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Original Article

Identification of Cylindrospermopsin and *Cylindrospermopsis raciborskii* from Anuradhapura District, Sri Lanka

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Cylindrospermopsin (CYN) is one of the potent cyanotoxin which affects the functions of kidney and liver. This study was performed to identify CYN in Anuradhapura water reservoirs using molecular and biochemical methods. Water samples were collected from Kala wewa, Nuwara wewa, Tissa wewa and Jaya ganga. Under microscope, *Cylindrospermopsis* species were recorded as the dominant cyanobacterial species along with *Microcystis*, *Anabaena*, *Chroococcus*, *Phormidium*, *Oscillatoria*, which were comparatively moderate to low. In molecular detection, the presence of cyanobacteria, the presence of *Cylindrospermopsis* and *Cylindrospermopsis* strains that have the genetic potential to produce CYN were detected using specific PCR primers targeting 16S rRNA gene, *C. raciborskii* specific cylindrospermopsin synthetase gene and cylindrospermopsin specific peptide synthase (PS) gene respectively. The presence of toxin producing *Cylindrospermopsis raciborskii* in those water samples was reconfirmed by a nested PCR using *C. raciborskii* specific primers cyl2, cyl4 and cyl-int and by direct sequencing. Further, ELISA detection kit confirmed the presence of CYN and the toxin concentration. All were positive for CYN with mean concentrations of 0.104, 0.091, 0.255 and 0.0967 ng/ml for Jaya ganga, Kala wewa, Nuwara wewa and Tissa wewa respectively. Water samples collected from water purification centre in Anuradhapura showed 0.245 and 0.154 ng/ml of CYN before purification and after addition of chlorine respectively.

Key words; Cyanotoxins, Cylindrospermopsin, CKDu

1. Introduction

Most of the world's population relies on surface freshwaters as its primary source for drinking. The drinking water industry is therefore constantly challenged with surface water contaminants that must be removed to protect human health. Most chemicals arising in drinking water are of health concern only after exposure of years, rather than months. A number of chemical contaminants have been shown to cause adverse health effects in humans. Moreover, the health effects of some contaminants in drinking water are not well understood. Among chemical contaminants, cyanotoxins which is produced by toxic cyanobacteria are well recognized as a cause for number of livestock and human poisonings (Sivonen and Jones 1999).Therefore, the presence of cyanobacteria and their toxins in surface waters used for drinking and recreation activities are now readily acknowledged as a serious human health risk.

Among cyanobacteria, over 46 species belong to cyanobacterial genera are known to produce cyanotoxins (Sivonen and Jones 1999). Exposure to such cyanobacterial toxins in freshwater systems, including both direct (drinking water) and indirect (recreational activities, bioaccumulation in food webs etc.) routes, is emerging as a potentially significant threat to human health.

Cyanotoxins can be categorized into hepatotoxins, neurotoxins, cytotoxins, irritants and gastrointestinal toxins. Additionally, other cyanotoxins whose toxicological or ecotoxicological profile is still only partially known (Funari and Testai (2008). So called cyanotoxins can be produced by more than one cyanobacterial species and likewise, the same species is able to produce more than one cyanotoxin (Funari and Testai (2008).

Among cyanotoxins, cylindrospermopsin (CYN) is one of the potent cyanotoxin which effects to the kidney and liver function (Humpage and Faconer 2003; Hawkins *et al.*, 1985).Liver damage is dose dependant and can be severe after exposure to acute doses. Although CYN is generally classified as a hepatotoxin, renal toxicity is another prominent effect and was clearly demonstrated in animals with sub chronic exposure (Humpage and Faconer 2003). The toxin is a general cytotoxin that blocks protein synthesis rapidly, completely and irreversibly (Froscio *et al.,* 2003). Additionally, *in-vitro* studies showed injury to the lungs, adrenals and intestine, indicating further toxicity (Hawkins *et al.*, 1985). Clinical symptoms may become manifest only several days

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after exposure, therefore, 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; Gutiérrez-Praena *et al*., 2012).

However, due to the lack of adequate data, no guideline value is set for concentrations of cylindrospermopsin by WHO for drinking water. Conversely, certain countries have fixed their own health alert levels for CYN in drinking water, for ex. Brazil 15 µg/L and New Zealand 1 µg/L (Burch, 2008). Further, Australia suggested it should be as 0.05µg/L of CYN in drinking water based on the study done by Shaw *et al.,* in 2004.

Chronic Kidney Disease of unknown aetiology (CKDu) prevailing in Sri Lanka also focuses on probable cause in drinking water. The disease is a major health problem in Sri Lanka. The geographical distribution of Chronic Kidney Disease of unknown Aetiology (CKDu) appears to be biased towards the North Central Region (NCR) of the country (Hittarage, 2004), and also part of North Western and part of Uva provinces. Many studies have conducted over the last decade with a view to elucidating the prevalence, nature and cause of CKDu in several parts of Sri Lanka. However, from the studies conducted so far, CKDu reported cannot be related to diabetes, hypertension, snakebite or any other known causes of traditional kidney diseases but likely triggered by an environmental factor (Wanigasuriya*et al.,* 2007). Therefore, we focused our study on cyanotoxin;CYN due to its potentiality to cause kidney diseases and other adverse health effects.

2. Materials and Methods

2.1 Site Selection and Sample collection

Kala wewa(8° 1' 0" North, 80° 31' 0" East), Tissa wewa (8° 20' 0" North, 80° 22' 0" East), Nuwara wewa (8° 21' 0" North, 80° 25' 0" East) and Jaya ganga (branch of Kala wewa) water reservoirs situated in District of Anuradhapura (8° 21' 0" North, 80° 23' 0" East), Sri Lanka were selected for water sample collection. All the samples were collected in sterile brown glass containers and the collections were carried out from both on the surface and down the water column (~ 0.1 - 15m) to represent the whole water body (three replicates from each site). Temperature and pH were also recorded. Additionally another set of water samples were selected from water purification centre in Anuradhapura District to test the CYN concentrations in water before and after purification. All the samples were stored at -20^{0}C until use. Morphological observations were also made from each sample using compound light microscope (Olympus BH-2) (400–1,000×) by standard morphological methods.

2.2 Molecular detection

DNA extraction and purification

500 µL of environmental sample 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 for DNA extraction. Above each sample was transferred to 500 µL of 1xTE buffer and three sequential heating (at 99 °C for 5 min) and freezing (at –5°C for 5 min) to achieve lyses. Samples were centrifuged (12,000 rpm, 5 min) and to each resulting pellet, 40 µL of TES and 20 µL of lysozyme (10 mg /mL) were added, and incubated for overnight at 37°C. Cells were then treated with 10 µL of proteinase K (20 mg/mL) and 40 µL of TE/ 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. Finally, nucleic acids were purified by Boom's method (Boom *et al.,* 1990) using silica particles and guanidium isothiocyanate.

Detection of cyanobacteria, cylindrospermopsin and *Cylindrospermopsis* raciborskii generating genes using Polymerase Chain Reaction (PCR) amplification

DNA amplification was performed for the 16S rRNA gene to identify the presence of cyanobacteria using the modified protocols of Nübel *et al.,* (1997). Cyanobacterial specific primers, forward primer CYA359F (5'-GGGGAATCTTCCGCAATGGG-3') along with the reverse primers CYA781Rb (5'GACTACAGGGGTATCTAATCCCTTT-3') and CYA781Ra (5'GACTACTGGGGTATCTAATCCC TT-3') and an equimolar mixture of reverse primers CYA781Ra + CYA781Rb were used for 16S rRNA 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, Madison, Wisconsin, USA) and 5 µL of template DNA.For the detection of cylindrospermopsin, forward primer M13 (5'-GGCAAATTGTGATAGCCACGAGC-3') and reverse primer M14 (5'GATGGAACATCGCTCACT GGTG-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.

Thermal cycling consisted of a single cycle of initial denaturation for 5 min at 94^{0}C followed by 35 incubation cycles each consisting of 1 min at 94^{0}C, 1 min at 60^{0}C and 1min at 72^{0}C followed by 15 min extension at 72^{0}C for 16S rRNA gene. A touchdown PCR was carried out with a single cycle of initial denaturation for 5 min at 94^{0}C followed by 5 incubation cycles each consisting of 1 min at 94^{0}C, 1

min at 62^{0}C and 1 min at 72^{0}C and another 5 cycles at 60^{0}C and 30 cycles at 58^{0}C followed by 15 min extension at 72^{0}C for the amplification of cylindrospermopsin specific peptide synthase (PS) gene.

In order to identify toxin producing *Cylindrospermopsis raciborskii,* a nested PCR was carried out according to the protocol of Wilson *et al.,* 2000 using *C. raciborskii* specific primers cyl2 (5'-GGC ATT CCT AGT TAT ATT GCC ATA CTA-3'), cyl4 (5'- GCC CGT TTT TGT CCC TTT CGT GC-3') and cyl-int (5'- TAT TGC CAT ACT ACC TGG TAA TGC TGA CAC ACT CG-3') targeting *C. raciborskii* specific cylindrospermopsin synthetase gene. PCR cycling consisted a single cycle of initial denaturation for 10 min at 95^{0}C followed by 35 incubation cycles each consisting of 30s at 94^{0}C, 30s at 45^{0}C and 30s at 72^{0}C followed by 15 min extension at 72^{0}C.

All the amplifications were carried out in Techine TC 3000 DNA Thermal Cycler. Aliquots of the resulted amplified products were electrophoresed in 1.5% agarose gels containing 10μgmL^{1}ethidiumbromide and documented through a Gel Documentation system (Syngene, UK).

Sequencing of amplified nested PCR products

Two PCR positive products were cleaned with GenElute^{TM} Gel Extraction Kit (SIGMA) according to the manufacturer's instructions and then directly sequenced at a commercial facility (Macrogen Inc., South Korea.). Sequences were identified using the National Center for Biotechnology Information (NCBI) database.

2.3 Quantification of cylindrospermopsin using ELISA method

With the purpose of detecting the concentrations of CYN in the reservoirs water samples and water samples collected from water purification centre, cylindrospermopsin ELISA kit (purchased from Abraxis LLC Warminster, PA 18974) was used for environmental water samples. Sample preparation and analysis procedures were performed according to the protocol provided with the kit. Absorbance of samples, standards and negative control were measured at 450nm using an ELISA Reader (Titertek Multiskan^R Plus).

The test kit governed the principle of direct competitive ELISA and is based on the recognition of cylindrospermopsin by specific antibodies. cylindrospermopsin, when present in a sample, and a cylindrospermopsin-HRP analogue compete for the binding sites of rabbit anti-cylindrospermopsin antibodies in solution. The cylindrospermopsin antibodies are then bound by a second antibody (sheep anti-rabbit) immobilized in the plate. The intensity of the color substrate is inversely proportional to the concentration of the cylindrospermopsin present in the sample.

3. Results and Discussion

3.1 Site Selection and Sample collection

This empirical study was performed to detect the cyanotoxins in three main reservoirs in Anuradhapura District in the North Central Province, Sri Lanka where there is a comparatively high prevalence of CKDu. According to the medical statistics of the Anuradhapura General Hospital, there was a 227% increase in live discharge patients with end stage CKDu whereas the death rate increased by 354% during the last few years (Chandrajith *et al*., 2011).The reservoirs under subject are often used for drinking and recreational purposes, including bathing, fishing and various water related activities. Furthermore, those reservoirs are the main water suppliers to the water purification centers in the District and therefore most of the people directly and indirectly rely on those reservoirs. On the other hand, they are the main water sources for their irrigation.

According to the data, mean temperatures of 28^{0}C, 28^{0}C and 27^{0}C and mean pH of 8.5, 8.34 and 8.7 were recorded from 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., *Lyngbya spp., Calothrix* spp., *Anabaenopsis* spp. and *Merismopedia* spp. which were comparatively moderate to low in numbers.

Table 1 Environmental samples selected for molecular detection

No	Sample Code	Collected Place	Water body	Tem ^{0}C	pH
1	AN 1	Nuwara Wewa	Bottom	28	8.6
2	AN 2	Nuwara Wewa	Bottom	28	8.4
3	AN 3	Nuwara Wewa	Surface	28	8.5
4	AT 1	Tissa Wewa	Surface	28	8.3
5	AT 2	Tissa Wewa	Bottom	28	8.4
6	AK 1	Kala Wewa	Bottom	27	8.5
7	AK 2	Kala Wewa	Surface	27	8.9

3.2 Molecular detection

According to the study done by Boutte *et al.,* 2005, the primers which are cyanobacterial specific CYA781Ra and 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 ~450bp (**Table 2** and **Fig. 1a**) indicating the presence of unicellular/ non heterocyst filamentous cyanobacterial community in those reservoirs. Further, the use of the forward primer CYA359F and the reverse primer CYA781Ra yielded ~450bp fragment for all the DNA samples except for AT2and AK2 (**Table 2 and Fig. 1b**). Therefore, this 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 ~450bp unique fragment with CYA359F and the equimolar mixture of CYA781Ra + CYA781Rb with different intensities (**Table 2**) signifying the presence of unicellular and filamentous cyanobacterial communities 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).

Table 2 PCR amplification results

No	Sample code	PCR Amplification				
		CYA359F with			M13/M14	cyl2, cyl4 and cyl-int
		Ra	Rb	Ra+Rb		
1	AN 1	+ve	+ve	+ve	-ve	-
2	AN 2	+ve	+ve	+ve	-ve	-
3	AN 3	+ve	+ve	+ve	+ve	-
4	AT 1	+ve	+ve	+ve	-ve	+ve
5	AT 2	-ve	+ve	+ve	-ve	+ve
6	AK 1	+ve	+ve	+ve	-ve	-
7	AK 2	-ve	+ve	+ve	-ve	-

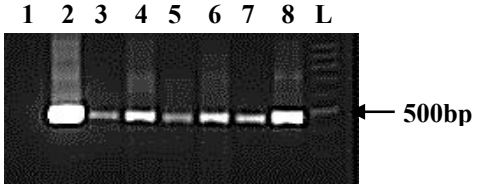

Fig. 1a Agarose gel profile (1.5%) obtained for DNA samples amplified with CYA359F and CYA781Rb primers. Lane L- 100bp DNA maker, Lane 1- (-) ve control (water), Lane 2 –7, AN1, AN2, AT1, AT2 and AK1 and AK2, Lane 8- (+) ve control (*M .aureginosa*)

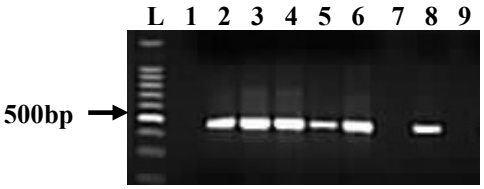

Fig. 1b Agarose gel profile (1.5%) obtained for DNA samples amplified with CYA359F and CYA781 Ra primers. Lane L- 100bp DNA maker, Lane 1- (-) ve control (water), Lane 2-8 AN1, AN2,AN3, AT1, AT2, AK1 and AK2
Lane 9- *M.aureginosa*

A specific ~597bp amplification was observed in AN3 DNA sample with M13 forward and M14 reverse primers (**Table 2**) indicating the presence of cylindrospermopsin specific peptide synthase (PS) gene and therefore confirmed the presence of *Cylindrospermopsis* species that have the genetic potential to produce cylindrospermopsin.

Use of *C.raciborskii* specific primers cyl2, cyl4 and cyl-int, yielded the unique fragment of ~250bp for all the Tissa wewa samples confirming the presence of C. *raciborskii* in that water reservoir (**Table 2 and Fig. 2**). Therefore, this nested PCR practice is a rapid method for the identification of *C. raciborskii* directly from the environmental water samples. Further, sequencing data reconfirmed the presence of *C.raciborskii* in the reservoir giving 97% similarity with a deposited partial sequence of the *C.raciborskii rpoC1* gene.

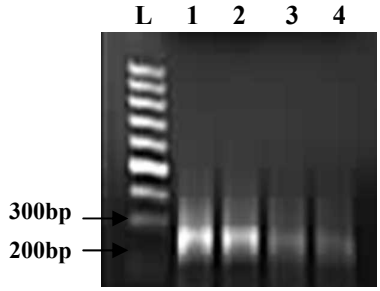

Fig. 2 Agarose gel profile (1.5%) obtained for Tissawewa DNA Samples amplified with cyl2, cyl4 and cyt-int primers. Lane L- 100bp DNA marker and lane 1-4 Tissawewa environmental samples

3.3 Quantification of cylindrospermopsin using ELISA method

According to the ELISA results, all the water samples were positive with mean concentrations of 0.104, 0.091, 0.255 and 0.0967 ng/ml for Jaya ganga, Kala wewa, Nuwara wewa and Tissa wewa respectively (**Table 3**). Further, water samples collected from water purification centre in Anuradhapura showed 0.245 and 0.154 ng/ml of CYN toxicity before purification and after addition of chlorine respectively. According to Shaw *et al*., 2004,

these values were much higher than the safe drinking water and therefore the relevant health authorities should be advised for appropriate remediation methods in CYN for the people who consume unpurified water. However, a sample treated with chlorine and alum collected from the same purification centre was negative (**Table 3**). Therefore, water purification processes cooperate in reduction of toxins and also protect lives around the peripheral area who consume the purified water. Moreover, the samples collected from Kandy area (CKDu unaffected areas) as control water samples also showed negative values for CYN proving the area discrepancy (**Table 3**).The ELISA method is simple, rapid and highly sensitive that allows detecting CYN concentration between 0.05-2.0 ng/ml which is more than the HPLC method. Further, the test kit does not require any sample processing and therefore water samples can be directly subjected to ELISA assay.

Table 3 CYN Concentrations for different water samples

Sample Code	Water source	Absorbance at 450nm B/B0(%)	Conc ng/ml
J (Surface)	Jaya Ganga	68	0.101
J (Bottom)	Jaya Ganga	67	0.117
K (Surface)	Kala Wewa	70	0.091
K (Bottom)	Kala Wewa	70	0.091
N(Surface)	Nuwara Wewa	47	0.301
N (Bottom)	Nuwara Wewa	54	0.209
T (Surface)	Tissa Wewa	71	0.087
T (Bottom)	Tissa Wewa	67	0.107
Before Purification		51	0.245
After Purification + Cl_2	Water purification Center - Anuradhapura	60	0.154
After Purification + Cl_2 + Alum		86	0.040
Autoclaved D/W	Kandy	88	0.036
Tap water (Kandy – IFS)	Kandy	84	0.044

4 Conclusions

In conclusion, molecular and biochemical analysis confirmed the presence of toxic cyanobacteria in all the subjected water reservoirs. Further, molecular data confirmed the presence of *Cylindrospermopsis raciborskii* and also the presence of cylindrospermopsin producing *Cylindrospermopsis* species. Biochemical results also confirmed the presence of CYN in water reservoirs in dry zone compared to CKDu unaffected areas. Therefore, molecular, and biochemical findings may be an answer to the prevalence of CKDu in North Central Province and lack of the disease among people in the peripheral areas who consume purified drinking water. Consequently, there is a need of extensive studies to identify the types of cyanobacteria and cyanotoxins present in those water reservoirs using molecular and analytical methods to prevent adverse health effects on humans.

References

1) Sivonen K. and Jones G.; Cyanobacterial toxins.*Toxic Cyanobacteria in Water: A Guide to Their* PublicHealth Consequences, Monitoring, and Management(eds. I. Chorus & J. Bartram), pp. 41–111. (1999). E & FN SponPublishers, London, UK.
2) Funari. E. and Testai, E.; Human Health Risk Assessment Related to Cyanotoxins Exposure, *Critical Reviews in Toxicology*,**38**, 97–125 (2008)
3) Humpage, A.R. and Falcorner, I.R.; Oral toxicity of the cyanobacterial toxin cylindrospermopsin male Swiss albino mice:Determination of no observable adverse effect level for deriving a drinking water guideline value. *Environmental Toxicology*, **18**,94-103 (2003).
4) Hawkins P. R., Runnegar, M. T., Jackson, A. B. and Falconer, I. R.; Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green algae) *Cylindrospermopsisraciborskii* (Woloszynska) Seenaya and SubbaRaju isolated from a domestic water supply reservoir. *Applied and Environmental Microbiology*, **50**, 1292-1295 (1985)
5) Froscio, S. M., Humpage, A.R., Bourcham, P.C. and Humpage, A.R.; Cylindrospermopsin- induced protein synthesis inhibition and its dissociation from acute toxicity in mouse hepatocytes, *Environmental Toxicology*, **18(4)**, 243-251 (2003).
6) Falconer, I.R. *Cyanobacterial Toxins of drinking Water Supplies*: Cylindrospermopsins and Microcystins. 2005. Chapter 5 Cyanobacterial poisoning of livestock and people. CRC press, Boca Raton, FI.
7) Gutiérrez-Praena, D., Pichardo, S., Jos, Á.J., Moreno, F. and CameánA.M.;Biochemical and pathological toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2.*Water Research* **46**(5), 1566–1575 (2012).
8) Burch, M.D. Effective doses, guidelines and regulations. 2008. Chapter 36 Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Hudnell H. K. (Eds.) Springer Science and Business Media, LLC.233 Spring Street, New York, USA
9) Shaw, G. R., McKenzie, R. A., Wickramasinghe, W. A., Seawright, A. A., Eaglesham, G. K., and Moore, M. R. Comparative Toxicity of the Cyanobacterial Toxin Cylindrospermopsin, Between Mice and Cattle: Human

Implications 2004. Harmful Algae 2002, Steidinger, K. A., J. H. Landsberg, C. R. Tomas and G. A. Vargo (Eds.) Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO.

10) Hittarage, A. Chronic Renal Disease in North Central Province of Sri Lanka,*Anuradhapura Medical Journal,* 3-5 (2004).
11) Wanigasuriya, K.P., Peiris-John, R.J., Wickremasinghe, R. andHittarage, A.; Chronic renal failure in North Central Province of Sri Lanka: an environmentally induced disease,*Transaction of the Royal Society of Tropical Medicine and Hygiene* **101,** 1013-1017,(2007).
12) Boom, R, Sol C.J.A. and Salimans, M.M. ; Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28(3), 495-503 (1990).
13) Nübel U., Garcia-Pichel F. andMuyzer G.; PCR primers to amplify 16S rRNA genes from cyanobacteria,*Applied and Environmental Microbiology* **63**(8), 3327−3332 (1997).
14) Schembri, M.A., Neilan, B.A. and Saint C.P.; Identification of genes implicated in toxin production in the cyanobacterium *Cylindrospermopsis raciborskii, Environmental Toxicology* **16**, 413 – 421 (2001).
15) Wison, K.M., Schembri, M.A., Baker, P.D. and Saint, C. P.; Molecular characterization of the toxic cyanobacterium Cylindrospermopsis raciborskii and design of a species-specific PCR. Applied and Environmental Microbiology 66, 333-338 (2000).
16) Cylindrospermopsin ELISA kit; Enzyme-Linked Immunosorbent Assay for the Determination of Cylindrospermopsin Water Samples. Abraxis LLC, 54 Steamwhistle Drive, Warminster, PA 18974. info@abraxiskits.com
17) Chandrajith, R., Nanayakkara, S., Itai, K., Aturaliya, T. N. C., Dissanayake, C. B., Abeysekera, T., Harada, K., Watanabe, T. and Koizumi, A.; Chronic KidneyDiseases of uncertain etiology (CKDue) in SriLanka: geographic distribution and environmental implications, *Environmental Geochemistry and Health* **33**, 267-78 (2011).
18) Boutte, C., Grubisic S., Balthasart, P. and Wilmotte, A.; Testing of primers for the study of cyanobacterial moleculardiversity by DGGE,*Journal of Microbiological Methods* **65**(3), 542−550 (2005).