RESEARCH ARTICLE

Determining the presence of cyanotoxins in water reservoirs of Anuradhapura, using molecular and bioassay methods

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Abstract: This study was performed to detect the presence of cyanotoxin producing organisms in the reservoirs of the Anuradhapura Distrct. Water samples were collected from Kala wewa, Tissa wewa and Nuwara wewa in Anuradhapura. Environmental and cultured samples were subjected to PCR to detect cyanobacteria, *Microcystis*, microcystin, *Cylindrospermopsis* and cylindrospermopsin, targeting the specific 16S rRNA and cpc, *mcyA* and *mcyE*, cylindrospermopsin synthetase (*rpoC1*) and cylindrospermopsin specific peptide synthase (PS) genes, respectively. All tested samples were positive for unicellular and five were positive for filamentous cyanobacteria. In all three reservoirs, positive amplification was observed for *mcy*A/*mcyE* genes indicating the presence of cyanobacterial strains with genetic potential to produce microcystins. A positive amplification resulted in samples cultured from Nuwara wewa for *rpoC1* gene confirming the presence of *Cylindrospermopsis* species in the reservoir. Further more, an environmental sample from Nuwara wewa was positive for PS gene confirming the presence of toxic *Cylindrospermopsis* species, and thereby cylindrospermopsin in that reservoir. Bioassay was performed using *Artemia salina* to test the toxicity of water samples. Filtrates and filters were assayed and in all three tanks, higher toxicities were recorded for filters of both environmental and cultured samples. Among filters, the highest toxicity was recorded in two environmental samples from Nuwara wewa with a mean LD_{50} value of -694 ppm. Among cultured samples, the highest toxicity of -1128.28 ppm was recorded from Kala wewa. According to molecular and bioassay findings, all three lakes have a wide distribution of cyanobacterial species with toxin generating ability and it might be a potential risk factor for the health issues that prevail in the Anuradhapura District.

Keywords: Bioassays, cyanotoxins, *Cylindrospermopsis*, *Microcystis,* PCR.

INTRODUCTION

Most chemical contaminants in drinking water become health concerns only after exposure for long periods. A number of chemical contaminants in drinking water, have been shown to cause adverse health effects in humans after prolonged exposure.

Cyanotoxins are well recognized as cause for a number of livestock and human poisonings (Sivonen *et al.,* 1990; Kuiper-Goodman *et al.,* 1999). There are several toxic cyanobacteria found in inland and coastal waters and at least 46 species have been shown to cause toxic effects in vertebrates (Sivonen & Jones 1999). The most common cyanobacteria in fresh water are colonial *Microcystis* spp. and filamentous *Cylindrospermopsis raciborski*, *Planktothrix* (syn. *Oscillatoria*) *rubescens*, *Planktothrix* (syn. *Oscillatoria*) *agardhii, Gloeotrichia* spp., *Anabaena* spp., *Nostoc* spp., *Lyngbya* spp., *Aphanizomenon* spp., *Synechococcus* spp., some *Oscillatoria* spp., *Schizothrix* spp., *Anabaenopsis* spp., and *Hapalosiphon* spp., (Sivonen & Jones, 1999). Among potentially toxic cyanobacterial species, several chemically and toxicologically diverse compounds have been isolated, purified and characterized (Lawton *et al.*, 1994; Sivonen & Jones, 1999).

Health impairments are shown in numerous anecdotal reports of irritations of the skin and/or mucous membranes and in documented cases of illness after exposure through drinking water as well as accidental swallowing or aspiration of scum material (Chorus *et al.*, 2000).

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Among the cyanotoxins, microcystins are the predominant toxins in freshwater lakes worldwide, which have been implicated in several cases of animal and human intoxications (Kuiper-Goodman *et al.,* 1999). Progressive active liver injury (Falconer *et al.*, 1983) and the potential for promotion of tumour growth are two aspects of microcystin damage. A number of human deaths have been reported resulting from exposure to the cyanobacterial toxin, microcystin and both cylindrospermopsin and microcystin have been detected in drinking water (Byth, 1980; Falconer *et al.*, 1983; Hawkins *et al.*, 1985; Falconer, 2005). Cylindrospermopsin is a general cytotoxin that blocks protein synthesis (Falconer, 1996), and the first clinical symptoms of cylindrospermopsin are kidney and liver failure. In contrast to the pure toxin, crude extracts of the organism also cause injury to the lungs, adrenals and intestine. This indicates that other toxins may also be present in the organism. Since clinical symptoms may become manifest only several days after exposure, it will often be difficult to determine a cause-effect relationship. Patients intoxicated with cylindrospermopsin via drinking water in an incident in Australia, escaped death only through skilled and intensive hospital care (Falconer, 2005).

Cyanobacteria are the dominant microbial inhabitants of rice fields. *Anabaena* spp., *Cylindrospermum* spp., *Hapalosiphon* spp., *Lyngbya* spp., *Nostoc* spp., *Phormidium* spp., *Oscillatoria* spp., *Aulosira* spp., *Rivularia* spp., *Gloeotrichia* spp., *Tolypothrix* spp., *Fischerella* spp., *Aphanothece* spp., *Gloeothece* spp., and some unidentified cyanobacteria were also recorded in paddy fields (Roger & Kulasooriya, 1980). Most of these genera have toxin generating ability and therefore, there is a high probability that these cyanotoxins can contaminate rice.

This research study was aimed at identifying the cyanotoxins present in certain water bodies of the Anuradhapura District.

METHODS AND MATERIALS

Site selection and sample collection: Kala wewa, Tissa wewa and Nuwara wewa in the District of Anuradhapura were selected for water sample collection in June 2010 (Table 1). All samples were collected in sterile brown glass containers. The samples were collected from the water surface and the water column (~ 0.1 - 15m), to represent the whole water body (three replicates were collected from each site). The temperature and pH were recorded using a thermometer and a pH meter, respectively. Morphological observations were also made of each sample, using a compound light microscope (Olympus BH-2) (400–1,000×) and standard morphological study methods.

Culturing of water samples: Collected water samples were concentrated by centrifugation (3500 rpm, 10 min) and 500 µL of the resulting pellet and 500 µL from the supernatant were inoculated into cyano specific BG11, $BG11_0$, $BG11_0C$ and MLA media. The cultures were incubated at room temperature with 12 - 12 light dark cycling. Morphological observations were made of each sample, using compound light microscope (Olympus

Table 1: Environmental and cultured samples selected for molecular detection

No	Sample code	Collected place	Environmental/ cultured	Water body	Temperature ^{0}C	pH
1	AN 6	Nuwara wewa	Environmental	Bottom – 1.5 m	28	8.60
2	AN 7	Nuwara wewa	Environmental	Bottom – 11 m	28	8.43
3	AN8	Nuwara wewa	Environmental	Surface- Plankton net	28	8.50
4	AT 5	Tissa wewa	Environmental	Surface	28	8.34
5	AT6	Tissa wewa	Environmental	Surface- Plankton net	28	8.35
6	AK 1	Kala wewa	Environmental	Bottom – 15 m	27	8.48
7	AK 3	Kala wewa	Environmental	Surface	27	8.93
8	AK1-C	Kala wewa	Cultured-MLA	-	-	-
9	AK3-C	Kala wewa	Cultured-MLA	-	-	-
10	AT5-C	Tissa wewa	Cultured-MLA	-	-	-
11	AT6-C	Tissa wewa	Cultured-MLA	-	-	-
12	AN6-C	Nuwara wewa	Cultured-MLA	-	-	-
13	AN7-C	Nuwara wewa	Cultured-MLA	-	-	-

BH-2) (400–1,000×) by standard morphological methods.

Molecular detection:

a) DNA extraction and purification: Five hundred microlitres of both environmental and cultured water samples from each site (Table 1) along with 500 μL of standard *Microcystis aeruginosa* culture [obtained from the Pasteur Culture Collection (PCC 7941), France] were subjected to DNA extraction. Each sample was transferred to 500 μL of 1x Tris EDTA (TE) buffer and subjected to sequential heating (at 99 °C for 5 min) and freezing (at – 5 °C for 5 min) three times, to achieve lysis. The samples were centrifuged (12,000 rpm, 5 min) and to each resulting pellet, 40 μL of N-Tris (Hydroxymethyl) methyl-2-amino-ethanesulfonic acid (TES) and 20 μL of lysozyme (10 mg/mL) were added, and incubated overnight at 37 °C. The cells were then treated with 10 μL of proteinase K (20 mg/mL) and 40 μL of TE/ Sodium dodecyl sulfate (SDS) and incubated at 55 °C for 2 h to lyse the organisms further. Subsequently, proteinase K was inactivated by heating the sample at 95 °C for 10 min. Nucleic acids were purified by Boom's method (Boom *et al*., 1990), using silica particles and guanidium isothiocyanate.

b) Detection of cyanobacteria, *Cylindrospermopsis*, *Microcystis*, microcystin and cylindrospermopsin generating genes using Polymerase Chain Reaction (PCR) amplification: DNA amplification was performed for the 16S rRNA gene and phycocyanin operon, which includes the genes responsible for coding of two phycobiliprotein subunits (*cpc*B and *cpc*A) and three linker polypeptides, to identify the presence of cyanobacteria using the modified protocols of Nübel *et al*. (1997) and Premanandh *et al*. (2006), respectively. Cyanobacterial specific primers, forward primer CYA 359F (5'-GGGGAATCTTCCGCAATGGG-3') along with the reverse primers CYA781Rb (5'-GACTACA GGGGTATCTAATCCCTTT-3') and CYA781Ra (5'-GACTACT GGGGTATCTAATCCCATT-3') and an equimolar mixture of reverse primers CYA781Ra + CYA 781Rb were used for 16S rRNA gene identification. Phycocyanin gene specific primers, forward primer cpc_arF (5'-TCGAAGATCGTTGCTTGAACG-3') and cpc_arR (5'-TTAGGTCCCTGC ATTTGGGTG-3') were used for *cpc* gene identification. The total 25 μL of reaction mixture contained 3 μL of 5 μM each primer, 2.5 μL of 1 μM each deoxynucleoside triphosphate, 5 μL of 5x PCR buffer, 2 μL of 25 mM MgCl$_2$, 1.25U of *Taq* DNA polymerase (Promega, Wisconsin, USA) and 5 μL of template DNA.

In order to detect the microcystin synthetase (*mcy*) gene cluster for the identification of toxic *Microcystis* strains in the water source, DNA amplification was performed for *mcy*E and *mcy*A genes using the general microcystin synthetase gene E forward primer mcyE-F2 (5'-GAAATTTGTGTAGAAGGTGC-3') and the genus-specific reverse primer for *Microcystis* MicmcyE-R8 (5'-CAATGGGAGCATAACGAG-3') (Vaitomaa *et al*., 2003) and primers, which code for *Microcystis* specific microcystin synthetase gene A forward primer McyAR47 (5'- CTCCCTCTAAAACCCGCAGTAAG- 3'), and the gene specific reverse primer for *Microcystis* McyAF19 (5'- AACATCCAGCAGTTGAGCAAGC- 3') (primers were self designed using Primer3 software) along with the other ingredients as mentioned above.

Detection of *Cylindrospermopsis raciborskii* was carried out according to the protocol of Kim *et al.* (2000), using rpoC1 – 1 forward primer (3'-GAGCTCCAAAAC CATCCACTCAGG-5') and rpoC1-T reverse primer (5'-GGTACCAAACGGAC AAATAGTTGG-3') (Palenik & Haselkorn, 1992), which codes for *C. raciborskii* specific cylindrospermopsin synthetase gene. Further, for the detection of cylindrospermopsin, forward primer M13 (5'-GGCAAATTGTGATAGCCACGAGC-3') and reverse primer M14 (5'-GATGGAACATCGCTCACTGGTG -3'), which codes for cylindrospermopsin specific peptide synthase (PS) gene were used according to the protocol of Schembri *et al.* (2001), along with the other ingredients as mentioned above.

All the amplifications were carried out in Techne TC 3000 DNA Thermal Cycler. A single cycle of initial denaturation for 5 min at 94 °C followed by 35 incubation cycles each consisting of 1 min at 94 °C, 1 min at 60 °C, 56 °C and 59 °C for 16S rRNA gene, *cpc* gene, *mcy*A gene and *mcy*E gene, respectively and 1min at 72 °C followed by 15 min extension at 72 °C. A touchdown PCR was carried out with a single cycle of initial denaturation for 5 min at 94 °C followed by 5 incubation cycles each consisting of 1 min at 94 °C, 1 min at 62 °C and 1 min at 72 °C and another 5 cycles at 60 °C and 30 cycles at 58 °C followed by 15 min extension at 72 °C for the amplification of cylindrospermopsin specific peptide synthase (PS) gene. Further, another touchdown PCR was carried out as above, with 5 cycles at 61 °C, 5 cycles at 58 °C and 30 cycles at 56 °C for the amplification of cylindrospermopsin synthetase gene. Aliquots of the resulted amplified products were electrophoresed in 1.5 % agarose gels containing 10 μg/mL ethidium bromide and documented through a gel documentation system (Syngene, UK).

*Bioassay detection***:**

a) Extraction of toxins: In order to detect the dissolved, intracellular and total cyanotoxins in water, environmental and cultured samples (Table 2) were filtered using Whatman's membrane filter papers. Each membrane with the residue was processed according to the protocol described by Fastner *et al.* (1998), vacuum dried at 45 ^{0}C and the toxin was extracted into 100 % methanol. Each filter and filtrate was then subjected to the bioassay.

Table 2:   Environmental and cultured samples selected for bioassay detection

No.	Sample code	Collected place	Environmental/ cultured
1	AN 1	Nuwara wewa	Environmental
2	AN 6	Nuwara wewa	Environmental
3	AN 7	Nuwara wewa	Environmental
4	AT 5	Tissa wewa	Environmental
5	AK 1	Kala wewa	Environmental
6	AK 3	Kala wewa	Environmental
7	AN 1	Nuwara wewa	BG11 and BG11$_0$C
8	AN 6	Nuwara wewa	BG11
9	AN 7	Nuwara wewa	BG11$_0$C and MLA
10	AT 5	Tissa wewa	BG11
11	AT 3	Tissa wewa	BG11
12	AK 1	Kala wewa	BG11, BG11$_0$C and MLA

b) Culture, harvesting and bioassay for *Artemia salina***:** *Artemia salina* bioassay was performed in 96 - well polystyrene plates. The *Artemia* cysts were incubated in 500 mL of artificial seawater for 48 h. Cysts were illuminated with a tungsten filament light and maintained at room temperature (27 ^{0}C), with continuous aeration. Ten individuals of *A. salina* nauplii were placed in each well of the polystyrene plate and the extracted toxins were added to each well with artificial seawater medium to get a concentration series in duplicates. A control was also performed with artificial seawater. The plates were incubated at room temperature for 24 h (Meyer *et al.*, 1982). After incubation, dead larvae in each well (or those swimming in a fixed position) were counted using a hand lens and the percentage mortality was calculated using the mean of two replicates. Results were expressed as percentage of mortality using the following formula and LD$_{50}$ value was analysed with StatPlus 2009 Probit Analysis software.

$$\% \text{ of mortality} = \frac{\text{Number of dead shrimps in well after 24 h} \times 100}{\text{Number of shrimps initially placed in well}}$$

% of final mortality = % of mortality - % of mortality in control

RESULTS AND DISCUSSION

Site selection and sample collection

This study was performed to determine the presence of cyanotoxins in three main reservoirs (Kala wewa, Tissa wewa and Nuwara wewa) in the Anuradhapura District. These reservoirs are often used for drinking, recreational purposes, bathing, fishing and various other water related activities. Furthermore, they are the main water sources for irrigation. According to the data, mean temperatures of 27 ^{0}C, 28 ^{0}C and 27 ^{0}C and mean pH of 8.5, 8.34 and 8.7 were observed in Nuwara wewa, Tissa wewa and Kala wewa, respectively (Table 1). Under microscope, *Cylindrospermopsis* species were recorded as the dominant cyanobacterial species in the environmental water samples collected from all three reservoirs along with *M. aeruginosa*, *Anabaena* spp., *Chroococcus* spp., *Phormidium* spp., *Microcystis* spp., *Oscillatoria* spp., *Limnothrix* spp., *Lyngbia* spp., *Calothrix* spp., Anabaenopsis spp., and *Merismopedia* spp., which were comparatively moderate to low in numbers. However, in culture samples in BG11, BG11$_0$ and BG11$_0$C, no *Cylindrospermopsis* species were observed except *Phormidium* spp., *Microcystis* spp., *Limnothrix* spp., *Lyngbia* spp., and *Merismopedia* spp., Moreover, in the cultures in MLA medium, which lacks the inorganic nitrogen, *Cylindrospermopsis* was observed only in Nuwara wewa sample and also were biased to other filamentous forms with a minority of *Merismopedia*. Although *Cylindrospermopsis* is a filamentous heterocyst former, the reason for this contrast can be due to the culture media used for culturing (BG11, BG11$_0$ and BG11$_0$C), which did not facilitate the growth of *Cylindrospermopsis* species. Further, it is well known that most of the microorganisms are non cultivable (Dadheech *et al.*, 2009).

Molecular detection

The method employed for genomic DNA extraction resulted in high quality DNA in satisfactory amounts for amplification. According to the study done by Boutte *et al.* (2005), the primers, which are cyanobacterial specific CYA781 Ra and CYA781 Rb were used separately to identify heterocyst forming filamentous and unicellular non heterocyst forming filamentous cyanobacteria, respectively and equimolar mixture of CYA781Ra + CYA781Rb to identify the presence of cyanobacterial community in those reservoirs. According to the gel profiles obtained, all DNA samples

submitted to PCR reactions with cyanobacterial specific oligonucleotide primers of CYA359F forward and CYA781Rb reverse primer yielded the unique fragment of ~450 bp (Table 3 and Figure 1a), indicating the presence of unicellular cyanobacteria in those reservoirs. However, the use of the forward primer CYA359F and the reverse primer CYA781Ra yielded ~450 bp fragment only in AN 8 from the environmental sample of the Nuwara wewa (Table 3) and AKI-C, AN7-C, AT5-C, and AT6-C samples, which were cultured in MLA medium (Table 3 and Figure 1b).These observations along with microscopic observations confirmed the presence of filamentous forms, which could be either heterocyst formers or non-heterocyst formers. Furthermore, all the samples yielded the ~450 bp unique fragment with CYA 359F and the equimolar mixture of CYA781Ra + CYA 781Rb with different intensities (Table 3 and Figure 1b) signifying the presence of unicellular and filamentous cyanobacteria in those water bodies. The reason for these different intensities might be due to the competition of reverse primers to the same template, resulting in less complete genetic fingerprints (Boutte *et al*., 2005). In addition, positive amplification of 500 bp in all the samples with cpc_arF and cpc_arR primers except AK3-C and AT5-C representing the presence of *phycocyanin* gene confirmed the presence of cyanobacteria (Table 3 and Figure 2).

AN8 and AT5 environmental samples gave positive amplification for the *mcy*A gene, yielding the unique
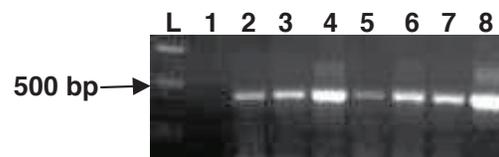

Figure 1a: Agarose gel profile (1.5 %) obtained for DNA samples amplified with CYA359F and CYA781Rb primers. Lane L- 1 kb DNA marker, lane 1- negative control (water), lane 2 – positive control (*M. aureginosa*), lanes 3-8 – AK1, AK3 AN6, AN7, AT5 and AT6 environmental samples.

Figure 1b: Agarose gel profile (1.5 %) obtained for DNA samples amplified with CYA359F and CYA781 reverse primers. Lane L- 1 kb DNA marker, lane 1- negative control (water), lane 2 – positive control (*M. aureginosa*), lanes 3 and 4- AKI-MLA and AN7-MLA amplified with CYA781Rb, lanes 5 and 6- AKI-MLA and AN7-MLA amplified with CYA781Ra, lanes 7 and 8- AKI-MLA and AN7-MLA amplified with CYA781Ra+Rb.

Table 3:   PCR amplification results

Sample no.	Sample code	CYA359F with			*cpc*	*Mcy*A	*Mcy*E	*rpo*C1	M13/M14
		Ra	Rb	Ra+Rb					
1	AN 6	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
2	AN 7	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
3	AN8	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve
4	AN6- C	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
5	AN7- C	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
6	AT 5	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
7	AT6	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
8	AT5- C	+ve	+ve	+ve	-ve	-ve	+ve	-ve	-ve
9	AT6- C	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve
10	AK 1	-ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
11	AK 3	-ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve
12	AK1- C	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve
13	AK3- C	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve

+ve = Positive amplification   -ve = Negative amplification

Figure 2: Agarose gel profile (1.5 %) obtained for DNA samples amplified with Cpc F and Cpc R primers. Lane L- 1 kb DNA marker, lane 1- negative control (water), lane 2 – positive control (*M. aureginosa*), lanes 3-8 – AK1, AK3 AN6, AN7, AT5 and AT6 environmental samples.
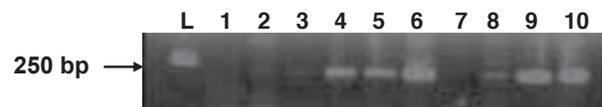
Figure 3: Agarose gel profile (1.5 %) obtained for DNA samples amplified with mcyE-F2 and MicmcyER8 primer pair. lane L- 1 kb DNA marker, lane 1- negative control (water), lane 2- positive control, lanes 3-6- AK1,AK3, AT5 and AN8 environmental samples respectively, lanes 7-10- AK3, AT6, AN6 and AN7 cultured in MLA medium, respectively.

Figure 4: Agarose gel profile (1.5 %) obtained for DNA samples amplified with rpoC1–1 forward and rpoC1-T primer pair. Lane L- 1 kb DNA marker, lanes 1-7 AK1, AK1-MLA, AK3, AT5, AT6, AN7, AN7-MLA.
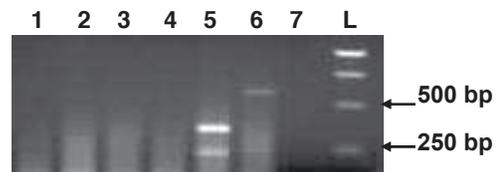
Figure 5: Agarose gel profile (1.5 %) obtained for DNA samples amplified with M13 and M14 primer pair. Lane L- 1kb DNA marker, lanes 1-7 AK1, AK3, AN6, AN7, AT5, AT6 and AN8 cultured in MLA medium.

fragment of 594 bp, using the microcystin synthetase gene A forward primer (McyAR47) and genus specific reverse primer for *Microcystis* (McyAF19) (Table 3). This indicates the presence of cyanobacterial strains that have the genetic potential to produce microcystins. Further, all the samples gave positive amplification for the *mcy*E gene, yielding the unique ~250 bp except AK1 environmental sample and AK3-C, which was cultured in MLA medium (Table 3 and Figure 3). This indicates the presence of *Microcystis* strains in all three reservoirs that have the genetic potential to produce microcystins.

Only AN7-C DNA sample, which was cultured in MLA medium gave positive amplification with rpoC1 –1 forward and rpoC1 - T reverse primer, confirming the presence of cylindrospermopsin synthetase gene and therefore, the presence of *Cylindrospermopsis* species. Although the expected size was 609 bp for the *Cylindrospermopsis raciborskii*, the only amplified product size was ~250 bp (Table 3 and Figure 4). The microscopic identification of the samples indicated the presence of *Cylindrospermopsis* species, and therefore, the resulting PCR product might be due to the presence of *Cylindrospermopsis* species other than

Cylindrospermopsis raciborskii. A specific ~597 bp amplification was observed in AN8 environmental DNA sample with M13 forward and M14 reverse primers, while a ~300 bp unspecific amplification was observed in AK1-C, AN7-C and AT6-C DNA samples, which were cultured in MLA medium (Table 3 and Figure 5), indicating the presence of cylindrospermopsin specific peptide synthase (PS) gene and therefore, confirmed the presence of toxin producing *Cylindrospermopsis* species and also cylindrospermopsin. However, unspecific amplifications might be due to the other strains of *Cylindrospermopsis*, which also contain the peptide synthase (PS) gene.

Bioassay detection

The bioassay detection is based on the lethal effect of cyanotoxins on the brine shrimp, *A. salina.* This bioassay provides a viable tool since it is a representative species of the zooplankton community. According to the results obtained for bioassay, higher toxicity for *A. salina* after 24 hours was recorded in the filters compared to the filtrates (Table 4 and 5). Moreover, 50 % of environmental samples showed higher toxicity compared to the cultured samples.

Among the filtrates, the highest mortality of 66 % was recorded in AK1 filtrate, which was cultured in MLA medium. A considerable amount of mortality (60 %) was also recorded in AK1 sample cultured in $BG11_0C$ (Table 4). Further, in filter samples, a sigmoid dose

Table 4: Percentage mortality of *Artemia salina* in the filtrate samples

Sample No.	Sample code	Total mortality after 24h	% of final mortality
1	Control	0	0
2	AK1	0.3	3
3	AK3	0.3	3
4	AN1	0	0
5	AN6	0	0
6	AN7	0.3	3
7	AN8	1.3	13
8	AT5	0	0
9	AK1-$BG11_0C$	6	60
10	AK1-BG11	0	0
11	AK1-MLA	6.6	66
12	AN1-BG11	0	0
13	AN1-$BG11_0C$	0	0
14	AN6-BG11	0	0
15	AN7-$BG11_0C$	0	0
16	AN7 - MLA	0	0
17	AT5-BG11	0	0
18	AT3-BG11	0	0

response curve was obtained for all the samples with percentage of mortality increasing with the concentration of toxins (Figures 6 and 7), except AN6 and AN7 environmental samples and AK1 and AT5, which were cultured in BG11 medium (Table 5). However, for the 2 samples AT5 and AK1 cultured in BG11 medium, the mortality was independent from the concentration (Table 5). Among cultured samples in BG11 and $BG11_0C$ media, the highest toxicity was recorded in the Nuwara wewa samples.

According to the LD_{50} values obtained for filter samples, the highest toxicity was recorded in AN6 (LD_{50}: − 694 ppm) and AN7 (LD_{50}: − 694 ppm) (Table 6), environmental samples collected from Nuwara wewa. Among cultured samples, the highest toxicity was recorded in AK1 (LD_{50}: -1128.28 ppm) sample, which was cultured in $BG11_0C$ medium (Table 6).

Beside chemical analyses of cyanobacterial toxins, a wide range of biological laboratory methods have been developed for detection and identification of the cyanotoxins in cyanobacterial biomass and raw waters. Since analytical analysis cannot describe the real toxic effect of complex mixtures of chemicals, relatively simple and low cost methods provide necessary additional information on overall toxicity, and allow rapid evaluation of the potential hazard for humans, animals or ecosystems. A specific biological screening method can be selected, depending on the type of information required. However,
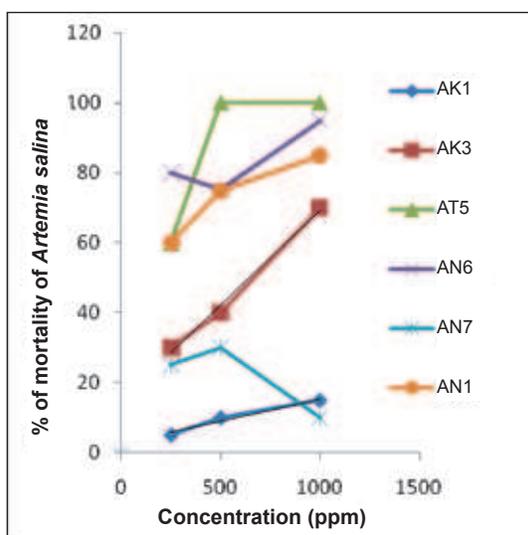

Figure 6: Percentage mortality of *Artemia salina* in different filters of environmental samples collected from Nuwara wewa (AN), Kala wewa (AK) and Tissa wewa (AT)
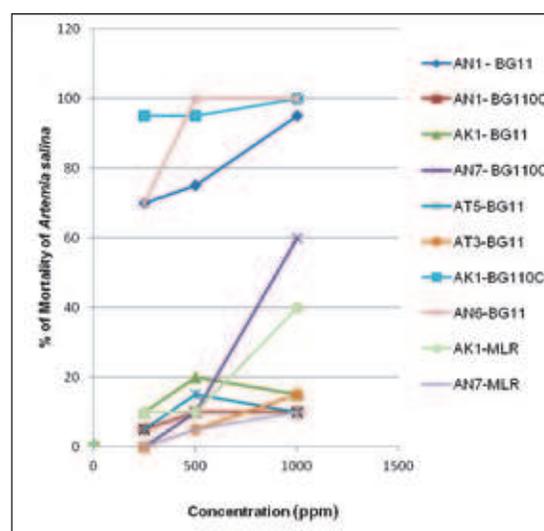

Figure 7: Percentage mortality of *Artemia salina* in different filters for cultured isolates originated from cyano specific culture media for water samples collected from Nuwara wewa (AN), Kala wewa (AK) and Tissa wewa (AT)

Table 5:   Percentage mortality of *Artemia salina* in the filter samples

Sample No.	Sample code	Toxin concentration-ppm	Total mortality after 24 hours	% of final mortility
1	Control	0	0	0
2	AK1	1000	1.5	15
3	AK1	500	1	10
4	AK1	250	0.5	5
5	AK3	1000	7	70
6	AK3	500	4	40
7	AK3	250	3	30
8	AT5	1000	10	100
9	AT5	500	10	100
10	AT5	250	6	60
11	AN6	1000	9.5	95
12	AN6	500	7.5	75
13	AN6	250	8	80
14	AN7	1000	1	10
15	AN7	500	3	30
16	AN7	250	2.5	25
17	AN1	1000	8.5	85
18	AN1	500	7.5	75
19	AN1	250	6	60
20	AN1- BG11	1000	9.5	95
21	AN1- BG11	500	7.5	75
22	AN1- BG11	250	7	70
23	AN1 -BG11$_0$C	1000	1	10
24	AN1 -BG11$_0$C	500	1	10
25	AN1 -BG11$_0$C	250	0.5	5
26	AK1-BG11	1000	1.5	15
27	AK1-BG11	500	2	20
28	AK1-BG11	250	1	10
29	AN7-BG11$_0$C	1000	6	60
30	AN7-BG11$_0$C	500	1	10
31	AN7-BG11$_0$C	250	0	0
32	AT5-BG11	1000	1	10
33	AT5-BG11	500	1.5	15
34	AT5-BG11	250	0.5	5
35	AT3-BG11	1000	1.5	15
36	AT3-BG11	500	0.5	5
37	AT3-BG11	250	0	0
38	AK1-BG11$_0$C	1000	10	100
39	AK1-BG11$_0$C	500	9.5	95
40	AK1-BG11$_0$C	250	9.5	95
41	AN6-BG11	1000	10	100
42	AN6-BG11	500	10	100
43	AN6-BG11	250	7	70
44	AK1 - MLA	1000	4	40
45	AK1 - MLA	500	1	10
46	AK1 - MLA	250	1	10
47	AN7-MLA	1000	1	10
48	AN7-MLA	500	0.5	5
49	AN7-MLA	250	0	0

Table 6: LD_{50} values for filter samples

Sample code	LD_{50} (ppm)
AK1	3,216.84
AK3	643.44
AT5	96.95
AN6	-694.97
AN7	-694.97
AN1	-118.08
AN1- BG11	-157.49
AN1 -BG11$_0$C	3,216.84
AK1-BG11	1539.92
AN7-BG11$_0$C	917.51
AT5-BG11	3216.84
AT3-BG11	2286.30
AK1-BG11$_0$C	-1128.28
AN6-BG11	-54.64
AK1 - MLA	1194.64
AN7-MLA	2286.30

it is important to note that there cannot be one single method providing adequate information on toxicity of all types of cyanotoxins. Therefore, analysis of chemical composition in the algal extract is important to verify the toxicity. However, the most widely used invertebrate bioassay available for cyanotoxins is the brine shrimp (*Artemia salina*) assay (Kiviranta *et al*., 1991; Lawton *et al*., 1994). This is a useful toxicity screening method used in very basic laboratories as it requires little expertise and does not require culture maintenance or special equipment.

This research study focused on cyanotoxins, because most males (from childhood to adulthood) in the Dry Zone, often use lake water for recreational purposes, bathing and washing after farming, fishing and various water related activities. Recreational use of lakes, rivers and canals is the most common route of exposure to cyanobacterial toxins. As an example, in 1959 in Canada, 12 people became ill with headaches, nausea, muscular pains and acute gastroenteritis symptoms (Dillenberg & Dehnel, 1959). The illness was noted in people who had been swimming in a lake contaminated by toxins from *Anabaena*, before the intoxication. In England in 1989, 10 of 20 soldiers became ill after swimming and canoe-training in water with a heavy bloom of *Microcystis* (Turner *et al*., 1990) and two cases of pneumonia were noted among the soldiers. According to Mehra *et al*. (2009), an additional aspect influencing the toxicity of cyanobacterial blooms is the age of the victim. Children are more vulnerable for several reasons: they drink more water per unit of body weight, are less likely to have

a choice of the source of drinking water and are more susceptible to damage that takes a considerable time to develop, such as environmentally induced carcinomas. It is a common sight in the Dry Zone tanks, where lots of children, mainly boys spend hours in the water and therefore, the direct ingestion of cyanotoxic water by the swimmers for a long period, might be the cause for the mysterious chronic kidney disease, cancers and other human illnesses recorded in the Dry Zone.

Healthy people are less susceptible to cyanotoxicosis than people with hepatitis, alcoholism or kidney damage. Patients who undergo kidney dialysis are especially susceptible to toxic damage from cyanobacteria. In 1996 in Caruaru, Brazil, 131 dialysis patients were exposed to microcystins *via* water used for dialysis; 56 of them died and the others had typical symptoms of harm from microcystins such as nausea, vomiting and painful, excessive enlargement of the liver (Jochimsen *et al.*, 1998). The episode of deaths of haemodialysis patients at the Caruaru dialysis clinic emphasized the importance of haemodialysis water as an exposure route for microcystins (Codd, 2000).

According to the investigations in all three reservoirs, *Cylindrospermopsis* species were recorded as the dominant cyanobacterial species along with a vast diversity of cyanobacterial species in moderate numbers. Except *Chroococcus*, the rest of the cyanobacterial species which have been recorded, has the genetic potential to generate toxins. Microcystins, which is a most common class of toxins worldwide, is mainly produced by *M. aeruginosa* and other species of *Microcystis*, and these were moderately present in all three lakes. The mechanisms of toxicity of the diverse cyanobacterial toxins include nerve ion-channel blocking, neuromuscular blockade, anti-acetyl cholinesterase activity, anti-phosphatase activity and protein synthesis inhibition. The liver is particularly damaged by the latter two activities, as well as the gastro intestinal tract and, in the case of protein synthesis inhibition, the kidneys. The anti-phosphatase activity of microcystins and nodularin has significant potential for stimulating tumour growth. This capacity is clearly demonstrated experimentally, and its mechanism is under detailed investigation. Cyanobacterial poisons can affect not only hepatocytes but also other types of cells. Microcystins and nodularins can inhibit the activity of protein phosphatases PP1 and PP2A in any eukaryotic cell they can get into, but usually need the bile acid carrier to cross cell membranes. Apoptotic changes have been observed after microinjection of microcystins or nodularins into human embryo kidney HEK 293, Swiss 3T3 mouse embryo fibroblast, breast carcinoma cell line MCF-7 and rat promyelocytic IPC-81 leukemia cells

(Fladmark *et al*., 1999). Morphological changes such as membrane budding, cell shrinkage and organelle redistribution have been seen in human fibroblasts, human endothelial cells, human epithelial cells, human lymphocytes and rat promyelocytes (Mcdermott *et al*., 1998; Mankiewicz *et al*., 2001). Microcystins also have a clastogenic effect in human lymphocytes, connected with a dose-related increase of chromosomal breakage (Repavich *et al*., 1990; Premazzi & Volterra, 1993). According to a previous study, the cytotoxic alkaloid cylindrospermopsin produced by *Cylindrospermopsis* species, were the dominant species in three lakes and it generally blocks the protein synthesis (Hawkins *et al*., 1985). The first clinical symptoms of poisoning are kidney and liver failure; it also causes damage to the spleen, intestine, heart and thymus. Cylindrospermopsin is especially dangerous because clinical symptoms may become manifest only several days after exposure, so the toxic effects can be difficult to correlate. Tumor promotion by exposure to cyanobacterial toxins poses an added risk, and in some areas of the Dry Zone the number of deaths due to cancer has increased sharply within the last few years.

According to the findings of this study, all three lakes show a wide distribution of cyanobacterial species with toxin generating ability and it might be a major risk factor for various human illnesses recorded in the Dry Zone. Therefore, more epidemiological studies are required to explore the relationship existing between human illnesses and cyanotoxins in water.

In conclusion, molecular detection data and bioassays have confirmed the presence of toxic cyanobacteria in all three sampled water reservoirs. Further, molecular data confirmed the presence of cyanobacterial strains that have the genetic potential to produce microcystins and more importantly, the presence of *Cylindrospermopsis* and also the presence of cylindrospermopsin producing *Cylindrospermopsis* species. The bioassay results confirmed the presence of lethal cyanotoxins in collected water samples. However, there is a need for extensive studies to identify the types of cyanobacteria, cyanotoxins, their variants, compositions and the level of toxicity present in those water reservoirs to correlate it with the present issue of human illnesses.

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