzingwane River; 14=Shashe River; 15=Limpopo River (next to Thuli coal mine); 16=Limpopo River (abstraction point at 0.0 m); 17=Limpopo River (abstraction point at 1.0 m); 18=Limpopo River (abstraction point at 1.68 m)

Table 2. Results of BLAST search showing the similarity between GenBank sequences with sample sequences from this study, Families of each species are shown on separate column.

Samples	Similarity %	Species similar to	Family	Accession No
S2	93	Uncultured *Leptolyngbya* sp. Clone	Leptolyngbyaceae	KM108695.1
S3	94	*Synechocystis PCC 6803*	Oscillatoriophycideae	CP012832.1
S5	97	*Anabaena oscillarioides*	Nostocaceae	AJ630428.1

S7	99	*Synechocystis* sp. *PCC 6803*	Oscillatoriophycideae	CP012832.1
S8	99	*Leptolyngbya boryana*	Leptolyngbyaceae	AP014642.1
S9	97	*Synechocystis PCC 6803*	Oscillatoriophycideae	CP012832.1
S9	100	*Cylindrospermopsis raciborskii CHAB3438*	Oscillatoriophycideae	KJ139743.1
S9	100	*Aphanizomenon sp*	Nostocaceae	GQ385961.1
S9	100	*Raphidiopsis curvata*	Nostocaceae	KJ139745.1
S10	96	*Spirulina laxissima SAG 256.80*	Spirulinaceae	DQ393278.1
S11	87	Uncultured Cyanobacterium clone		AM159315.1
S12	83	Uncultured Cyanobacterium clone		HQ189039.1
S13	90	Uncultured Cyanobacterium clone		JX041703.1
S14	98	*Leptolyngbya boryana*	Leptolyngbyaceae	AP014642.1
S16	83	*Leptolyngbya*	Leptolyngbyaceae	KJ654311.1
S18	96	Alkalinema pantanalense	Pseudanabaenaceae	KF246497.2

2.5 Detection of genes involved in toxin production

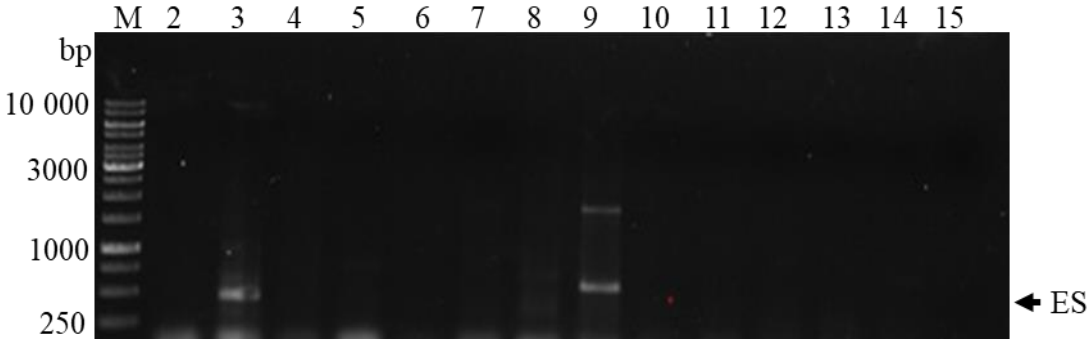

Figure 6. PCR products using PKS primers for cylindrospermopsin polyketide synthetase gene. ES (estimated fragment); M (Standard Marker), 2-18 Samples number. Lane 2=Notwane River; 3=Sand River upstream; 4=Mogalakwena River; 5=Mawoni River; 6=Lephalale River; 7=Mokolo River; 8=Crocodile River downstream of Hartbeespoort Dam; 9=Nzhelele River downstream; 10=Sand River downstream;11=Crocodile River downstream (near bridge on road D1235); 12=Nzhelele River upstream; 13=Mzingwane River; 14=Shashe River; 15=Limpopo River (next to Thuli coal mine);

Table 3. Results of BLAST search showing the similarity between GenBank sequences with sample sequenced using PKS and HEP primers for toxin gene identification.

Primers	Sample No	Similarity%	Species similar to	Accession No
PKS	S3	100	*Aphanizomenon sp. 10E6*	GQ385961.1
	S3	100	*Raphidiopsis curvata*	KJ139745.1
	S3	100	*Cylindrospermopsis raciborskii*	AF160254.1
	S3	100	*Arthrospira sp. str. PCC 8005*	FO818640.1
	S3	100	*Nostoc sp. NIES-4103*	AP018288.1
	S9	93	*Calothrix sp. 336/3*	CP011382.1
	S9	89	*Oscillatoria nigro-viridis PCC 7112*	CP003614.1
	S9	100	*Gloeocapsa sp. PCC 7428,*	CP003646.1
	S9	100	*Cylindrospermum sp. NIES-4074*	AP018269.1
HEP	S8	100	Uncultured *Microcystis sp. clone msp microcystin synthetase E (mcyE) gene, partial cds*	KF687998
	S8	100	*Microcystis panniformis FACHB-1757*	CP011339.1
	S8	100	*Microcystis aeruginosa PCC 7806*	AF183408.1
	S8	100	*Nostoc sp. 152*	KC699835.1
	S8	100	*Planktothrix rubescens NIVA-CYA 98*	AM990462.1
	S13	100	*Nostoc sp. 152*	KC699835.1
	S13	100	*Planktothrix rubescens NIVA-CYA 98*	AM990462.1
	S13	100	Uncultured *Microcystis sp. from Uganda*	FJ429839.2
	S13	100	*Microcystis aeruginosa PCC 7806SL*	CP020771.1
	S13	100	Uncultured *Microcystis sp. clone mw microcystin synthetase E (mcyE)gene, partial cds*	KF687997.1

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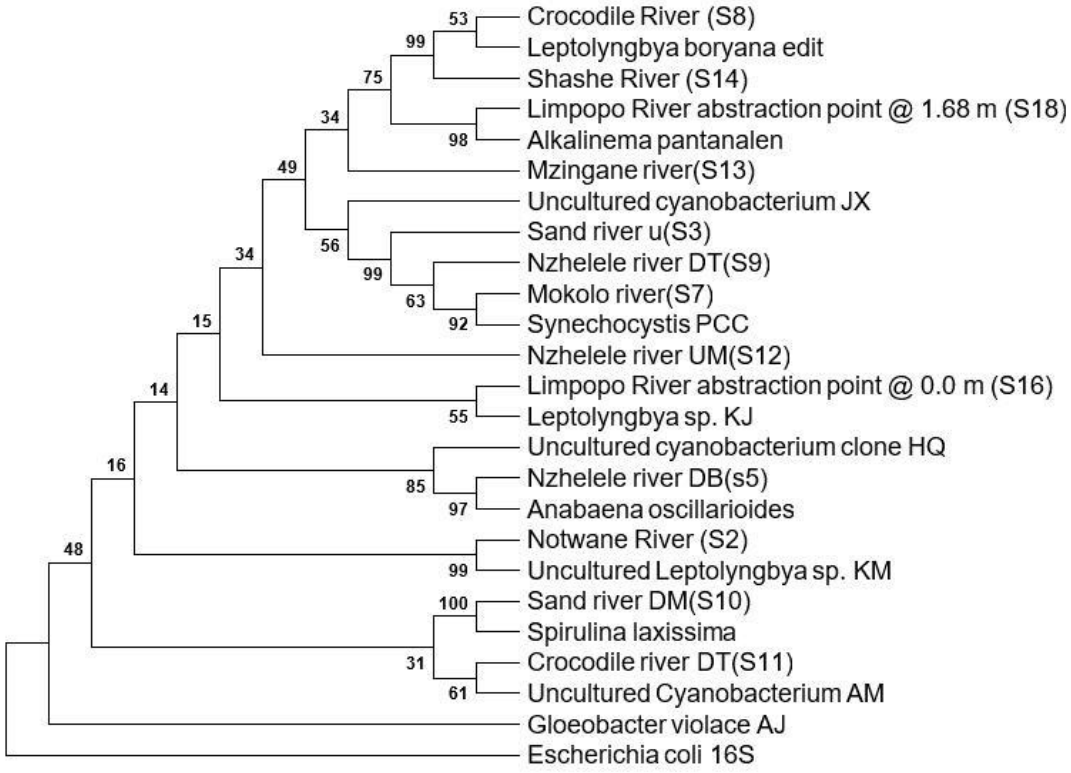
151 *2.6 Phylogenetic relationship*



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153 **Figure 7.** The evolutionary history was inferred using the Neighbor-Joining method

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158 **Table 4.** Divergence matrix for reflection of similarity

	S2	S3	S5	S7	S8	S9	S10	S11	S12	S13	S14	S16	S18
Notwane River (S2)	–												
Sand River (S3)	0.216	–											
Nzhelele River (S5)	0.191	0.187	–										
Mokolo River (S7)	0.167	0.064	0.130	–									
Crocodile River (S8)	0.166	0.160	0.149	0.119	–								
Nzhelele River (S9)	0.184	0.095	0.152	0.028	0.140	–							
Sand River (S10)	0.155	0.216	0.153	0.156	0.169	0.169	–						
Crocodile River (S11)	0.257	0.295	0.280	0.244	0.278	0.254	0.236	–					
Nzhelele River (S12)	0.391	0.394	0.365	0.351	0.350	0.361	0.364	0.492	–				
Mzingwane River (S13)	0.190	0.180	0.184	0.130	0.134	0.139	0.168	0.267	0.377	–			
Shashe River (S14)	0.173	0.163	0.156	0.119	0.006	0.140	0.173	0.278	0.355	0.134	–		
Musina borehole (S16)	0.376	0.359	0.312	0.314	0.342	0.321	0.343	0.414	0.555	0.371	0.347	–	
Musina borehole (S18)	0.183	0.184	0.179	0.136	0.128	0.150	0.186	0.285	0.366	0.173	0.131	0.348	–

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3. Discussion

3.1 The physical-chemical characteristics of the river sediments

The physical characteristics of the river sediments showed considerable variation from the different tributaries of the Limpopo River and the Limpopo River itself (Figure 1). The river sediments electrical conductivity (EC) and total dissolved solids (TDS) values varied between 21.2 and 1269 μS cm^{-1} throughout the sampling sites. The temperatures measured during the sampling trips were more than 22 °C. High temperatures due to climate change have been also reported as an important factor in the global expansion of harmful algal bloom worldwide [32]. Rising temperature and exceeding 20°C, the promotes the growth rate of cyanobacteria whereas other freshwater eukaryotic phytoplankton growth rate decreases and this is regarded as a competitive advantage for cyanobacteria [33]. A study done O'Neil et al. [9] by reported that higher temperatures not only promote the dominance of cyanobacteria, but also favor the production of microcystins and result in an increase in their concentration.

The pH values were in the range between 6.4 and 8.5. Higher pH value has a competitive advantage for many cyanobacteria, because of their strong carbon-concentrating abilities compared to eukaryotic phytoplankton species [34]. A laboratory experiment done by Jahnichen et al. [35] with *Microcystis aeruginosa* showed that microcystin production started when pH exceeded 8.4, indicating a lack of free carbon dioxide (CO_2).

3.2 The abundance of nutrients in the river sediments

The increased input of nutrients into surface water is the main factor responsible for massive proliferations of cyanobacteria in fresh water, brackish and coastal marine ecosystem. However, phosphorus and nitrogen nutrients in high levels lead to accelerated growth of cyanobacteria [36-37].

3.2.1 Total phosphorus

The total phosphorus concentration values in the river sediments ranged from 0.5 mg/l to 6.3mg/l (Figure 2). The highest value was recorded for Nzhelele River (S12) near Mphephu resort and downstream of Siloam oxidation ponds. The presence of phosphorus may be due to the discharge of sewage effluent from Siloam oxidation pond [38]. The low value of phosphorus for Lephalale River (S6) may be due less anthropogenic activities upstream of sample site [39]. Phosphorus has been implicated more widely than nitrogen as a limiting nutrient of phytoplankton including cyanobacteria in freshwater systems [40]. A minute amount of phosphorus entering or becoming soluble in a water body can trigger a significant algal bloom [41]. The impact of excess phosphorus in the receiving river streams is shown the green colour presence of cyanobacteria. Limpopo River (S1) receives inflows from Notwane and Crocodile Rivers and this contributes to the phosphorus loading of the river. Notwane River (S2) receives municipal discharge from Glen Valley sewage plant and from agricultural runoff from irrigated farms and livestock ranching [23,42]. The Crocodile River receives sewage effluent from upstream catchment activities as discharge of sewage effluent into tributaries of Crocodile River and or discharge into Crocodile River itself and agricultural runoff [43,44]. Sand River (S3) receives municipal nutrient discharges from the Polokwane sewage plants and from rainfall runoff from fertilizer on agricultural land around the river [45]. The sample point on Mogalakwena River (S4) is downstream of sewage plants of Mokopane, Modimolle and Mookgophong and golf course, game farming, livestock farming and irrigated farmlands [46]. After Mokopane, the Nyl River is renamed Mogalakwena River. Mzingwane River (S13) receives municipal discharge from Filabusi, Gwanda, West Nicholson sewage plants and agricultural runoff from irrigated farms and livestock ranching [47]. The sample point at Shashe River (S13) was second highest phosphorus content and this may be attributed to sewage plants upstream in Francistown and agricultural runoff from irrigated farms and livestock ranching [44,48]. These rivers are some of tributaries of the Limpopo River and they contribute the successive loading of phosphorus in the Limpopo River (S15-S16). At sample point S18, the phosphorus is 1.2 ± 0.5 mg/l is available to a depth of 1.68 m into Limpopo River [49].

3.2.2 Total Nitrogen

The nitrogen concentration values in the river sediments were variable ranging from 1.5 mg/l to 6.5 mg/l (Figure 3). The highest value was recorded for Nzhelele River (S12) near the Mphephu resort and downstream of Siloam hospital oxidation ponds. The reason for the highest value at Nzhelele River was possible due the discharge of sewage effluent from Siloam hospital [38]. Filamentous Cyanobacteria can obtain the nitrogen by fixing the atmospheric nitrogen gas and convert to nitrate for their growth [50]. Nitrogen is a common gas (79%) that is found in the atmosphere. Thus cyanobacteria such as *Anabaena* are able to utilize atmospheric nitrogen in addition to nitrate originating from the river sediments [50,51]. The other sample sites with nitrates in excess of 2 mg/l are Sand River (S4); Mawoni River (S5); Crocodile River (S11); Mzingwane River (S13); Limpopo River (S16 to S18). These tributaries have one in common upstream of the sample sites, there is a municipal sewage plant and also surrounded by farmland where commercial irrigation farming is practiced as the case with Crocodile, Notwane, Shashe, Mzingwane & Sand Rivers and subsistence agriculture is practiced as the case of Mawoni and Mzingwane Rivers [42,44-48]. The Crocodile River also receives inflows from eutrophic Hartbeespoort dam [43]. The Limpopo River (S16) is downstream of all the sample points and this shows the cumulative discharge of nitrates originating from the tributaries. At sample point S18, the total nitrogen is 6.25 mg/l is available to a depth of 1.68 m into Limpopo River [49]. The Musina local municipality has drilled 8 boreholes in the Limpopo river bed and most of these boreholes are located close to S16 [52].

Botha and Oberholster [53] performed a survey of South African freshwater bodies between 2004-2007, using RT-PCR and PCR technology to distinguish toxic and non-toxic *Microcystis* strains bearing the *mcy* genes, which correlate with their ability to synthesize the cyanotoxin, microcystin. The study revealed that 99 % of South Africa's major impoundments contained toxicogenic strains of *Microcystis*. The study of Su et al.[54] in the Shanzi impoundment, China showed that the sediments were the source of cyanobacteria inoculum. This has implications that the cyanobacteria flocculates in the sediments during periods of adverse environmental conditions such as cessation in river flows. These cyanobacteria cysts or spores than reactivate during periods of river flow. This is feasible when considering the Limpopo river basin where the majority of tributaries are perennial and river flows commence during period of summer rainfall. The river flows disturb the sediments thus bringing into water column the cyanobacteria species [54]. The source of nutrients in the Limpopo river basin may be attributed directly to sewage discharge of municipal waste water plants, from Botswana side such as Glen Valley, Mahalpye, from South Africa side it is western and northern parts of Johannesburg to Musina and indirect to agricultural practices of fertilizer application and animal waste [42,44-48].

3.3 The presence of cyanobacteria in the river sediments

The study showed the presence of toxic and non-toxic cyanobacteria species in the Limpopo river basin (Table 1). The dominate cyanobacteria was the filamentous *Leptolyngbya* species followed by *Synechocystis species (*non-toxic and toxic strains), toxigenic *Microcystis* species, and toxigenic *Cylindrospermopsis raciborskii* species. The FlowCam showed the presence of cyanobacteria species in the Limpopo River basin (Table 1; Figure 4). The dominant cyanobacteria species identified from the samples were *Microcystis* species, followed by *Cylindrospermopsis raciborskii Calothrix, Phormidium and Planktothrix* species. There were no cyanobacteria species that were detected in Mokolo River (S7).

Cyanobacteria undergo distinct developmental stages [55]. For example, they differentiate into resting cells, spores, akinetes and cysts which represent a survival strategy under unfavorable environmental conditions [51,56]. Under favorable conditions the cell will germinate again [57]. The ability of cyanobacteria to adapt to adverse dry periods allows them to in habitat the river sediments as shown by studies by Perez at al. [56], Kim et al. [51] and this study (Figure 8). The study of Kim et al. [51] further illustrated the viable nature of cysts and akinetes in providing the next inoculum of *Microcystis, Anabaena, Aphanizomenon* and *Oscillatoria* is Bukhan, Namhan Rivers and Lake Paldang and Kyeongan stream, in South Korea.

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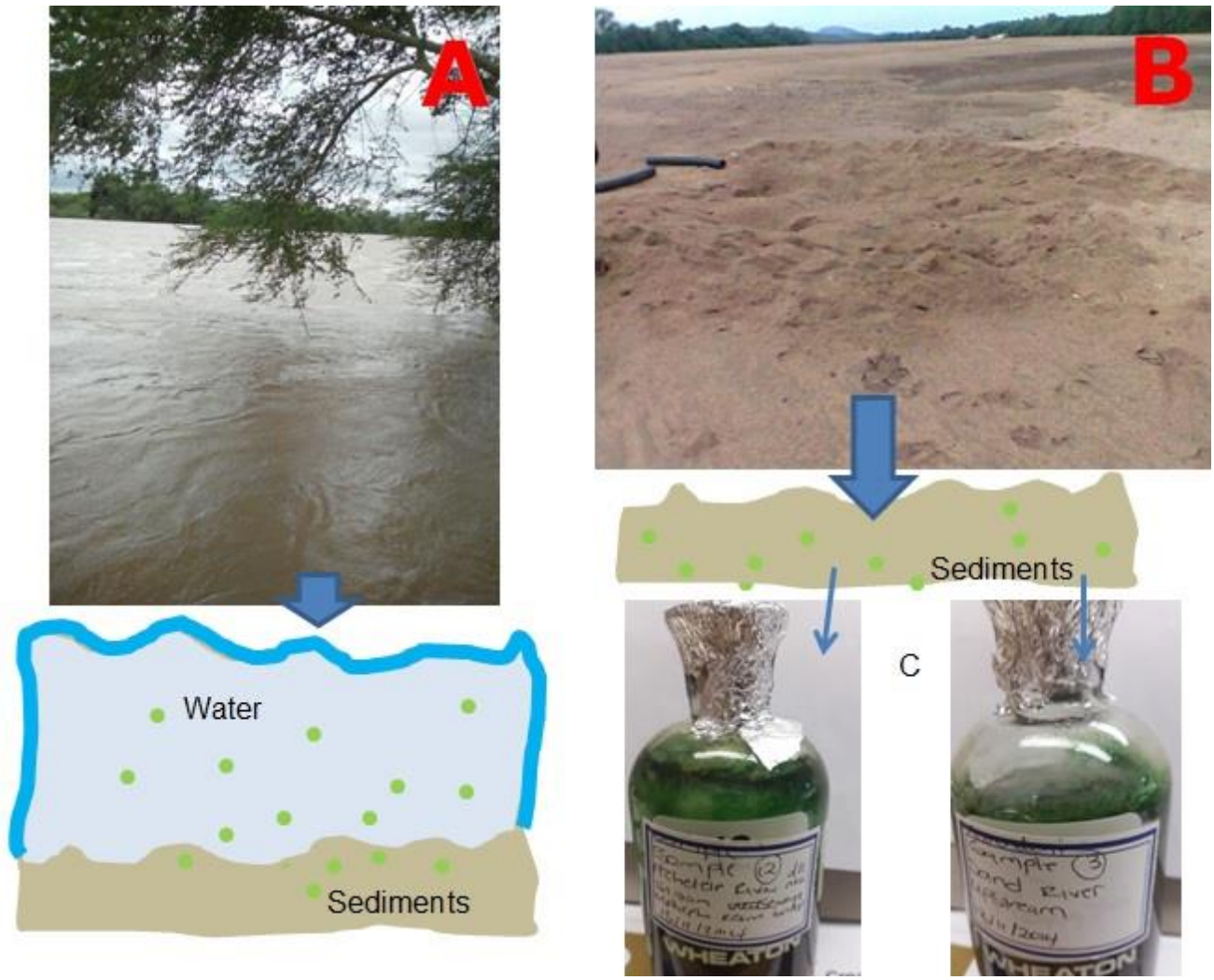

Figure 8: Scenario involving sedimentation of cyanobacteria (green dots) cysts and akinetes (A) during flood and flow conditions in Limpopo River and (B) during non-flow (DRY) conditions in the Limpopo River and (C) growth of cyanobacteria under continuous lighting and provision of BG medium at room temperature.

3.4 Pcr analysis of 16S rRNA gene

PCR products separated by gel electrophoresis are shown above in the Figure 5. The presence of bands is indicative of positive amplification whereas a blank sample indicates negative amplification. The blank samples where repeated several times and failed to amplify. Almost all the samples showed positive amplification which confirmed the presence of cyanobacterial DNA in the samples. The two samples which showed no amplification were from Mogalakwena (S4) and Lephalale Rivers (S6).

For each sample, multiple fragments obtained by sequencing with both forward and reverse primers; were edited and assembled using the Staden package [58]. All assembled sequences were aligned in BioEdit v7.0.9 [59]. However, the sample collected from Limpopo River (S1), is not shown in figure above because it was used as the test sample. Also noted was the fact that the amplified fragment from that test sample failed to sequence only producing only 100 bp while around 650 bp is expected. Other samples like the sample Limpopo River (S15), and sample Limpopo River (S17), Musina borehole abstraction point (S16) did amplify but failed to assemble in Staden package [58]. Assembled sequences were run on BLAST algorithm [60] to identify their closely similar sequences already deposited in GenBank via NCBI and the outcomes shown in Table 4.

From BLAST algorithm [60] it must be understood that more than 98% similarity obtained matches the sample to the correct species, more than 90% similarity obtained matches the sample to the correct genus, more

than 80% similarity obtained matches the sample to the correct the correct Family level. From that is confirmed by BLAST that cyanobacteria from samples of Mokolo River (S7); Crocodile river downstream of Hartbeespoort dam (S8) and Shashe River (S14) have been identified to the correct species, while cyanobacteria from samples of Notwane River (S2); Sand River upstream (S3); Mawoni River (S5); Nzhelele River downstream (S9); Sand river downstream and Limpopo river (S18)(abstraction point at 1.68m) have been identified to correct genus, the cyanobacteria from sample Limpopo river (S16) (abstraction point at 0.0m) been identified to the correct family. However, samples of Crocodile River downstream (S11) (near bridge on road D1235); Nzhelele River upstream (S12) and Mzingwane River (S13) have been identified to be similar to the clones and no families could be detected. The first to be noted was that Uncultured Cyanobacterium clone HQ189039.1 could not be used for phylogenetic tree because of its length (about 480 bp), with the reason that complete deletion option of gaps and missing information in MEGA 7 [61] was used. The second to be noted was that two outgroups sequences have been used in phylogenetic alignment.

3.5 Detection of genes involved in toxin production

Cyanotoxins detection was also based on PCR by amplification of microcystin/nodularin synthetase using the HEP primer pairs and cylindrospermopsin polyketide synthetase genes using PKS primer pair. The *mcy*A-Cd primer pair and M13 & M14 primer pair were also used for toxins detection but did not produce a positive result. Hence they were excluded in the discussions. For most of the samples, no amplification of any of the toxin genes was obtained. Nevertheless, few samples showed the amplification of cylindrospermopsin polyketide synthetase gene such as Sand River (S3) upstream and Nzhelele River (S9) downstream (Figure 6). This confirmed the presences of cyanotoxin, cylindrospermopsin in the sediment samples and was attributed to the cyanobacteria species, *Cylindrospermopsis raciborskii* (Table 3).

The HEP primer pair produced two positive results for samples from Crocodile River (S8) and Mzingwane River (S13) and was attributed to the presence of toxigenic *Microcystis* sp. (Table 3). As expected the toxigenic *Microcystis* species was found in the Crocodile River, downstream of the Hartbeespoort dam, a eutrophic water impoundment known for the regular occurrence of *Microcystis* dominated harmful algal blooms [36]. However our two toxigenic *Microcystis* strains were different from the seventeen toxigenic *Microcystis* strains studied by Mbukwa et al. [24] from the Hartbeespoort dam. The differences may be explained by the different use of *mcy* primers in identifying the genes expressing toxicity and differences in experimental approach. Our study on the *mcy*A-Cd primer did not amplify and whereas the work of Mbukwa et al. [24] their *mcy*A-Cd did amplify and produced the seventeen toxigenic *Microcystis* strains. However during our study, the *mcyE* genes were positive based on the HEP primer did amplify but these were also different from toxigenic *Microcystis* strains studies by Mbukwa et al. [24]. During our study the total genomic DNA was not extracted directly from the sediments but from cyanobacteria that was cultured in the laboratory. The laboratory culture conditions have been known to alter the toxicity of *Microcystis* species as shown by the study of Scherer et al. [62]. The authors mimicked a temperature increase in 10 °C, the *Microcystis* under laboratory conditions was able to express *mcy*B gene instead of the *mcy*D in expressing toxicity. This may imply that the biodiversity of toxigenic *Microcystis* strains in Hartbeespoort dam and the Crocodile River and the Limpopo river basin.

Mbukwa et al. [24] used DNA molecular techniques to identify the two species of *Microcystis* as *M. aeruginosa* (origins from Hartbeespoort dam, South Africa) and *M. novacekii* (origins from Phakalane effluent, Gaborone, Botswana). The molecular techniques showed the presence of *mcy* genes that is responsible for microcystin encoding, thus confirming that the two *Microcystis* species were indeed toxic. The Phakalane ponds effluent is discharged into the Notwane River, tributary of the Limpopo [23]. An earlier study by Basima [47] upstream of the sample point, Mzingwane (S13), showed the abundance of cyanobacteria genera dominated by *Microcystis* species followed by *Anabaena* and *Nostoc* species in water impoundments situated inside the Mzingwane River. In lower Limpopo River, in Mozambique, at Chokwe irrigation scheme received irrigation waters from Maccaretane Dam, Pedro et al. [25] showed the presence of *Microcystis* species and microcystin LR levels of 0.68 ppb linked to presence of *mcy*B gene and *mcy*A gene in collected water samples. Mikalsen et al. [63] identified eleven *Microcystis* species containing different variants of the *mcy*ABC (toxic species) and seven *Microcystis* species that lacked the *mcy*ABC gene (non-toxic species). Davies et al. [64] in the

northwest of the U.S.A of four temperate lakes showed that the increase in water temperature contributed to increase of toxic *Microcystis* species (possessing the *mcy*D gene). Yamamoto [65] and Oberholster et al. [66] have shown the *Microcystis* species adopt survival strategies to mitigate harsh external environments such as reduced river flow, a major characteristic of Limpopo River by sinking into the sediments.

The Limpopo river basin is characterized by extreme weather events such as heatwaves, floods and drought [67] and could do these weather events contribute toxic or non-toxic *Microcystis* species? Hence there is a need to determine the presence *mcy* gene that is responsible for the production of microcystins by toxic *Microcystis* species. The presence of microcystins in the rivers may constituent a health risk especially for the communities that may be in contact or drink the polluted water without any form of treatment or suitable treatment that can be able to remove the toxins in the water. Convectional method for water treatment is not convenient for the removal of microcystins in water [68]. Drinking water treatment processes might trigger the release of hepatoxin into drinking water by disrupting the trichomes of cyanobacteria [69]. The water supplies from the Limpopo river basin are used by water utilities for drinking water supplies, commercial and subsistence irrigation farmers for growing food crops and livestock watering (Figure 9). Thus presence of cyanotoxins can also poison the livestock and game animals (wildlife) in transfrontier parks such as Kruger National Park, Gona-re-zhou National Park and Mapungubwe National Park [70]. Already the microcystins have been implicated in the death of wildlife in the Kruger National Park [71]. The cyanotoxins have been implicated in the negative growth (stunting) of plants and this may be serious repercussions for the irrigation farmers [72].

Figure 9: A scenario involving boreholes drilled inside the Limpopo river channel and contamination with cyanobacteria (green dots) cysts and akinetes for (A) irrigation farmers & (B) water utility raw water supply for human consumption

357
358 *3.6 Phylogenetic relationship*
359 The evolutionary tree was constructed could not be used for phylogenetic purposes because of two
360 important things; the number of samples used for PCR per river site was not enough to make conclusive
361 argument; and the cyanobacteria were the expected products which needed to be identified. Hence the tree
362 was used to verify the identification as done by BLAST search, however the phylogenetic relationship was
363 basically done by divergence matrix and combined discussion followed the divergence matrix.
364 The relationship between the samples and their most similar species as from BLAST search was confirmed
365 by phylogenetic tree, and the relationships between some cyanobacteria species from different samples have
366 been confirmed (Figure 7). The first was the confirmation of similarity of samples from Crocodile River (S8)
367 downstream Hartbeespoort dam and Shashe River (14) to *Leptolyngbya boryana* with 99 % bootstrap confidence.
368 Then the similarity of Musina borehole extraction (S16) sample to *Alkalinema pantanalense* with 98 % bootstrap,
369 then the similarity of samples from Sand River (S3) upstream. Nzhelele River (9) downstream near Tshipise
370 and Mokolo river to *Synechocystis* sp. PCC 6803. The other similarity was Mawoni River (5) downstream of
371 Makhado oxidation pond to *Leptolyngbya* sp. with 97 % bootstrap confidence, then Notwane River (2) to
372 uncultured *Leptolyngbya* sp with 99 % bootstrap confidence, and lastly Sand River (10) downstream to *Spirulina*
373 *laxissima* with 100 % bootstrap confidence.
374 However the cyanobacteria at these two sites, Musina borehole extraction (S16) and Sand River (S3) may
375 imply that there is movement of aquatic animals such as fish from the entrance (mouth) of Sand River towards
376 the Musina abstraction point (Figure 10). In simple terms there is an upstream movement of cyanobacteria
377 species being carried by aquatic animals but this requires further investigation.
378 The other which matched their supposed to match from BLAST search includes Musina Borehole
379 extraction point (S16) to *Leptolyngbya* sp. and Crocodile River (S11) near bridge on road D1235 and upstream to
380 Thabazimbi to Uncultured Cyanobacteria clone though they bootstrap confidence level was little about 55 %
381 and 61 % respectively. Following the BLAST search results and Phylogenetic tree the divergence matrix can be
382 used to verify the truth of the two, i.e. BLAST search and phylogenetic tree. Divergence matrix confirms that
383 cyanobacteria from Crocodile River (S8) downstream of Hartbeespoort dam and from Shashe River (S14) are
384 same species which is *Leptolyngbya boryana,* they both show at least 98 % similarity to this species in BLAST and
385 bootstrap confidence is 99% forming a clade in phylogenetic tree, while they have the least difference in the
386 divergence Matrix.
387 Thus based on this study there is DNA evidence to suggest that the cyanobacteria at the Musina abstract
388 point is similar to cyanobacteria from the Crocodile River system. This may be possible because the Musina
389 abstraction point is downstream to Crocodile River which flows into the Limpopo River (Figure 9).

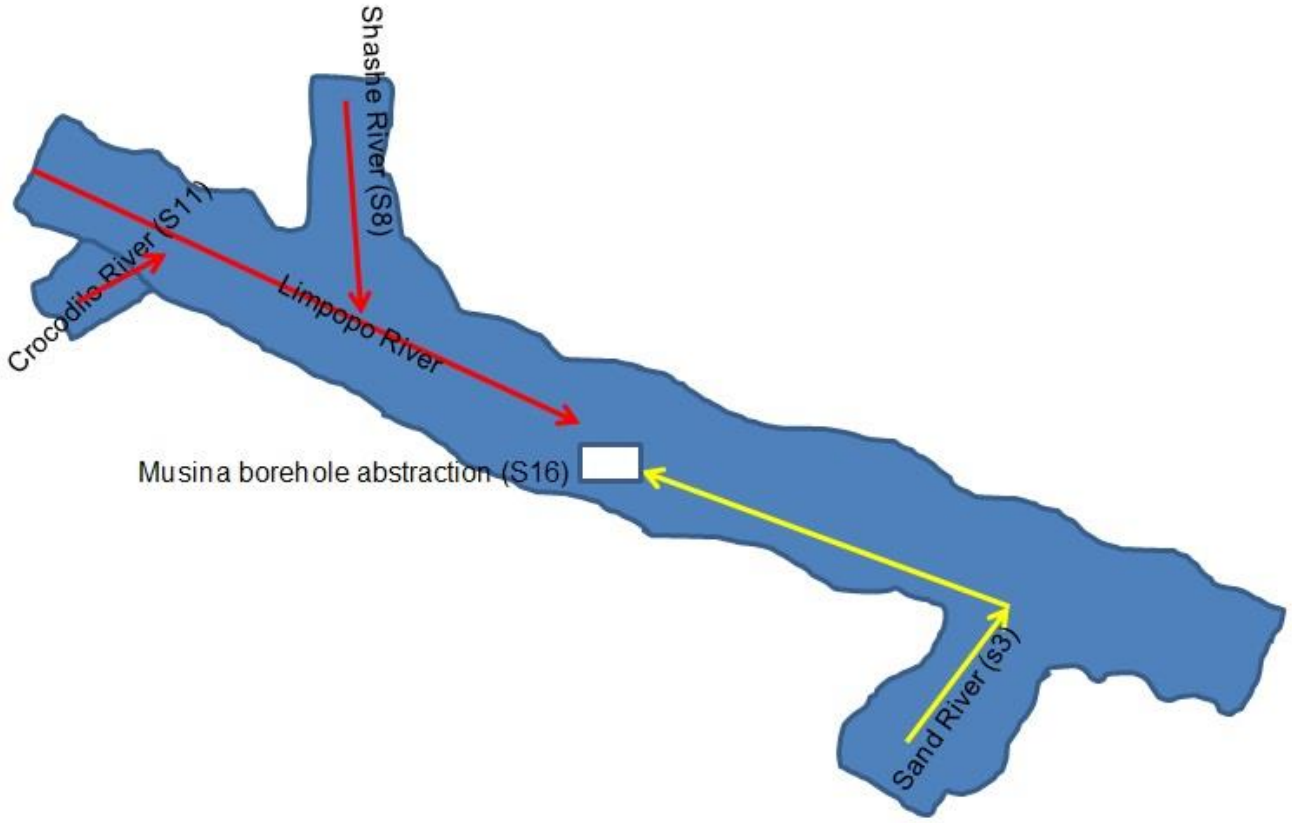

Figure 10: A scenario involving the movement of cyanobacteria species during water flows in the Limpopo River (red arrow) towards the Musina abstraction borehole (White Square). The possible upstream movement (yellow arrow) from Sand River (S3) to Musina borehole (S16) may involve cyanobacteria 'hiking a ride' on aquatic animals such as fish, crocodiles, etc.

Samples from Nzhelele River upstream near Mphephu resort and Mzingwane River (Zimbabwe) never attempted to make a clade with their supposed matches. The relationship between the cyanobacteria species as identified from specific locations where they were collected from have been identified by Divergence Matrix (Table 4). Same species are detected by the difference co-efficient of 0.00 whereas the complete unrelated species are detected by the co-efficient of 1.00.

The cyanobacteria species from Crocodile River (S8) is the same species as the cyanobacteria from sample Shashe River (S14), they have less than 1 % difference (0.006) and their differences to other species are approximately the same. This may be expected since Shashe River is downstream of Crocodile River (Figure 10). The cyanobacteria species from Mokolo River (S7) and the cyanobacteria species from sample Nzhelele River (S9) share undetectable difference but by comparing their difference to the cyanobacteria species from sample Notwane River (S2) and Mawoni River (S5) there is a slight difference of about a percent hence they are not one species. The cyanobacteria which are much diverged from others are the cyanobacteria species from sample The Notwane River (S2) and Limpopo River (S16) and they themselves are the most different from each other with 28% difference, their comparison co-efficient range from 0.17 to 0.28. The Nzhelele River upstream (S12) and Limpopo River (S16) and they themselves are the most different from each other with 28% difference, their comparison co-efficient range from 0.312 to 0.492 which is the highest for all species.

4. Conclusions

Limpopo River is a source of water supply to Musina Township after some sort of water treatment. Chemical and physical parameters analysis showed a highly chance of cyanobacteria growth in the rivers. Enrichment of the nutrients in the rivers will be responsible for the growth of cyanobacteria blooms which can lead into production of cyanotoxins. These toxins will cause severe impacts to human and animals using water

from the rivers. Many countries in Africa have reported cases of intoxication and deaths of animal that may have been caused by cyanobacterial toxins. Monitoring of the nutrients loads into the river system will decrease the threat of cyanobacteria blooms to human and animal health. The results obtained in this study indicated the presence of toxic and non-toxic cyanobacteria species in the Limpopo Rivers and its tributaries. The molecular tools were used to presence of non-toxic and toxic cyanobacteria based genes that code for the production of cyanotoxins. The presence of nutrients, phosphates and nitrates in the river sediments, encourages the growth of the cyanobacteria should river flows commence. Also the presence of toxic genes, expressing cylindrospermopsin and microcystin/nodularin in the river sediments points to worrisome trend in the Limpopo river basin. The cyanotoxins are harmful to humans who consume the water originating from boreholes located inside the Limpopo river basin or drilled along the Limpopo river basin. Secondly the water supplies from the Limpopo river basin are used by commercial and subsistence irrigation farmers for growing food crops and livestock watering. Thus presence of cyanotoxins can also poison the livestock and game animals (wildlife) in transfrontier parks. Cyanotoxins have been implicated in the negative growth (stunting) of plants and this may be serious repercussions for the irrigation farmers.

Future research work

From the matrix, it is convenient to conclude that the cyanobacteria species diversity in these sources is huge. It can be recommended that a project wherein the diversity of this cyanobacteria can be done using more advanced technology, Next Generation Sequencing to be specific, wherein almost all cyanobacteria form each sample will be sequenced and determined. Also further research is required on the different cyanobacterium species, *Leptolyngbya* which was found in all sediments samples. Also further research is required to determine the levels of cyanotoxins in the Limpopo River basin and also to compare the cyanobacteria species and their cyanotoxins in downstream side of the Limpopo in Mozambique using molecular techniques. It must be recommended that drinking water supplies at Musina should be monitored for the presence of cyanotoxins to manage/ minimize the risk of intoxication.

5. Materials and Methods

5.1 The study area

The study area is the Limpopo River and its major tributaries (Figure 10). Limpopo River basin consists of four countries: Botswana, South Africa, Zimbabwe and Mozambique [73].

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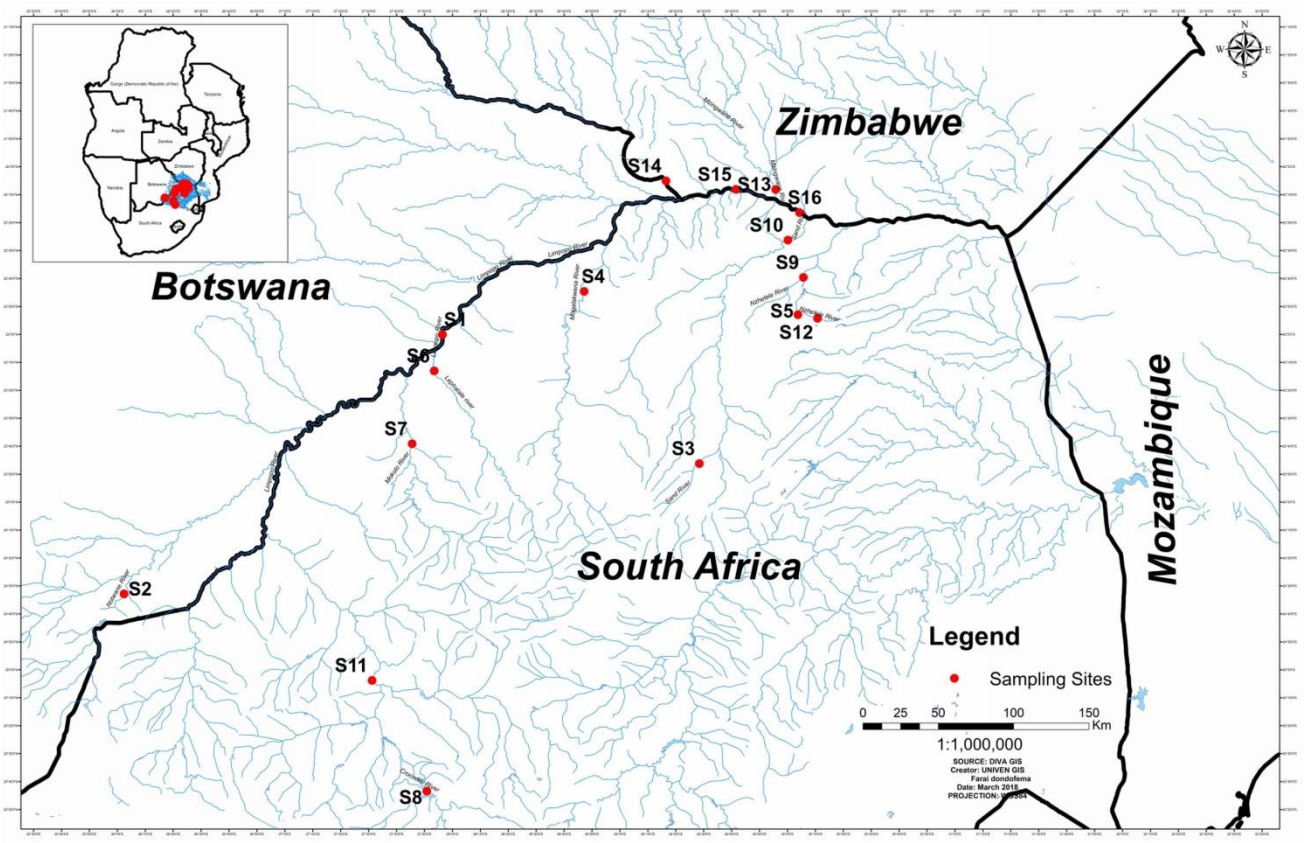

Figure 10. The location of sediment sample sites on some of the tributaries of the Limpopo River

The Limpopo River basin is an arid to semi-arid region where water is of strategic importance to development. Water has a potential limiting effect on all future development in the region. The Limpopo River basin is home to almost 14 million people in four riparian states [74].

5.2 Sampling

The 18 grab river sediment samples were collected in October and November, 2014. The river sediment samples (~500g) were collected in sterile glass containers from rivers and tributaries of the Limpopo River (Table 7). The use of river sediments was chosen because most suspended material including cyanobacteria spores and cysts settles at the river bottom where they became part of sediments in the river.

Table 7: The location of sample sites and sample codes

River Names	Samples Numbers
Limpopo River (Groblers' bridge)	S1
Notwane River (Odi Bridge-Matabeleng)	S2
Sand River upstream	S3
Mogalakwena River next to Tolwe	S4
Mawoni River downstream Makhado oxidation ponds	S5
Lephalale river	S6
Mokolo River	S7
Crocodile River downstream Hartbeespoort dam	S8
Nzhelele River downstream near Tshipise	S9
Sand River downstream (at bridge on N1 road towards Musina)	S10
Crocodile River downstream (near bridge on road D1235) near Thabazimbi	S11
Nzhelele River upstream near Mphephu resort (downstream of Siloam oxidation ponds)	S12

Mzingwane River (Zimbabwe)	S13
Shashe River (near Irrigation scheme, Zimbabwe)	S14
Limpopo River next to Thuli coal mine	S15
Limpopo River abstraction point @ 0.0 m	S16
Limpopo River abstraction point @ 1.0 m	S17
Limpopo River abstraction point @ 1.68 m	S18

462
463 *5.3 Physical-chemical measurements*
464 In the laboratory, the physical measurement of pH, Total dissolved solids (TDS) and electric conductivity
465 (EC) were carried out using Portable pH meter Crison MM40 on the river sediments. It was first be calibrated
466 per the manufacturer's guidelines. The pH, TDS and EC of the sediments were determined by the method of
467 Islam et al.[75] of which 50g of sediment was mixed with 50ml of distilled water in 100 ml beaker to produce a
468 ratio of 1:1. The mixture was stirred with a stirring rod to homogenize the mixture and was then left for 30 min
469 to settle. EC, pH and TDS were then measured by inserting the electrodes in the soil solution and readings
470 were taken.
471
472 *5.4 Nutrients analyses*
473 The air dried sediments were subjected to nutrients analyses and this involved determining Total
474 Phosphate (TP) and Total Nitrogen (TN). The analyses were done in duplicates and the aliquot of all digested
475 samples were analyzed with Merck Spectroquant® Pharo 100 spectrophotometer with a wavelength of
476 320-1100 nm purchased from Merck (Darmstadt, Germany).
477

478 5.4.1. Total Phosphorus Analysis

479 Total phosphorus was determined by using the perchloric acid digestion method as described by
480 APHA[76]: 2 g of air dried sediment was acidified to methyl orange with concentrated HNO_3, another 5 ml of
481 concentrated HNO_3 was added and evaporated on a hot plate until the dense fume appear. 10 ml each of
482 concentrated HNO_3 and $HClO_4$ was added and evaporated gently until the dense white fumes of $HClO_4$
483 appear. The solution was then neutralized with 6N NaOH and made up to 100 ml with distilled water.
484 Aliquots of the samples were then analysed with spectrophotometer using phosphate cell test kit (Merck,
485 Darmstadt, Germany).
486 .

487 5.4.2. Total Nitrogen Analysis

488 Total Nitrogen was determined per APHA [76] as ammonia: 1 g of each air-dried sediment sample was
489 treated with 2 ml of Sulphuric acid. The sample was heated on a hotplate for 2 hours. Aliquots of 50 ml of
490 deionized water were added to each sample. The sample was filtrated through No. 41 Whatman filter paper.
491 The filtrate of each sample was made up to 250 ml with deionized water and 55 ml of 1 M sodium hydroxide
492 solution. Aliquots of the samples were then analyzed with spectrophotometer using nitrate cell test kit (Merck,
493 Darmstadt, Germany).
494 .
495
496 *5.5 Data analysis*
497 The physico-chemical and cyanotoxins measurements were conducted in duplicates, the standard
498 deviation and the mean were calculated, using Microsoft (MS) Excel 2010 spread sheet for each sampling point.
499 The graphs were plotted using MS Excel spread sheet.
500
501 *5.6 The culture of cyanobacteria species in river sediments*
502 The modified BG11 medium was laboratory prepared as per Gumbo et al. [77] for cyanobacteria culturing.
503 The 200 ml sterile modified BG 11 medium was transferred to sterile 250 ml laboratory jars under sterile

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conditions and then 200 g of river sediments was added. A total of 18 laboratory jars were incubated for 30 days under continuous light (1100 lux) fluorescent lamps at room temperature. The harvested cyanobacteria cells were subsequently used for identification and molecular characterization.

5.7 The identification of cyanobacterial species using the Flowcam

The harvested cyanobacteria cells were used to identify cyanobacterial species present in the samples, a bench top FlowCAM (Model VS IV) was used. In the FlowCAM system, sample is drawn into the flow chamber by a pump. Using the laser in trigger mode, the photomultiplier and scatter detector monitor the fluorescence and light scatter of the passing particles. When the particles passing through the laser fan has sufficient fluorescence values and/or scatter the camera is triggered to take an image of field view. The fluorescence values were then saved by the Visual Spreadsheet. The computer, digital signal processor, and trigger circuitry work together to initiate, retrieve and process images of the field of view. Groups of pixels that represented the particles were then segmented out of each raw image and saved as separate collage image. The image was then captured and compared image of cyanobacteria as per procedure of van Vuuren et al. [78].

5.8 The identification of cyanobacterial species using molecular characterization

The cyanobacteria cells were harvested and also used for molecular characterization and following procedures outlined below:

5.8.1. DNA extraction and purification

Samples were freeze-dried and stored at -20 °C for DNA extraction. Total genomic DNA was extracted using the ZR-Duet™ DNA/RNA Miniprep DNA extraction kit from Inqaba Biotech Laboratories South Africa. Sample preparation and DNA extraction was carried out following the protocol supplied by the manufacturer.

5.8.2. Detection and amplification of 16S rRNA by Polymerase chain reaction

The PCR method was performed for detection and amplification of 16S rRNA as described briefly by Frazao et al. [3]. The PCR amplification of the cyanobacteria 16S rRNA gene was determined using set of primers 27F/809R (Table 2). Thermal cycling conditions were 1 cycle at 95 ºC for 5 min, 35 cycles at 95 ºC for 30 s, 55.4 ºC for 30 s and 72 ºC for 60 s and 1 cycle at 72 ºC for 10 min. Reactions were carried out in 50 µl reaction volume that consisted of 0.5 pmol of each primer (10 pM/µl), 25 µl of Dream Taq master mix (Inqaba Biotech), 19 µl sterile ultra-pure water and 5 µl of DNA sample.

5.8.3. Toxin genes detection

The presence of cyanotoxins were determined by PCR using primers that have been used for detection of genes involved in the production of nodularins (NOD), microcystins (MC) and cylindrospermopsin (CYN) (Table 8). The NOD gene cluster, *nda*, consists of nine open reading frames (*ndaA-I*) [79]. The MC gene cluster, *mcy*, comprises 10 genes in two transcribed operons, *mcyA-C* and *mcyD-J* [80]. The HEP primer pair was used for detection of genes involved in MC and NOD production.

These primers are responsible for sequencing the aminotransferase (AMT) domain, which is located on the modules *mcyE* and *nda*F of the MC and NOD synthetase enzyme complexes, respectively [80,81]. Primers *mcyA-C* were used to detect the *mcyA*, *mcyB* and *mcyC* genes [82-83]. For detection of CYN production (*cyr*) genes the polyketide synthase PKS M4 and M5 primers and the peptide synthetase M13 and M14 primers were used as designed by Schembri et al. [84] who demonstrated a direct link between the presence of the peptide synthetase and polyketide synthase genes and the ability of cyanobacteria to produce CYN.

PCR reaction conditions that were used were those described for the amplification of the 16S rRNA gene [81]. Concerning the cycling conditions, for mcyA-Cd genes the thermal cycling conditions were 1 cycle at 95 ºC for 2 min, 35 cycles at 95 ºC for 90 s, 56 ºC for 30 s and 72 ºC for 50 s and 1 cycle at 72 ºC for 7 min. For HEP and CYN as genes, the thermal cycling conditions were as those for the amplification of the 16S rRNA with an exception for HEP gene annealing temperature of 58.15 ºC for 30 s. Positive control was used.

4.8.3.1 Electrophoresis

PCR products were electrophoresed in 0.8% agarose gel by adding prepared 1.2 g of agarose powder into 150 ml 1X TAE buffer (48.4g Tris, 11.4 ml Glacial acetic acid, 3.7g EDTA disodium salt topped up to 1000 ml with deionised water). The mixture was heated until there was complete dissolution. Exactly 10µl of Ethidium bromide was added and mixed thoroughly. The mixture was transferred to the gel-casting tray with the comb already in position and allowed to solidify. The solidified gel was transferred to the running trays. The gel in the tray was covered with 1X TAE buffer. In the first well 3 µl 100 bp of the molecular weight marker was loaded and the samples were loaded from the second well onwards. The gel was run at 100 V and 250 mA for 60 min. The gel was viewed using the Gel doc (Biorad, USA) and the picture was taken.

5.8.4. Pcr purification and sequencing purification and sequencing

PCR products were purified using the GeneJet Gel Extraction Kit Thermo Scientific (South Africa) under room temperature as per protocol provided by kit manufacturer. The purified DNA was stored at -20 ^{0}C. PCR products were sent for sequencing at Inqaba biotech laboratory (South Africa). Sequences were analyzed using the BLAST system (http://www.ncbi.nlm.nih.gov/BLAST/).

4.8.4.1 Primers

Primers used for PCR amplification were synthesized at Inqaba Biotech (South Africa). Details of primer sequences, their specific targets and amplicon sizes are summarized (Table 8) below:

Table 8.PCR primers which will be used for amplification of 16S rRNA gene for cyanobacteria identification and for the amplification of genes related to cyanotoxins production. A—Individual annealing temperature, B—Reference annealing temperature, bp = base pairs.

Primers	Target genes	Sequence (5′-3′)	A	B	Size (bp)	Amplified gene	Ref
27F 809R		AGAGTTTGATCCTGGCTCAG GCTTCGGCACGGCTCGGGTCGATA	52 64	60	780	16S rRNA	[85] [86]
*mcy*A-Cd F *mcy*A-Cd R	*mcyA*	AAAATTAAAAGCCGTATCAAA AAAAGTGTTTTATTAGCGGCTCAT	51 43	59	297	Microcystin synthetase	[83]
HEPF HEPR	*mcyE/ndaF*	TTTGGGGTTAACTTTTTTGGGCATAGTC AATTCTTGAGGCTGTAAATCGGGTTT	57 55	52	472	Microcystin/nodularin synthetase	[81]
PKS M4 PKS M5	cyr	GAAGCTCTGGAATCCGGTAA AATCCTTACGGGATCCGGTGC	52 56	55	650	Cylindrospermopsin polypeptide synthase	[84]
M13 M14	*ps*	GGCAAATTGTGATAGCCACGAGC GATGGAACATCGCTCACTGGTG	57 57	55	597	Cylindrospermopsin peptide synthetase	[84]

5.8.5. Phylogenetic relationship

Additional sequences were downloaded in FASTA format from GenBank through NCBI and combined with assembled sequences. The evolutionary history was inferred using the Neighbor-Joining method [87]. The bootstrap consensus tree inferred from 1000 replicates [88] is taken to represent the evolutionary history of the taxa analyzed [88]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [88]. The evolutionary distances were computed using the Kimura 2-parameter method [61] and are in the units of the number of base substitutions per site. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions

containing gaps and missing data were eliminated. There were total of 640 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [60].

5.8.6. Divergence matrix

PCR products for 16S rRNA gene, identified on agarose gels, were selected for subsequent identification by sequencing (Inqaba Biotech, South Africa). The obtained sequenced data were used to conduct homology searches on GenBank using BLAST (http:// blast.ncbi.nlm.nih.gov/blast.cgi)[89], and for further bioinformatic analyses to perform divergence matrix using BioEdit v7.0.9 [59]). Sequences were exported to and analysed with the MEGA 7 package [60].

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: title, Table S1: title, Video S1: title.

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Conflicts of Interest: The authors declare no conflict of interest.

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