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

Murendeni Magonono1, Paul Johan Oberholster2 Addmore Shonhai3, Stanley Makumire4 and Jabulani Ray Gumbo5,*

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...
2. Results

2.1. The physical-chemical characteristics of the river sediments
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.
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

<table>
<thead>
<tr>
<th>Cyanobacteria species/sample sites</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
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<th>S10</th>
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<th>S15</th>
<th>S16</th>
<th>S17</th>
<th>S18</th>
</tr>
</thead>
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<tr>
<td>Raphidiopsis curvata</td>
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<td>Synechocystis PCC 6803</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Similarity %</th>
<th>Species similar to</th>
<th>Family</th>
<th>Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>93</td>
<td>Uncultured <em>Leptolyngbya</em> sp. Clone</td>
<td>Leptolyngbyaceae</td>
<td>KM108695.1</td>
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<tr>
<td>S3</td>
<td>94</td>
<td><em>Synechocystis PCC 6803</em></td>
<td>Oscillatoriophycideae</td>
<td>CP012832.1</td>
</tr>
<tr>
<td>S5</td>
<td>97</td>
<td><em>Anabaena oscillarioides</em></td>
<td>Nostocaceae</td>
<td>AJ630428.1</td>
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</tbody>
</table>
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.

<table>
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<th>Primers</th>
<th>Sample No</th>
<th>Similarity%</th>
<th>Species similar to</th>
<th>Accession No</th>
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<td>S3</td>
<td>100</td>
<td><em>Raphidiopsis curvata</em></td>
<td>KJ139745.1</td>
</tr>
<tr>
<td></td>
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<td>100</td>
<td><em>Cylindrospermopsis raciborskii</em></td>
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</tr>
<tr>
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<td>100</td>
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<tr>
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<td>CP003614.1</td>
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<td></td>
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<td>100</td>
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<tr>
<td></td>
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<td>HEP</td>
<td>S8</td>
<td>100</td>
<td>Uncultured <em>Microcystis sp.</em> clone msp microcystin synthetase E (<em>mcyE</em>) gene, partial cds</td>
<td>KF687998</td>
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<tr>
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<td>S8</td>
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<td><em>Microcystis panniformis</em> FACHB-1757</td>
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<td><em>Microcystis aeruginosa</em> PCC 7806</td>
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<td>S8</td>
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<td><em>Nostoc sp.</em> 152</td>
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<td>S8</td>
<td>100</td>
<td><em>Planktothrix rubescens</em> NIVA-CYA 98</td>
<td>AM990462.1</td>
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<tr>
<td></td>
<td>S13</td>
<td>100</td>
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<td>S13</td>
<td>100</td>
<td><em>Planktothrix rubescens</em> NIVA-CYA 98</td>
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<tr>
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<td>Uncultured <em>Microcystis sp.</em> clone mw microcystin synthetase E (<em>mcyE</em>) gene, partial cds</td>
<td>KF687997.1</td>
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</table>
2.6 Phylogenetic relationship

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

Table 4. Divergence matrix for reflection of similarity

<table>
<thead>
<tr>
<th></th>
<th>S2</th>
<th>S3</th>
<th>S5</th>
<th>S7</th>
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<td>0.166</td>
<td>0.167</td>
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<td>0.095</td>
<td>0.152</td>
<td>0.028</td>
<td>0.140</td>
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<td>0.169</td>
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<td>0.166</td>
<td>0.167</td>
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<td>0.167</td>
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<td>0.173</td>
<td>0.163</td>
<td>0.156</td>
<td>0.119</td>
<td>0.006</td>
<td>0.140</td>
<td>0.173</td>
<td>0.278</td>
<td>0.355</td>
<td>0.134</td>
<td>-</td>
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<tr>
<td>Musina borehole (S14)</td>
<td>0.376</td>
<td>0.359</td>
<td>0.312</td>
<td>0.314</td>
<td>0.342</td>
<td>0.321</td>
<td>0.343</td>
<td>0.414</td>
<td>0.555</td>
<td>0.371</td>
<td>0.347</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Musina borehole (S15)</td>
<td>0.183</td>
<td>0.184</td>
<td>0.179</td>
<td>0.136</td>
<td>0.128</td>
<td>0.150</td>
<td>0.186</td>
<td>0.285</td>
<td>0.366</td>
<td>0.173</td>
<td>0.131</td>
<td>0.348</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Musina borehole (S16)</td>
<td>0.183</td>
<td>0.184</td>
<td>0.179</td>
<td>0.136</td>
<td>0.128</td>
<td>0.150</td>
<td>0.186</td>
<td>0.285</td>
<td>0.366</td>
<td>0.173</td>
<td>0.131</td>
<td>0.348</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Musina borehole (S17)</td>
<td>0.183</td>
<td>0.184</td>
<td>0.179</td>
<td>0.136</td>
<td>0.128</td>
<td>0.150</td>
<td>0.186</td>
<td>0.285</td>
<td>0.366</td>
<td>0.173</td>
<td>0.131</td>
<td>0.348</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Musina borehole (S18)</td>
<td>0.183</td>
<td>0.184</td>
<td>0.179</td>
<td>0.136</td>
<td>0.128</td>
<td>0.150</td>
<td>0.186</td>
<td>0.285</td>
<td>0.366</td>
<td>0.173</td>
<td>0.131</td>
<td>0.348</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4. Divergence matrix for reflection of similarity
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 (CO₂).

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 pond. 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 Sioloam hospital oxidation ponds. The reason for the highest value at Nzhelele River was possible due the discharge of sewage effluent from Sioloam 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 dominant 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 inhabit the river sediments as shown by studies by Perez et 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.
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 mcyA-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 mcyA-Cd primer did not amplify and whereas the work of Mbukwa et al. [24] their mcyA-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 *mcyB* gene instead of the *mcyD* 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 *mcyB* gene and *mcyA* 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 *mcyD* 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 constitute 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. Conventional method for water treatment is not convenient for the removal of microcystins in water [68]. Drinking water treatment processes might trigger the release of hepatotoxin 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
3.6 Phylogenetic relationship

The evolutionary tree was constructed could not be used for phylogenetic purposes because of two important things; the number of samples used for PCR per river site was not enough to make conclusive argument; and the cyanobacteria were the expected products which needed to be identified. Hence the tree was used to verify the identification as done by BLAST search, however the phylogenetic relationship was basically done by divergence matrix and combined discussion followed the divergence matrix.

The relationship between the samples and their most similar species as from BLAST search was confirmed by phylogenetic tree, and the relationships between some cyanobacteria species from different samples have been confirmed (Figure 7). The first was the confirmation of similarity of samples from Crocodile River (S8) downstream Hartbeespoort dam and Shashe River (14) to *Leptolyngbya boryana* with 99 % bootstrap confidence. Then the similarity of Musina borehole extraction (S16) sample to *Alkalimema pantanalense* with 98 % bootstrap, then the similarity of samples from Sand River (S3) upstream Nzhlele River (9) downstream near Tshipise and Mokolo river to *Synechocystis* sp. PCC 6803. The other similarity was Mawoni River (5) downstream of Makhado oxidation pond to *Leptolyngbya* sp. with 97 % bootstrap confidence, then Notwane River (2) to uncultured *Leptolyngbya* sp with 99 % bootstrap confidence, and lastly Sand River (10) downstream to *Spirulina laxissima* with 100 % bootstrap confidence.

However the cyanobacteria at these two sites, Musina borehole extraction (S16) and Sand River (S3) may imply that there is movement of aquatic animals such as fish from the entrance (mouth) of Sand River towards the Musina abstraction point (Figure 10). In simple terms there is an upstream movement of cyanobacteria species being carried by aquatic animals but this requires further investigation.

The other which matched their supposed to match from BLAST search includes Musina Borehole extraction point (S16) to *Leptolyngbya* sp. and Crocodile River (S11) near bridge on road D1235 and upstream to Thabazimbi to Uncultured Cyanobacteria clone though they bootstrap confidence level was little about 55 % and 61 % respectively. Following the BLAST search results and Phylogenetic tree the divergence matrix can be used to verify the truth of the two, i.e. BLAST search and phylogenetic tree. Divergence matrix confirms that cyanobacteria from Crocodile River (S8) downstream of Hartbeespoort dam and from Shashe River (S14) are same species which is *Leptolyngbya boryana*, they both show at least 98 % similarity to this species in BLAST and bootstrap confidence is 99% forming a clade in phylogenetic tree, while they have the least difference in the divergence Matrix.

Thus based on this study there is DNA evidence to suggest that the cyanobacteria at the Musina abstraction point is similar to cyanobacteria from the Crocodile River system. This may be possible because the Musina 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].
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

<table>
<thead>
<tr>
<th>River Names</th>
<th>Samples Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limpopo River (Grobler’s bridge)</td>
<td>S1</td>
</tr>
<tr>
<td>Notwane River (Odi Bridge-Matabeleng)</td>
<td>S2</td>
</tr>
<tr>
<td>Sand River upstream</td>
<td>S3</td>
</tr>
<tr>
<td>Mogalakwena River next to Tolve</td>
<td>S4</td>
</tr>
<tr>
<td>Mawoni River downstream Makhado oxidation ponds</td>
<td>S5</td>
</tr>
<tr>
<td>Lephalale river</td>
<td>S6</td>
</tr>
<tr>
<td>Mokolo River</td>
<td>S7</td>
</tr>
<tr>
<td>Crocodile River downstream Hartbeespoort dam</td>
<td>S8</td>
</tr>
<tr>
<td>Nzhelele River downstream near Tshipise</td>
<td>S9</td>
</tr>
<tr>
<td>Sand River downstream (at bridge on N1 road towards Musina)</td>
<td>S10</td>
</tr>
<tr>
<td>Crocodile River downstream (near bridge on road D1235) near Thabazimbi</td>
<td>S11</td>
</tr>
<tr>
<td>Nzhelele River upstream near Mphephu resort (downstream of Siloam oxidation ponds)</td>
<td>S12</td>
</tr>
</tbody>
</table>
5.3 Physical-chemical measurements

In the laboratory, the physical measurement of pH, Total dissolved solids (TDS) and electric conductivity (EC) were carried out using Portable pH meter Crison MM40 on the river sediments. It was first be calibrated per the manufacturer’s guidelines. The pH, TDS and EC of the sediments were determined by the method of Islam et al.[75] of which 50g of sediment was mixed with 50ml of distilled water in 100 ml beaker to produce a ratio of 1:1. The mixture was stirred with a stirring rod to homogenize the mixture and was then left for 30 min to settle. EC, pH and TDS were then measured by inserting the electrodes in the soil solution and readings were taken.

5.4 Nutrients analyses

The air dried sediments were subjected to nutrients analyses and this involved determining Total Phosphate (TP) and Total Nitrogen (TN). The analyses were done in duplicates and the aliquot of all digested samples were analyzed with Merck Spectroquant® Pharo 100 spectrophotometer with a wavelength of 320-1100 nm purchased from Merck (Darmstadt, Germany).

5.4.1 Total Phosphorus Analysis

Total phosphorus was determined by using the perchloric acid digestion method as described by APHA[76]: 2 g of air dried sediment was acidified to methyl orange with concentrated HNO₃, another 5 ml of concentrated HNO₃ was added and evaporated on a hot plate until the dense fume appear. 10 ml each of concentrated HNO₃ and HClO₄ was add and evaporated gently until the dense white fumes of HClO₄ appear. The solution was then neutralized with 6N NaOH and made up to 100 ml with distilled water. Aliquots of the samples were then analysed with spectrophotometer using phosphate cell test kit (Merck, Darmstadt, Germany).

5.4.2 Total Nitrogen Analysis

Total Nitrogen was determined per APHA [76] as ammonia: 1 g of each air-dried sediment sample was treated with 2 ml of Sulphuric acid. The sample was heated on a hotplate for 2 hours. Aliquots of 50 ml of deionized water were added to each sample. The sample was filtrated through No. 41 Whatman filter paper. The filtrate of each sample was made up to 250 ml with deionized water and 55 ml of 1 M sodium hydroxide solution. Aliquots of the samples were then analyzed with spectrophotometer using nitrate cell test kit (Merck, Darmstadt, Germany).

5.5 Data analysis

The physico-chemical and cyanotoxins measurements were conducted in duplicates, the standard deviation and the mean were calculated, using Microsoft (MS) Excel 2010 spreadsheet for each sampling point. The graphs were plotted using MS Excel spreadsheet.

5.6 The culture of cyanobacteria species in river sediments

The modified BG11 medium was laboratory prepared as per Gumbo et al. [77] for cyanobacteria culturing. The 200 ml sterile modified BG 11 medium was transferred to sterile 250 ml laboratory jars under sterile conditions.
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 °C for 5 min, 35 cycles at 95 °C for 30 s, 55.4 °C for 30 s and 72 °C for 50 s and 1 cycle at 72 °C for 7 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 ndaF 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 °C for 2 min, 35 cycles at 95 °C for 90 s, 56 °C for 30 s and 72 °C for 50 s and 1 cycle at 72 °C 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 °C for 30 s. Positive control was used. 

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 10 May 2018 doi:10.20944/preprints201805.0169.v1
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 °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>
<thead>
<tr>
<th>Primers</th>
<th>Target genes</th>
<th>Sequence (5’-3’)</th>
<th>A</th>
<th>B</th>
<th>Size (bp)</th>
<th>Amplified gene</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td></td>
<td>AGAGTTTGGATCCTGGCTCAG</td>
<td>52</td>
<td>64</td>
<td>780</td>
<td>16S rRNA</td>
<td>[85]</td>
</tr>
<tr>
<td>809R</td>
<td>meca</td>
<td>GCTTGGCAGGCGGCTCAATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mczA-Cd F</td>
<td>mczA</td>
<td>AAAATAAAGGCGATACAAA</td>
<td>51</td>
<td>59</td>
<td>297</td>
<td>Microcystin synthetase</td>
<td>[83]</td>
</tr>
<tr>
<td>mczA-Cd R</td>
<td></td>
<td>AAAAGTGTATATAGCGGTCAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPE</td>
<td>mczE/n</td>
<td>TTTGGGTTGACTTTTTTTGCGGATAGTC</td>
<td>57</td>
<td>55</td>
<td>472</td>
<td>Microcystin/nod</td>
<td>[81]</td>
</tr>
<tr>
<td>HEPR</td>
<td>daF</td>
<td>AATTCTTGAGCCGTGAAATCGGTTT</td>
<td></td>
<td></td>
<td></td>
<td>ularin synthetase</td>
<td></td>
</tr>
<tr>
<td>PKS M4</td>
<td>cyr</td>
<td>GAAGCTCTGGAAATCGGGTAA</td>
<td>52</td>
<td>56</td>
<td>650</td>
<td>Cylindrospermopsin polypeptide synthase</td>
<td>[84]</td>
</tr>
<tr>
<td>PKS M5</td>
<td></td>
<td>AATCTTACGGATCCTCGTTGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13</td>
<td>ps</td>
<td>GGCAAATTTGTGATAGCCACGCC</td>
<td>57</td>
<td>57</td>
<td>507</td>
<td>Cylindrospermopsin peptide synthetase</td>
<td>[84]</td>
</tr>
<tr>
<td>M14</td>
<td></td>
<td>GATGAAACATCGGCTACAGTGTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

References

1. Christiansen, G., Molitor, C., Philmus, B., & Kurmayer, R. Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. Molecular Biology and Evolution, 2008, 25(8), 1695-1704.


7. Paerl, H.W., Fulton, R.S., Moisander, P.H., & Dyble, J. Harmful freshwater algal blooms with an emphasis on cyanobacteria. Sci. World, 2001, 1, 76-113


33. Peperzak, L. Climate change and harmful algal bloom in the North Sea, Acta *Oecologica* 2003,24,139-144.


46. Musa, R., Greenfield, R. Nutrient loads on an important watercourse. Pre-and Post-Acid spill. In7th International Toxification Symposium in Africa, 2015, 89.
52. Dzebu W, statement in relation to water supply challenges in Musina, 14 March 2017


