KKU Res. J. 2013; 18(1):1-8
http : //resjournal.kku.ac.th

Detection of toxic cyanobacteria and quantification of microcystins in four recreational water reservoirs in Khon Kaen, Thailand

Theerasak Somdee[*], *Kamonchanok Kaewkhiaw and Anchana Somdee*

Department of Microbiology, Faculty of Science, Khon Kaen University
[*]*Correspondent author: thesom@kku.ac.th*

Abstract

Toxic cyanobacterial communities of four recreational water reservoirs (Bueng Kaen Nakhon, Bueng Thung Sang, Bueng Nong Khot, and Bueng See Than) in Amphur Muang, Khon Kaen, Thailand were investigated, with particular importance given to genus *Microcystis*. Water samples were collected seasonally, viz. summer, monsoon, and winter for studies of diversity and density of toxic cyanobacteria, and toxin quantification. The main toxic cyanobacteria present in these reservoirs were *Cylindrospermopsis* sp., *Microcystis* sp., *Oscillatoria* sp., and *Pseudanabaena* sp. The biovolume of *Microcystis* sp. attained a maximum of $447x10^3$, $132x10^3$, and $108x10^3$ mm^3 m^{-3} in the water samples collected from Bueng Nong Khot in summer, monsoon, and winter, respectively. Application of 16S rRNA analyses to *Microcystis* sp. isolate NK1 and NK3 show that they are most closely related to *M. aeruginosa* 2LT27S08 (96% similarity), and *M. aeruginosa* FC-070 (97% similarity), respectively. ELISA assays specific for hepatotoxic microcystins were carried out for detection of microcystin quantification. The highest values were found in the water samples collected from Bueng Nong Khot in summer, monsoon, and winter with 1.091, 1.209, and 1.235 μg L^{-1}, respectively.

Keywords: toxic cyanobacteria, *Microcystis*, microcystins

1. Introduction

Toxic cyanobacterial blooms are frequently observed in eutrophicated fresh water bodies throughout the world. Several genera are known to produce toxic secondary metabolites-cyanotoxins (1). Microcystins (MCs) are the most commonly found and widespread cyanotoxins, being associated with several bloom-forming genera, especially *Microcystis* (2-3). These toxins are cyclic peptides hepatotoxins and share the common structure cyclo-(-D-Ala$^{(1)}$-X$^{(2)}$-D-MeAsp$^{(3)}$-Z$^{(4)}$-Adda$^{(5)}$-D-Glu$^{(6)}$-Mdha$^{(7)}$), where X and Z are variable L-amino acids, D-MeAsp is D-erythro-β-iso-aspartic acid, Adda is (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methyl-2,6,8 trimethyl-10-phenyldeca-4,6-dienoic acid, and Mdha is *N*-methyl-dehydroalanine. MCs nomenclature is based on the variable amino acids, e.g. microcystin-LR (MC-LR) contains leucine and arginine in position X

and Z, respectively (4). As well as variation in amino acids there are a number of MCs with minor chemical modifications, giving a current total in excess of 80 MCs structures (2).

The toxicity of MCs is associated with the inhibition of serine/threonine protein phosphatase 1 and 2A, which induces the overphosphorelation of cytoskeletal proteins in the liver cells, leading to the collapse of liver tissue organization, hepatic hemorrhage, and hepatocytes necrosis (5-8). Contamination of MCs in water bodies has led to fatalities in wild and domestic animals worldwide (9), and the toxins have also been associated with primary hepatocellular carcinoma in human (10-11). Incidences of animal and human fatalities caused by MCs have led to the introduction of guideline values for drinking water by the World Health Organization (WHO), with a recommended limit of a 1.0 μg of MC-LR per liter (12). Recently, MCs have been classified by the International Agency for Research on Cancer (IARC) as "possibly carcinogenics to humans" group 2B (13).

In Thailand, a country with tropical climate, cyanobacterial blooms have been observed in water bodies used for fisheries, recreational and/or drinking water reservoirs. Several cyanobacterial species occurred in the blooms, but *Microcystis* sp. has frequently been found (14-16). The present study aimed to survey and monitor toxic cyanobacteria, with particular to genus *Microcystis*, in four important recreational water reservoirs in Khon Kaen, Thailand. Quantitative analyses of MCs in those reservoirs were also investigated.

2. Materials and Methods

2.1 Site description and sample collection

Bueng Kaen Nakhon, Bueng Thung Sang, Bueng Nong Khot, and Bueng See Than are located in Amphur Muang, Khon Kaen, Thailand, with surface area of 1.05, 2.40, 1.92, and 0.08 km^2, respectively. These reservoirs are recreational reserve to general public in Khon Kaen City. Physicochemical and bacteriological pollutants and heavy metals were surveyed and reported (17). Bueng Nong Khot was less polluted when compared with the other three, classified as Water Quality Class 2-3. Bueng Kaen Nakhon failed in to Class 3, while Bueng Thung Sang and Bueng See Than were Class 4.

Water samples from four reservoirs (Bueng Kaen Nakhon, Bueng Thung Sang, Bueng Nong Khot, and Bueng See Than) were collected seasonally, viz. summer, monsoon, and winter, between Jan-Dec 2011, using a 10 μm mesh phytoplankton net. These samples were collected at the deepest point of the reservoirs and made up of three individual sub-samples taken from the surface to 1 and 2 m depth. The water samples were filtered from 10 litre water samples to give about 100 ml. Subsamples were preserved with Lugol's solution for morphological identification and the reminder frozen (-20 °C) for MCs analysis.

2.2 Light microscopy and morphological descriptions

Light microscopy was performed on the Lugol's preserved samples from all reservoirs using a Motic BA300 compound microscope with a digital camera. Motic Image Plus 2.0 software was used for image analyses. The morphological identification of toxic cyanobacteria was done according to Komárek and Anagnostidis (18). The cell numbers of the dominant genera of toxic cyanobacteria were count from Lugol fixed samples using a haemacytometer under the light microscope. Cyanobacterial biovolumes were calculated from cell numbers and cell size measurements.

2.3 Isolation and purification of *Microcystis* sp.

Microcystis sp. was isolated and purified according to the micropipette-washing method (19), and

all were cultured in solid MLA (20). All cultures were maintained at room temperature under light intensity of 20 μmol photons $m^{-2}s^{-1}$ with a 12/12 h light/dark cycle. Two successfully isolated strains were used for genetic analysis.

2.4 Isolation of DNA

Colonies of *Microcystis* sp. were used as inoculums for cultivation in 250 mL flasks containing MLA broth. The cultures were maintained with the conditions previously described. Cell cultures were harvested at the late exponential phase (7 days after inoculation, approx.) for isolation of DNA.

Genomic DNA from *Microcystis* isolates were extracted by a standard CTAB genomic DNA isolation method according to Doyle and Doyle (21).

2.5 DNA amplification and sequencing

PCR amplification of the 16S rRNA gene was performed in 10 μL reaction volume containing 20 ng of genomic DNA, 0.5 μM of each primer (27F1 5′AGAGTTTGATCCTGGCTCAG3′ and 1494Rc 5′TACGGCTACCTTGTTAC GAC3′; Invitrogen, Singapore), 1x GoTaq® Green Master Mix (Promega, USA), and 1 mM $MgCl_2$ (Promega, USA). Thermal cycling was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA). The initial denaturation step at 94 °C for 2 min was followed by 30 cycles of DNA denaturation at 94 °C for 10 s, primer annealing at 55 °C for 20 s, strand extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min.

Sequencing of the partial 16S rRNA genes was performed using the primer pair 27F1/1494Rc. Automated DNA sequencing was performed using the MegaBACE 1000 sequencing system (Amersham Biosciences, USA). Sequence data were analyzed using the Cimarron 3.12 software. The sequences were compared in a multiple alignment using the ClustalW2 version 2.1 software. Reference strain data were obtained via BLAST searches with BLASTN sequence on NCBI GenBank (www.ncbi.nlm.nih.gov/blast).

A phylogenetic tree was constructed using the Neighbor-Joining algorithm (NJ) with 1000 bootstrap replicates (MEGA 5.05 software) (22).

2.6 MCs analysis

Frozen subsamples of water from four reservoirs were analyzed by a Microcystins-Adda ELISA kit (Abraxis, USA) for quantification of MCs, according to the manufacturer's protocol.

3. Results and discussion

3.1 Bloom forming cyanobacteria in recreational water reservoirs

Toxic cyanobacteria dominated the phytoplankton community in all recreational reservoirs. *Microcystis* sp. and *Cylindrospermopsis* sp. were the main cyanobacteria found in water samples. Other toxic cyanobacteria present included *Pseudanabaena* sp. and *Oscillatoria* sp. No *Microcystis* sp. was observed in Bueng Kaen Nakhon and Bueng Thung Sang (Fig. 1a and 1b). Cell densities of *Microcystis* sp. and other cyanobacteria varied between sampling sites and seasons. The total cyanobacterial biovolumes were generally higher in summer than in other (Fig. 1). *Microcystis* biovolume attended a maximum of $447x10^3$ mm^3 m^{-3} in the water sample collected from Bueng Nong Khot in summer. This result corresponds to the studies of Prommana et al. (16) and Xu et al. (23). They reported that *Microcystis* sp. was frequently found in summer, and its amounts found during summer were higher than in other seasons. Water temperature is an important factor determining cyanobacterial biomass in water bodies. Robarts and Zohary (24) reviewed that photosynthetic capacity, specific respiration rate, and growth rate of bloom-forming cyanobacteria are temperature-dependent with optimum usually at 25 °C or greater.

These temperatures are not suitable for growth of green algae and diatoms since their optimum temperature are lower than those of cyanobacteria. This could explain why most *Microcystis* sp. and other cyanobacteria bloom during summer.

In recent years the toxic phytoplankton bloom, *Cylindrospermopsis* sp., has replaced *Microcystis* sp. (14, 25). We also found that *Cylindrospermopsis* sp. was the dominant cyanobacteria in three of four reservoirs (Bueng Kaen Nakhon, Bueng Thung Sang, and Bueng See Than). The dominances of *Microcystis* sp. and *Cylindrospermopsis* sp. agree with their occurrence as the most common bloom-forming cyanobacteria in water reservoirs worldwide including Thailand (14-16, 26-27).

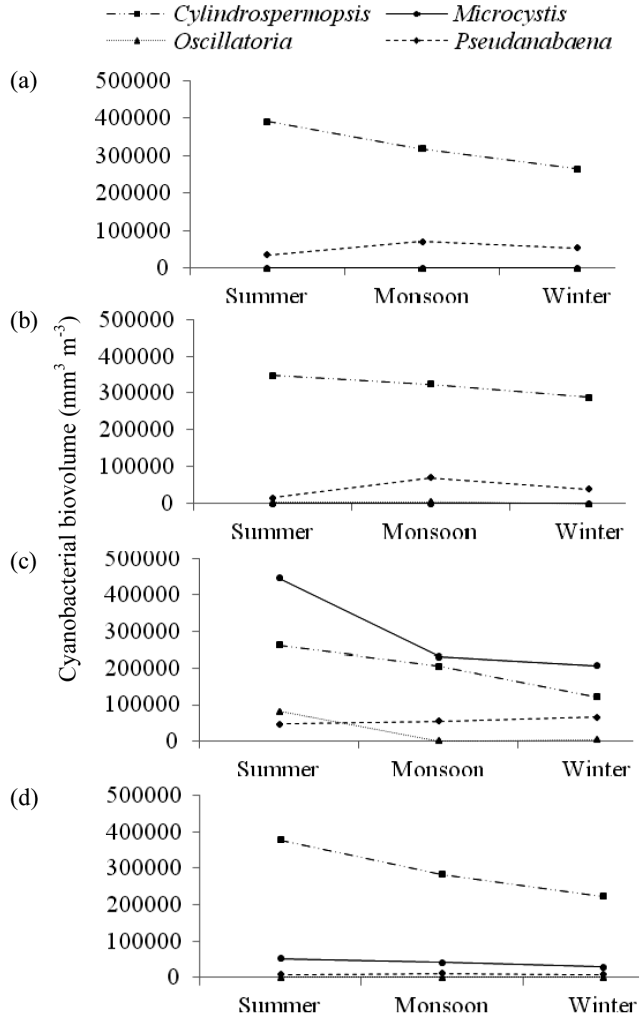

Figure 1. Biovolume of toxic cyanabacteria detected from four recreational water reservoirs

(a): Bueng Kaen Nakhon; (b): Bueng Thung Sang; (c): Bueng Nong Khot; and (d) Bueng See Than

3.2 MCs analysis

MCs concentrations of water samples were analyzed by ELISA method. The highest concentrations of total dissolved MCs were found in the water samples collected from Bueng Nong Khot in summer, monsoon, and winter with 1.091, 1.209, and 1.235 μg L^{-1}, respectively (Fig. 2). This study found a trend of negative correlation between cell densities of *Microcystis* sp. and total MCs dissolved in water. The concentration of total MCs in water was high when the cell density of *Microcystis* sp. was low. Prommana et al. (16) reported that most (>80%) of MCs are intracellular in healthy growing cells, and that the release of toxins to surrounding water occurs during cell senescence, death, and lysis. This could explain why the peak of MCs concentration tended to occur later than the peak of *Microcystis* sp. biomass.

According to the ELISA assay, MCs was found in all water reservoirs, even though *Microcystis* sp. was not detected (Bueng Kaen Nakhon and Bueng Thung Sang), or was not the dominant genus (Bueng See Than). It is possible that other genera of cyanobateria could be sources of MCs. Several bloom-forming cyanobateria are reported to produce MCs e.g. *Anabaena* sp., *Nostoc* sp., *Oscillatoria* sp. (*Planktothrix* sp.), *Pseudanabaena* sp., *Scytonema* sp., and *Synechocystis* sp. (9, 28-29). This indicates that *Oscillatoria* sp. and *Pseudanabaena* sp. found in these reservoirs tend to be the potential MCs producing genera.

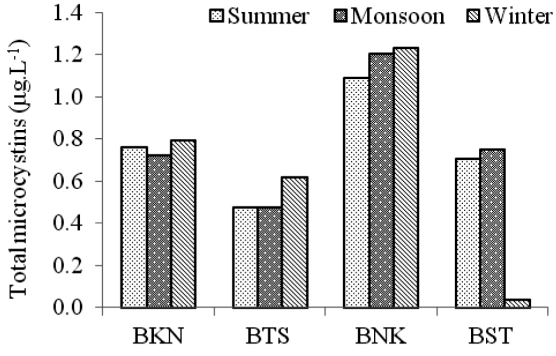

Figure 2. Total dissolved MCs in the water samples collected from four recreational water reservoirs
BKN: Bueng Kaen Nakhon; BTS: Bueng Thung Sang; BNK: Bueng Nong Khot; and BST: Bueng See Than

3.3 Genetic analysis

Partial 16S rRNA genes from two isolates of *Microcystis* sp. were PCR amplified and sequenced. The sequences were aligned using the ClustalW2 version 2.1 software. All sequences were subjected to BLAST searches to identify other highly homologous sequences (30). The partial sequence of 16S rRNA genes of *Microcystis* sp. isolate NK1 matched with 96% sequence homology to *M. aeruginosa* 2LT27S08 (GenBank FM177497), and isolate NK3 matched with 97% sequence homology to *M. aeruginosa* FC-070 (GenBank DQ887510).

The phylogenetic tree was constructed using 16S rRNA sequences from a variety of Chroococcales and Nostocales. The tree revealed that both isolates (NK1 and NK3) were grouped into *M. aeruginosa* cluster (Fig. 3).

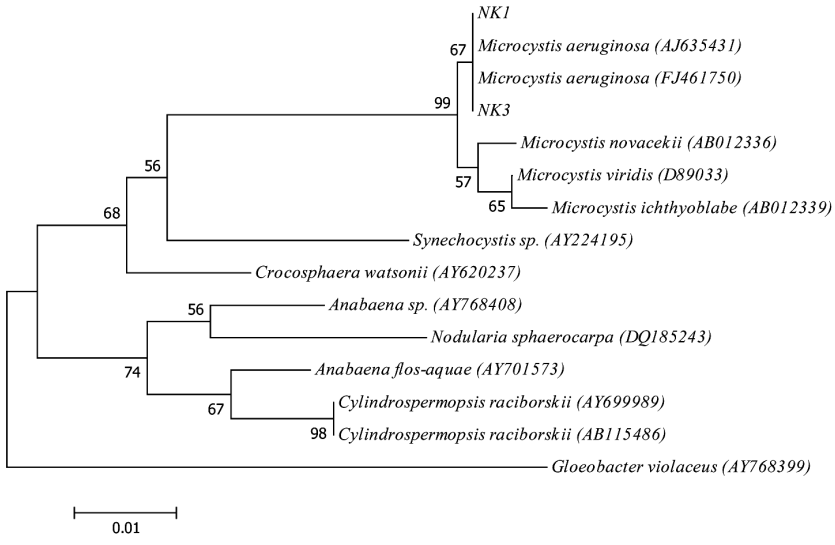

Figure 3. Phylogenetic tree of *Microcystis aeruginosa* isolates NK1 and NK3, and related taxa, obtained by neighbor-joining analysis of the 16S rRNA. Percentage bootstrap values of 1,000 replicates are given at each node. The scale bar denoted branch lengths. NCBI accession numbers are indicated after the species nomenclature.

4. Conclusion

The results of this study clearly demonstrate that MCs were detected from all four recreational water reservoirs. The highest MCs content and also *Microcystis* sp. biovolume were found in Bueng Nong Khot, with negative correlation. Based on the morphological characteristics and 16S rRNA analysis, the dominant bloom-forming species in Bueng Nong Khot was identified as *M. aeruginosa*. However, the potentially toxic tropical cyanobacterium, *Cylindrospermopsis* sp. was found as the dominant cyanobacteria in Bueng Kaen Nakhon, Bueng Thung Sang, and Bueng See Than. This finding suggests that many cyanobacteria which are growing in water reservoirs may constitute a source of cyanotoxins. To prevent intoxications continuous monitoring of these reservoirs is strongly recommended. This has to be taken into account because human health and animal hazards may be involved.

5. Acknowledgement

The authors would like to thank Prof. Dr. Nison Sattayasai for assistance with the genetic analysis and useful discussions. Financial support from the new researcher project of Khon Kaen University, Thailand is also gratefully acknowledged.

6. References

(1) Diltmann E, Wiegand C. Cyanobacterial toxins-Occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res. 2006; 50(1): 7-17.

(2) Codd GA, Morrison LF, Metcalf JS. Cyanobacterial toxins: Risk management for health protection. Toxicol Appl Pharmacol. 2005; 203(3): 264-72.

(3) Falconer IR. Cyanobacterial toxins of drinking water supplies-cylindrospermopsins and microcystins. Boca Raton: CRC Press; 2005.

(4) Carmichael WW, Beasly V, Bunner DL, Eloff JN, Falconer I, Gorham P, et al. Naming cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon. 1988; 26(11): 971-3.

(5) Falconer IR, Yeung DS. Cytoskeletal changes in hepatocytes induced by *Microcystis* toxins and their relation to hyperphosphorelation of cell proteins. Chemico-Biological Interactions. 1992; 81(1-2): 181-6.

(6) MacKintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatase 1 and 2A from both mammals and higher plants. FEBS Lett. 1990; 264(2): 187-92.

(7) McElhiney J, Lawton LA. Detection of the cyanobacterial hepatotoxins microcystins. Toxicol Appl Pharmacol. 2005; 203(3): 219-30.

(8) Yoshizawa S, Matsushima R, Watanabe MF, Harada K, Ichihara A, Carmichael WW, et al. Inhibition of protein phosphatases by microcystins and noduralin associated with hepatotoxicity. J Cancer Res Clin Oncol. 1990; 116(6):609-14.

(9) Sivonen K, Jones G. Cyanobacterial toxins. In: Chorus I, Bertram J, editors. Toxic cyanobacteria in water. London: E and FN Spon; 1999. p. 41-111.

(10) Kuiper-Goodman T, Falconer IR, Fitzgerald J. Human health aspects. In: Chorus I, Bertram J, editors. Toxic cyanobacteria in water. London: E and FN Spon; 1999. p. 113-53.

(11) Ueno Y, Nagata S, Tsutsumi T, Hasegawa A, Watanabe MF, Park HD. Detection of microcystins, a blue-green algal hepatotoxins, in drinking water sample in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis. 1996; 17(6): 1317-21.

(12) World Health Organization. Guidelines for drinking-water quality. Addendum to volume 2, health criteria and other supporting information. 2nd ed. Geneva: World Health Organization; 1998.

(13) Grosse Y, Baan R, Straif K, Secretan B, Ghissassi FEl, Cogliano V. Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Lancet Oncol. 2006; 7(8): 628-9.

(14) Mahakhant A, Sano T, Ratanachot P, Tong-a-ram T, Srivastava V, Watanabe MM. Detection of microcystins from cyanobacterial water blooms in Thailand freshwater. Phycol Res. 1998; 46 Suppl 2: S25-9.

(15) Peerapornpisal Y, Sonthichai W, Suckchotiratana M, Lipigorngoson S, Ruangyuttikarn W, Ruangrit K, et al. Survey and monitoring of toxic cyanobacteria in water resources for water supplies and fisheries in Thailand. Chiang Mai J Sci. 2002; 29(2): 71-9.

(16) Prommana R, Peerapornpisal Y, Whangchai N, Morrison LF, Metcalf JS, Ruangyuttikarn W, et al. Microcystins in cyanobacterial blooms from two freshwater prawn (*Macrobrachium rosenbergii*) ponds in Northern Thailand. Sci Asia. 2006; 32(4): 365-70.

(17) Sungsitthisawad W, Inmuong U, Nienvitoon T, Thiramanus T. Pollutants and heavy metals in recreational waters in Khon Kaen City. KKU Res J. 2000; 5(2): 70-80.Thai.

(18) Komárek J, Anagnostidis K. Cyanoprokaryota 2. Teil/2nd part: Oscillatoriales. In: Büdel B, Gärtner G, Krienitz L, Schagerl M, editors. Süsswasserflora von Mitteleuropa, Vol 19(2). Heidelberg: Elsevier; 2005. p. 759.

(19) Hoshaw R, Rosowski JR. Method for microscopic algae. In: Stein JR, editor. Handbook of phycological methods, culture methods and growth measurements. London: Cambridge University Press; 1973. p. 53-6.

(20) Bolch CJS, Blackburn SI. Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa* Kütz. J Appl Phycol. 1996; 8(1): 5-13.

(21) Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus. 1990; 12(1): 13-5.

(22) Tamira K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28(10): 2731-9.

(23) Xu Q, Chen W, Gao G. Seasonal variations in microcystin concentrations in Lake Taihu, China. Environ Monit Assess. 2008; 145(1-3):75-9.

(24) Robarts RD, Zohary T. Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. NZ J Mar Freshwat Res. 1987; 21(3): 391-9.

(25) Chapman AD, Schelske CL. Recent appearance of *Cylidrospermopsis* (cyanobacteria) in five hypereutrophic Florida Lakes. J Phycol. 1997; 33(2): 191-5.

(26) Peerapornpisal Y, Sonthichai W, Somdee T, Mulsin P, Rott E. Water quality and phytoplankton in the Mae Kuang Udomtara Reservoirs Chiang Mai, Thailand. Chiang Mai J Sci. 1999; 26(1): 25-43.

(27) Pongswat S, Thammathaworn S, Peerapornpisal Y, Thanee N, Somsiri C. Diversity of phytoplankton in the Rama IX Lke, a man-made lake Pathumthani province, Thailand. Sci Asia. 2004; 30(3): 261-7.

(28) Brittain S, Mohamed ZA, Wang J, Lehmann VKB, Carmichael WW, Rinehart KL. Isolation and characterization of microcystins from a River Nile strain of *Oscillatoria tenuis* Agardh ex Gomont. Toxicon. 2000; 38(12): 1759-71.

(29) Oudra B, Loudiki M, Vasconcelos V, Sabour B, Sbiyyaa B, Oufdou K. Detection and quantification of microcystins from cyanobacterial strains isolated from reservoirs and ponds in Morocco. Environ Toxicol. 2002; 17(1): 32-9.

(30) Zhang Z, Schwart S, Wagner L, Miller W. A greedy algorithm for aligning DNA sequences. J Comput Biol. 2000: 7(1-2): 203-14.