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Isolation and Characterization of Microcystins (Heptapeptides Hepatotoxins) from *Microcystis aeruginosa* Bloom in a Homestead Pond, Dhaka, Bangladesh

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Abstract: A bloom of *Microcystis aeruginosa* occurred in a homestead pond in Rupgoanj, Dhaka. Bloom sample was collected and filtered through a glass fiber filter. Methanol-water extract of filtered cells were analyzed by High Performance Liquid Chromatography (HPLC) with UV, MS and MS-MS detection, detected three types of microcystins viz., Microcystin-RR, Microcystin-YR and Microcystin-LR and those were confirmed by HPLC-MS. The amount of MC-LR was the highest (34.8 μg L^{-1}) followed by MC-RR (16.8 μg L^{-1}) and MC-YR (10.9 μg L^{-1}). The concentration of microcystins was well above the WHO provisional guideline value of 1 μg L^{-1} MC-LR. Further investigations need to characterize other types of microcystins from bloom forming cyanobacteria and their effect on human health and cultured fish in Bangladesh.

Key words: Cyanobacteria, HPLC-MS, microcystin-LR, drinking water

INTRODUCTION

The occurrence of heavy water blooms of blue-green algae (Cyanobacteria) in eutrophic freshwaters is a worldwide problem. The production and release of cyanotoxins is often associated with cyanobacteria blooms. Most of the toxic cyanobacteria genera have been recognized to produce a range of hepatotoxic toxins-microcystins (Carmichael *et al*., 1988). Among these toxic cyanobacteria, *Microcystis* is the most common and cosmopolitan genus from which 35 variants of microcystins have been isolated (Sivonen and Jones, 1999).

In recent years, for a variety of reasons, the harmful impact of cyanobacteria on human health has been more reported by Figueiredo *et al*. (2004). The most dramatic example is the human poisoning case attributed to direct exposure to high concentrations of microcystins which occurred in Brazil in 1996 (Jochimsen *et al*., 1998). Concern about the microcystins health risk to humans through drinking water, led the World Health Organization (WHO) to develop and suggest a provisional guideline level of Microcystin-LR at 1 μg L^{-1}. Up to now this value has been considered as a safe level in drinking water (Falconer *et al*., 1999).

The supply of clean and safe drinking water is one of the main challenges of public health care in Bangladesh. Traditionally, surface water is the main source of drinking water and consumed without any treatment or after boiling when fuel is available. Resulting, about 4.5 million people suffered from watery diarrhea every year, among them a considerable number affected with cholera (Siddique *et al*., 1996). Man-made ponds are one of the main characteristic of rural Bangladesh and almost every house or group of houses is located near a pond (Islam *et al*., 2000). Pond water is directly used intensively or extensively for personal hygiene, washing of clothes and dishes, bathing of cattle, cooking rice and aquaculture. In many of these intensively used eutrophic ponds

cyanobacteria blooms are common and microcystins have been detected occasionally in pond water from several regions, mainly associated with high abundance of *Microcystis* sp. (Aziz, 1974; Welker *et al*., 2005; Ahmed *et al*., 2007). Recently, there has been an increased use of surface water for human consumption due to arsenic contamination in ground waters. In the case of Dhaka district and perhaps other regions of Bangladesh such a practice could amount of replacing one health hazard with another. Nevertheless, studies dealing with toxins and toxicology of cyanobacteria in Bangladesh waters are not very abundant (Welker *et al*., 2005; Ahmed *et al*., 2008).

This study deals with isolation and characterization of microcystins from a natural bloom of *M. aeruginosa* occurring in a homestead fish culture pond in Rupgoanj, Dhaka.

MATERIALS AND METHODS

The study pond is located in Dhaka district (90°30' E longitude 23°45' N latitude) 20 km southeast from Dhaka city. The pond is 0.1 ha in size and is mainly used for fish culture and domestic purposes. *Microcystis aeruginosa* bloom was initiated in the last week of February, 2005 and the highest cell density (95% *Microcystis*) was recorded on 02 March 2005. The bloom sample was collected with plankton net of 20 μm mesh size. A portion of the concentrated samples were filtered through an 0.45 μm glass fiber filter (Whatman GF/C, 47 mm diameter) and dried in an oven at 60-80°C. Dried filters covered with algae cells were transported to the Alfred Wegner Institute, Sylt, Germany for analysis.

Extraction

GF/C filters and 1.0 mL of a mixture of water and methanol (50:50; v:v) was sonicated for 20 min and centrifuged (3000 g). The supernatant was filtered through a nylon filter with 0.45 μm pore size.

Chemical Analysis

The HPLC/UV determination of microcystins was carried out following the methods of Lawton *et al*. (1994) with some modifications (C18 column: Phenomenex prodigy, ODS (3), 250×4.6 mm, 5 μm, mobile phases: acetonitrile/water/0.05%TFA)(Hummert *et al*., 2001a). Detection of microcystins was done by the use of an UV detector (Shimadzu SPD-10AV; λ = 238 nm). HPLC/MS and HPLC/MS-MS analysis were applied to ensure the identity of the toxin peaks in the chromatograms. The HPLC was coupled by means of an electrospray interface to a single quadrupol mass spectrometer (API 150, PE Sciex Instruments, Canada) and additionally to a triple quadrupol mass spectrometer (API 365, PE Sciex Instruments, Canada). The detection was carried out in Selected Ion Monitoring (SIM) mode using LC/MS and multiple Reactions Monitoring Mode (MRM) using LC/MS-MS (Hummert *et al*., 2001b).

Microcystins and Nodularin Standards

Standards of Microcystin-RR, Microcystin-LR, Microcystin-YR, Microcystin-LA and Nodularin were purchased from Calbiochem/Novabiochem (La Jolla, CA, USA).

Chemicals

HPLC grade acetonitrile and HPLC grade methanol were from Baker (Deventer, Netherlands). Water was purified to HPLC grade quality with a Millipore-Q RG Ultra Pure Water System (Millipore, Milford, USA).

RESULTS AND DISCUSSION

In the original bloom sample the cell density of *M. aeruginosa* was 5.12×10^7 cells L^{-1}. During the bloom the dissolved oxygen, free carbon dioxide and nitrite nitrogen of pond water was recorded as 4.4, 15.0 and 0.68 mg L^{-1}. The pH was 8.5 and the water temperature was between 20-24°C.

The main factors leading to periodic *Microcystis* proliferations were pointed out as increased dissolved organic nutrients, long sunshine hours and favorable water temperature (Ahmed *et al*., 2007). As in other tropical country, cyanobacteria blooms phenomena will often last year round occurring in many eutrophic lakes and ponds in Bangladesh when the climate permits. HPLC analysis of *M. aeruginosa* extract showed three peaks, the retention time of which agreed well with standard MC-RR, MC-YR and MC-LR (Fig. 1). The results of HPLC-MS revealed the identification of three variants of microcystins (Fig. 2), according to their corresponding molecular weight: MC-LR (at m/z 995.0 $(M+H)^+$), MC-RR (at m/z 519.5 $(M+2H)^{2+}$) and MC-YR (at m/z 1045.0 $[M+H]^+$). In *M. aeruginosa* sample the amount of MC-LR was the highest (34.8 μg L^{-1}) followed by MC-RR (16.8 μg L^{-1}) and MC-YR (10.9 μg L^-).[1] A small amount of MC-LA was also detected. The concentration of different types microcystins detected in the present study was much higher than the previously detected microcystins from an aquaculture pond in Bangladesh (Ahmed *et al*., 2008). In Welker *et al*. (2005) study at three different regions in Bangladesh detected microcystins in 39 ponds, mostly together with varying abundance of potentially microcystin-producing genera such as *Microcystis, Planktothrix* and *Anabaena*. Total microcystin concentrations in their study ranged between <0.1 and up to >1000 μg L^{-1} and more than half of the positive samples contained high concentrations of more than 10 μg L^{-1}. The present study clearly showed that the concentration of microcystin MC-LR (34.8 μg L^{-1}) is much above the WHO provisional guideline value of 1 μg L^{-1} MC-LR for drinking water. A child of 10 kg body weight will already be exposed to the TDI (Tolerable Daily Intake) through consumption of 11.5 mL of water containing 34.8 μg L^{-1} MC-LR (as proposed by WHO 4 mL of water containing 100 μg L^{-1} of MC-LR) (Chorus and Faster, 2001). In Australia, a safety factor for tumor promotion is 1.0 μg microcystins or nodularins L^{-1} (Falconer *et al*., 1999). In Canadian drinking water maximum accepted concentration for MC-LR is 0.5 mg L^{-1} and for other microcystins, 1 μg L^{-1} of total microcystins (Carmichael, 1995).

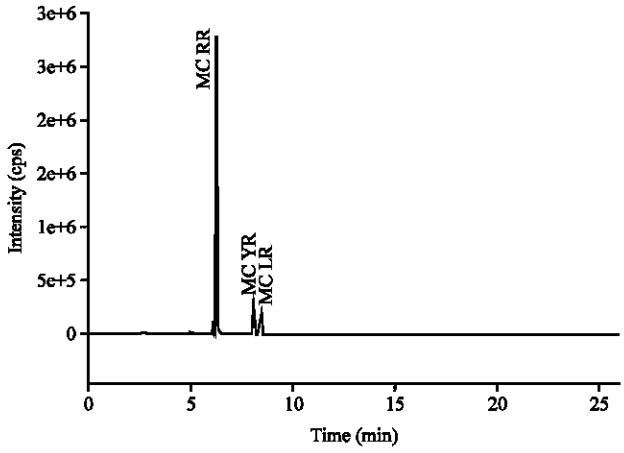

Fig. 1: HPLC-MS chromatograms of *Microcystis aeruginosa* (filtered cells) collected from Rupgoanj, #Dhaka

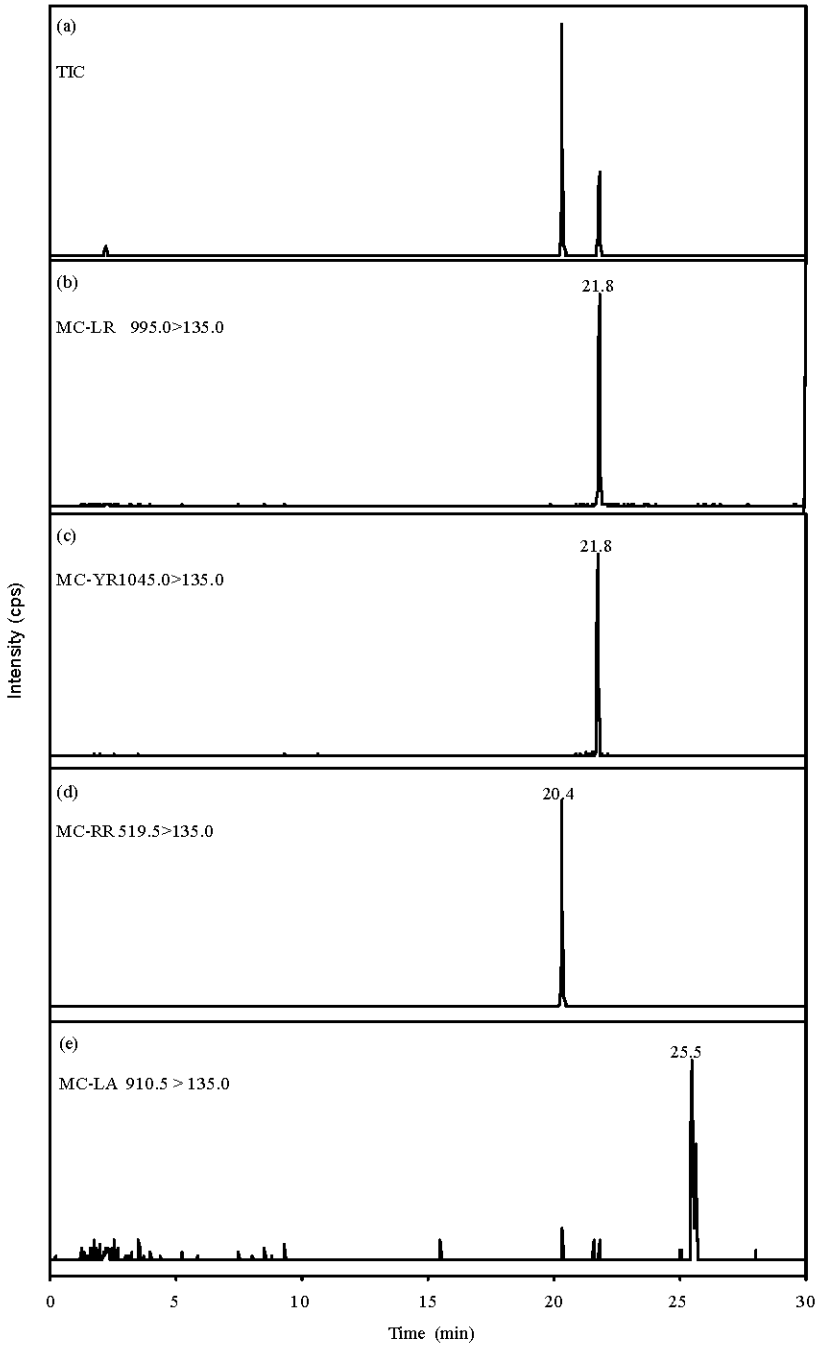

Fig. 2: HPLC/MS-MS chromatogram of microcystins detected from *Microcystis aeruginosa* (filtered cells), (a) TIC, (b) Microcystin-LR, $(MC\text{-}LR+H)^+$ 995.0>135.0, (c) Microcystin-YR, $(MC\text{-}YR+H)^+$ 1045.0>135.0, (d) Microcystin-RR $(MC\text{-}RR+2H)^{2+}$ 519.5>135.0 and (e) Microcystin-LA $(MC\text{-}LA+H)^+$ 910.5>135.0

The occurrence of *M. aeruginosa* blooms in lake/pond that produce hepatotoxic microcystins is a problem, especially if the water is utilized as a drinking supply and/or for recreational purposes. Epidemiological investigations have demonstrated that microcystins cause stomach and intestinal inflammation, liver cancer and disease of the spleen in humans who drink water containing microcystins (McDermott *et al*., 1998; Ding *et al*., 2000; Zhou *et al*., 2002). In Bangladesh, rural people use pond/lakes water for drinking or domestic uses even when bloom or scum is formed as they have no knowledge about toxicity and in some cases they have no alternative. They also culture and consume fish from the same waters without any hesitation.

Recently, it is also evident that microcystins does accumulate in the liver and muscle of fish when they are exposed to toxic cyanobacteria bloom (Magalhaes *et al*., 2001; Deblois *et al*., 2008). In tropical and subtropical climates, where blooms can be permanent, chronic year-round exposure is likely to occur, leading to potentially high levels of microcystin contamination. Accumulation of microcystin in fish is a potentially important route of exposure for humans. So, the effect of cyanobacteria blooms (microcystins) on aquatic animals and human through direct exposure or food chain in Bangladesh waters remains to be identified.

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