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Effect of Environmental Factors on Cyanobacterial Abundance and Cyanotoxins Production in Natural and Drinking Water, Bangladesh

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ABSTRACT
Cyanobacterial blooms commonly appear during the summer months in ponds, lakes and reservoirs in Bangladesh. In these areas, fish mortality, odorous water and fish and human skin irritation and eye inflammation have been reported. The influence of physicochemical factors on the occurrence of cyanobacteria and its toxin levels were evaluated in natural and drinking water in Bangladesh. A highly sensitive immunosorbent assay was used to detect microcystins (MCs). Cyanobacteria were found in 22 of 23 samples and the dominant species were *Microcystis aeruginosa*, followed by *Microcystis flosaquae*, *Anabeana crassa* and *Aphanizomenon flosaquae*. Cyanobacterial abundance varied from 39 to 1315×10^3 cells mL^{-1} in natural water and 31 to 49×10^3 cells mL^{-1} in tap water. MC concentrations were 25-82300 pg mL^{-1} with the highest value measured in the fish research pond, followed by Ishakha Lake. In tap water, MC concentrations ranged from 30-32 pg mL^{-1}. The correlation between nitrate-nitrogen (NO_3-N) concentration and cyanobacterial cell abundance was $R^2 = 0.62$ while that between cyanobacterial abundance and MC concentration was $R^2 = 0.98$. The increased NO_3-N from fish feed, organic manure, poultry and dairy farm waste and fertilizer from agricultural land eutrophicated the water bodies and triggered cyanobacterial bloom formation. The increased amount of cyanobacteria produced MCs, subsequently reducing the water quality.

Key words: Cyanobacteria, environmental factors, microcystins, natural and drinking waters

INTRODUCTION

Water toxicity generated from freshwater cyanobacteria has been documented in at least 45 countries, including Australia, China, throughout the former USSR, Europe, India, Israel, Japan, Latin America, North America, South Africa, Thailand and Sri Lanka (Carmichael and Falconer, 1993; Codd *et al*., 1999). Several toxigenic species of freshwater blue-green algae (Cyanobacteria) have been isolated which are associated with toxicity to humans and livestock. As causal agents of these acute intoxications, microcystins (MCs) have been identified from *M. aeruginosa* (Kütz) and other cyanobacteria that commonly form blooms in lakes, ponds, reservoirs and rivers (Carmichael, 1992; Ueno *et al*., 1996). The species responsible for the majority of poisonous outbreaks are *M. aeruginosa*, *Anabaena* sp. (Lyngb. Bréb) and *A. flosaquae* (L. Ralfs). *Microcystis aeruginosa* toxins have caused issues worldwide and have been well-characterized (Gorham and Carmichael, 1988). Among the toxic species of freshwater cyanobacteria, *M. aeruginosa* produces blooms year round in Bangladesh; however, their intensity increases during the spring, summer and autumn.

In Bangladesh, fish farmers apply feeds and fertilizers in their intensive and semi-intensive aquaculture ponds, making the water body eutrophic. This problem is exacerbated by increasing nitrogen or phosphate loads which ultimately

accelerate cyanobacteria blooms. Moreover, fertilizers are applied to agricultural lands to increase crop yields and poultry and dairy farms often dump waste in open locations. During the monsoon months, the nutrients from these sources spread due to surface run-off and monsoon floods and these nutrients may trigger noxious and toxic cyanobacterial blooms in inland seas (beels, haors and even in floodplains) and further reduce the water quality. Blue-green algal blooms are endemic to Bangladesh, but they have become widespread in recent years. Fish mortality, irritating odors from decaying algae, skin irritation and eye inflammation and odorous fish meat after cooking have been reported in association with local ponds affected by algal blooms. Incidents of hepatitis, dysentery, diarrhea and liver cancer are also increasing in densely populated, developing countries, both in rural and urban areas which may be associated with blue-green algal toxins, as has been reported previously by Bell and Codd (1994) and Ueno *et al*. (1996).

In many such areas, arsenic contamination of groundwater has encouraged the use of surface waters for various purposes such as drinking, cooking and bathing. Despite the water crisis in rural and urban areas of Bangladesh, limited studies have been performed. Therefore, this study was conducted to investigate the effect of environmental factor on the occurrence of cyanobacteria and their toxins production in different water resources in Bangladesh. In addition, three major factors that affect MCs in natural and drinking water were evaluated, namely, the abundance of cyanobacteria in relation to environmental factors, the link between the abundance of cyanobacteria and production of toxins and the presence and levels of toxins.

MATERIALS AND METHODS

Sampling: Samples were collected from 23 water sources in Mymensingh district in Bangladesh (Fig. 1): 16 from ponds of domestic and recreational use and fish culture, 2 from tap water and 1 each from an intensive fish culture pond, a poly culture fish pond, a tube-well, the Old Brahmaputra River and an experimental fish culture pond near the Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh. Samples were collected twice from each area during the

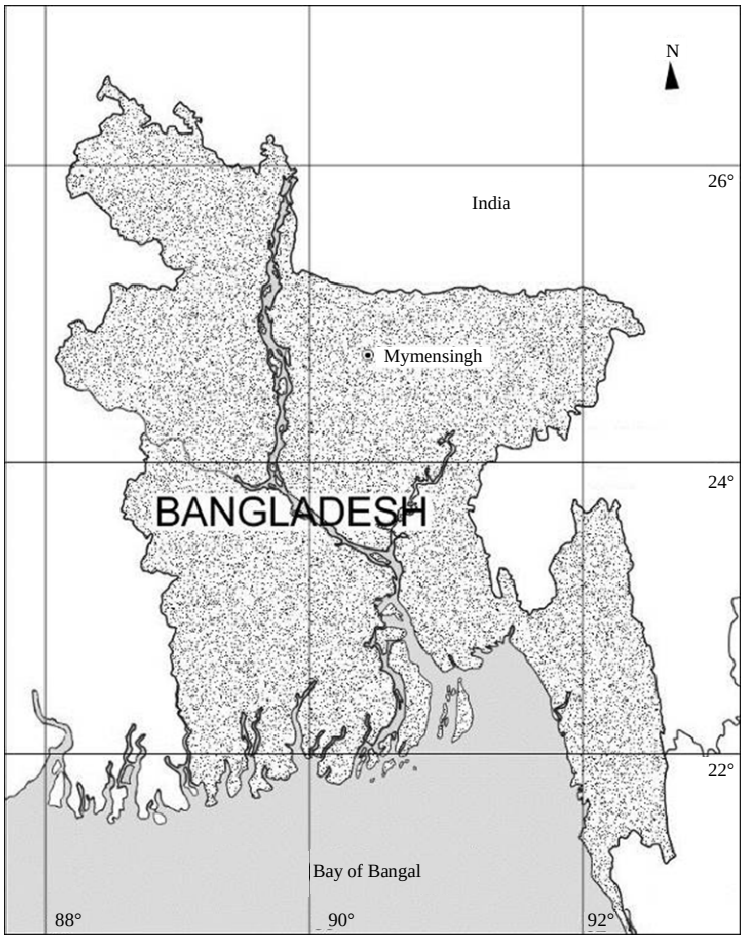

Fig. 1: Sampling sites in the Mymensingh district (24°45'14"N and 90°24'11"E) in Bangladesh

summer months which was in July and August 2000. The samples were collected in 1 L plastic bottles for further study. A 40 mL sample was directly collected in a double-capped plastic tube to estimate microcystin concentrations. Next, a 1/100 volume of 10% sodium azide was added and maintained at 20°C (Nagata *et al*., 1996).

Water quality parameters analysis: Water temperature and pH were measured using a Celsius thermometer and electronic pH meter (Jenwary, 3020). Nitrate-nitrogen (NO_3-N) and phosphate-phosphorus (PO_4-P) concentrations were measured using the Hach kit (DR/2010, a direct-reading spectrophotometer) with high range chemicals (Nitra Ver. 5 Nitrate Reagent Powder Pillows for 25 mL sample for NO_3-N and Phos. Ver. 3 Phosphate Reagent Powder Pillows for 25 mL sample for PO_4-P analysis).

Taxonomic identification of cyanobacteria: For species identification, a sample was gently shaken and 2-3 drops were placed on a glass slide. A cover slip was placed on the slide and visually inspected. Taxonomic identification of cyanobacteria was performed using an Olympus phase-contrast light microscope at 100-400X, with bright-field and phase-contrast illumination on living material and samples preserved with 10% formaldehyde. The quantitative estimation of cyanobacteria was performed using a Sedgewick-Rafter (S-R) counting chamber under the same microscope. Each cyanobacterial colony was counted as a single cell.

Enzyme-linked immunosorbent assay: From each sample, 10 mL water was poured in a 12 mL polyvinyl vial and 1/100 volume of 10% sodium azide was added and frozen in a deep freezer. The samples were freeze-thawed twice and then filtered through glass fiber filters (Whatman GF/C, 25 mm in diameter) and used for Enzyme-Linked Immunosorbent Assay (ELISA). The water samples or microcystins LR (MC-LR) standard were mixed with an appropriate dilution of the anti-MC-LR MAb M8H5 and then added to a 96 well microtiter plate coated with MC-LR-bovine serum albumin conjugate. After washing, the bound MAb was detected with horseradish peroxidase-labeled goat anti-mouse IgG (TAGO 4550) plus substrate (0.1 mg mL^{-1} 3,3'-5,5'-tetramethyl benzidine, 0.005% H_2O_2 in 0.1 M acetate buffer pH 5), resulting in an absorption measurement at 450 nm. The concentrations of MCs used when examining the ELISA data were an average of two triplicate estimations expressed as picogram per milliliter (pg mL^{-1}) by MC-LR (Nagata *et al*., 1996).

RESULTS

Cyanobacterial abundance: A total of 11 cyanophyceae phytoplankton species belonging to 3 families were identified: 7 Chroococcaceae, 3 Nostocaceae, 1 Oscillatoriaceae (Table 1). Cyanophyceae cell abundance and their percent compositions are shown in Table 2. Cell abundance varied from 31×10^3 to 1315×10^3 cells mL^{-1}, with the highest in the fish research pond (sample No. 4). The second (955×10^3 cells mL^{-1}) and third (903×10^3 cells mL^{-1}) higher cell abundance were in Ishakha Lake in samples No. 1 and 2, respectively. The dominant species were *M. aeruginosa*, followed by *M. flosaquae*, *A. crassa* and *A. flosaquae* in all samples excluding tube-well water. The highest contribution of *M. aeruginosa* was 98.0% among cyanobacterial in the BAU fish research pond (samples No. 4) in July. In Ishkah Lake, the contribution of *M. aeruginosa* was 86.0 and 93.0% among cyanobacteria in July and August, respectively (samples No. 1 and 2).

Environmental factors: Water temperature varied from 25.0-32.1°C in July to August, with the highest in the Ishakha lake at BAU in August (sample 2, Table 2). There was no significant difference in water temperature among the ponds and rivers, whereas, the temperatures of the tube-well (sample No. 8) and tap water (samples No. 9 and 12) were significantly lower ($p<0.05$). The pH ranged from 6.6-10.5 and the lowest and highest pH was found in tap water (sample No. 12) and in the polyculture pond (sample No. 10), respectively. The pH was higher in ponds where the cyanobaterial bloom was visible to the naked eye as a green hue or scum on the water surface (Fig. 2).

Table 1: List of blue-green algal species observed among the samples of natural and drinking water at Mymensingh district in Bangladesh from July to August 2000

Order	Family	Genus	Species
Class: Cyanophyceae			
Hormogonales	Nostocaceae	*Anabaena* Bory	*Anabaena crassa* Lemm.
			Anabaena spiroids Klebahn
		Aphanizomenon Morren	*Aphanizomenon flosaquae* Linné
Chroococcales	Chroococcaceae	*Aphanocapsa* sp. Nägeli	
		Cyanodictyon Pascher	*Cyanodictyon imperfectum* Corberg
		Microcystis Lemmermann	*Microcystis aeruginosa* (Kützing) Lemm.
			Microcystis botrys Teiling
			Microcystis flosaquae (Wittrock) Kirchner
			Microcystis natans Lemmermann
			Microcystis wesenbergii (Komárek)
Oscillatoriales	Oscillatoriaceae	Oscillatoria Vaucher ex Gomont	*Oscillatoria agardhii* Gomont

Fig. 2(a-d): Cyanobacterial blooms in (a-b) BAU fish research pond and (c-d) Ishakha Lake at Bangladesh Agricultural University, Mymensingh

NO_3-N concentrations ranged from 0.1-3.1 mg mL^{-1} with the lowest and highest in tube well water (sample No. 8) and the BAU fish research pond (sample No. 4), respectively. The NO_3-N concentration was greater than 2.0 mg mL^{-1} in ponds where cyanobacterial abundance was higher than 325.0×10^3 cells mL^{-1}, especially in samples 1, 2, 4, 7 and 10. The PO_4-P concentrations varied from 0.1-2.7 mg L^{-1} with the highest in the BFRI fish culture pond (sample No. 13) (Table 2).

MCs concentrations: The ELISA data revealed that 22 out of 23 samples were contaminated with MCs. The concentrations of MCs were 25-82,300 pg mL^{-1} with the highest in the BAU fish research pond (sample No. 4) where a thick mat of cyanobacterial bloom was observed during sampling (Fig. 2). MCs concentrations were 27,800 and 37,460 pg mL^{-1} in Ishakha Lake (samples No. 1 and 2) in July and August, respectively (Table 2).

DISCUSSION

Influence of environmental factors: Sampling was completed during the summer and the temperature was approximately 30.0°C at most of the sampling sites. Water temperature is an important factor for the growth and bloom formation of cyanobacteria (Davis *et al*., 2009; Preubel *et al*., 2009; Pavlova *et al*., 2010). Relatively higher cyanobacterial cell abundance, especially *M. aeruginosa*, was found at sites where the water temperature was 30.0-32.1°C. Similarly, Eloff (1981) found optimal growth of *M. aeruginosa* within a temperature range of 28.8-30.5°C. Watanabe and Oishi (1985) also reported that *M. aeruginosa* growth increased at temperatures of 32.0°C under culture. Thus, the increased abundance of *Microcystis* spp. in ponds and lakes was influenced by high temperatures (28.0-30.0°C). The MCs concentrations were also relatively high at the above mentioned sites, similar to the results of Davis *et al*. (2009), who found that MCs levels increased two to three-fold with increasing temperature from 26.9±1.7 to 30.6±1.4°C in lake and river waters in a laboratory culture. In addition, Watanabe and Oishi (1985) found that the highest toxin production was from cyanobacteria cultured at temperatures of 18-25°C in the laboratory. Taken together, these results suggest that summer temperature played an important role in bloom formation of toxic cyanobacteria (e.g., *M. aeruginosa*) as well as MCs production at our study.

In addition, pH is an important indicator in the growth of cyanobacteria and the pH can also play vital role of toxin production. Cyanobacterial cell abundance was more than 350×10^3 cells mL^{-1} in water bodies where the pH was greater than 8.0. The higher concentrations of MCs were also found in locations where pH values were relatively high

Table 2: Cyanobacterial cell abundance ($\times 10^3$ cells mL^{-1}) at different sampling sites at Mymensingh district in Bangladesh from July to August 2000

No.	Sampling site	Purpose of use	$\times 10^3$ cells mL^{-1}	Dominant species (%)	Temp. (°C)	pH	NO_3-N (mg L^{-1})	PO_4-P (mg L^{-1})	MCs (pg mL^{-1})
1	Ishakha Lake (J)	Fish culture, swimming, bathing, etc.	903	*Microcystis aeruginosa* (86), *Microcystis wesenbergii* (12)	31.0	9.7	2.7	0.3	27800
2	Ishakha Lake (A)		955	*Microcystis aeruginosa* (93)	32.1	9.7	2.7	0.3	37460
3	Brahmmaputra River (A)	Recreational, drinking, cooking, etc.	145	*Microcystis natans* (31), *Snowella litoralis* (22)	30.5	8.4	1.3	0.2	30
4	BAU Pond -1 (A)	Fish research pond (intensive carps poly culture)	1315	*Microcystis aeruginosa* (98)	30.6	9.3	3.1	0.7	82300
5	BAU Pond-2 (A)	Fish culture	213	*Microcystis aeruginosa* (21), *Microcystis natans* (15)	30.9	7.4	1.8	0.2	30
6	BAU Pond-3 (A)	Domestic use and extensive fish culture	157	*Microcystis aeruginosa* (32), *Microcystis flos-aquae* (28)	30.7	8.6	1.6	0.1	30
7	Adjacent to BAU (A)	Farmer's pond (domestic use and extensive fish culture)	715	*Anabaena crassa* (52), *Aphanizomenon flos-aquae* (23)	31.0	9.9	2.0	1.9	350
8	BAU tube well water (A)	Exclusively for drinking and cooking	ND*	ND*	25.0	7.3	0.1	0.4	N.D.
9	BAU tap water (A)	Drinking, bathing and kitchen use	31	*Microcystis aeruginosa* (41), *Mirocystis wesenbergii* (19)	29.0	7.3	1.3	0.4	30
10	Pond at Mymensingh city (A)	Farmer's pond (Intensive carps poly culture)	893	*Microcystis aeruginosa* (56), *Microcystis wesenbergii* (38)	32.0	10.5	2.2	0.4	21600
11	A.M. College Pond (A)	Recreational and fish culture	196	*Microcystis flos-aquae* (28), *Microcystis wesenbergii* (19)	31.0	6.9	1.4	0.3	40
12	Mymensingh City tap water (A)	Drinking, bathing and kitchen use	49	*Microcystis aeruginosa* (38), *Anabaena spiroids* (29)	27.0	6.6	2.0	0.8	30
13	BFRI Pond-1 (A)	Fish culture	155	*Aphanizomenon flos-aquae* (25), *Anabaena crassa* (11)	32.0	9.5	2.1	2.7	120
14	BFRI Pond-2 (A)	Brood fish rearing pond	175	*Microcystis aeruginosa* (41), *Microcystis wesenbergii* (25)	31.0	8.3	1.7	0.1	40
15	Sutiakhali Pond-1	Farmer's pond (semi-intensive fish culture)	108	*Microcystis botrys* (38), *Microcystis aeruginosa* (24)	31.0	6.7	1.2	0.2	40
16	Sutiakhali Pond-2 (A)	Farmer's pond (domestic use and small scale fish culture)	187	*Microcystis flos-aquae* (45), *Microcystis aeruginosa* (31)	30.8	6.8	1.6	0.2	70
17	Bailor Pond-1 (A)	Commercial fish farm (intensive catfish culture)	325	*Aphanizomenon flos-aquae* (45), *Microcystis aeruginosa* (39)	31.7	7.5	2.1	0.9	600
18	Bailor Pond-2 (A)	Farmer's pond (domestic use and small scale fish culture)	198	*Microcystis flos-aquae* (49), *Snowella litoralis* (26)	30.2	6.9	1.8	0.1	40
19	Bailor Pond-3 (A)	Farmer's pond (domestic use and extensive fish culture)	263	*Microcystis aeruginosa* (47), *Anabaena spiroids* (22)	30.6	7.7	1.9	0.8	210
20	Kalirbazar, Trisal Pond-1 (A)	Farmer's pond (domestic use and small scale fish culture)	252	*Aphanizomenon flos-aquae* (53), *Anabaena crassa* (28)	30.0	6.8	1.3	1.1	170
21	Kalirbazar, Trisal Pond-2 (A)	Farmer's pond (domestic use and small scale fish culture)	221	*Microcystis natans* (38), *Cyanodictyon imerfectum* (26)	30.1	6.8	1.7	0.2	30
22	Chattrapur Pond-1 (A)	Farmer's pond (domestic use and small scale fish culture)	39	*Microcystis natans* (41), *Aphanocapsa* sp. (27)	31.0	6.7	1.1	0.2	60
23	Chattrapur Pond-2 (A)	Farmer's pond (domestic use and small scale fish culture)	187	*Microcystis aeruginosa* (39), *Microcystis natans* (34)	27.0	7.6	2.0	0.8	80

*ND: Not detected, J: July, A: Agust. pH: NO_3-N (mg L^{-1}), PO_4-P (mg L^{-1}) and MCs (pg mL^{-1})

(Table 2, Fig. 3). Bouvy *et al.* (1999) and Briand *et al.* (2002) both reported that cyanobacterial blooms occurred at a pH range of 8.1-9.4 in freshwater. The pH and cyanobacterial cell abundance showed a positive correlation in samples No. 1, 2, 4 and 10 (Fig. 3). The concentrations of NO_3-N appeared to be the most important factor for the occurrence and abundance of cyanobacteria. The cell abundance was found to be higher at those locations where the NO_3-N concentration was higher than 2 mg L^{-1} (Table 2). Increasing the dissolved NO_3-N favored the growth of *Microcystis* spp. at Suwa Lake, Japan (Park *et al.*, 1993) and the extent of a toxic bloom of *Microcystis* spp. decreased with decreasing of NO_3-N concentrations in Lake Akersvatnet, Norway (Utkilen *et al.*, 1996). NO_3-N concentration and cell abundance showed a positive correlation in samples No. 1, 2, 4, 7, 10 and 17 (Fig. 4) which suggests that nitrogen plays strong role in cyanobacterial growth and bloom formation. The increase in NO_3-N concentration which may be related to the application of fertilizer, fish feed and organic manure in those bodies of water, triggered the cyanobacterial bloom during the summer. The strong positive relationship between NO_3-N and MC concentrations was observed in samples No. 1, 2, 4, 7, 10 and 17 (Fig. 4), suggesting that high concentrations of NO_3-N influenced toxin production by increasing *M. aeruginosa* propagation. Watanabe *et al.* (1992) found that increased in NO_3-N concentration favors *M. aeruginosa* bloom formation and MCs production) and a lack of NO_3-N causes a ten-fold decrease in toxicity

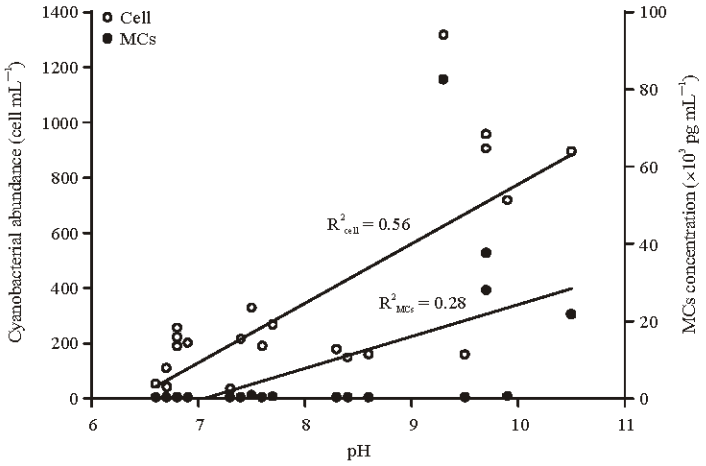

Fig. 3: Relationship between pH cyanobacterial abundance and microcystin concentrations in July and August

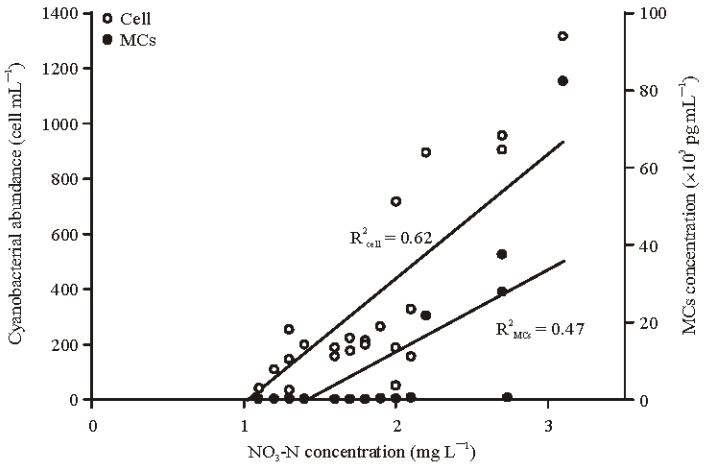

Fig. 4: Relationship between NO_3-N concentrations cyanobacterial abundance and microcystins in July and August

(Codd and Poon, 1988). Therefore, the released NO_3-N from fertilizer and manure favored the growth of cyanobacteria and then decaying cyanobacterial cells released MCs into the water bodies.

In a previous study, chain-forming cyanobacteria such as *A. flosaquae* and *A. crassa* dominated at high PO_4-P concentrations (more than 0.9 mg L^{-1}) with moderate concentrations of NO_3-N in the water and an *A. flosaquae* bloom was found in a high-phosphate environment with nitrogen-depleted water during the summer in the Baltic Sea (Lehtimaki *et al*., 1997). Other studies have found that low orthophosphate concentrations limit the growth of *A. flosaquae* and *A. crassa* while the addition of phosphorus increases the growth of these nitrogen-fixing species (Rinne and Tarkiainen, 1978; Rapala *et al*., 1993).

Microcystins: The strong positive relationship between cyanobacterial cell abundance and MCs concentration (R^2 = 0.98 with cubic regression, Fig. 5) suggests that MCs concentrations rely on cyanobacterial cell abundance. A similar relationship in freshwater sources has been reported, because MCs are produced by the activity of cyanobacteria such as *Microcystis* spp. (Vasconcelos *et al*., 1996; Pawlik-Skowronska *et al*., 2004; Rinta-Kanto *et al*., 2009). At the present study sites, cyanobacterial blooms occur every year but *Microcystis* spp. bloomed more frequently, especially in samples 1 and 2 where the waters were eutrophicated with decomposed organic materials. Fish mortality and bad odors are common at sampling sites 1 and 2 during the summer. Furthermore, residents of the lake have reported eye inflammation, skin irritation and other allergenic responses after bathing or washing clothes in the Ishakha Lake water, similar to reports by Prescott (1948), Sevrin-Reyssac and Pletikosic (1990) and Baxter (1991). Generally, this is caused by relatively high amounts of cyanotoxins from cyanobacterial cells in Ishakha Lake. Fish mortalities in the research and farmer's ponds (samples 4 and 10) were also associated with an increased abundance of *Microcystis* spp. and MCs concentration. Nutrients from the increased input of organic and inorganic fertilizers used for better fish production may have influenced the *Microcystis* spp. bloom which released high concentrations of MCs after cell decay.

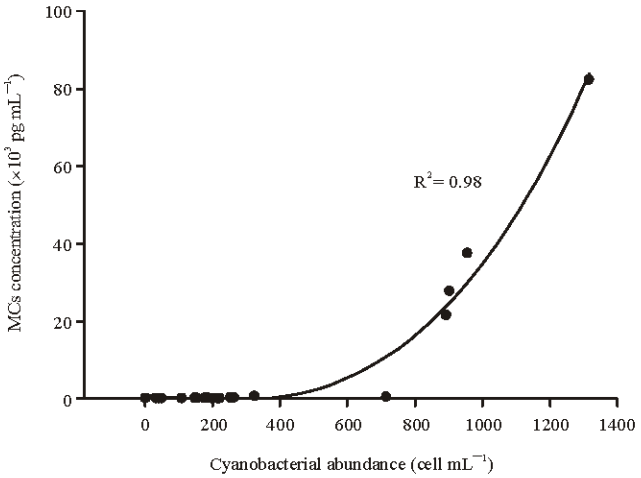

Fig. 5: Cubic relationship between cyanobacterial abundance and microcystin concentrations in July and August

Microcystins level: High concentrations of MCs were found in ponds, rivers and tap water, all of which are used for drinking, cooking and recreational purposes. The high levels of cyanobacteria found in the water are likely due to nutrient input from urban sources and agricultural fertilizer mixing with the river water during floods. Thus, the waters favored cyanobacteria growth which released MCs and deteriorated the water quality. Local people suffer from cholera, dysentery, typhoid and hepatitis in Mymensingh and on the BAU campus (personal observation); the high levels of MCs in those areas could be to blame. In China and South Africa, hazardous concentrations of MCs in both natural and drinking water have been reported, ranging from 100-1558 pg mL^{-1} (Ueno *et al*., 1996; Oberholster *et al*., 2004), similar to present study. There have also been reports that the high levels of primary liver cancer in Haimen and Fusui, China, may be related to the intake of drinking water contaminated with cyanobacteria (Yu, 1989, 1995; Yu *et al*., 1989). Turner *et al*. (1990) found that 10 of 20 army recruits showed symptoms indicative of intoxication (e.g., vomiting, diarrhea, central abdominal pain, blistering of the lips and sore throats) after swimming and canoe training in water with a dense bloom of *Microcystis* spp. and two of them developed severe pneumonia. Recently, incidents of cholera, liver cancer, hepatitis, dysentery, diarrhea, skin irritation, eye inflammation and asthmatic problems have occurred more frequently in this densely populated country which may be related to the occurrence of MCs. Freshwater from natural sources is used for various purposes in Bangladesh, where water quality is reduced by toxigenic cyanobacteria. The use of this water could pose a risk to human health. Lakes and rivers commonly used for recreation should have signs alerting users to the dangers of algal blooms, or even prevent use in extreme cases, although the safety limits of MCs in Bangladesh have not been established. According to the WHO. (1998), water containing 1 μg L^{-1} MCs can be used as drinking water and the MCs concentrations of all drinking water samples (3, 8, 9 and 12) in the present study were lower than that value. However, considerably higher concentrations of MCs can be ingested during swimming, bathing and recreational activities.

CONCLUSION

This study was performed in 1 district out of 64 in Bangladesh, where untreated freshwater is used for drinking or recreational purposes due to a lack of proper treatment facilities. However, officials from the Bangladesh Water Development Board suggest using natural water sources for drinking and other domestic use due to arsenic contamination of groundwater. Therefore, further studies are required to determine whether MCs have contaminated natural and drinking water throughout the country and to investigate the effect of MCs on freshwater ecosystems.

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