Cyanobacteria bloom dynamics in eutrophic ponds and their toxic effects on *Oreochromis niloticus* and *Hypophthalmichthys molitrix*

A thesis submitted to the University of Dhaka, Bangladesh in the fulfillment of requirements for the degree of Doctor of Philosophy

SUBMITTED BY

Sumaiya Ahmed

Registration number: 130/2010-2011
Session: 2010-2011
Re-registration number: 162/2015-16
Session: 2015-2016

Department of Zoology
University of Dhaka
Bangladesh

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CERTIFICATION

The dissertation entitled **"Cyanobacteria bloom dynamics in eutrophic ponds and their toxic effects on *Oreochromis niloticus* and *Hypophthalmichthys molitrix*"** submitted to the Department of Zoology, Faculty of Biological Sciences, University of Dhaka, Bangladesh in partial fulfillment of the requirements for the degree of Doctor of Philosophy. I certified that the candidate, **Sumaiya Ahmed** (Registration No. 130/2010-2011, Re-registration No. 162/2015-2016) has been completed her research under my supervision and suggestions. I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy. The work has not been and will not be presented for any other degree. It is further certified that to the best of our knowledge the thesis contains original research.

Supervisor

Dr. Md. Sagir Ahmed
Department of Zoology
University of Dhaka
Dhaka

DECLARATION

I do hereby declare that the research work entitled "**Cyanobacteria bloom dynamics in eutrophic ponds and their toxic effects on *Oreochromis niloticus* and *Hypophthalmichthys molitrix*"** submitted to the Department of Zoology, Faculty of Biological Sciences, University of Dhaka, Dhaka, Bangladesh, for the degree of Doctor of Philosophy is the results of my own observations and analysis. The thesis or part of it has not been presented before for any other degree.

Sumaiya Ahmed
Ph.D. Student
Registration No.: 130/2010-2011
Re-registration No.: 162/2015-16
Department of Zoology
University of Dhaka
Dhaka

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The Author

Abstract

Cyanobacteria are the most ancient form of life and create harmful algal blooms in freshwater, estuarine and marine ecosystems. It produces some secondary metabolites known as cyanotoxins which pose threat to humans, animals and the environment. Cyanobacterial blooms are common phenomenon in freshwater eutrophic ponds and lakes in Bangladesh. A study was conducted to assess the plankton diversity especially the cyanobacteria and their bloom in four eutrophic ponds in Mymensingh Sadar Upazila and their relationship with physicochemical parameters in both spatial and temporal scales. Plankton samples were collected along with water quality parameters from January 2012 to December 2013. A total of 22 plankton genera of six families were identified. Among them, Ceratiacea (81.36%), Cyanophyceae (72.74%), Euglenophyceae (47.23%), Peridiniaceae (28.67%) found highest in number in Pond 1 (P1), Pond 3 (P3), Pond 4 (P4) and Pond 2 (P2), respectively. Species composition of phytoplankton was typical of eutrophic conditions (high PO_4-P, $N0_3$-N and NH_4) was frequently characterized by the presence of Cyanobacterial bloom but it was also found in scarcity of nitrogen (*Microcystis aeruginosa*, 30,000 colony/L, June 2013, P3). Plankton diversity status was analyzed by using PAST (Paleontological Statistics version 2.17) software. Result showed that *Microcystis* sp., *Ceratium* sp., *Tracheolomonas* sp., *Lepocinclis* sp., and *Spirulina* sp. were the major contributing species (17%) for season all basis. Analysis of Similarity (ANOSIM) results showed that spatial differences and low temporal similarity in species community structure with a diverse assemblage. Canonical Correspondence Analysis (CCA) has been carried out to show the relationship among spatial and temporal data. With the CCA analysis, Conductivity, Ammonium, Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), Nitrate Nitrogen had positive correlation with several phytoplankton species Cyanophyceae (*Microcystis* sp., *Spirulina sp.*, *Lepocinclis* sp., *Merismopodium sp.*, *Anabaenopsis* sp.) and Cholorophyceae (*Senedesmus* sp., *Phacus* sp., *Pandorina* sp.). A bloom of *Microcystis aeruginosa* was occurred in P3 in May 2013. Bloom sample was collected and analyzed with High

performance liquid chromatography (HPLC). Five types microcystins (MC) were detected. The concentration of MC-RR was the highest (5.4µg/L) followed by MC-YR (1.14 µg/L), MC-WR (0.46 µg/L), dm-MC-RR (0.36 µg/L) and MC-LR (2.0 µg/L) which was much higher than the WHO provisional guide value (MC-LR 1 µg/l) for drinking water. Another *Microcystis aeruginosa* bloom sample was collected from P4 during June 2013. Two types microcystins were detected and the highest concentration was MC-RR (1.0 µg/l) followed by and dm-MC-RR (0.04 µg/l). Among the organs tested of fish (*Oreochromis niloticus*) sample MCs were only detected from the liver and the concentration was MC-RR (0.049 µg/gm) which was much higher than provisional guideline of WHO 0.04 µg/kg TDI (tolerance daily intake). No MCs was detected from the gut and muscle of the fish (*Oreochromis niloticus*). Histopathological study confirmed the damage of liver cell of fish exposed with *Microcystis aeruginosa* bloom in vitro experiment. In exposed fish (*Oreochromis niloticus*), histopathological alternations were characterized by swollen and granular cytoplasm, vascular proliferation, bile stasis, fatty change and focal necrosis. The present study thus suggested that fish farms should be monitored for the presence of toxic cyanobacterial blooms to minimize the exposure of potent hepatotoxins to fish and humans through the food chain. So, sustained integrated monitoring system for aquaculture and domestic ponds is strongly recommended.

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ABBREVIATIONS

ACIA	acetylcholinesterase inhibition assay
Adda	(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6decadienoicacid
ANOSIM	One way Analysis of Similarities
BOD	biological oxygen demand
CCA	Canonical Correspondence Analysis
CE	capillary electrophoresis
CRI	German Walch's Global Climate Risk
DAD	photodiode-array UV-detectors
DAF	dissolved air flotation
DBTC	di-n-butyltindichloride
Dha	dehydroalanine
D-MeAsp	D-erythro-β-methyllaspartic acid
DO	dissolved Oxygen
DOF	department of fisheries
ELSIA	enzyme - linked immunosorbent assay
FLD	fluorescence detection
FRSS	fisheries Resources Survey System
GC	gas chromatography
GDP	gross domestic product
HE	eosin
HPLC	high performance liquid chromatography
HABs	harmful algal bloom
ISRP	internal surface reversed phase

LD_{50}	lethal dose
LPS	lipopolysaccharides
MCs	microcystins
Mdha	*N*-methaldehydroalanine
Mdhb	*N*-methaldehydro-α-aminoisobutyric acid
MMPB	3- methoxy-2-methy 1-4 phenylbutyric acid
MTD	mean time to death
PAST	statistical Analysis was performed by paleontological Statistics
PLC	primary liver cancer
pp	protein phosphatases
PPIA	protien phosphatase inhibition assay
PSP	paralytic selfish poisoning
Ser/Thr	Serine/thereonine
SIMPER	Similarity percentage analysis
TA	temperature of air
TBTC	tri-n-butylin chloride
TBTO	bis (tri-n-butyltin) oxide
TDI	tolerable daily intake
TPA	tumor – promoting activity
TW	temperature of water
UNESCO	the united nations educational, scientific and cultural organisation
UNICEF	united nations children's emergency fund
USEPA	united states of environment protection agency
UV	ultra violate
WHO	world health organization

1.1. Cyanobacteria

The blue-green algae are prokaryotes having cell walls composed of peptidoglycan and lipopolysaccharide layers instead of the cellulose walls of green algae (Carmichael and Falconer, 1993). These blue green algae are known as cyanobacteria (Staley et al., 1989). Cyanobacteria are a diverse group of phototrophic prokaryotes. The versatile physiology and adaptive means of cyanobacteria to respond to changes in growth conditions contribute to their dominance over other phytoplankton especially in eutrophicated freshwater (Waterbury, 1992). The wide ecological tolerance makes cyanobacteria more competitive over the water community from early evolutionary history (Giovannoni et al., 1998; Schopf, 1994). Fossil evidence suggests that cyanobacteria were among the first living organism on earth and that they dominated the biota in the Precambrian era as long as 3500 million years ago (Schopf, 1994). Their oxygenic photosynthesis led to the gradual conversion of the earth's atmosphere from an anaerobic to an aerobic one. Cyanobacteria are unicellular, colonial or filamentous (Siddique et al., 2007).They are unique in their special ability to simultaneously carry out oxygenic photosynthesis and oxygen labile nitrogen fixation (Kulasooriya, 2011). Many cyanobacterial species possess gas vacuoles that allow them to regulate their position in water column and give them a distinct ecological advantage over other planktonic species (Reynolds et al., 1987).

1.2. Bloom Formation

Cyanobacteria are found worldwide in inland, coastal and marine environments. Cyanobacteria may accumulate in surface as blue-green have been "scum". Mass developments of cyanobacteria and especially surface scums pose the risks. More than 2,000 species, belonging to 150 genera, at least 40 species have been shown to be toxin producers (Skulberg et al., 1993)). More toxic cyanobacterial species have been recorded from freshwater than from brackish or marine water.

Growth and proliferation of these algae forms not only depend upon the supply of nutrients nitrogen (N) and phosphorus (P), but also on geographical and

environmental factors (Xu et al., 2010).

Cyanobacteria have some adaptive means to dominant over other phytoplankton. Cyanobacteria are unique in that they are capable of performing respiration and photosynthesis in the same compartment. Accumulation of cellular nutrient (Glycogen, cyanophycin and phycobiliproteins, phosphate granules, sugar, phophates) and buoyancy regulation through gas vesicles play an important role in several adaptive responses (Rapala, 1998). In general, most cyanobacteria are considered as shade-adapted organisms (Mur, 1983; Donkor and H¨ader, 1995) but they can tolerate a wide range of irradiance. Some survive at irradiance of only few µmolm^{-2} s^{-1}, and some tolerate direct sunlight (Tandeau and Houmard, 1993; Schoof, 1994). Floating cyanobacteria shade other phototrophic organisms, thus having a competitive advantage (Rapala, 1998). Cyanobacteria grow well at temperature 20-35˚c (Robarts and Zohary, 1987). Nitrogen and phosphorus are the most important element for growth and Redfield (1934) has been recognized a ration for N: P is 16:1. Though nitrogen is an essential element but cyanobacteria can overcome nitrogen starvation. Nitrogen starvation induces the degradation of cyanophycin granules, the storage compound of nitrogen (Grossman et al., 1994a; 1994b), certain cyanobacteria can overcome nitrogen starvation by their ability to fix atmospheric N_2, which gives them a competitive advantage over other species but requires a lot of energy (Bothe, 1982). Nitrogen fixation occurs via the nitrogenase complex. Nutrogenase is irreversibly inhibited by oxygen (Fay and Cox, 1967). Cyanobacteria protect themselves from the harmful effects of oxygen by several ways. By the formation of specialized cells (heterocysts), heterocystous species generate an environment of low partial pressure of oxygen and nitrogen fixation occur (Stewart, 1980). Others like *Microcystis* sp. achieve the same goal by rapid and continuous biochemical consumption of oxygen before it can inhibit nitrogenase, spatial or temporal separation of nitrogen fixation occurs (Kangatharalingam et al., 1991; Flores and Herrero, 1994). Besides them, light irradiance, P^H and availability of external CO_2 have been reported to regulate the nitrogen fixation through post translational modification of the iron-protein in the nitrogenase complex. Although carbon is quantitatively the most important inorganic element in cyanobacteria (Rapala, 1998), they are capable of utilizing low levels of

the CO_2 more effectively than other phytoplankton due to their carbon concentration system. Observations that cyanobacteria do not generally dominant in lakes in which the pH does not increase significantly in the summer would support the hypothesis that low carbon dioxide availability has a major role in determining cyanobacterial dominance (Shapiro, 1990). Iron, molybdenum, sulphate are explanatory factory for the dominance of cyanobacteria.

1.3. Climate change and Cyanobacteria

In recent decades, frequency, severity and geographic distribution of harmful cyanobacteria have increased worldwide. Eutrophication and climate change are the main cause of cyanobacterial growth and bloom potentials in freshwater and marine ecosystems. A variety of mechanisms including warmer water temperatures, changes in salinity, increases in atmospheric carbon dioxide concentrations, changes in rainfall patterns, intensifying of coastal upwelling, sea level rise may promote the growth and dominance of harmful algal bloom (Fig.1.1.).

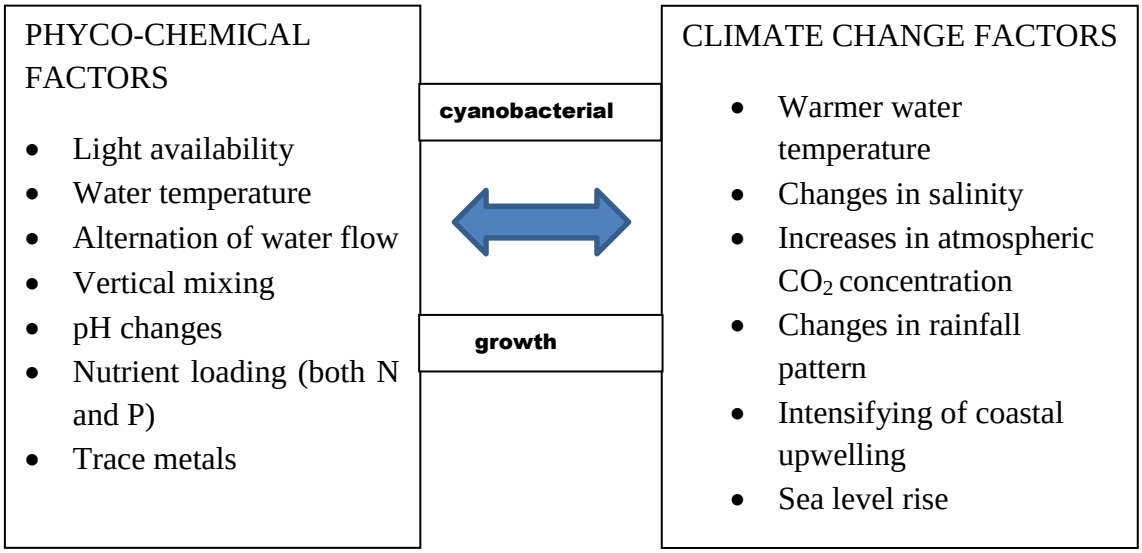

Fig. 1.1. The factors influence cyanobacterial growth

Warmer temperature can promote certain types of cyanobacterial growth as they grow faster at high temperature. Optimal temperature for highest cyanobacterial growth, respiration rate and photosynthetic capacity is 25˚C or more (Robarts and Zohary, 1987). Moreover, warmer temperature increases the strength, frequency and duration of stratification. In stratified waters, cyanobacteria can float upwards to form dense surface blooms that block sunlight for other algae and increasing their competitive advantage. Most bloom forming cyanobacteria can form gas vesicle, regulates buoyancy (Walsby, 1994) and migrate up and down to get nutrients from deeper waters while returning to the surface as blooms. Warmer temperatures decrease the velocity of water which make easier for small cyanobacteria to float on surface water whereas larger algae and organisms cannot (Kardinaal et al., 2007). Intensity and duration of summer drought increase by the effects of climate change which causes salinity increase day by day. Moreover, rising sea levels and increasing demand on freshwater for drinking water and irrigation purposes have promote increase level of salinity (Paerl and Paul, 2012). Vertical stratification is one of impact of salinization which would beneficial for cyanobacterial growth. In addition, some harmful cyanobacterial blooms like *Microcystis*, *Anabaena*, *Anabaenopsis* and *Nodularia* relatively can tolerate more salinity. For example the growth of toxic strains of *Microcystis aeruginosa* remains unaffected by salinities ranging from 0 g /L to 10 g/L, or 30% of seawater salinity (Tonk et al., 2007). Increase in atmospheric carbon di oxide in marine and freshwater ecosystems, favoring cyanobacteria to grow faster in elevating dissolved carbon di oxide condition (Paerl and Ustach, 1982). The incidence of storms will increase which causes more precipitation results transport of nutrients from land into water bodies via run off eventually promote the possibilities of cyanobacterial bloom formation.

1.4. Cyanotoxins

A bloom forming cyanobacteria in fresh, brackish and marine waters produce some toxins which is known as cytotoxins. According to their mode of action cytotoxins are classified into hepatotoxins, neurotoxins and dermatotoxins (Carmicheal, 1994). The

main toxic cyanobacterial genera include filamentous *Anabaena*, *Aphanizomenon*, *Nodularin*, *Oscillatoria* and unicellular colonial *Microcystis* (Skulberg et al., 1979).

1.4.1. **Hepatotoxins**

1.4.1.1. Microcystins

Microcystins are a group of cyclic heptapeptides. Their general structure is characterized as cyclo (D-Ala^{1}-X^{2}-D-MeAsp^{3}-Z-Adda^{5}-D-Glu^{6}-Mdha^{7}) in which X and Z are variable L-amino acids. D-MeAsp is D erythro- β-methylaspartic acid, Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadicnoic acid and Mdha is N-methyldehydroalaninie (Carmichael et al., 1988a) (Fig. 1.2). Variation in the chemical structure is common, and it has been reported for every amino acid (Rinehart et al., 1994; Sivonen, 1996). The main differences between the compounds are in the variable two L-amino acids, X (leucine, alanine, tyrosine, homoisoleucine, arginine, phenylalanine, methionine S-oxide, homotyrosine, tetrahydrotyrosine, or tryptophan) and Z (alanine, aminoisobutyric acid, leucine, arginine, phenylalanine, tyrosine, homoarginine, or methionine S-oxide) and in the presence or absence of a methyl group in amino acids 3 and /or 7 (Rapala, 1998).

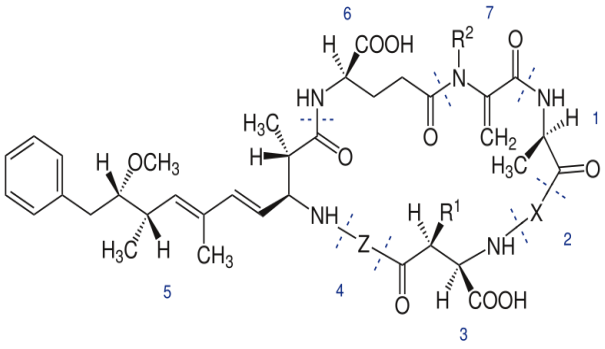

Fig. 1.2. The general structure of microcystins

The acute toxicity (LD_{50}, i.p.mouse) of most microcystins is between 50-600 µg kg^{-1} (Stotts et al., 1993; Rinehart et al., 1994).

1.4.1.2. Nodularins

Nodularin is a cyclic pentapeptide that is produced by *Nodularia spunigena*, and it has been detected in Australia (Runnegar et al., 1988), Baltic Sea (Sivonen et al., 1989b) and North Sea coastal lakes and basins (Nehring, 1993). It contains the same three amino acids as microcystins, D-MeAsp[1,] Adda[3] and D-Glu[4]. In addition to these, the ring structure consists of L-Arg[2] and Mdhb[5] (N-methyldehydro-alpha-aminobutyric acid). Nodularin show significantly less variation than microcystins. Thus far, seven different nodularins have been characterized with demethylation of D-MeAsp, substitution of Mdhb with D or L-methyl-aminoisobutyric acid, and changes in Adda and D-Glu (Rinehart et al., 1994) (Fig. 1.3.).

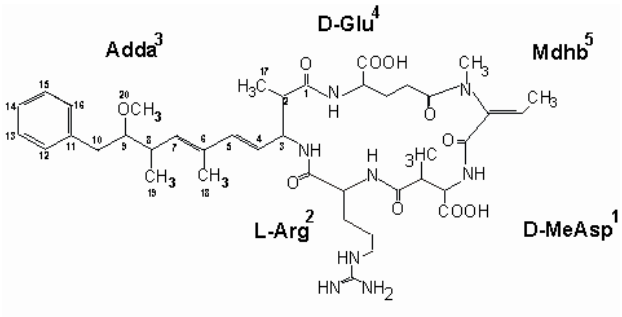

Fig. 1.3.The general structure of nodularins

The acute toxicity (LD_{50}, i.p.mouse) of most nodularins is between 50-150 µg kg^{-1} (Rinehart et al., 1994). Nodularin penetrates into hepatocytes more easily than microcystins, and is itself a liver carcinogen (Ohta et al., 1994).

1.4.1.3. Cylindrospermopsin

A new cyanobacterial hepatotoxin, cylindrospermorphin (Ohtani et al., 1992; Moore et al., 1993) (Fig. 1.4.) has been identified from *Cylindrospermopsis raciborscii* in the late 1970 (Hawkins et al., 1997). The main target of this alkaloid toxin is the liver but it also affects the thymus, kidneys and heart (Terao et al., 1994).

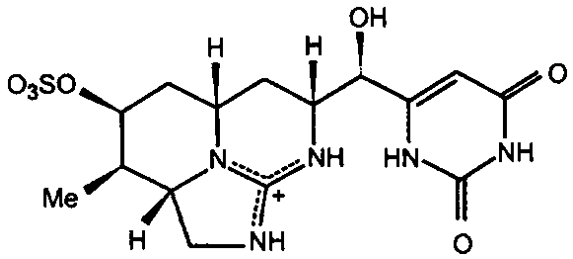

Fig. 1.4. Cylindrospermopsin

1.4.2. Neurotoxins

1.4.2.1. Anatoxin-a and homoanatoxin-a

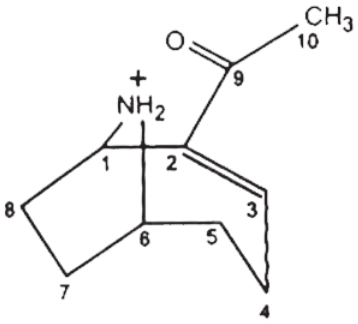

Fig. 1.5. Anatoxin-a

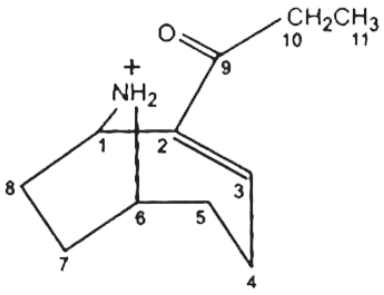

Fig. 1.6. Homoanatoxin-a

Anatoxin-a is a low molecular weight secondary amine, 2-acetyl-9-azabicyclo (4-2-1) non-2-ene (Huber, 1972, Devlin et al., 1977) (Fig. 1.5.) and isolated from *Anabaena flos-aquae* (Gorham, 1964a, 1964b, 1965; Carmichael et al., 1977). It acts as a postsynaptic depolarizing neuromuscular blocking agent (Carmichael et al., 1975, 1979; Valentine et al., 1991). Homoanatoxin-a (Fig. 1.6.), a homologue of anatoxin-a with similar toxicity (Wonnacott et al., 1992), isolated from *Oscillatoria formosa* (Skulberg et al., 1993)

The LD_{50} of the toxins for mouse is 250µg kg^{-1} and the death is due to respiratory arrest occurs within a few minutes (Devlin et al., 1977).

1.4.2.2. Anatoxin-a(S)

Anatoxin-a(S) is a phosphate ester of ancyclic N-hydroxyguanine (Matsunaga et al., 1989) (Fig. 1.7.). It is an inhibitor of cholinesterase (Mahmood and Carmichael, 1987) with an LD_{50} (mouse) of 20-50 µg Kg^{-1}(Mahmood and Carmichael, 1986b) .

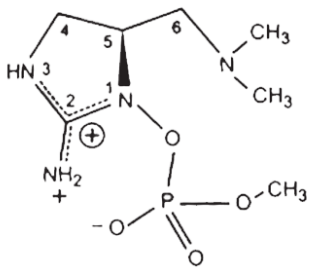

Fig. 1.7. Anatoxin-a(S)

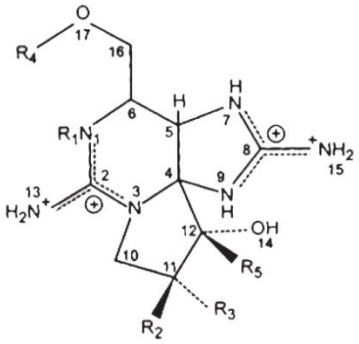

Fig. 1.8.The general structure of PSP-toxins

1.4.3. Paralytic shellfish poisons

Paralytic shellfish poisons, PSP – Toxin are a group of compound that causes death by respiratory arrest through blocking sodium channel (Adelman et al., 1982). The n – sulfocarbamoy 11 -hydroxysulfate C – toxins are the least potent among the PSP toxin singularly sulfated gonyautoxins have a wide range of toxicities and the nonsulfated saxitioxins (Fig. 1.8.) are highly potent the neurotoxins.

The LD_{50} of the toxin for mouse is 10µg kg^{-1}(Mahmood and Carmichael, 1986b).

1.4.4. Other toxins

Aphysiatoxin, debromoplysiatoxin and lyngbyatoxin A are produced by strains of *Lyngbya majuscule* (Mynderse et al., 1977; Cardellina et al., 1979), and have been associated with inflammatory dermatitis and with gastrointestinal disorders. Aplysiatoxin and debromoaplysiatoxin act as protein kinase C activators and are

tumor promotors (Fujiki et al., 1990; Moore et al., 1996).

Lipoplysaccharides of cyanobacteria have been suspected to be cause of gastrointestinal disorders and bathwater fever outbreaks (e.g. Muittari et al., 1980; Keleti et al., 1981). Unlike other bioactive compounds, they are not secondary metabolites.

1.5. Toxicology

Protein phosphorylation is a principal mechanism in the regulation of cytoskeletal structure and organization. Serine/thereonine (Ser/Thr) specific protein phosphatases are the vital importance in maintaining cytoskeletal integrity (Eriksson et al., 1992 a,b). Microcystins and nodularins are inhibitors of protein phosphatases 1 and 2a (PP1, PP2A) in decreasing order of potency (Honkanen et al., 1990; MacKintosh et al., 1990; Yoshizawa et al., 1990). In the case of microcystins, it has been suggested that covalent binding to cysteine-273 and cysteine-226 on PP1 and PP2A, respectively, is responsible for this effect (Mackintosh et al., 1995). PP1 and PP2A dephosphorylate phosphoseryl or phosphothreonyl protein and their inhibition leads to hyper phosphorylation of cytoskeletal protein resulting in the deformation of hepatocyte (Runnegar et al., 1988; Eriksson, et al., 1989, 87). The liver- targeted effects of microcystins are due to selective uptake (Meriluoto et al., 1997) through a hepatocyte specific organic anion carrier bile acid transport system (Eriksson et al., 1990b, Runnegar et al., 1991). At tissue level, microcystins induce extensive hepatic hemorrhage with a complete disruption of the lobular and sinusoidal liver architecture, leading to rapid death by hemodynamic shock (Falconer et al., 1983). The cynobacterial cyclic peptides possess tumor promoting activity (TPA) by a TPA independent pathway (Fujiki and Suganuma, 1999). Inhibition of protein phosphatases, which was referred to the apparent “activation” of protein kinases, is assumed to be involved in tumor promoting activity in the liver (Fujiki et al., 1991; Yoshizawa et al., 1990). Microcystin LR has a potent tumor promoting activity in rat

liver initiated with diethyl nitrosamine (Fujiki et al., 1991).

1.6. Detection and Analysis of cyanobacterial toxins

Mouse bioassay has been traditionally used to screen for the presence or absence of toxins. It can easily distinguish between hepatotoxic and neurotoxic samples, and identifies also anatoxin-a(S) which causes salivation in test animals. Among other bio tests, the use of brine shrimp *Artemia salina* larvae seems promising since this method can also distinguish between hepatotoxic, neurotoxic and non-toxic samples (Kiviranta et al., 1991b; Campbell et al., 1994; Lathi et al., 1995; Vezie et al., 1996). A drawback in the use of the method is that unknown compounds in cyanobacteria may cause false positive reactions (Kiviranta et al., 1991b). However, the non-specific toxicity can be removed by pretreatment of samples with solid phase fractionation (Lathi et al., 1995). Other bioassay methods suggested for the screening of toxicity include the use of the mouse hepatocytes (Aune and Berg, 1986), mosquito larvae (Kiviranta et al., 1993), adult mosquitos (Turell and Middlebrook, 1988) and the mustard seedling growth test (kos et al., 1995). Enzyme - linked immunosorbent assay (ELSIA) using polyclonal (Brooks and Codd, 1988; Chu et al., 1990, An and Carmichael, 1994) or monoclonal (Ueno et al., 1996b) antibodies for microcystins is a highly sensitivity (concentration detection limit, 25 pg ml^{-1}) and a quick method. The protien phosphatase inhibition assay (PPIA) is able to detect less then pg levels of microcystins (Lambert et al., 1994; Mackintosh and Mackintosh, 1994). A colorimetric application that is less expensive and more convenient than the radioisotope method has also been developed (An and Carmichael, 1994). PPIA is nonspecific for microcystins, and the cyanobacterial sample itself may contain phosphorylase phosphatase activity that marks the presence of toxins (Sim and Mudge, 1993). An acetylcholinesterase inhibition assay (ACIA) (Ellman et al., 1961) can be used for screening the presence of anatoxin -a(S). Thin layer chromatography (TLC) has been used in screening of anatoxin-a microcystins and nodularin (Ojanpera et al., 1991; Pelander et al., 1996). Screening of the total amount of microcystins has been successfully performed with separation by gas chromatography or by liquid

chromatography of an oxidation product of microcystins, 3- methoxy-2-methy 1-4 phenylbutyric acid (MMPB) with fluorescence (Sano et al., 1992) or mass spectrometric (Harada et al., 1996; Kondo and Harada, 1996) detection. The method is sensitive enough at the Pico mole determination level of microcystins.

High performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC) can be used for the separation of toxins (Meriluoto, 1997). Microcystins (Lawton et al., 1994b), anatoxin -a (Edwards et al., 1992) and cylindrospermopsin (Harada et al., 1994) each have characteristic UV - absorption spectra, and can thus be distinguished from other compounds by using photodiode-array UV-detectors (DAD). Fluorescence detection (FLD) after derivatization of the compounds increases the sensitivity and methods have been developed for microcystins (Sano et al., 1992), anatoxin-a (James and Sherlock, 1996) and PSP - toxins (Lawrence et al., 1996). Several published methods describe quantitative analysis of cyanobacterial hepatotxins and neurotoxins. Most of them are based on reversed-phase liquid chromatographic separation with detection by UV, FLD or MS. An advantage of internal surface reversed phase (ISRP) liquid chromatography over other methods is the elimination of the sample purification steps.

1.7. Treatment and control measures

Nuisance bloom of toxic cyanobacteria and their potential health hazard create great problem worldwide. Advanced technology tried to develop to control this bloom. Environmentally sustainable technology should be implemented for bloom suppression. A number of methods such as chemical, physical and biological has implement for mitigation of harmful cyanobacterial bloom.

1.7.1 Physical Methods

Microcystins are largely cell-bound, with usually more than 95% of the toxin contained within healthy cells. Dying and decaying cyanobacteria may release microsystins into the water, but the data available indicate that usually biodegradation will be sufficiently effective to preclude the build-up of high concentrations of

extracellular microcystin dissolved in water, unless cell lysis is induced artificially. A very effective way to deal with high microcystin concentrations therefore is to remove the cells intact and without damage (Drikas et al., 2001; Hart et al., 1998). Any damage such as that caused by peroxidation, may lead to cell leakage and consequently in an increase of the dissolved toxin concentration entering the treatment plant. This may be critical as dissolved toxin is not removed by conventional treatment technology.

The standard drinking water treatment processes (coagulation, flocculation, sedimentation and filtration), have shown to be effective in removing intracellular cyanotoxins. Coagulation, flocculation and dissolved air flotation (DAF), are more effective than sedimentation. Conventional treatment using coagulation will remove cyanobacteria cells; however, sludge containing toxic cyanobacteria should be isolated from the treatment process as cells contained in sludge can break down rapidly and release dissolved toxin (Chow et al., 1999). Experimental and full scale studies for the removal of cyanobacteria using membranes are scarce. In general, micro and ultrafiltration membranes could be expected to remove cyanobacterial cells effectively. Membrane filtration of toxic cyanobacteria should be carried out with frequent backwashing and isolation of the backwash water from the plant due to the risk of the cells releasing dissolved toxin (Chow et al., 1997). The treatments mentioned above will not remove extracellular or dissolve toxin to a significant extent.

Dissolve microcystins have been shown to be removed by some reverse osmosis and nanofiltrationm embranes. As removal will depend of membrane pore size distribution and water quality, site specific tests are recommended (Smith et al. 2002).

Riverbank filtration and slow sand filtration have proven very effective in removing microcystins as cyanobacterial cells are retained and dissolved toxin is degraded in the uppermost substrate layers. Grötzmacher et al. (2006) showed that a travel time of several days is likely to suffice, particularly if the underground consist of fine to middle-grained sand and conditions are aerobic, not below 10° C and some clogging

layer (i.e. biofilm) is present.

1.7.2 Chemical Methods

Most of the common microcystin variants are well removed by activated carbon (Hart et al., 1998; UKWIR, 1996; Cook and Newcombe, 2002). The expectation is microcystin LA which is not readily removed and other processes are recommended (Cook and Newcombe, 2002). For other microcystins wood-based, chemically activated carbon is the most effective or a carbon with similar physical properties. Doses of powdered activated carbon required for removal to below the guideline value will depend on water quality and site specific tests are recommended. This can vary between two months to more than one year depending on the type of toxin and the water quality (Newcombe, 2002; UKWIR, 1996).

Chlorination and ozonation are effective for the removal of microcystins. A residual of at least 0.3 gmL^{-1} of ozone for 5 minutes will be sufficient for all of the most common microcystins. For chlorine a dose of 3 mgL^{-1} applied to obtain a residual of 0.5 mgL^{-1} for at least 30 minutes will be effective (Nicholson et al., 1994; Newcombe, 2002; Rositano et al., 1998; Rositano et al., 2001; Ho et al., 2006a; Acero et al., 2005).

Microcystins LA may require a higher residual as it is slightly less susceptible to oxidation by chlorine (Ho et al., 2006a). Potassium permanganate is effective for microcystins and chlorine dioxide and chloramine are ineffective (Rositano et al., 1998). Currently, 98.99% of MCLR is removed by microgel Fe (III) complex (Dai et al., 2012). Slaked lime [$Ca(OH)_2$] or Calcite ($CaCO_3$) is known to remove cyanobacteria community. Aluminum has also been reported to remove the nutrient from industrial and domestic waste waters (Auvray et al., 2006). Salt of copper ($CuSO_4.5H_2O$) is frequently used as an algaecide (McGuire et al., 1984). The herbicide diuron together with copper sulfate has been approved by the United States of Environment Protection Agency (USEPA) for use as algaecide in fish production pond (Schrader et al., 2004).

1.7.3 Biological methods

Biological filtration can be very effective for the removal of most toxins. However, factors affecting the removal such as biofilm mass and composition acclimation periods, temperature and water quality cannot be easily controlled (Ho et al., 2006b). The gastropod *Radix swinhoei* and a submerged plant (*Potamogeton lucens*) in eutrophic waters can eliminate cyanobacterial bloom by minimizing the eutrophication (Zhang et al., 2014). Some aquatic plants release different allelochemicals such as *Miriophyllum* sp., Barly straw have negative affect on cyanobacteria (Welch et al., 1990). Biodegradation using different strains of bacteria possibly the most effective process to control cyanobacterial bloom (Zhang et al., 2008).

1.8. Risk assessment

Risk assessment is the scientific discipline of the risk management process and includes the wellbeing of individuals and populations. The risk assessment process includes four steps: the hazard identification, exposure assessment, dose-response relationships in likely target individuals and populations (Duffus et al., 2007) and Risk characterization. A schematic representation of the steps involved in rick assessment of cyanotoxins in depicted in Fig. 1.9.

Human and animal poisoning episodes as well as toxicological studies show that cyanobacterial toxins can cause adverse human health effects (Codd et al., 2005). Toxicities in human is diverse, ranging from mild to fetal, and includes symptoms of gastroenteritis, abdominal pain, kidney and liver damage, nausea, vomiting, sore throat, blistered mouth, flu-like symptoms, ear and eye irritation, rashes etc. (Codd, 2000; Codd et al., 2005)

The toxicity of microcystin and nodularins is due to inhibition of the catalytic sub unit of protein phosphatases 1 and 2A (PP1, PP2) (Gulledgea, 2002). The cyanobacterial cyclic peptides possess tumor promoting activity (TPA). Most acute effects observed

in China, where consumption of Microcystin contaminated drinking water has been associated with a high incidence of Primary liver cancer (PLC)(Yu, 1995; Ueno et al., 1996a) and colorectal cancer (Zhou et al., 2002). The human exposure to cyanobacterial cells or its toxins through three routes, direct contact of exposed parts of the body such as the ears, eyes, mouth, throat, accidental uptake of water containing cells by swallowing and by aspiration.

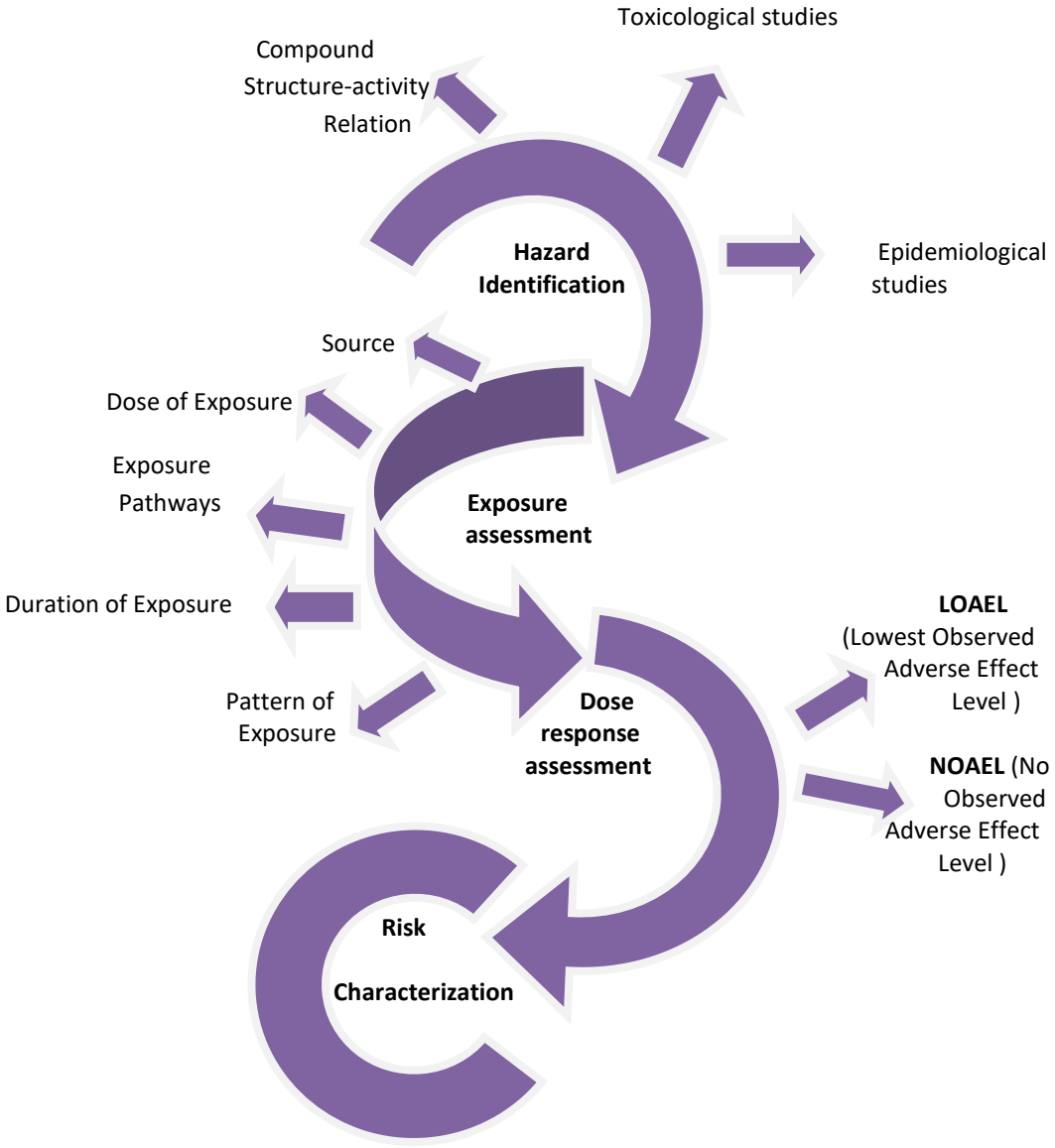

Fig. 1.9. Organizational chart of the steps involved in risk assessment (adapted from Dolah et al. 2001)

Table 1.1. WHO guideline values for safe practice in managing bathing waters that may contain cyanobacterial cells, according to the level of probability of adverse health effects (WHO, 2003).

WHO guideline levels	Cyanobacterial cells and chlorophyll levels	Health risks	Recommended action
Low	<20,000 of total cyanobacterial cells mL^{-1} OR < 10 ugL^{-1} chlorophyll-a with dominance of cyanobacteria OR <2.5 mm^3 L^{-1} cyanobacterial biomass	Short term adverse health outcomes unlikely	Continue monitoring
Moderate	200,000-100,000 of total cyanobacterial cells mL^{-1} OR 10-50 ug L^{-1} chlorophyll-a with dominance of cyanobacteria OR 2.5-12.5 mm^3 L cyanobacterial biomass	Short term adverse health outcomes,e.g.skin irritation, gastrointestinal illness, probably at low frequency	Add signs to indicate MODERATE alert level - increased health risk for swimming and other water contact activities
High	Cyanobacterial scumformation in contact recreation areas OR >100,000 of total cyanobacterial cells mL^{-1} OR >50 ug L^{-1} chlorophyll-a with dominance of cyanobacteria OR >12.5 mm^3 L^{-1} cyanobacterial	Short term adverse health outcomes such as skin irritations or gastrointestinal illness following contact or accidental ingestion Severe acute poisoning is possible in worst ingestion case	Immediate action to prevent contact with scums Add signs to indicate HIGH alert level - warning of danger for swimming and other water contact activities

In exposure assessment, three parameters the, e.g dose of toxins, length of exposure to toxins and abundance of different MC congeners are observed. WHO has been

regularly monitoring the occurrence of toxigenic cyanobacteria and its significant toxic effect on animal health and developed guidelines for drinking and recreational water environments (WHO, 1998, 2003) (Table 1.1.). Characterization of human hazards has done by mainly on animal studies (Churro et al., 2012) and guideline value has been estimated on a tolerable daily intake (TDI) of animals.

The final phase in the risk assessment is risk characterization. It has been determined by the consideration of former phases: hazard identification, dose-response assessment and exposure assessment. There are insufficient data to determine a health based guideline value for most cyanobacterial toxins (Donohue and Orme-zavaleta, 2008). Regarding microcystins there is a wide variation in the toxicity due to the several variants (Ferreira et al., 2010). Moreover, risk assessment has done for only one microcystin variant MC-LR. There is a need to increase the toxicity database for microcystin variants other than MC-LR.

Additive, synergistic, potentiating or antagonistic effects caused by other compounds in a cyanobacterial extract complicate further the estimation of toxicity (Donohue and Orme-zavaleta, 2008; Jokela et al., 2010). Best et al. (2002) showed that toxicity of Lipopolysaccharides (LPS) has intensifying by the co-exposure of MC-LR. Majsterek et al. (2004) reported on the increased toxicity of a MC-LR containing extract compared to a MC-LR standard employing a cytochrome C oxidase assay using mammalian mitochondria from *Bos taurus*. So, characterization of toxic, its toxicity and synergistic effect of total toxins have to calculate.

1.9. Cyanobacteria in Bangladesh

Surface water is one of the main sources of drinking water in Bangladesh. Traditionally water is consumed without any treatment or after boiling when fuel is available. An increasing population density and inadequate sanitation, surface water sources have been contaminated with microorganisms, causing a significant burden of diseases like (cholera, diarrhea, typhoid, dysentery) and mortality. Moreover, *Vibrio cholerae* 01 is isolated from patients as well as from surface water and peoples especially infants and children suffered from acute gastrointestinal disease like

cholera. During 1970s the United Nations Children's Fund (UNICEF) worked with the Department of Public Health Engineering and install tube-wells to provide safe drinking water. In 1997, UNICEF reported that to reach their goal by providing 80% of the population safe drinking water in the form of tubewells, ring-wells and taps (Smith et al., 2000). But, in 1993 arsenic contamination of water in tubewells was confirmed in the Nawabganj district. According to the UNICEF in 2008, there are approximately 8.6 in Bangladesh, of them, 4.75 million tube wells (55%) have been tested for arsenic among which 1.4 million (16%) were marked red indicating that they are unsafe to use as sources of drinking water. The World Bank has estimated that about 20 million people in Bangladesh are using tube-wells contaminated with arsenic over the permissible level (>50ppb) (Smith et al., 2000). So, surface water is recommended to use as drinking and household purposes.

There are about 1.3 million ponds and lakes in the country. The increase of human population and the consequent intensification of agricultural and industrial activities along with deficient water management have led to the enhancement of eutrophication (nutrient enriched) in freshwater bodies used for domestic purposes and as drinking water sources (Ahmed et al., 2007). The occurrence of phytoplanktonic blooms is also becoming more frequent in these ponds and lakes. Environmental conditions such as higher temperature and pH values, low turbulence, and high nutrient inputs (particularly phosphorus, as well as nitrogen) enhance the development of planktonic cyanobacteria in lakes and reservoirs, leading to formation of surface blooms that may accumulate as scum.

About 307 different species of cyanobacteria has been reported from all kinds of water sources (river, canal, ponds, ditches, lakes etc.) in Bangladesh (Siddiqui et al., 2007). Among them 13 species frequently form blooms (Islam, 1991). Affan et al. (2001) has reported microcystin LR from Mymensingh and amount was 27.8 µg/l. Welker et al. (2004) in a study at three different region in Bangladesh detected microcytins in 39 ponds, mostly together with varying abundance of. potentially microcystin-production genera such as *Microcystis*, *Planktothrix* and *Anabaena* and total mycrocystin concentration in their study ranged between < 0.1 and up to >1000

µg /l and more than half of the positive samples contained high concentrations of more than 10.

The incidence and intensity of cyanobacterial bloom in aquatic ecosystem create great concern for Bangladesh. Eutrophication, global climate change which include warmer water temperature, changes in salinity, increase in atmospheric carbon di oxide concentration, changing rainfall pattern, intensifying coastal upwelling, sea level rise might aggravated the frequency, intensity and spreading of such bloom. Very few researches have done about the classification of species and their toxins of cyanobacteria. According to German Walch's Global Climate Risk (CRI) of 2017, Bangladesh is the most vulnerable to global change in the world. As cyanobacteria are adaptable by these changes, tomorrow Bangladesh will faced great problem of safe water as well as safe food. Government and Research organization should concern about it and more research should conduct on cyanobacterial bloom to evaluate its status of hazard in Bangladesh. About 307 different species of cyanobacteria has been reported from all kinds of water sources (river, canal, ponds, ditches, lakes etc.) in Bangladesh. Among them 13 species frequently form blooms (Islam, 1991). Affan et al. (2001) has reported microcystin LR from Mymensingh and amount was 27.8 µg/l. Welker et al. (2004) in a study at three different regions in Bangladesh detected microcytins in 39 ponds, mostly together with varying abundance of. potentially microcystin-production genera such as *Microcystis, Planktothrix* and *Anabaena* and total mycrocystin concentration in their study ranged between < 0.1 and up to >1000 µg /l and more than half of the positive samples contained high concentrations of more than 10.

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- To determine the monthly fluctuation of physicochemical parameters of ponds;
- To determine the qualitative and quantitative analysis of cyanobacteria;
- To determine the effects of some physicochemical parameters on abundance and distribution of phytoplankton community;
- To estimate correlation among physical, chemical and biological factors;
- To determine the mechanisms and contributing factors related to the seasonal dynamics of cyanobacteria blooms in eutrophic pond;
- Isolation and characterization of cyanotoxins (microcystins) from cyanobacteria blooms and
- To know the effect of cyanotoxins (microcystins) on fish liver and tissues.

Cyanobacteria are known as the oldest fossils, dating back 3.5 billion years. They were the organisms that caused the mass extinction on earth, but also the reason for the earth as we know it today. Today, cyanobacteria found all the water bodies in the world. And cyanobacterial bloom is a common phenomenon for this world, it is believed that the first written reference (1000 BC) to a harmful algal bloom appears in the Bible…..all the water that were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river (Exodus 7: 20-1). In this case, a bloom forming algae became densely concentrated that it generated anoxic condition or toxins resulting in indiscriminate kills of both fish and invertebrates. One of the first recorded fetal cases of human poisoning after eating shell fish contaminated with dinoflagellate toxins was in 1793 when Captain George Van couver and his crew landed in British Colombia in an area now known as Poison Cove. He noted that for local Indian tribes it was taboo to eat shell fish when the seawater became bioluminescent due to dinoflagellate blooms (Dale and Yentsch, 1978). On the global scale, close to 2000 cases of human poisoning (15% mortality) through fish and shellfish consumption are reported each year (Hallegraff, 2003).

2.1. History of exposure

Human health risk from exposure to cyanobacteria and their toxins during water use arises through three routes of exposure; dermal contact, swallowing or drinking, ingestion or aspiration.

2.1.1 Exposure through dermal contact

A number of cyanobacterial genera *Anabaena*, *Aphanizomenon*, *Oscillatoria*, *Nodularia*, *Gleotrichia* have been reported to cause allergic reaction after dermal contact (Grauer and Arnold 1961). Some marine filamentous cyanobacteria, *Lyngbya majusuela*, *Schizothrix calcicole* and *Oscillatoria negroviridis* causes severe dermatitis results from contact. Actually it is not allergic reactions but skin inflammation caused by lyngbyatoxin and aplysiatoxins (Moore et al., 1986). According to the Cohen and Reif, 1953, an allergic

reaction has been reported from USA and the cyanobacterial pigment phycocyanin found responsible for this reaction.

2.1.2 Exposure through water

The earliest reported cases of gastro-enteritis from cyabacteria were in the population of a series of towns along the Ohio River in 1931. Low rainfall causes the development of cyanobacteria bloom. As this water moved downstream a series of outbreaks of illness was reported, which could not be attributed to infectious agent (Tisdale, 1931).

In Harare, Zimbabwe, children in area of the city supplied from a particular water reservoir, developed gastro-enteritis each year at the time when a natural bloom of *Microcystis* was decaying in the city with different water supplies were not affected and no infectious agent was identified (Zilberg, 1966).

In 1979, a major outbreak of hepato-entiritis has detected among the children on a tropical island off the coast of Queenland, Australia. Although 140 children and 10 adults required treatment where clinical examination showing malaise, anorexia, vomiting, headache, painful liver enlargement, initial constipation followed by bloody diarrhea and varying levels of severity of dehydration, which is known as “Plam Island Mystery Disease” (Byth, 1980). Later *Cylindrospermopsis rociborkii* was suspected for disease outbreak which formed bloom in the Solomo Dam, the only source of drinking water for the affected people. *C. raciborskii* was identified as the cyanobacterium responsible for this episode.

In Armidale, Australia, the water supply reservoir had been monitored for blooms of toxic *Microcystis* for several years, when a particularly dense bloom occurred. An epidemiological study of the local population indicated liver damage occurring simultaneously with the termination of the bloom by copper sulphate (Botes et al., 1985).

A severe gastro enteritis epidemic in the Paulo Afonso region of Bahia State in Brazil followed the flooding of the new constructed Itaparica Dam reservoir in 1988. Same 2000

gastro- enteritis causes 88 of which resulted in death were reported over a 42-day period. *Anabaena* and *M. genus* was identified as the responsible for this episode. (Teixeira et al., 1993).

2.2. Animal poisoning

In Lake Alexandrina, Australia, first report of cyanobacterial poisoning has reported. Cattle, sheep, dogs, horses and pigs were death after drinking a scum of *Nodularia spumigena* (Francis, 1878). Selected example of different animal poisoning, types of toxins and cyanobacterial species with country reference are enclosed in table 2.1.

Table 2.1. Selected example of animal poisoning associated with cyanobacteria (WHO, 1999)

Country	Species killed	Pathology	Organisam	Reference
Argentina	Cattle	Hepatotoxicity	*Microcystis aeruginosa*	Odriozola et al., 1984
Australia	Sheep	Hepatotoxicity	*Microcystis aeruginosa*	Jackson et al., 1984
Australia	Sheep	Neurotoxicity, PSPs	*Anabaena circinalis*	Negri et al., 1995
Canada	Cattle	Neurotoxicity, anatoxin-a	*Anabaena flosaquae*	Carmichael and Gorham, 1978
Canada	Waterfowl	Neurotoxicity, anatoxin-a	*Anabaena flosaquae*	Pybus and Hobson et al.,1986
Finland	Dogs	hepatotoxicity, nodularin	*Nodularia spumigene*	Person et al.,1984

Finland	Waterfowl, fish, muskrats	Hepatotoxicity, gill damage	*Planktothrix agardhii*	Eriksson et al., 1986
Norway	Cattle	Hepatotoxicity, microcystin	*Microcystis aeruginosa*	Skulberg, 1979
England	Shepherd dogs	Hepatotoxicity, microcystin	*Microcystis aeruginosa*	Pearson et al., 1990
Scotland	Dogs	Neurotoxicity, anatoxin-a	*Oscillatoria* spp.	Gunn et al., 1992
Scotland	Fish (trout)	Gillinjury, microcystin	*Microcystis aeruginosa*	Bury et al., 1995
USA	Dogs	Neurotoxicity, anatoxin-a(s)	*Anabaena flosaquae*	Mahmood et al., 1988

Hepatoxicosis (liver necrosis and hemorrhage) has reported in Monkey (Tustin et al., 1973) and Rhinoceros (Soll and Williums, 1985) by cyanobacterial toxins. Freshwater mussels accumulate both microcystins (Prepas et al., 1997) and saxitoxins (Negri and Jones, 1995) and which can bio accumulated in the food chain. An extensive list of poisoning incidents, and discussion of them, is included in Ressom et al., 1994. Fish kills have been reported by Philips et al., 1985 by immersion of rainbow trout in a culture of *M. aeruginosa.* The severity of bird kills have ranged from a few individual to several thousand birds per incident. In Califonia, high mortality in birds wintering at the Salton Sea has been linked to microcystins (Carmichael and Li, 2006).

3.1. Introduction

Ponds and lakes were natural feature prominently in the landscape of rural area in Bangladesh. Ponds are usually build for raising homesteads above the flood levels and serve as reservoir of excessive water during monsoon. In the rural household, ponds are used for multiple purposes like fish culture, water source for washing, bathing and other household requirements. Bangladesh has about 2.5 million ponds, and about 65% of them have already been brought under commercial fish culture (DOF, 2006). According to Dey et al. (2008), ponds are mainly three types in Bangladesh; cultured pond (using proper fish culture techniques), culturable pond (infrastructure development is needed) and derelict pond (extensive investment is needed). People produce fish in culturable and derelict pond but do not follow the proper culture system due to lack of knowledge and money. Traditional fish culture system, pesticide and fertilizer run off from the field, multipurpose uses of pond cause nutrient upload which lead a great problem are known as eutrophication. It is well known that phytoplankton is the principal bio indicators of pollution in aquatic ecosystem. Due to inconsistent input of different nutrient causes nutrient enrichment in the ecosystem which influences the phytoplankton species structure and production and may affect the ecosystem composition and function (Smith et.al., 1999). Moreover, time-space deviation in physical and chemical parameters of water influences the plankton production (UNESCO, 1981). So study of spatial and temporal dynamics of phytoplankton considered as structural and functional features of aquatic ecosystems (Armengol et al., 1999).

Aquaculture is the most important sector contributing 4.39% to GDP in Bangladesh (FRSS, 2014). Mymenshing District is the most favorable for fish culture in regards to climate, soil topography and water condition. According to DOF (2006), around 19,882 hector are pond area and 84.3% are cultured area in Mymensingh. Most of household ponds are cultarable or derelict pond in Mymensingh. Uneven management of these ponds creates seasonal bloom (Affan, 2001, 2015; Jewel, 2003; Welker et al., 2004; Jahan et al., 2010) and deteriorates water quality. So, the aim of the study to analyses the temporal and spatial variation in phytoplankton structure, abundance and richness in addition their relationship with physicochemical condition to better understand reason behind the phytoplankton bloom.

3.2. Material and Methods

3.2.1. Study Area

Four shallow and small ponds were taken as sampling site (Fig. 3.1.). The geographical locations of four ponds are:

Pond 1 (P1) and Pond 2 (P2) were situated at Anandomohon College (24˚45´35.75″N and 90˚23´41.60″E) (Fig. 3.2.) and Bidyamoyei school campus (24˚45´37.39″N and 90˚24´18.08″E) (Fig. 3.3.) in Mymensingh town. P1 was surrounded by Anandomohon student hostel and P2 was beside the Bidyamoyei School. Area of P1 and P2 were 0.4774 hector (ha) and 0.2808 ha respectively. Though different household activities were prohibited in both of pond but sometimes used for washing and bathing and received wastage from campus. Periodic fish fingerlings were released but no cultural method was followed.

Pond 3 (P3) and Pond 4 (P4) were located at Kaowatkhali area (24˚43´59.96″N and 90˚25´24.94″E; 24˚43´51.87″N and 90˚25´11.19″E) (Fig. 3.4.; 3.5) at Mymensingh. Area of P3 and P4 were 0.1197 ha and 0.2433 ha respectively. P3 was semi cultured pond. Regularly fishes were cultured and artificial fish feed had been given but it is also used as household activities like personal hygiene, washing of cloths and dishes, bathing of cattle and other household work.

All three ponds (P1, P2 and P3) had no sewerage connection from neighboring but surface runoff and slum wash also entered the water system.

P4 was totally derelict ponds which was surrounded by several neighborhoods and received domestic and organic wastage including soap, detergent and sewer waste through sewage connection from the neighboring household. This pond was used for fish culture but no household activities had done for its color, texture and odor.

3.2.2. Sampling protocol

Samples were collected from January 2012 to December 2013 at four stations in the study area. Field visits were conducted monthly and a total twenty four collections

was made over this period. Collection time was in between 10:00 A.M. to 11:00 A.M. Surface water was collected and 20µm mesh size plankton net was used for collection plankton and preserved in 20ml glass bottle with 4% formalin. Phytoplankton was enumerated as cells per liter using a Sedgewick-Rafter counting chamber (S-R cell) under a compound microscope at ×400 magnifications. Phytoplankton were identified by following the methodology described by Stirling (1985), Using a range of reference (Whitford and Schumacher, 1973; Bellinger, 1992), the qualitative identification of phytoplankton was carried out up to the genus while the colony forming cyanobacteria *(Microcystis)* was be counted as number of colonies.

Quantitative analysis of plankton was performed using methods by Welch (1952). Seasonal averages of phytoplankton abundance was formulated for fixed time periods throughout the year [December-February: winter, March-May: pre monsoon (summer), June-September: monsoon (Rainy), October-November: post monsoon (autumn)] (Shahjahan, et al., 2012).

Quantitative analysis of phytoplankton has done by following formula (Welch, 1952):

Plankton enumeration formula: $N = \{(A \times C)/L\} \times 1000$

Where, N= Total number of plankton per liter of original water;

A= Average number of plankton counted per ml of concentrated sample;

C= Volume of concentrated sample;

L=Volume of original water passed through the plankton net.

3.2.3. Measurement of environmental parameters

Alkalinity, acidity, pH, ammonia, Dissolved Oxygen (DO), Free Carbon di oxide (CO_2) were determined by ecological HACH test kit (Model FF2, cat.no.2430-01), nitrate nitrogen, phosphate phosphorus was estimated by Colorimeter (DR/850) and conductivity is determined by Digital conductivity meter (conductivity probe: model CDC 4010). BOD was determined by BOD track machine (BOD Trak™ 11 DOCO22.53.90072).

Air temperature and rainfall data was collected from Dhaka Meteorological Office.

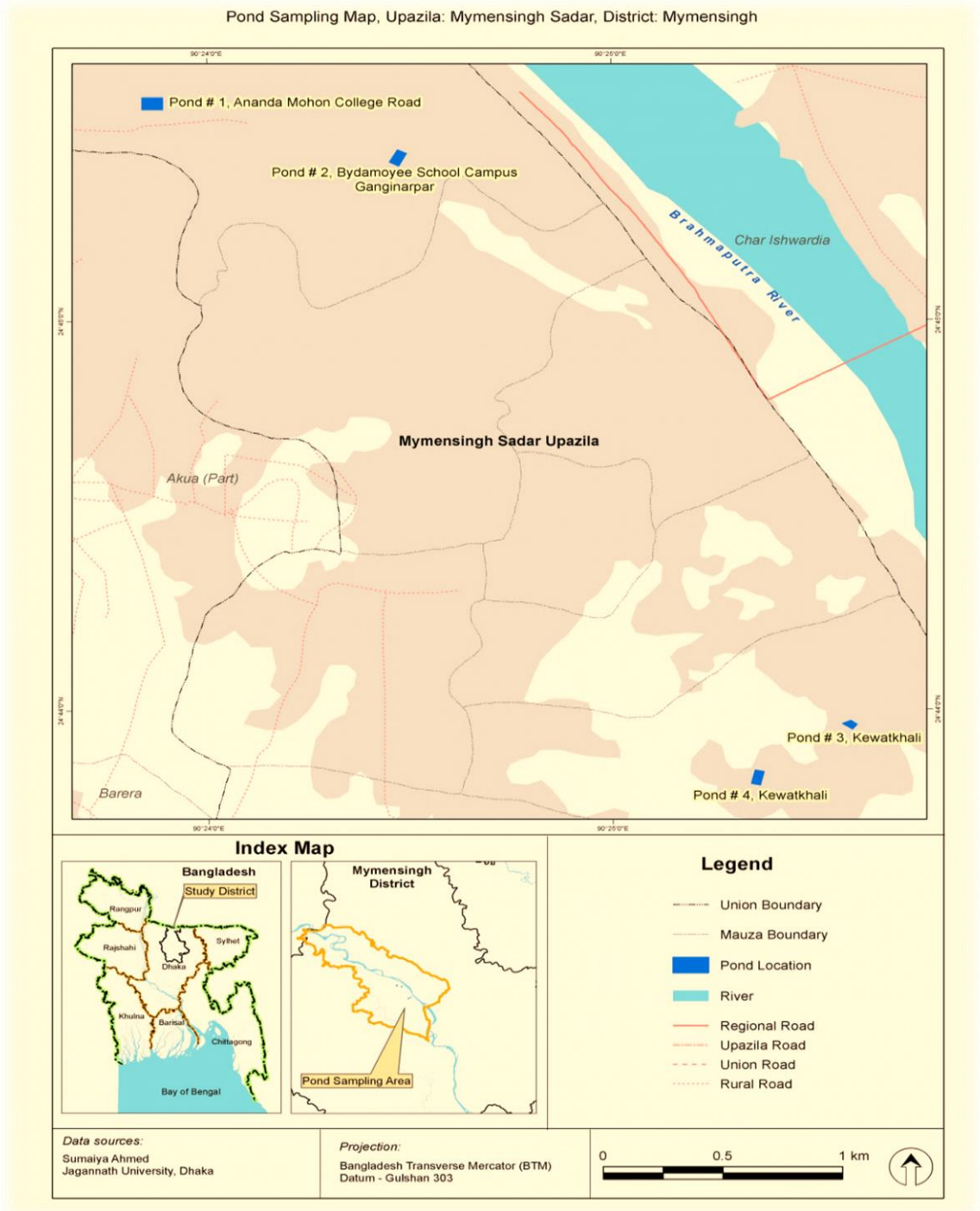


Fig. 3.1. Map of the Mymenshigh Saadar Upazila showing location of sampling stations (P1, P2, P3 and P4).

Fig. 3.2. Pond 1 (Anandomohon College Hostal campus)

Fig. 3.3. Pond 2 (Byadmoyae school campus)

Fig. 3.4. Pond 3 (Khawatkhali Area)

Fig. 3.5. Pond 4 (Khawatkhali Area)

3.2.4. Statistical Analysis

Statistical Analysis was performed by Paleontological Statistics (PAST) version 2.17, a software package (Ryan et al., 1995). Species Diversity was performed using four different indices viz., Species richness, Dominance Indices, Shannon-Wiener Diversity and Evenness in temporal and spatial spectrum.

The dominance index (Harper, 1999) was measured to determine the dominate species in a particular aquatic system. It would be a beneficial index of assessing monopolization of single or group of taxa over a community.

Dominance index;

$D = \sum\left(\frac{n1}{n}\right), D,$

where n =total number of individuals

n_1 is the total number of individual of taxon

Diversity of species expressed by Shannon Weiner diversity index (Shannon, 1949; Shanon and Weaver, 1963) using the following formula;

H= $sum(\frac{n_1}{n})\log n(\frac{n1}{n})$,

where n=total number of individuals

n_1 is the total number of individual of taxon

Margalef index (*d*) (Margalef, 1968) was used to measure species richness.

$d = (\frac{S-1)}{In\,(n)})$;

where *S* is total species and

n is total individuals.

Buzas and Gibson's evenness (Harper, 1999) was measured by following formula;

$E = \frac{e^H}{S}$

where, S= species richness,

H= Shannon index diversity,

E= evenness,

e= natural logarithm base

One-way Analysis of Variance (ANOVA) (Fisher and Mackenzie, 1923) was performed of environmental parameters- alkalinity, acidity, pH, hardness, ammonia, DO, Free CO_2, nitrate nitrogen, phosphate phosphorus, conductivity, BOD, water temperature, air temperature, rainfall and turbidity. It was also performed to find significance difference between station-phytoplankton and season-phytoplankton. Simple correlation was tested to find any significant correlation ship among physicochemical data. Correlation was also tested in between physicochemical and biological data.

One way Analysis of Similarities (ANOSIM) (Clark and Warwick, 1994) is a non-Parametric Test of significant difference between two groups or more. It was used to determine the significance of temporal and spatial difference in the structure assemblage. The test was based on a Bray-Curtis rank similarity matrix were calculated using log (x+1) transformed data. Similarity percentage analysis (SIMPER) (Clarke, 1993) was used to observe the percentage contribution of each taxon to the average dissimilarity between samples of various seasons and stations pair combination. The hierarchical clustering (Clarke and Warwick, 1994) was calculated to produce a dendogram for investigating similarities among the genus according to the number. This analysis was based on Bray-Curtis similarity measure (Bray and Curtis, 1957). Canonical Correspondance Analysis (CCA) (Legendre and Legendre, 1998) was calculated to find out the association between species and environmental parameters. The ordination axes are linear combinations of the environmental variable. CCA is the direct gradient analysis, where the gradient in environmental variables is known a priori and the species abundance (presence/absence) is considered to be response to the gradient. The ordination axes of CA are termed Eigenvectors. Each Eigenvectors has a corresponding Eigenvalue, often denoted by λ. The Eigenvalue is actually equal to the maximized dispersion of the species scores on the ordination axis and is thus a measure of importance of the ordination axis. The first ordination axis has the largest Eigenvalue (λ_1), the second axis the second largest Eigenvalue (λ_2), and so on (Rashed-Un-Nabi et al., 2011). The Eigenvalues of CA all lie between 0 and 1. Values over 0.5 often denote a good separation of the species along axis (Jongman et al., 1995).

3.3. Results and Discussion

3.3.1. Environmental Parameters

The measured environmental parameters summarized in Table 3.1. The highest water temperature (33˚c) was measured in August 2013 at P4, while the lowest (19 ˚c) was measured in January 2013 (mean= 27±4.33 at P4). No statistically significant difference was found among ponds (F=0.12, p > 0.05). Water pH value varies between 6.9 mg/L (Sep 13 at P2) to 9.0 mg/L (Jan 13 at P4). Mean water pH is 8.44± 41 for P4 and significant difference was found among ponds (F=3.52, P < 0.05). Alkalinity values (means 199.37±55.51 at P4), ranged from 40 mg/L (Jan 13, P2) to 315 mg/L (Nov 12, P4). Acidity showed highest value 125 mg/L (P4) to lowest value 5 mg/L at P3 (mean 69.25 ±28.24 at P4). Hardness aliened maximum in 180 at P3 and minimum in 44 at P2. All the three parameters (alkalinity, Acidity, Hardness) showed statistically significance difference among ponds (F= 33.45, P < 0.05; F=16.69, P < 0.05; F=26.55, P < 0.05). Dissolved oxygen concentration attained maxima in (11 mg/L at P4) and minima in (2mg/l at P1) whereas mean value 8.27±1.32 at P4. Free CO_2 ranged from 5 mg/L at P1 to 54 mg/L at P4 (Mean value 33.85 ±11.44, P4). The highest nitrate nitrogen (NO_3-N) (0.55 mg/l) was observed in P4, while lowest NO_3-N (0) was observed at P3 (Mean 0.17±0.18 at P4). Phosphate phosphorus (PO_4-P) was recorded at highest value (0.9 mg/l) at P4 (Mean=0.83±0.16, P4). Ammonia (NH_3) value was ranged from 0.1 mg/L at P2 to 5 mg/L at P4 (Mean 3.75±1.37). All the parameter's (DO, Free CO_2, NO_3-N, NH_3) showed high significance difference among ponds (F=13.45, P< 0.05; F=22.28, P < 0.05; F=12.05, P < 0.05; F=106.09, P < 0.05) except PO_4-P (F= 1.18; p > 0.05). It is known that eutrophication is an

increasing problem for aquatic ecosystem in Bangladesh (Ahmed, 2007) and our study ponds received constant excess nutrient from various anthropogenic sources which may cause the phosphate enrichment. Biological Oxygen Demand (BOD) showed highest value 190 mg/L at P4 lowest value 29 mg/L at P2 (mean 59.42±28.48,

Table 3.1. Physical chemical parameters of four study ponds during the study period (Jan 2012 to Dec 2013).

Stations	**P1**	**P2**	**P3**	**P4**
Parameters	**Range Mean±SD**			
Alkalinity	92-197 143.75±28.95	40-159 92.62±28.87	92-229 170.62±34.10	87-315 199.37±55.51
Acidity	23-90 50±22.44	10-64 32.79±13.81	5-63 33.25±14.86	17-125 69.25±28.24
Hardness	63-171 118.08±31.51	44-141 73.96±28.89	67-180 120.04±31.06	85-179 151.58-29.24
pH	7.1-9 8.08±0.42	6.9-9.5 8.08±o.68	7-10 8.4±.61	7.4-9 8.47±.41
Dissolved Oxygen(DO)	2-9.83 5.38±2.10	4-10.3 6.05±1.60	4-11 7.17±1.66	5.89-11 8.27±1.32
Free CO_2	5-51.8 27.98±14.08	5-20 12.71±4.28	5-27 18.17±6.54	15.2-54 33.85±11.44
Nitrite-Nitrogen(NO_3-N)	0-0.2 0.03±.05	0-0.17 0.04±0.05	0-0.1 0.02±0.02	0.01-0.55 0.17±0.18
Phosphate-Phosphorus(PO_4-P)	0.11-0.9 0.77±0.27	0.22-0.9 0.78±0.23	0.6-0.9 0.87±0.08	0.38-0.9 0.83±0.16
Ammonia	0.2-2.4 0.72±0.55	0.1-0.85 0.46±0.20	0.4-1.1 0.60±0.17	0.6-5 3.75±1.37
BOD	31-36 33.08±1.64	29-47 35.46±4.56	38-48 42.12±2.86	40-190 59.42±28.48
Temperature of water(TW)	19-31 26.09±3.94	19.4-31 26.36±3.9	20-39 26.79±4.64	20-33 27±4.33
Temperature of air(TA)	16.35-30.75 25.09±4.64	16.35-30.75 25.09±4.64	16.35-30.75 25.09±4.64	16.35-30.75 25.09±4.64
Conductivity	445-544 499.04±29.51	245-511 373.92±84.42	600-911 702.75±112.28	623-1290 952.58±206.88
Turbidity	1-28 9.71±7.24	2-28 14.12±7.12	8-107 51.83±30.84	60-188 119.42±51.67
Rainfall	0-409 134.08±136.28	0-409 134.08±136.28	0-409 134.08±136.28	0-409 134.08±136.28

P4). Turbidity aliened maximum in 188 FAU at P4 and minimum in 01 FAU at P1. BOD and turbidity showed highly significance difference among ponds ($F=16.12$, $P < 0.05$; $F=66.39$, $P < 0.05$). No significance difference was found in temperature of air ($F=0.00$, $p > 0.05$) and rainfall $F=0.00$, $p > 0.05$) among ponds because data was collected from same region (Mymensingh Sadar Upazila).

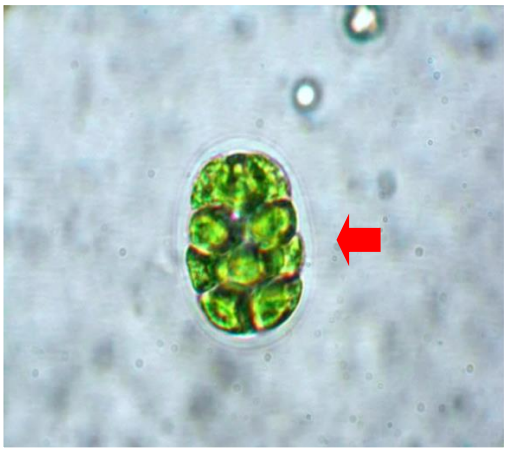
3.3.2. Phytoplankton community

A total of 22 phytoplankton genera, representative of 6 families, were identified during the study periods (Table 3.2.). *Arcella* sp. is not phytoplankton but it has been studies for its seasonal abundance in the study area.

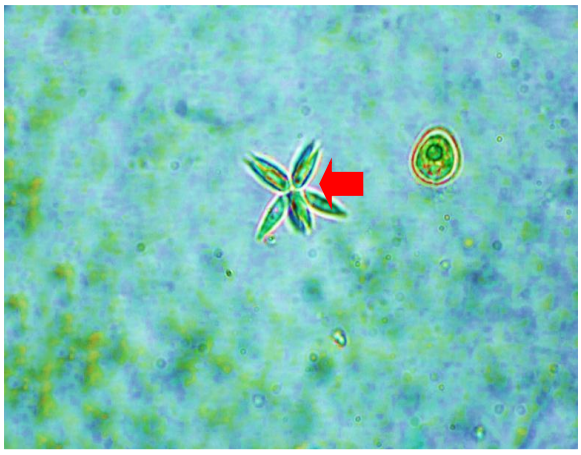
Table 3.2. Dominant Phytoplankton genus recorded from Jan 12 to Dec 13 in Mymenshing (Photomicrograph of phytoplankton, Plate 1.1. to 1.6.)

Phytoplankton Class	**Name of the genera and their code**
Cyaonphyceae	*Anabaena* (Ana) , *Anabaenopsis* (Ans),*Chroococcus* (Chr), *Merismopedia* (Mer), *Microcystis* (Mic), *Spirulina* (Spi)
Euglenophyceae	*Euglena* (Eug), *Lepocinclis* (Lep), *Phacus* (Pha), *Trachelomonas* (Tra)
Chlorophyceae	*Actinastrum* (Act), *Coelastrum* (Coe), *Crucigenia* (Cru), *Dictyospharium* (Dic), *Pandorina* (Pan), *Pediastrum* (Ped), *Senedesmus* (Sen)
Bacillariophyceae	*Cyclotella* (Cyc), *Syndra* (Syn), *Navicula* (Nav)
Ceratiaceae	*Ceratium* (Cer)
Peridiniaceae	*Peridinium* (Per)
Arcellidae	*Arcella*(Arc)

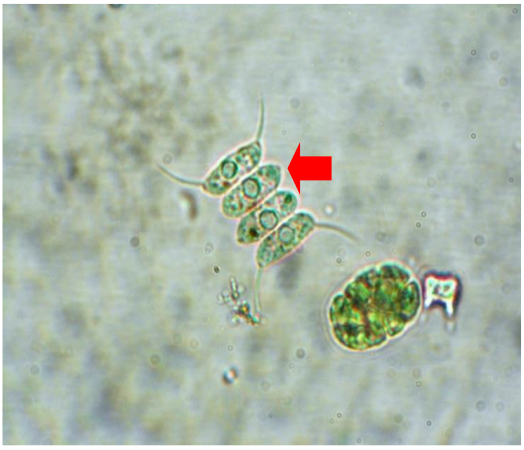
Among the phytoplankton *Microcystis* sp. showed significance difference between ponds ($F= 7.78$; $p=0.0001$)



Pandorina sp.

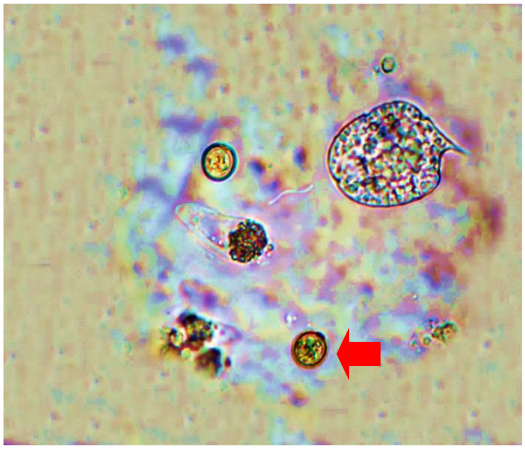


Actinastrum sp.

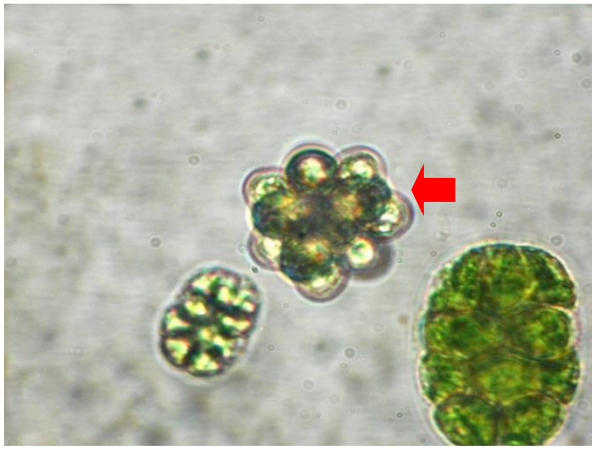


Senedesmus sp.

Phacus sp.

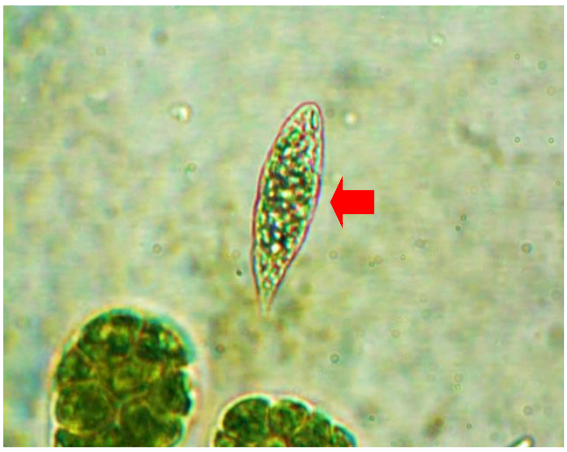


Trachelomonas sp.

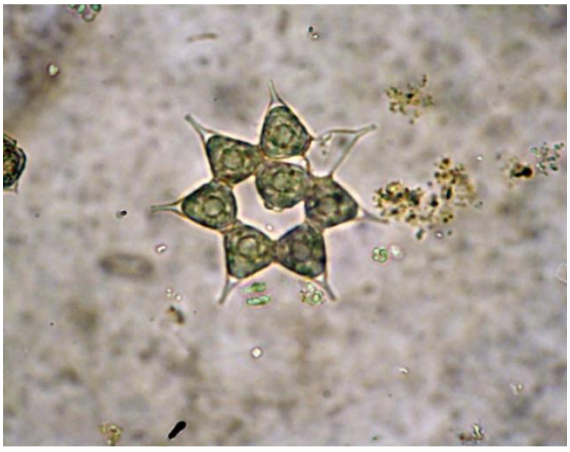


Coelastrum sp.

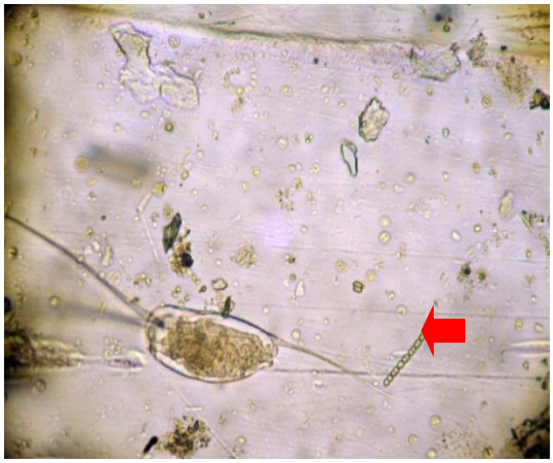
Plate1.1. Identified phytoplanktons during the study periods (Jan 12 to Dec 13).



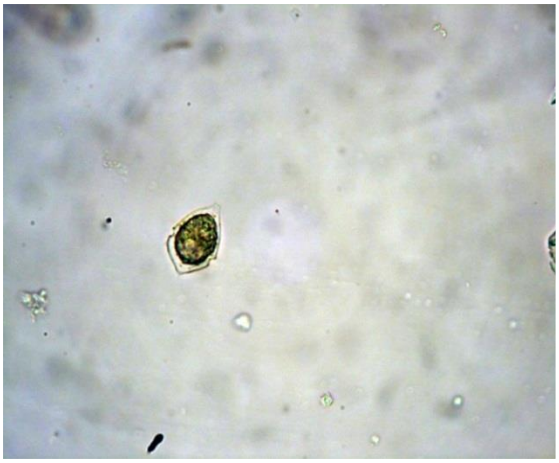
Euglena sp.



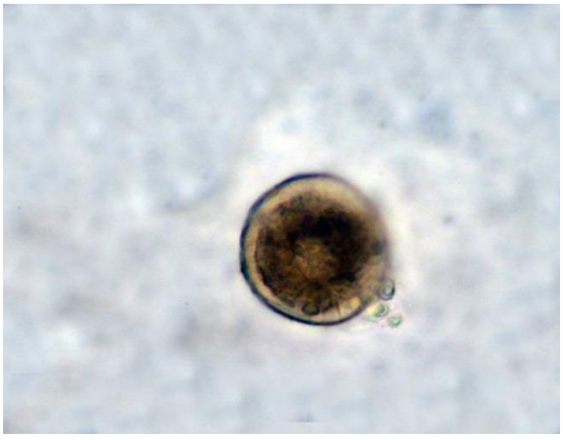
Pediastrum sp.



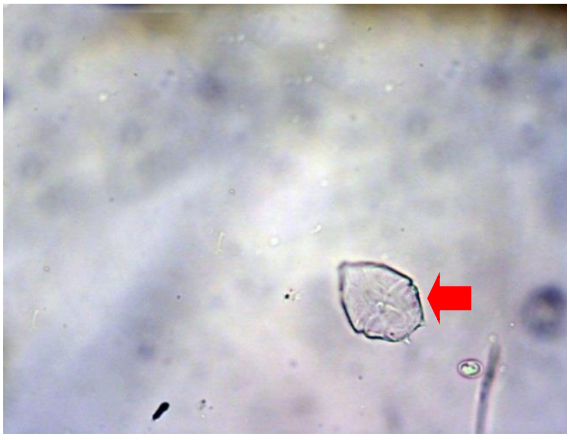
Anabaenopsis sp.



Peridinium sp.

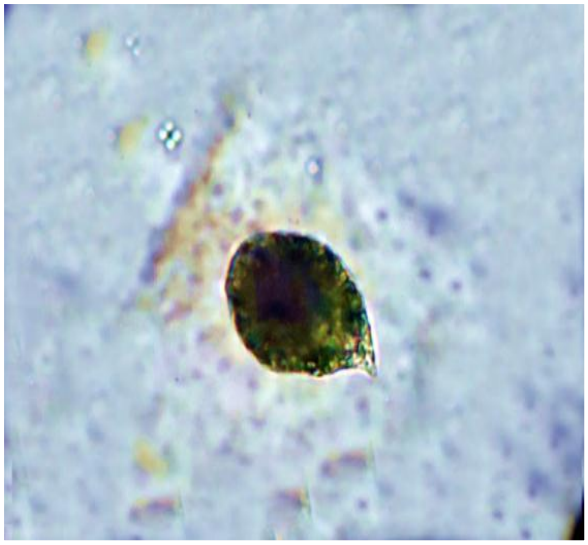


Arcella sp

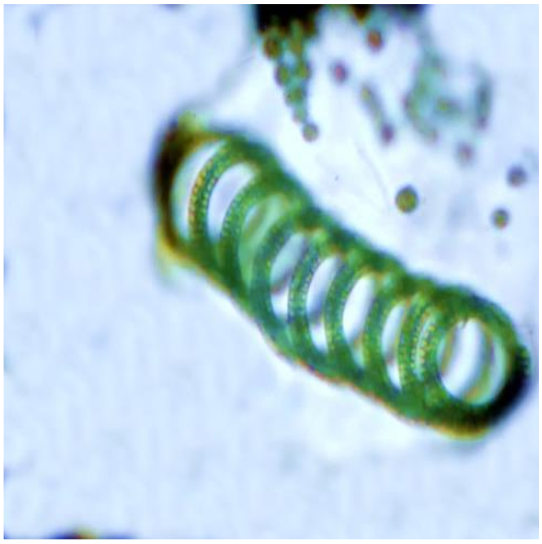


Peridinium sp.

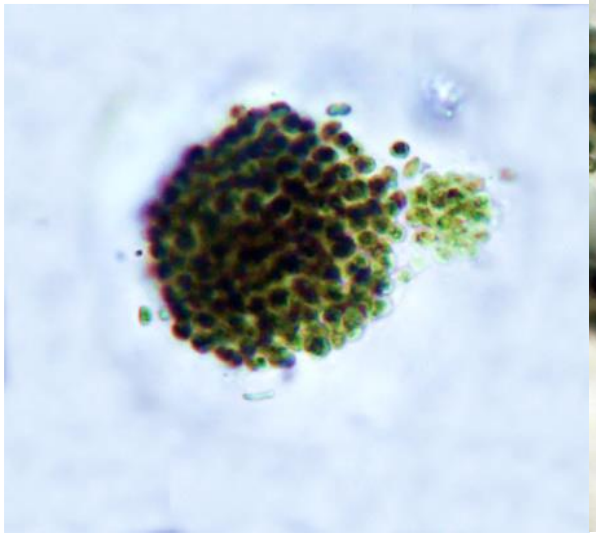
Plate 1.2. Identified phytoplankton during the study periods (Jan 12 to Dec 13).



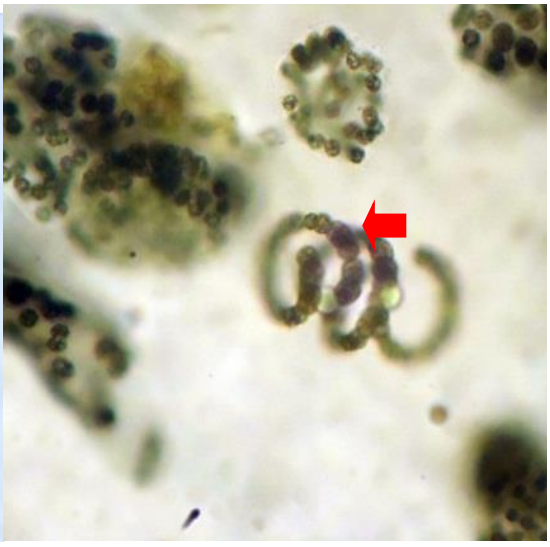
Lepocinclis sp.



Spirulina sp.

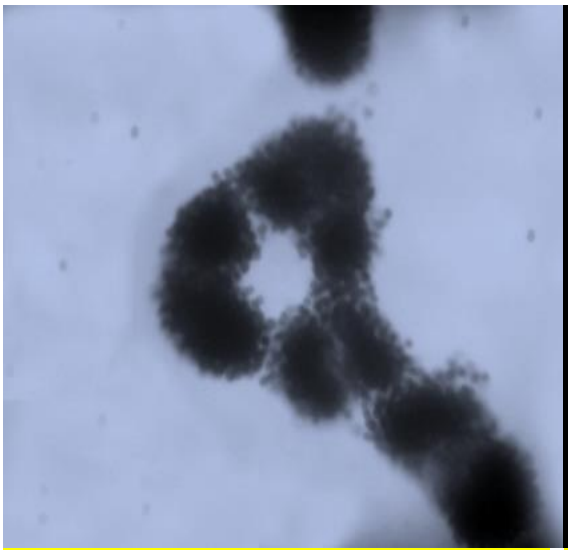


Microcystis sp.

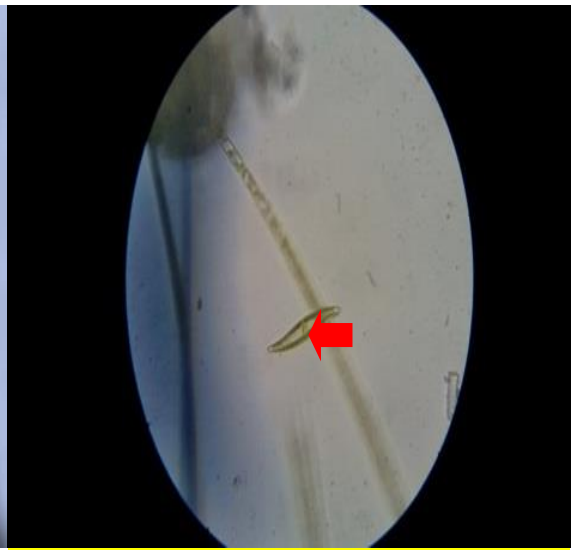


Anabaena sp.

Plate 1.3. Identified phytoplankton during the study periods (Jan 12 to Dec 13).

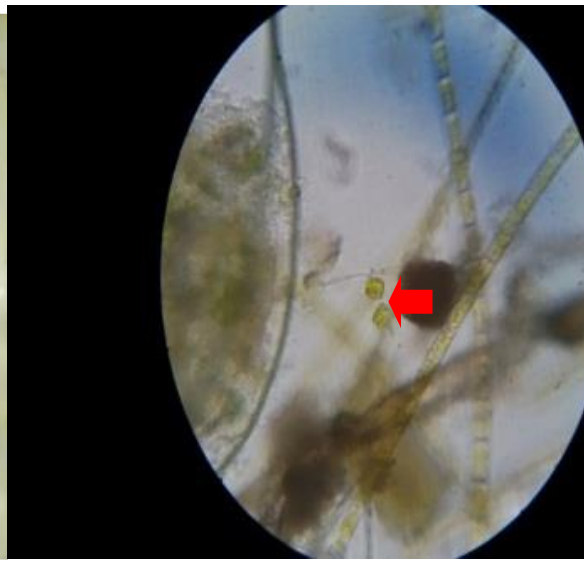


Microcystis sp.



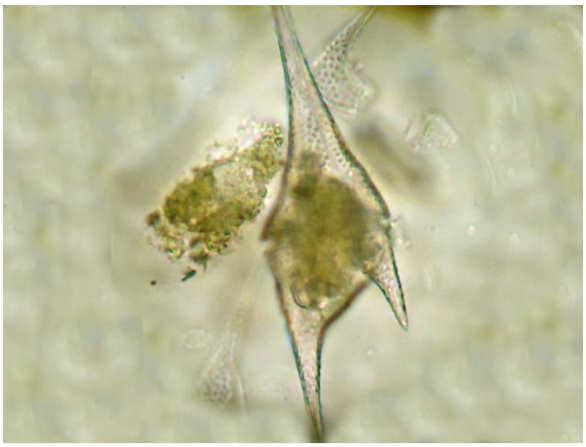
Syndra sp.

Ceratium sp.

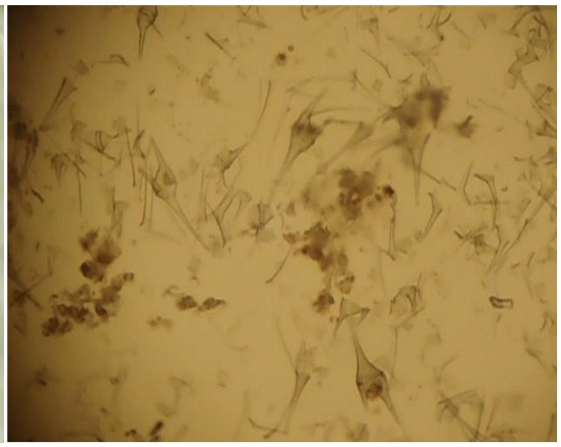


Cyclotella sp.

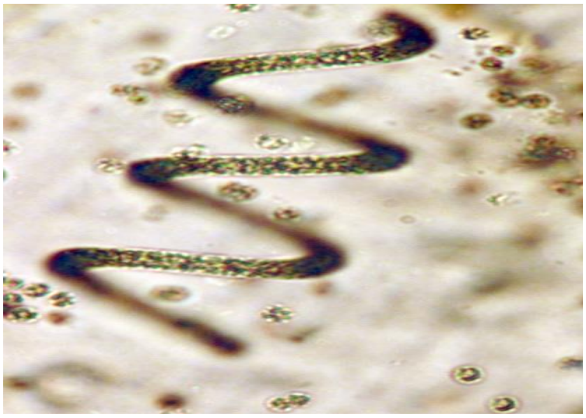
Plate 1.4. Identified phytoplankton during the study periods (Jan 12 to Dec 13).



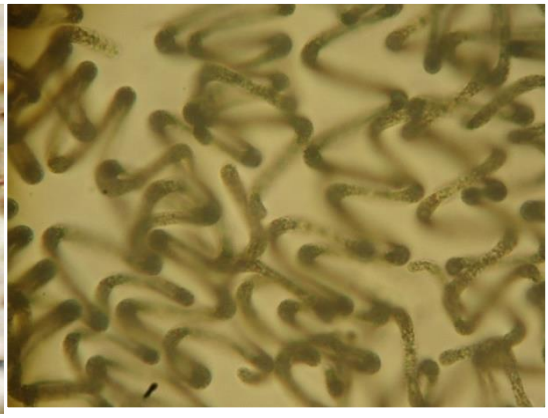
Ceratium sp.



Bloom of *Ceratium* sp.

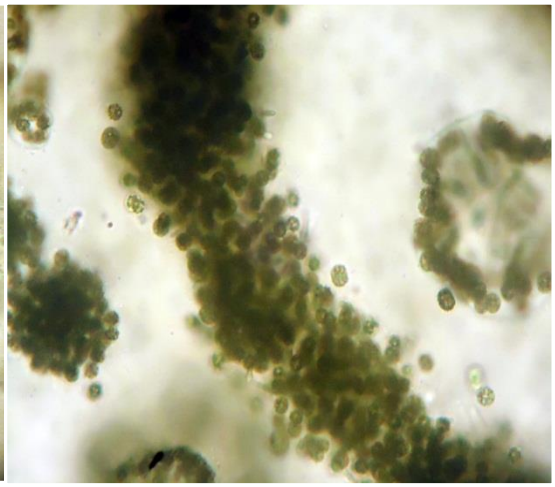


Spirulina sp



Bloom of *Spirulina* sp.

Microcystis sp



Bloom of *Microcystis* sp

Plate 1.5. Identified phytoplankton during the study periods (Jan 12 to Dec 13).

In case of P1, among plankton families Ceratiacea (81.36%) represent the highest number followed by Euglenophyceae (11.76%), Chlorophyceae (5.14%), Peridiniaceae (0.63%), Bacillariophyceae (0.40%) and Cyanophyceae (0.06%). For P2, Peridiniaceae (28.67%) represent the highest number followed by Ceratiacea (26.67%), Euglenophyceae (25.67%), Cyanophyceae (5.31%), Chlorophyceae (1.36%) and Bacillariophyceae (0.40%). For P3, Cyanophyceae (72.74%) represent the highest number followed by Euglenophyceae (17.43%), Chlorophyceae (5.32%), Peridiniaceae (0.63%) Bacillariophyceae (0.18%) and Ceratiacea (0.18%). In P4, only three families found, Euglenophyceae (47.23%) represent the highest number followed by Cyanophyceae (45.04%) and Chlorophyceae (7.38%) (Table 3.3.).

Table 3.3. The percentage of phytoplankton according to their families.

	P 1	**P2**	**P3**	**P4**
Cyanophyceae	0.06%	5.31%	72.74%	45.04%
Euglenophyceae	11.76%	25.05%	17.43%	47.23%
Chlorophyceae	5.14%	1.36%	5.32%	7.38%
Bacillariophyceae	0.40%	0.27%	0.18%	0
Ceratiaceae	81.36%	26.67%	0.18%	0
Peridiniaceae	0.63%	28.07%	0.63%	0

It is well known that nitrogen and phosphorus are the most important inorganic element for plankton production. Redfield (1934) has been recognized a ration for N: P is 16:1 for cyanobacterial growth. During study, range of nitrate nitrogen for P1, P2, P3 and P4 are 0-0.2, 0-0.17, 0-0.1, and 0.1-0.55. In the study, a successful *Microcystis* sp. winter bloom has developed in the scarcity of nitrogen in P4. It is noted that P3 and P4 were totally eutrophic ponds and periodic cyanobacterial bloom has occurred. Though cyanobacteria grow well in high nitrogen concentration but cyanobacteria have capacity to survive in unfavorable condition. L. Mhlanga and W. Mhlanga (2013) showed that as Chlorophytes and Cryptophytes population has replaced by *Microcystis aeruginosa* in high ammonium and low nitrite concentration. Ammonia

concentration was found 0.4-1.1 mg/l in P3 and 0.6-5 mg/l in P4. By the glutamine synthetase, ammonium is converted in glutamate which is the main product in the synthesis of nitrogen-containing metabolites (Flores & Herrero, 1994). Moreover, certain cyanobacteria can overcome nitrogen deficiency by nitrogen fixation occurred in specialized cell, heterocyst (Stewart, 1980). *Microcystis* sp. also achive same purpose by continuous and rapid biochemical intake of oxygen for nitrogen fixation. In addition phosohate-phosphorus level is high in all ponds which are symbol of eutrophication and almost all ponds received anthropogenic wastage from other sources. Though N-P concentration is moderate high in P1 and P2 but these ponds had low cyanobacterial concentration. During the site selection P1 was covered by *Microcystis* bloom and had two inlets opening from which external water introduced into the pond and bring heavy nutrient loading. During the beginning of the study, this inlet was closed which causes reduction of loading. From that *Microcystis* bloom is totally disappeared and *Ceratium* had introduced and formed bloom on March-April 2013. Similarly finding had reported in lake Biwa, Japan, eutrophicated from 1950-1970 due to human activities. A number of control measures in sewage treatment have taken and TP was reduced to < 10 μgL^{-1} and plankton biovolumn was low as <0.07 mm^3. Instead of cyanobacteria dominancy, a dinoflagellate *Ceratium sp.* in May and June. It is pointed that, changes in the plankton community are the good indicator of environmental quality. *Ceratium* sp, is known as harmful phytoplankton and causing eutrophication in marine ecosystem (Waterbury, 1992). Toxicity of *Ceratium* sp. in freshwater not yet reported. So, further research should carryout to identify it. Again percentage of Peridiniaceae in different ponds was P1 (0.63%), P2 (28.07%) and P3 (0.63%). P4 was devoid of this species. This species is good for water body because *Perimidium* has algicidal value. *Peridinium gutanense*, a bloom forming dinoflagellate in Lake Kimmeret, Israel, influences toxin production in *Microcystis* sp., bloom forming cyanobacteria in this lake (Vardi et al., 2002). *P. bipes eas* has shown to have an algicidal effect on *M. aeruginosa* (Wu et al., 1998). In the presence of *P. gatunensis*, *Microcystis* cells lost buoyancy, followed by cell lysis and a dramatic increase of McyB, a subunit of the peptide synthetase complex involved in microsystin biosynthesis (Vardi et al., 2002).

Euglenophyceae was the second dominant class in this study (Table.3). Euglenophyta are mixotroph and can alternate the carbon source. For example *Euglena* has the

advantage to keep a double ecological niche (Reynolds, 2006). They can feed on primary production or from particulate organic matter present on water.

3.3.3. Bloom and bloom forming species

May and June 2013 and cell concentration was 15000 colony/L and 11000 colony/L respectively. In P4 mixed algal bloom was found from September 2012 to July 2013. *Spirulina* (5500 cells/L) and *Lepicinclis* (81000 cells/L) were appeared in September 2012. *Microcystis (*9000 cells/L), *Spirulina (*4000cells/L) and *Trachelomonas* (70050 cells/L) were found in December 2012. Only *Microcystis* bloom was found in June 13 where cell concentration was 30,000 colony/L (Table 3.4.).

Table 3.4. Number and species responsible for bloom formation in different months (2012-2013).

Months	**species**	**P1**	**P2**	**P3**	**P4**
July 12	*Chroococcus*	-	-	-	85000 cells/L
Aug 12	*Trachelomonas*	21600 cells/L	-	-	-
Sep 12	*Spirulina* *Lepocinclis*	-	-	-	55000 cells/L 81000 cells/L
Nov 12	*Coelastrum*	8200 cells/L	-	-	-
Dec 12	*Microcystis* *Spirulina* *Trachelomonas*				9000 colony/L 4000 cells/L 70050 cells/L
Jan 13	*Microcystis* *Spirulina*	- -	- -	- -	10000 colony/L 8500 cells/L

Months	species	P1	P2	P3	P4
Mar 13	*Ceratium*	90000cells/L	-	-	-
Apr 13	*Ceratium*	8000 cells/L	-	-	-
May 13	*Microcystis*	-	-	15000 colony/L	-
Jun 13	*Microcystis*	-	-	11000 colony/L	30000 colony/L

3.3.4. Diversity status

The value of Shannon Weiner diversity index (H′), Dominance (D), Margalef's richness (M) and Buzas and Gibson's evenness was calculated as per station and season (Fig. 3.6.). The maximum margalef's richness value was observed 1.701 at P3 and lowest was 1.21 at P2.

Buzas and Gibson's evenness was ranged between 0.125 (at P1) to 0.3911 (at P4). Highest dominance value (0.6769) was observed in P1 and lowest value (0.1682) was observed in P4. According to Shanon diversity index showed P1 was less diversified and had highest dominance. Other ponds were moderately diversified but Buzas and Gibson's evenness was low (0.12-0.39) which means species were not evenly distributed. Low value of Buzas and Gibson's evenness (< 1) indicates polluted community. On the other hand, about 22 genera were found in study period where as 15 genera were pollution tolerant genera according to Palmer Pollution Tolerant Index (1969). *Euglena, Senedesmus, Navicula, Phacus, Cyclotella, Pandorina, Lepocinclis, Anabaena, Padiastrum, Arthrospira, Trachelomonas, Actinastrum, Coelastrum, Dictyosphaerium, Crucigenia* were pollution tolerant species which were found during study period.

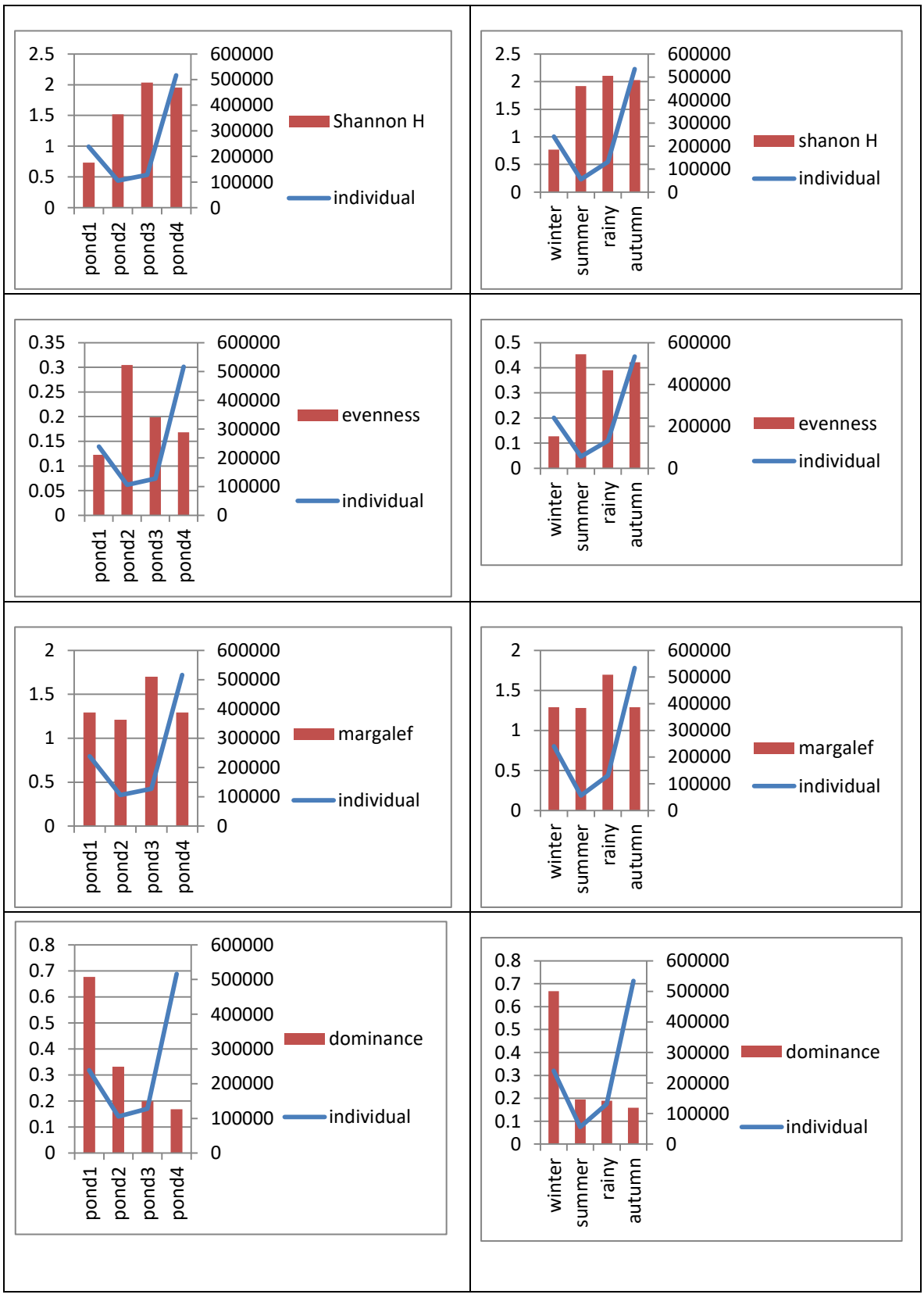


Fig. 3.6. Diversity indices of phytoplankton at different ponds in relation to seasons.

3.3.5 Spatial and temporal relationship of phytoplankton community

The analysis of similarity (ANOSIM) showed significance difference (5%) in assemblage structure among ponds (R= 0.05955; P=0.001) and months (R= 0.2149; P=0.001) (Fig. 3.7.; 3.8.). Among the seasons winter-summer and winter-rainy season showed significance difference among them, whereas other season shows similarity among them (Table 3.5.).

Table 3.5. Overall average dissimilarity and discriminating species in ponds and seasons using SIMPER analysis.

POND					
ANOSIM			**SIMPER**		
Groups	**R**	**p 5% level of significance**	**Average dissimilarity (%)**	**Most discriminating species**	**Contribution (%)**
P1×P2	0.189	0.000	93.44%	*Ceratium* sp.	20.1%
P1×P3	0.118	0.001	94.34%	*Ceratium* sp.	13.64%
P1×P4	0.348	0.000	98.82%	*Microcystis* sp.	26.62%
P2×P3	0.127	0.000	91.48%	*Microcystis* sp.	13.19%
P2×P4	0.443	0.000	97.73%	*Microcystis* sp.	28.28%
P3×P4	0.156	0.000	91.25%	*Microcystis* sp.	30.27%
Seasons					
W×S	0.068	0.021	94.28%	*Microcystis* sp.	20.19%
W×R	0.083	0.008	94.61%	Microcystis sp.	14.34%
W×A	0.067	0.982	93.49%	*Peridinium* sp.	29.4%
S×R	0.005	0.370	91.52%	*Microcystis* sp.	22.85%
S×A	0.065	0.077	93.41%	*Microcystis* sp.	19.76%
R×A	0.013	0.368	92.28%	*Microcystis* sp.	14.53%

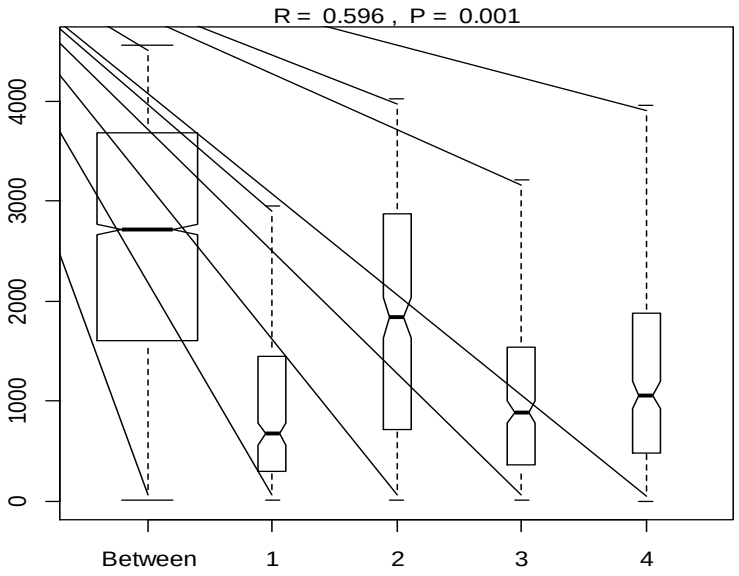


Fig. 3.7. Overall dissimilarity analysis among ponds by ANOSIM analysis.

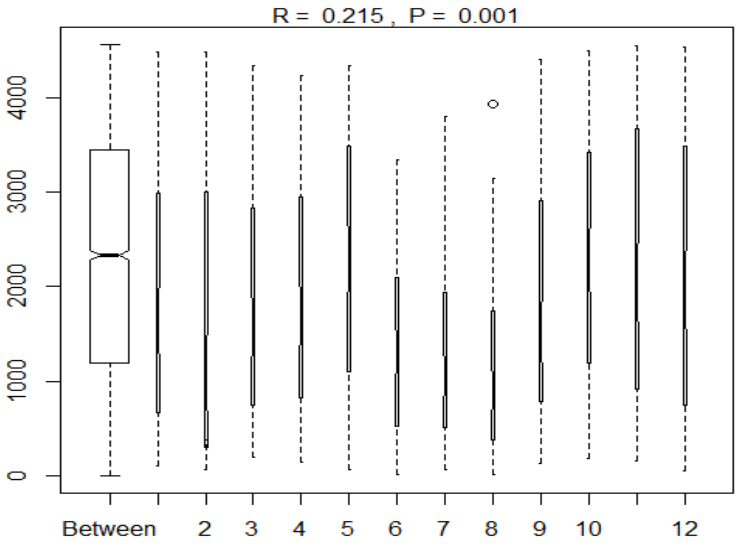


Fig. 3.8. Overall dissimilarity analysis among months by ANOSIM analysis.

Table 3.6. Average dissimilarity and discriminating species in ponds and seasons.

PONDS (overall)		**SEASONS (overall)**	
Average dissimilarity	**94.52%**	**93.24%**	
species	**contribution**	**species**	**contribution**
Microcystis sp.	18.52%	*Microcystis* sp.	17.31%
Trachelomonas sp.	8.88%	*Ceratium* sp.	9.55%
Ceratium sp.	8.82%	*Trachelomonas* sp.	8.78%
Lepocinclis sp	8.73%	*Lepocinclis* sp.	8.41%
Spirulina sp.	8.21%	*Spirulin*a sp.	8.05%
Pediastrum sp.	5.9%	*Pediastrum* sp.	5.98%
Arcella sp.	5.49%	*Peridinium* sp.	5.92%
Merismopedia sp.	5.44%	*Euglena* sp.	5.61%
Euglena sp.	5.37%	*Merismopedia* sp.	5.57%
*Peridiniu*m sp.	5.15%	*Arcella* sp.	5.29%

According to similarity percentage (SIMPER) overall 94.52% dissimilarity was found among ponds and major contributory species were *Microcystis* sp. (18.52%), *Trachelomonas* sp. (8.88%), *Ceratium* sp.(8.82%), *Lepocinclis* sp. (8.72%), *Spirulina* sp.(8.21%), *Padiastrum* sp.(5.91%), *Arcella* sp.(5.49%), *Merismopedia* sp.(5.44%), *Euglena* sp.(5.37%) and *Peridinium* sp.(5.15%). On the other hand, 93.24% dissimilarity were observed among seasons and major contributing species were *Microcystis* sp. (17.31%), *Ceratium* (9.55%), *Trachelomonas* sp.(8.78%), *Lepocinclis*

sp.(8.41%), *Spirulina* sp.(8.05%), *Pediastrum* (5.92%), *Euglena* sp.(5.61%), *Merismopedia* sp.(5.57%) (Table 3.6.)

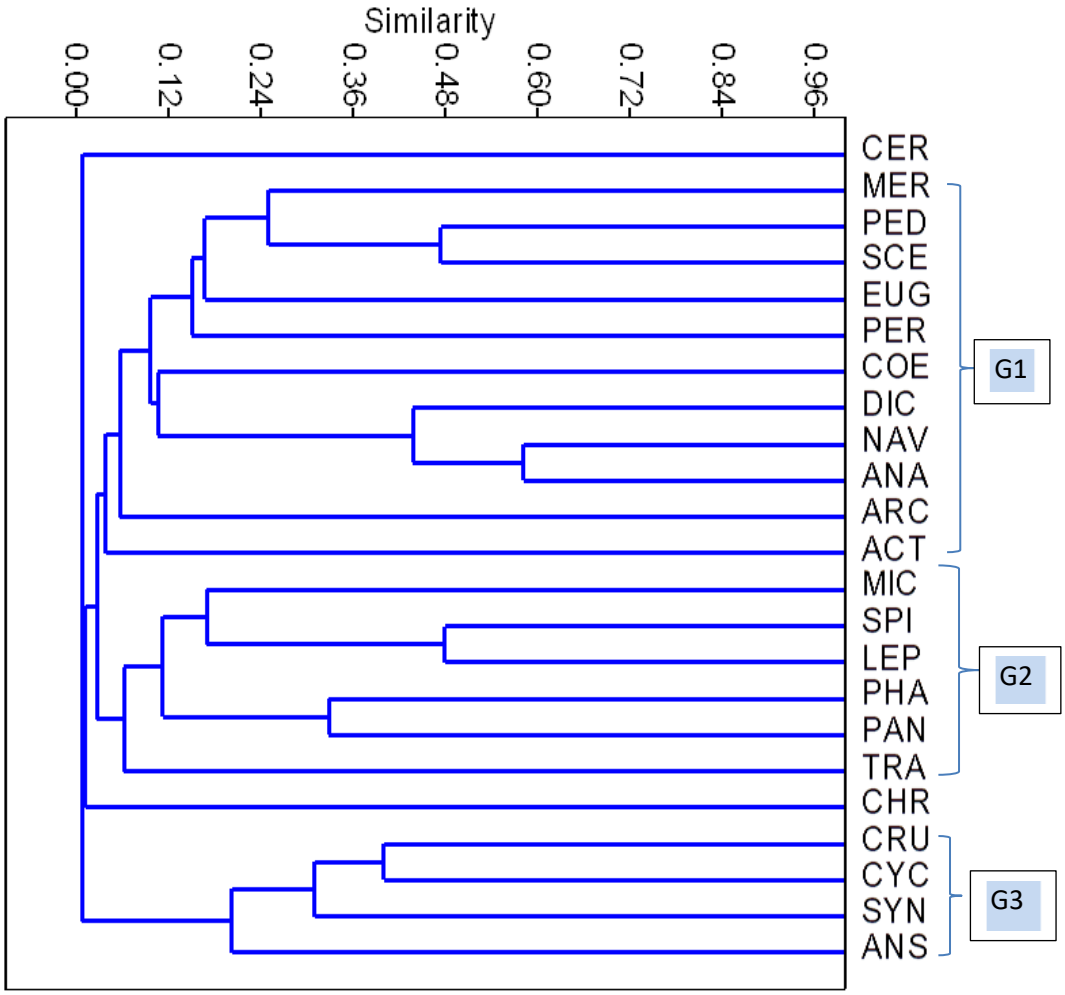


Fig. 3.9. Dendogram showing cluster based on Bray-Curtis similarity matrix on each-composition.

At the level of 6% similarity for ponds, plankton was classified by cluster analysis (Fig. 3.9.). No marked separation of the genera was observed except *Ceratium* sp. and *Chroococcus* sp.. At the similarity of 6%, three groups were attained while two genera (*Ceretium* sp., *Chroococcus sp.*) were remained isolated. *Merismopedia* sp.,

Pediastrum sp., *Senedesmus* sp., *Euglena* sp., *Peridinium* sp., *Coelastrum* sp., *Dictyospharium* sp., *Navicula* sp., *Anabaena* sp. , *Arcella* , sp. *Actinastrum* sp. were in group one (G1). Among the three groups, second group (G2) contains *Microcystis* sp., *Spirulina* sp., *Lepocisclis* sp., *Phacus* sp., *Pandorina* sp. and *Tracheolomonas* sp. where first three genera found bloom forming species in the study or most contributing species in the SIMPER analysis. *Crucigenia* sp., *Cyclotella* sp., *Syndra* sp., *Anabaenopsis* sp. were in the third group (G3).

ANOSIM showed all the ponds were significantly different from each other. From the SIMPER analysis overall 94.52% dissimilarity found between the pond and the most five dominating species for this dissimilarity are *Microcystis* sp., *Spirulina* sp., *Lepocinclis* sp., *Trachelomonus* sp., *Ceratium* sp., which were mostly found bloom forming species in the study.

3.3.6. Canonical Correspondence Analysis

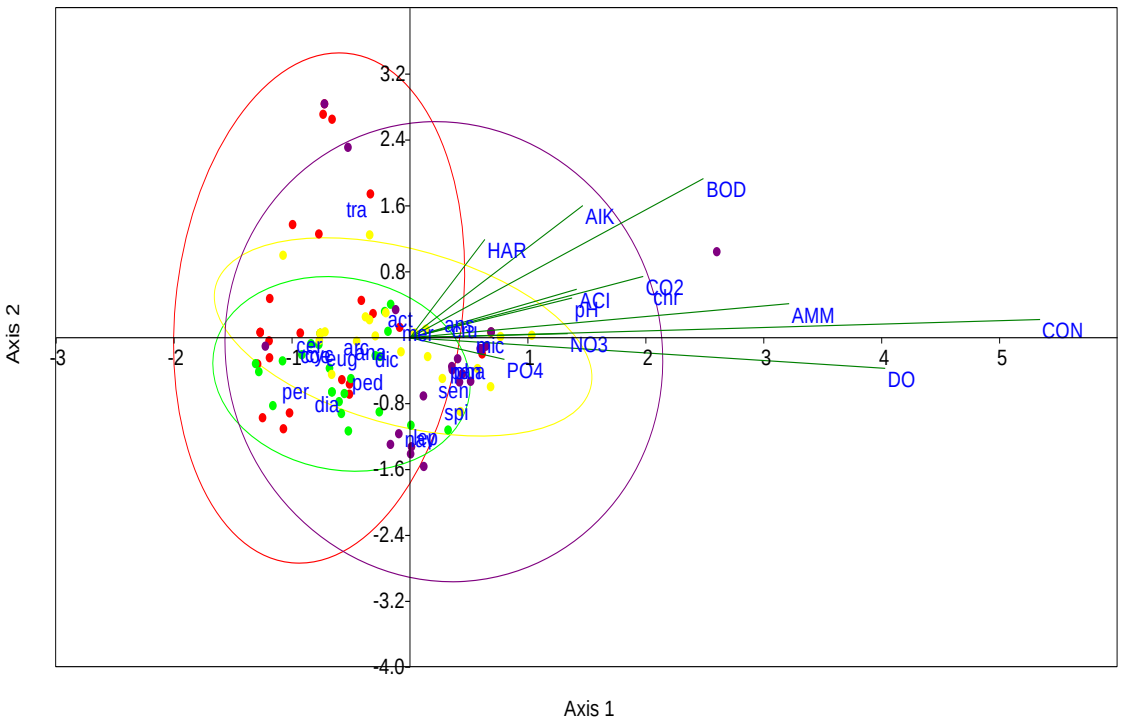


Fig. 3.10. The CCA ordination of species abundance and environmental parameters.

Eigenvalues of CCA percentage value and p-value (hydrological parameters) for the first eleven axes has been given in table 3.7. The result obtain from the first two axes were plotted in Fig. 3.10..

Table 3.7. Eigenvalues of CCA percentage value and p-value (hydrological parameters) for the first eleven axes.

Axis	Eigenvalue	Percentage	p-value
1	0.78	28.39	0.16
2	0.60	21.82	0.16
3	0.54	19.84	0.03
4	0.22	8.19	0.61
5	0.19	6.97	0.15
6	0.18	6.73	0.02
7	0.08	3.06	0.07
8	0.07	2.63	0.01
9	0.04	1.51	0.02
10	0.02	0.85	0.01
11	5.798E-05	0.00	0.15

The CCA sample biplots shows environmental parameters potentially influencing the phytoplankton. The length of the environment parameters indicated the influencing the phytoplankton. The length of the environmental variable arrowed represented the relative explanatory of each variable in relation to individual sample positions within the ordination. For the analysis of relationship between the phytoplankton and environmental parameter pearson correlation and CCA (Canonical Correspondence Analysis) has done. NO_3-N, BOD, ammonium and turbidity showed positive significance relationship (Pearson Correlation) with phytoplankton (Table 3.8.). Conductivity, Ammonium, DO, BOD, NO_3-N had positive correlation with axis one. Several phytoplankton species such as *Microcystis* sp., *Spirulina*, sp. *Lepocinclis* sp., *Merismopodium* sp., *Anabaenopsis* sp. (Cyanophyceae) and *Senedesmus* sp., *Phacus*

sp., *Pandorina* sp. (Chlorophyceae), which distributed at the right side of Axis1, were positively related correlated with the content of nutriments such as ammonia, NO_3-N, PO_4. *Ceratium* sp., *Cyclotella* sp., *Euglena* sp. *Pediastrum* sp., *Peridinium* sp., *Trachelomonas* sp. negatively related with the nutrient.

Table 3.8. Pearson correlation showing correlation between physico-chemical parameters and phytoplankton at 1% level of significance.

		ALK	ACI	HAR	pH	DO	CO2	NO3-N	PO4-P
PHYTO-PLANK-TON	PEARSON CORRELATION	0.147	0.146	0.168	0.111	0.148	0.128	0.316	0.121
	SIG (2-TAILED)	0.153	0.157	0,101	0.282	0.151	0.215	0.002	0.240
	N	96	96	96	96	96	96	96	96
		BOD	AMM	TOW	TOA Max	TOA Min	CON	TUR	RAI
	PEARSON CORRELATION	0.421	0.290	0.081	0.046	0.038	0.408	0.350	0.050
	SIG (2-TAILED)	0.000	0.004	0.433	0.633	0.711	0.000	0.000	0.630
	N	96	96	96	96	96	96	96	96

Correlation is significant at the 0.01 level (2-tailed).

Harmful cyanobacterial bloom (HABs) are diagnostic of accelerating eutrophication in freshwater ecosystems (Paerl, 1996; Stahl-Delbanco, 2004). Presence of harmful algal bloom species like *Microcystis* sp. and other toxic plankton e.g. *Ceratium* sp., *Tracheolomonas* sp. in leading state in the ecosystem/community is not a good sign. However, *Arthrospira/Spirulina* bloom was found in combine with *Microcystis* bloom

at P4. *A. fusiformes* was identified as one source of microcystins and anatoxin-a in Lake Bogoria and Lake Nakuru. In a cultured strain of *A. fusiformes* from Lake Bogoria, microcystis-LR and anatoxin-a and anatoxin-a in a strain from Lake, Nakuru, Kenia was identified. There are important finding, because the genus *Arthrospira/Spirulina* is regarded as nontoxic (Ciferri, 1983; Jassby, 1988). Several members of this genus are widely used in mass culture as a source of food, animal feed, and specific chemicals in subtropical and tropical countries (Vonshak, 1987, 1997).

It is reported harmful Algal bloom found most frequent in the Mymensingh area (Welker et al., 2005; Roksana et al.,. 2009). Present study also found harmful algal blooms. The development of cyanobacteria blooms has become serious problem because in the past decades many cyanobactria have been reported to be able to produce secondary metabolic toxic to many organisms, including humans (Gorham and Carmichael, 1988; Codd et al., 1995; Codd, 2000; Briand et al., 2003; Haider et al., 2003; WHO, 2003). Knowledge of temporal and spatial dynamics of phytoplankton and relationship with water parameters necessary to understand eutrophication effects. Present study demonstrated that nutritional effects may lead certain type specially cyanobacteria production. So sustained integrated monitoring of the water body is strongly recommended to mitigate eutrophication problem in Bangladesh.

4.1. Introduction

Bloom formation due to excessive growth of certain cyanobacteria followed by the production of toxic compounds have been reported in many eutrophic to hypertrophic lakes, ponds, and rivers throughout the world (Rastogi et al., 2014). Anthropogenic eutrophication and global climate change have created harmful algal blooms and contaminated the surface waters. Cyanobacterial blooms and the accumulation of several toxins, called cyanotoxins, in water bodies pose ever ecological consequences with high risk to aquatic organisms and global public health (Rastogi et al., 2015). The cyanotoxins are responsible for intermittent but repeated widespread poisonings of wild life, domestic animals, fish (Carbis et al., 1996) and human (Carmicael, 1994). One of the most studied groups of cyanotoxins is the cyclic heptapeptides called Microcystins (MCs). There are 80 different variants of these MCs. Microcystin-LR and LA are the most common and the most toxic forms which occur more often in cyanobacterial blooms (Dawson,1998). MCs have strong affinity to serine/threonine protein phosphatases thereby acting as an inhibitor of type 1 and type 2A phosphatases (Richard et al., 1990; Campos and Vasconcelos, 2010). This cyclic peptide interacts with the mitochondria of animal cells triggering oxidative stress and apoptosis (Campos and Vasconcelos, 2010). In China, incidences of primary liver cancer (Ueno et al., 1996) and colorectal cancer (Zhou et al., 2002) have been associated with MCs contaminated drinking water. Additionally, tumor promotion and liver injury caused by oral consumption of MCs pose serious health risks (Falconer, 1991). The World Health Organization (WHO) has set a tolerable daily intake (TDI) for chronic exposure to MC-LR of 0.04 μg/kg body weights and a provisional guideline value of 1.0 μg/L MC-LR for drinking water (WHO, 1998, Falconer et al., 1999). The guideline values of cyanotoxins in drinking water for different countries are shown in Table 4.1.

Moreover, MCs are heat stable compounds, and neither boiling water nor cooking fish prior to consumption is expected to reduce the potential for exposure (Harada, 1996, Zhang et al., 2010). Magalhaes et al. (2003) showed that aquatic animals can

bioaccumulate MCs (cyanobacteria hepatotoxins). High concentrations of MCs have been detected in piscivorous and phytoplanktivorous fish (Xie et al., 2005). Studies have demonstrated that MCs accumulate in several fish tissues, such as gut, liver, gills, kidney, bile, muscle, blood, heart, and brain (Mohamed et al., 2003; Xie et al., 2005; Cazenave et al., 2005; Deblois et al., 2008; Lei et al., 2008).

Table 4.1. Guideline value of cyanotoxins in drinking water different countries.

Toxin	**Drinking water guideline values**	**Countries using the GV**	**Reference**
MC-LR	1.0 µg/L (most generally accepted)	Brazil, Czech Republic, Denmark, France, Great Britain, Greece, Italy, New Zealand, Poland, Portugal, South Africa, Spain and U.S.A.,	Chorus, 2005; Codd et al., 2005; Van Apeldoorn et al., 2007;
MC-LR	1.3 µg/L	Australia, Canada	Chorus, 2005; Van Apeldoorn et al., 2007;

Toxin	Drinking water guideline values	Countries using the GV	Reference
Nodularin	No guideline, However, hazard assessment can be guided by that for microcystins		Fitzgerald et al., 1999; Chorus,2005; Van Apeldoorn et al., 2007;
	1.0µg/L	New Zealand	
Anatoxin-a	3.0µ/L (no official guideline)		Codd et al.,2005; Svrcek & smith, 2004;
	6.0µg/L	New Zealand	Chorus, 2005;
Homoanatoxin-a	2.0 µg/L	New Zealand	Chorus, 2005;
Anatoxin-a	nd		Chorus,2005;
	1.0µg/L	New Zealand	
Cylindrospermopsin	1.0 µg/L-2.0 (suggested)	Canada, New Zealand	Humpage & Falconer, 2003; Svrcek & smith, 2004; Chorus,2005;
	15.0µg/L	Brazil	
STX	3.0 µg STX eq/L	Australia, Brazil, New Zealand	Svrcek & smith, 2004; Chorus, 2005; Codd et al.,2005;
Aplysiatoxins	nd		
Lyngbyatoxins	nd		

nd-not detected

About 307 different species of cyanobacteria have been reported from all kinds of water sources (river, canal, ponds, ditches, lakes etc.) in Bangladesh (Siddiqui et al., 2007). Among them 13 species frequently form blooms (Islam, 1991). MCs have been reported in freshwater ponds from different locations of the country (Ahmed et al., 2000; 2008; 2009; 2014) and their concentrations were well above the provisional WHO guideline value (1µg/L MC-LR). Ahmed et al. (2000) first characterized MCs from Chandpur pond and also reported MCs from other parts of the country (Ahmed, 2008; 2009). Recently Affan et al. (2015) have studied 23 water sources in Mymensingh district and 22 cyanobacterial bloom samples were found while microcystin concentrations ranged from 25-82300 pg/ml. Even in tap water, microcystins were detected in concentrations ranging from 30 to 32 pg/ mL, which were very alarming for public health safety (Table 4.2.).

Table 4.2. Harmful algal blooms (HABs) species composition and amounts of microcystins in eutrophic pond, Bangladesh.

City	**HABs Species (abundance)**			**Microcystins**				**References**
	Microcystis	***Plankto-thrix***	***Anabaena***	**Total MCYST**	**MC-LR**	**MC-RR**	**MC-YR**	
Mymen-singh	1.1-6.425 mm^3L^{-1}	3-897 mm^3L^{-1}	1.2-109.7 mm^3L^{-1}	0.1-1.390				Welker et al., 2004
Chadpur	0.4-510.6 mm^3L-1	0.7-22.1 mm^3L^{-1}	2.3-2.9 mm^3L^{-1}	0.14-268				Welker et al., 2004
Gazipur	6.22×108 Cell L^{-1}				33.2	9.03	5.23	Ahmed et al., 2007
Dhaka	+++				34.8	16.8	10.9	Ahmed et al., 2009
Mymen-singh (farm pond)	+++				27.8			Affan et al, 2001

Mymen-singh (research pond)	+++				82.3			Affan et al., 2001
Mymen-singh (rural pond)	+++				21.6			Affan et al., 2001
Mymen-singh	303.13×103 Cells L^{-1}	739×103 Cells L^{-1}	2468.75×103 Cells L^{-1}	No Data				Jahan et al., 2010
Mymen-singh	72.80×103 Cells L^{-1}	*Aphanizomenon flos-aqua(*130.5×105 cells L-1)		No Data				Jewel et al., 2003
Mymen-singh				25-82300 Pg/mL				Affan et al., 2015

Freshwater fish farming plays an important role in rural livelihood and contributes 55% to total fish production (FRSS, 2014) in Bangladesh. Excessive use of artificial feed and lack of scientific management create eutrophication leading to cyanobacterial bloom formation (Ahmed et al., 2014). Cyanobacterial bloom at time commonly encountered in close or semi closed freshwater and brackish water bodies in Bangladesh like any other countries. These toxins (Microcystins) may find their excess into human body through various aquatic foods including finfish and shellfish. Thus, potential health hazard from consumption of fish cultured in eutrophicated pond is a major concern. So, detection of microcystins from different blooms of cyanobacteria and evaluates the possible health risk is time earnest issue.

4.2. Materials and Methods

4.2.1. Collection of *Microcystis aeruginosa* bloom

M. aeruginosa bloom was started in March 2013 and the highest cell density (95%) was observed in May 2013 in P3. On the other hand, *M. aeruginosa* bloom started in November 2012 and the highest cell density (95%) was observed in June 2013 in P4. The bloom sample was collected with plankton net (no. 55 bolting silk plankton net). The concentrated samples were filtered through an 0.45µm glass fiber filter (Whatman GF/C, 47 mm diameter) and dried in an oven at 60-70˚C. In the original bloom sample the cell density of *M. aeruginosa* was 3×10^4 colony/l recorded by a Sedgewick-Rafter counting chamber (S-R cell) under a compound microscope at ×400 magnifications.

4.2.2. Extraction of toxins

4.2.2.1. *Bloom filter*

The GF/C filters were extracted with 2.0 ml water/methanol (50:50; v:v) per filter by ice-cooled sonication for 4 min with an ultrasonic probe GM 70 (Bandelin, Berlin, Germany).The extract was sonicated for another 15 min in an ultrasonic bath. Extracts were centrifuged (10000 g, 15min) and the supernatants were filtered using 0.22 µm nylon syringe filters (Roth, Karlsruhe, Germany). The extracts were directly subjected to the liquid chromatography.

4.2.2.2. *Chemical analysis*

The HPLC/UV determination of microcystins was carried out following the method of Lawton *et al.* (1994a) with some modifications. Separation was performed using a C18 column (Phenomenex prodigy, ODS (3), 250 x 4.6mm, 5 µm) and acetonitrile /water/0.05% TFA as the mobile phase.Microcystinswere detected using an UVdetector

(Shimadzu SPD-10AV; λ=238 nm). HPLC/MS-MS analyses were applied to confirmthe identity ofthe toxins.HPLC-MS/MS was performed using Shimadzu HPLC LC-20A coupled to an ABSciex 4000 QTrapwith an electrospray interface. Analytes were separated on a Phenomenex Gemini 5 µm C18 column (150*3 mm) with a guard column and a mobile phase consisting of 5 mM ammonium acetate in water/acetic acid (99:1; v/v) (eluent A) and 5 mM ammonium acetate in methanol/water/acetic acid (97:2:1; v/v/v) with a flow rate of 0.4 ml/min. Elution started with 60% eluent A and 40% eluent B.

4.2.2.3. *Quantification*

Since reference materials for desmethylated MCs are not available commercially, determination of the concentrations of desmethyl-MCs, [D-Asp3, Dha7] MC-LR, and [Dha7] MC-LR, was performed using the standard calibration curves of MC-LR.

Chemicals

Reference standards of MC-RR, -LR, -YR, -LA, LF and –LW were purchased from Calbiochem/Novabiochem (La Jolla, CA, USA). Acetonitrile and methanol obtained from VWR (Leuven, Belgium) were HPLC grade. All chemicals were at least analytical grade.

4.3. Results and discussion

4.3.1. Microcystis toxins characterization

4.3.1.1. *Dry bloom filtered cell Sample A* (collected from P3 during May'2013)

HPLC analysis of *M. aeruginosa* extract showed five peaks, the retention time of which agreed well with standard MC-RR, MC-YR, MC-LR, MC-WR, dm-MC-RR (Fig. 4.1.). The results of HPLC-MS revealed the identification of five variants of microcystins, according to their corresponding molecular weight MC-LR (at m/z 950.0 $(M+H)^{+}$), MC-RR (at m/z 519.5 $(M+2H)^{2+}$), MC-YR (at m/z 1045.0 $(M+H)^{+}$), MC-WR, dm-MC-RR. In *M. aeruginosa* sample, the concentration of MC-RR was the highest (5.4µg/L) followed by MC-YR (1.14 µg/L), MC-LR (2.0 µg/L), MC-WR (0.46 µg/L) and dm-MC-RR (0.36 µg/L) (Table 4.3.).

Table 4.3. Characterization and concentration of microcystins in *M. aeruginosa* bloom sample A colleted from P3.

	Level of microcystin (µg/L)												
Types of Micro-cystins	MC-RR	MC-YR	MC-LR	MC-LW	MC-LF	MC-LA	Nodularine	MC-WR	dm MC-RR	dm MC-LR	MC-Hty R	MC-LY	Total
Total	**5.4**	**1.14**	**2.0**	**<0.04**	**<0.07**	**<0.07**	**<0.01**	**0.46**	**0.36**	**<0.08**	**<0.1**	**<0.08**	**9.36**

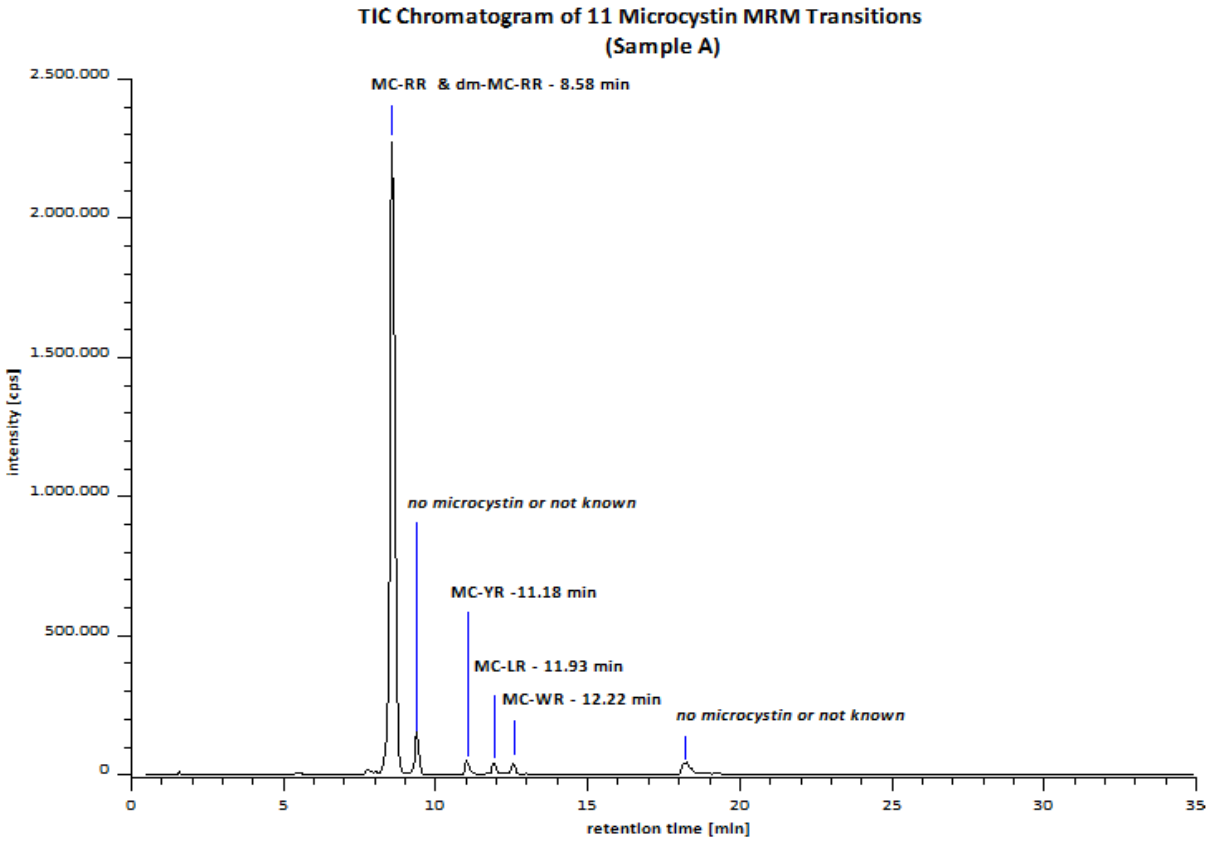


Fig. 4.1. TIC Chromatogram of Microcystins MRM Transition (Bloom filter)

4.3.1.2. *Dry bloom filtered cell Sample B* (collected from P4 during June'2013)

HPLC analysis of *M. aeruginosa* extract showed two peaks, the retention time of which agreed well with standard MC-RR, dm-MC-RR (Fig. 4.1.). The results of HPLC-MS revealed the identification of two variants of microcystins, according to their corresponding molecular weight MC-RR (at m/z 519.5 $(M+2H)^{2+}$), dm-MC-RR. In *M. aeruginosa* sample, the concentration of MC-RR was the highest (1.0 mg/l) followed by and dm-MC-RR (0.04 µg/l) (Table 4.4.).

Table 4.4. Characterization and concentration of microcystins in *M. aeruginosa* bloom sample B collected from P4.

	Level of microcystin (µg/L)												
Types of Micro-cystins	MC - RR	MC - YR	MC - LR	MC - LW	MC - LF	MC - LA	Nodul a-rine	MC - WR	dm MC-RR	dm MC-LR	MC-Hty R	MC - LY	Total
Total	**1.0**	**<0.1**	**<0.07**	**<0.04**	**<0.07**	**<0.07**	**<0.01**	**<0.1**	**0.04**	**<0.08**	**<0.1**	**<0.08**	**1.04**

Microcystis is the most available microcystin producing cyanobacterial genus in Bangladesh. There are almost 80 different MCs congeners are released from different cyanobacterial strains (Fastner et al, 2002). The most common, and also the most widely studies MCs are MC-LR (position 2, Leucine; position 4, arginine), MC-YR (position 2, tyrosine; position 4, arginine), MC-RR (position 2, arginine; position 4, arginine). Several studies in Bangladesh reported MCs-RR, -YR,-LR, -LA as major microcystins in bloom of *M. aeruginosa* (Ahmed, 2000; Affain, 2001; Welker 2015). Our present studies also detected MC-RR, MC-YR, MR-LR, MC-WR, dm MC-RR in Pond 3. Sample was collected in the highest bloom period and bloom was filter dried. Five MCs congeners were detected. The concentration of MC-RR was the highest (5.4µg/L) followed by MC-YR (1.14 µg/L), MC-LR (2.0 µg/L), MC-WR (0.46 µg/L) and dm-MC-RR (0.36 µg/L; Table 4.3). It is reported that MC-RR found in highest amount which is less toxic than MC-LR and -YR. MC-LR is the most toxic form of microcystin than MC-YR and MC-RR (MC-LR>MC-YR>MC-RR). Eriksson et al. (1990) and Fastner et al. (1995) reported that MC-RR (EC50 1500-4300 nM) is less susceptible than MC-LR (EC50 60-200 nM) in primary rat hepatocytes. In a laboratory toxicity tests, the median lethal dose (LD_{50}) of

MC-LR, -RR and -YR (43, 235.4 and 110.6 µg/kg body weight, respectively), and biochemical and histological variables were determined at 30 min post-treatment and mean time to death (MTD) (Gupta et al., 2003). Based on biochemical and histological studies, MC-LR was found to be the most potent toxin followed by MC-YR and MC-RR. WHO has adopted a provisional guideline value for microcystin-LR based on tolerable daily intake is 1 µg/l for the drinking water. In this studies total MCs was 9.36 µg/g where MC-LR is 2.0 µg/g which is high above than the provisional guideline. More over other microcystins congener are present in the bloom. But risk assessment calculation based on the toxic kinetic and distributive properties of one single microcystins congener (MC-LR) is not adequate. Additionally, Pond 4 with the highest *Microcystis* bio volumes, very little amount microcystins (1.04 µg/L) was found. This is probably due to differences in the predominating species/genotypes in the water body or may dominated by less toxic genotypes or the period /stage when species produce more toxic or not. Numerous MC congeners can concurrently exist in an algal bloom but very little work has been done about the estimation of toxicological potency in bloom episode. So, to evaluate the true toxic potency, detection of all the possible MC variants and evaluate their toxicity is necessary.

5.1 Introduction

Cyanobacterial toxins (Hepatotoxins) are the most frequently found toxins in fresh and brackish water bodies all over the world (WHO, 1999). Microcystins (MCs), the cyclic heptopeptide, are produced by different cyanobacterial genera such as *Microcystis*, *Planktothrix*, *Nostoc*, *Anabaena*, *Oscillatoria*, *Anabaenopsis* and *Hapalosiphon* (Carmicheal and Li, 2006). Among the 80 different variants of this Microcystins, Microcystin-LR is the most common and toxic forms which occurs more often in cyanobacterial blooms (Dawson, 1998). Exposure to cyanotoxins represents a health risk to aquatic organisms, wildlife, domestic animals and humans through drinking, ingestion or contact with either cyanobacteria or toxins from the water (Dietrich and Hoeger, 2005). The toxicity of microcystins is due to inhibition of the catalytic subunits of protein phosphateses1 and 2A (PP1, PP2A). Inhibition of PP1 and PP2A dephosphorylate phosphoseryl or phosphothreonyl proteins lead to hyper phosphorylation of cytoskeletal proteins resulting which causes deformation of hepatocytes (Runnegar et al., 1981). Additionally, tumor promotion and liver injury caused by oral consumption of microcystins and poses serious health risk (Falconer, 1991). Indirect evidence of possible promotion of primary liver cancer, associated with the contamination of surface drinking water supplies by MCs producing cyanobacteria have been studied (Ueno et al., 1996). Moreover, accumulations of MCs in aquatic animals have been reported by many authors (Mohammed et al., 2003; Xie et al., 2005; Deblois et al., 2008; Lei et al., 2008). High concentrations of MCs have been detected in piscivorous and phytoplanktivorous fish (Xie et al., 2005). MCs are heat stable compounds, and neither boiling water nor cooking fish prior to consumption is expected to reduce the potential for exposure (Harada, 1996; Zhang et al., 2010). Fish consumption is the potential route of human exposure to microcystins, as they standing at the top of the aquatic food chain which may create high risk to human. However, the extend of hazard caused by the consumption of fish and shell fish has not yet been assessed in Bangladesh. The potential health hazard through consumption of contaminated fish (aquatic food) should be seriously taken since cooking of such food does not deactivate the said toxins (Zhang et al., 2010). So, this experiment was conducted to know the accumulation of microcystins on liver tissue of *Oreochromis niloticus.*

5.2 Materials and Methods

A eutrophicated pond in Dhaka was selected for studying accumulation of microcystins on liver tissue and their toxic effects on *Oreochromis niloticus* and *Hypopthalmicthys militrix.*

5.2.1. Location of fish farm

Nazirabazar pond is located at the old Dhaka city, Bangladesh (23˚43'41"N & 90˚24'11"). The pond is 0.25 ha in size and is used for fish culture. *Microcystis aeruginosa* bloom is very common in this pond. Local people culture and consume fish from this pond.

5.2.2. Collection of *Microcystis aeruginosa* bloom and sample fish

M. aeruginosa bloom started in February 2015 and the highest cell density (95%) was observed in June 2015. The bloom sample was collected with a plankton net (no. 55 bolting silk plankton net).The concentrated samples were filtered through an 0.45µm glass fiber filter (Whatman GF/C, 47 mm diameter) and dried in an oven at 60-70˚C. The fish samples were harvested by local fisherman. For the analyses of fish tissues five fishes were dissected and livers, stomachs and muscles were pooled out and dried in an oven at 60-80˚C. Dried tissues were grinded in a kitchen grinder. In the original bloom sample the cell density of *M. aeruginosa* was 3×10^4 colony/l recorded by a Sedgewick-Rafter counting chamber (S-R cell) under a compound microscope at ×400 magnifications. Water quality parameters were determined by ecological HACH test kit (Model FF2).

5.2.3. Extraction of toxins

5.2.3.1. *Fish tissue sample*

5 ml extraction solution (0.3% acetic acid in methanol/water (8:2 v/v)) was added to the

dried and ground tissue samples. After sonication in an ultrasonic bath for 10 min the solution was shaken overnight. Solids were removed by centrifugation. 2.5 ml extract were concentrated to 1 ml using a rotary evaporator (180 mbar, 45°C). The concentrated extracts were frozen at -20°C for at least 3 hours. After thawing and filtering through a 0.45 µm PTFE filter the extract was ready for LC-MS/MS analysis.

5.2.3.2. *Bloom filter*

The GF/C filters were extracted with 2.0 ml water/methanol (50:50; v:v) per filter by ice-cooled sonication for 4 min with an ultrasonic probe GM 70 (Bandelin, Berlin, Germany).The extract was sonicated for another 15 min in an ultrasonic bath. Extracts were centrifuged (10000 g, 15min) and the supernatants were filtered using 0.22 µm nylon syringe filters (Roth, Karlsruhe, Germany). The extracts were directly subjected to the liquid chromatography.

5.2.3.3. *Chemical analysis*

The HPLC/UV determination of microcystins was carried out following the method of Lawton et al. (1994a) with some modifications. Separation was performed using a C18 column (Phenomenex prodigy, ODS (3), 250 x 4.6mm, 5 µm) and acetonitrile /water/0.05% TFA as the mobile phase. Microcystinswere detected using an UVdetector (Shimadzu SPD-10AV; λ=238 nm). HPLC/MS-MS analyses were applied to confirmthe identity ofthe toxins.HPLC-MS/MS was performed using Shimadzu HPLC LC-20A coupled to an ABSciex 4000 QTrapwith an electrospray interface. Analytes were separated on a Phenomenex Gemini 5 µm C18 column (150*3 mm) with a guard column and a mobile phase consisting of 5 mM ammonium acetate in water/acetic acid (99:1; v/v) (eluent A) and 5 mM ammonium acetate in methanol/water/acetic acid (97:2:1; v/v/v) with a flow rate of 0.4 ml/min. Elution started with 60% eluent A and 40% eluent

B.

5.2.3.4. *Quantification*

Since reference materials for desmethylated MCs are not available commercially, determination of the concentrations of desmethyl-MCs, [D-Asp3, Dha7] MC-LR, and [Dha7] MC-LR, was performed using the standard calibration curves of MC-LR.

5.2.3.5. *Chemicals*

Reference standards of MC-RR, -LR, -YR, -LA, LF and –LW were purchased from Calbiochem/Novabiochem (La Jolla, CA, USA). Acetonitrile and methanol obtained from VWR (Leuven, Belgium) were HPLC grade. All chemicals were at least analytical grade.

5.3. Results and discussion

Nazirabazar fish pond was covered with a heavy bloom of *M. aeruginosa* during the warm season (March-September). During the bloom and fish collection period, the water quality parameters were as follows: alkalinity 172 mg/l, acidity 52 mg/l, pH 9.4, hardness 160 mg/l, carbon dioxide (CO_2) 30mg/l, nitrate-nitrogen (NO_3-N) 0.01 mg/l, phosphate phosphorus (PO_4-P) 0.78mg/l, ammonia (NH_3) >3.0 mg/l, dissolved oxygen (O_2) 4.8 mg/l at the surface, conductivity 940 FAU and temperature 30˚C (±2˚C).

Table 5.1. Amount of MCs and nodularine in different organ of fish (*Oreocromis niloticus*)

Sample name/type:	Fish Tissue			Bloom Filter (µg/g)
	Fish Stomach (µg /g)	Fish Liver (µg /g)	Fish Muscle (µg /g)	
MC-RR	<0.01	**0.049**	<30	240
MC-YR	<0.06	<0.19	<180	5.6
MC-LR	<0.05	<0.14	<140	30
MC-LW	<0.02	<0.07	<80	<0.6
MC-LF	<0.04	<0.13	<130	<1.1
MC-LA	<0.04	<0.13	<130	12
Nodularine	<0.01	<0.02	<30	<0.2
MC-WR	<0.06	<0.19	<200	16
dm- MC-RR	<0.01	<0.02	<30	15
dm- MC-LR	<0.05	<0.15	<150	<1.3
MC-HtyR	<0.06	<0.19	<200	<1.7
MC-LY	<0.05	<0.15	<150	<1.3
Total				325.6

Microcystin- RR was only found on liver and concentration was 0.049 µg/g (Table 5.1.). Several studies have shown that MCs were detected in 57% of the fish liver and viscera sample. In liver, the concentration varied from 0-15 µg/g with an average of 6.5 µg/g. In viscera it varied from 0 to 7.16 µg/g (average of 14.6 µg/g) and muscle contain a

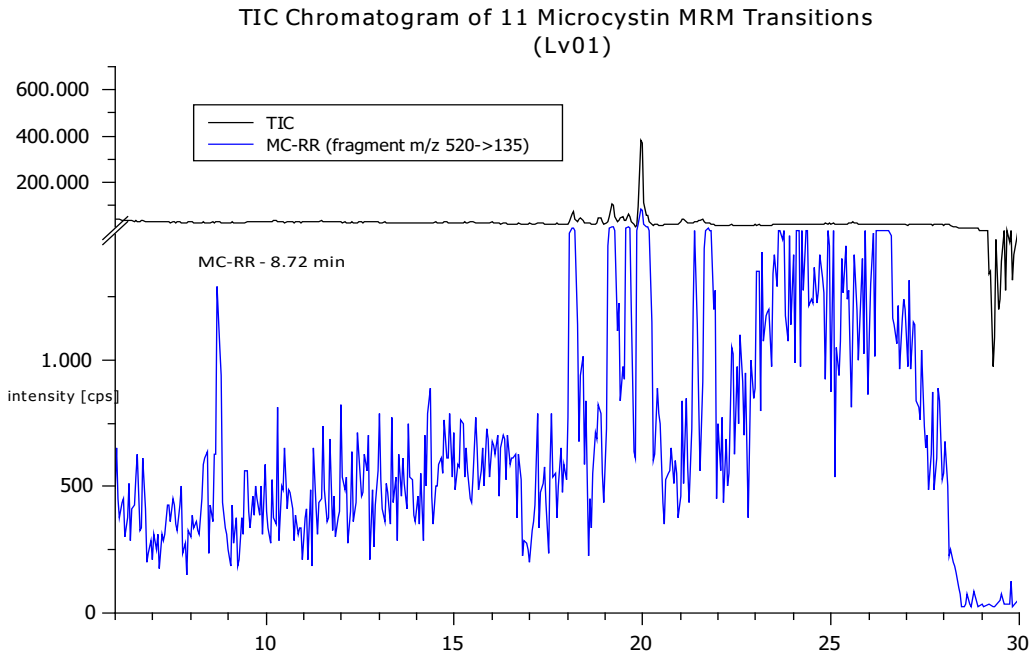


Fig.5.1 TIC Chromatogram of 11 Microcystin MRM Transitions of liver tissue

maximum of 0.337 µg/g of microctstins (Magalhaes, 2001). Mohamed et al., also detected MCs highest in the guts (0.821 µg/g fresh weight) of *Oreochromis niloticus* followed by livers (0.531µg/g) and kidneys (0.40µg/g) and muscles (0.102µg/g.) (Table 5.2.).

Table 5.2. Amount of microcystis in different organ of fish

	Magalhaes, 2001	Mohamed et al., 2003	Present study
Fish species	*Tilapia rendalli*	*Oreochromis niloticus*	*Oreochromis niloticus*
Bloom species	*Microcystis aeruginosa*		
Gut	14.6 µg/g	0.821 µg/g	-
Liver	6.5 µg/g	0.5318 µg/g	0.049 µg/g
Muscle	0.33 µg/g	0.102 µg/g	-

Among the organs tested MCs were only detected from the liver *O. niloticus*. No MCs was detected from the gut and muscle of the fish. Only MC-RR was found in liver and concentration was 0.049 µg/g (Fig. 5.1.). MC-LR is the most common and highly toxic variant in the environment. Most toxicological experiments have been done for MC-LR and provisional tolerable daily intake TDI of 0.04µg/kg body weight per day (Chorus and Bartnam, 1999) has been established. MC-LR, MC-RR, MC-YR and demethyleted variants of MC-LR and MC-RR are the most frequently detected MCs. MC-LF and MC-LW have been reported less often. But recent investigation showed that MC-LF and MC-LW are even more toxic than MC-LR (Faassen and Lürling, 2013) (Table 5.3.). Eriksson et al. (1990) and Fastner et al. (1995) reported that MC-RR (EC50 1500-4300 nM) is less susceptible than MC-LR (EC50 60-200 nM) in primary rat hepatocytes. Fisher et al, 2010 compared PP-inhabiting capabilities of MC-LR, MC-RR, MC-LW and MC-LF and stated that MC-LR is more toxic than MC-RR.

Table 5.3. IC50, EC50 values of the investigated MC congeners (Fisher et al., 2010).

	IC50(nM)		EC50 (nM)	
	PP1	PP2	Human hepatocytes (donor1)	HEK293-OATP1B1
MCLR	1.2	0.9	24.6	257.1
MCRR	1.5	0.9	900.2	1267
MCLW	1.9	1.1	0.4	4.0
MCLF	1.8	1.1	0.6	3.7

MCs can be accumulated in the food chain. Several authors have reported the accumulation of MCs, especially in aquatic invertebrates, which are essential elements of

the diet of many different fishes (Eriksson et al., 1989; Kotak et al., 1996; Mohammed, 2003). The accumulation of MCs by tilapia in a natural environment has been reported by Magalhaes et al., 2001 and Mohammed et al., 2003. In Bangladesh as a developing country, tilapia has taken an important role in the commercial fish farming business and contributes 8.1% in fish production in Inland water (FRSS, 2014). Tilapia culture has been promoted in small, seasonal ditches (Hussain et al., 1989; Gupta et al., 1992) because of rapid growth, good flavor, its high resistance to poor water quality and its ability to convert the organic and domestic waste into high quality protein (Balarin and Hallar, 1982). The present study is the first report on accumulation of MCs in fish tissue up to levels that pose a health risk for humans in Bangladesh. Total amount of MCs detected in the bloom was 146.28 µg/L which is higher than WHO provisional guideline. 0.049 µg/g MC-RR was detected in the liver sample. It should be noted that in Bangladesh especially in the rural areas people consume fish liver along with fish muscle. The average portion of fish muscle eaten by a person is about 100-300 g. If a man with a body weight of 80 kg consumes 100 g of contaminated tissue (4.9µg MCs) would ingest 0.061 µg/kg body weight of MCs- more than the TDI of 0.04 µg/kg body weight for MC-LR.

It was also observed that liver cells exposed with *M. aeruginosa* bloom were damaged both *in situ* and *ex-situ* (Ahmed et al., 2017). Zhang et al. (2010) have reported substantial amounts of MCs in boiling water suggesting that eating soup of MC contaminated fish also poses a potential hazard to humans. MC-concentrations increased upon boiling probably due to the release of phosphate- bound microcystin (Zhang et al., 2010). Thus, proper regulation and a monitoring system should be developed for fish farms and household ponds in Bangladesh to prevent potential public health hazards.

6.1. Introduction

Fish liver is an excellent organ for the study of environmental quality biomarkers, due to its role in the specimen's metabolism, which include the production of proteins, the oxidation, conjugation, methylation, inactivation or detoxication of substances, or rather the excretion of pollutants (Brusle and Anadon, 1996). Different laboratory studies have shown the effects of microcystins in different organ of fishes by histopathology (Fisher et al. 2000, Fisher and Dietrich 2000, Gupta and Guha, 2006, Atencio *et al.* 2008, Ferrira et al. 2010) Histopathological change has been observed in liver of tilapia fish (*Oreochromis* sp.) exposed to a single intraperitoneal (i.p.) injection of the pure standards (MC-LR and MC-RR) at a dose of 500µg/kg and changes are megolocystsis, necrotic process, and micro vesicular steatosis in liver (ATencio et al., 2008). Hepatic tumor and strong hepatic hemorrhages has also been reported (Tencalla et al., 1994; Fisher et al., 2000).

Most studies have done by intraperitoneal injection (extracted microcystins) or oral exposure (freeze-dried cyanobacterial cell). However, IP injections of microcystins are not analogous to field exposures since the toxin is absorbed faster and metabolized differently when administrated into the abdominal cavity (as with the IP route) as compared to oral administration (Ibeling et al., 2007). 550 µg MC of intraperitonial injection dose showed mortality in rainbow carp whereas a same oral dose showed no mortality and minor pathological change has been identified (Tencalla et al., 1994). Lower and higher severity has observed in histopathological changes with application of 300 and 500 µg/kg ip MC-LR while 400 µg/kg oral dose of MC-LR resulted same change (Fisher and Dietrich, 2000). Not only exposure route, susceptibility of fish to the toxins is different. In comparison to the pathological events in salmonids exposure to MCs, where a slower development of pathology and primarily necrosis cell death prevails, and the pathology in carp rapidly develops in lower toxins (Fisher et al., 2000). So, specific toxicity thresholds and effects are different for fish species.

Freshwater fish farming plays an important role in rural livelihood and contributes 55% to total fish production (FRSS, 2014) in Bangladesh. Excessive use of artificial feed and lack of scientific management create eutrophication leading to cyanobacterial bloom formation (Ahmed et al., 2014). Mainly planktivorous fish (e.g., common carp) and omnivorous fish (e.g., tilapia) are popular for aquaculture. Zurawell et al. (2005) stated that cyanobacteria are an important component of tropical cichilids (e.g., tilapia, *Oreochromis niloticus*) and cyprinids (e.g., silver carp, *Hypophthalmicthys molitrix*). In this study, natural unicellular *Microcystis aeruginosa* bloom, tilapia fish (*Oreochromis niloticus*) and silver carp (*Hypophthalmicthys molitrix)* have been selected. Tilapia (*Oreochromis niloticus*) and silver carp (*Hypophthalmicthys molitrix)* have a commercial and aquaculture importance in Bangladesh which contributes about 11.28% and 6.63% of total inland fish production (FRSS, 2014). Thus, potential health hazard from consumption of fish cultured in eutrophicated pond is a major concern. Although MCs have been detected from different aquaculture ponds and lakes in Bangladesh, the accumulation of toxins in fish tissue and their toxic effect on aquatic lives have not been reported before. Present study reports the accumulation of MCs in fish tissue and evaluates the possible public health risk in the country.

6.2. Materials and Methods

6.2.1. Collection of fish and Cyanobacteria bloom

Tilapia, *O. niloticus* and *Hypopthalmicthyes militrix* were collected from Babul Fish Farm, located at Chittagong Road, Dhaka. The average weight of fish was 10.23±1 g (mean ± SD). Unialgal bloom of *Microcystis aeruginosa* was collected from Nazira Bazar Pond, Old Dhaka city (23˚43'26"N and 90˚24'24"E).

6.2.2. Experimental design

Four aquarium of 100L each designated as A_1, A_2, A_3, A_4 (Fig. 6.1.) were setup for experiments. Fish were acclimated for 7 days prior to experiment. Aquariums were containing dechlorinated tap water, temperature was maintained at 24 ± 1˚C, pH 7.8 ± 2 and dissolved oxygen was 7.5 mg/L, photoperiod of 12 h and continuous aeration was given with submerged pumps. Fish were feed with artificial commercial fish feed during acclimatization period.

6.2.3. Extraction and determination of MCs

M. aeruginosa bloom started in February and the highest cell density (95%) was observed in June, 2015. The bloom sample was collected with plankton net (no. 55 bolting silk plankton net). The concentrated samples were filtered through an 0.45µm glass fiber filter (Whatman GF/C, 47 mm diameter) and dried in an oven at 60-70˚C. The GF/C filters were extracted with 2.0 ml water/methanol (50:50; v/v) per filter by ice-cooled sonication for 4 min with an ultrasonic probe GM 70 (Bandelin, Berlin, Germany). The extract was sonicated for another 15 min in an ultrasonic bath. Extracts were centrifuged

(10000 g, 15min) and the supernatants were filtered using 0.22 µm nylon syringe filters (Roth, Karlsruhe, Germany). The extracts were directly subjected to the liquid chromatography.

The HPLC/uv determination of microcystins was carried out following the method of Lawton *et al.* (1994a) with some modifications Separation was performed using a C18 column (Phenomenex prodigy, ODS (3), 250 x 4.6mm, 5 µm) and acetonitrile /water/0.05% TFA) as the mobile phase. Microcystins were detected using an uv detector (Shimadzu SPD-10AV; λ=238 nm). HPLC/MS-MS analyses were applied to confirm the identity of the toxins. HPLC-MS/MS was performed using Shimadzu HPLC LC-20A coupled to an ABSciex 4000 QTrap with an electrospray interface. Analytes were separated on a Phenomenex Gemini 5 µm C18 column (150*3 mm) with a guard column and a mobile phase consisting of 5 mM ammonium acetate in water/acetic acid (99:1; v/v) (eluent A) and 5 mM ammonium acetate in methanol/water/acetic acid (97:2:1; v/v/v) with a flow rate of 0.4 mL/min. Elution started with 60% eluent A and 40% eluent B.

Since reference materials for desmethylated microcystins are not available commercially, determination of the concentrations of desmethyl-MCs, [D-Asp3, Dha7] MC-LR, and [Dha7] MC-LR, was performed using the standard calibration curves of MC-LR.

aeration. Temperature was maintained at 24 ± 1˚C, pH 7.8 ± 2, dissolved oxygen 7.5 mg/L with a photoperiod of 12 h darkness. Every alternative day 20% of water was exchanged and added same concentration of *M. aeruginosa* bloom. Total exposure period was fifteen days. Five fishes were sacrificed by anesthesia (0.02% Clove oil; Hilltech Canada Inc. Vankleak Hill, Ontario, Canada) at five day intervals from four different aquaria (Fig. 6.1.). Stomach analysis has done for detection of food both for control and treated fish (Fig. 6.2., 6.3., 6.4., 6.5.). Exposed fish liver samples were taken for histological study.

6.2.4.Histopathology

Liver tissues were fixed washed in physiological saline and preserved in Bouin’s fluid for

18 hours. The sample were dehydrated with ethyl alcohol, cleaning with xylene, impregnated and embedding in paraffin and cut into 3-4 µm thick sections by a microtome machine (KD 2258, Kedee, China). Fixed and prepared slide were held overnight. The sections were stained with hematoxylin and eosin (HE), then mounted with PBX and observed under a light microscope (Olympus CX41 co-observation microscope; Humansan, 1997).

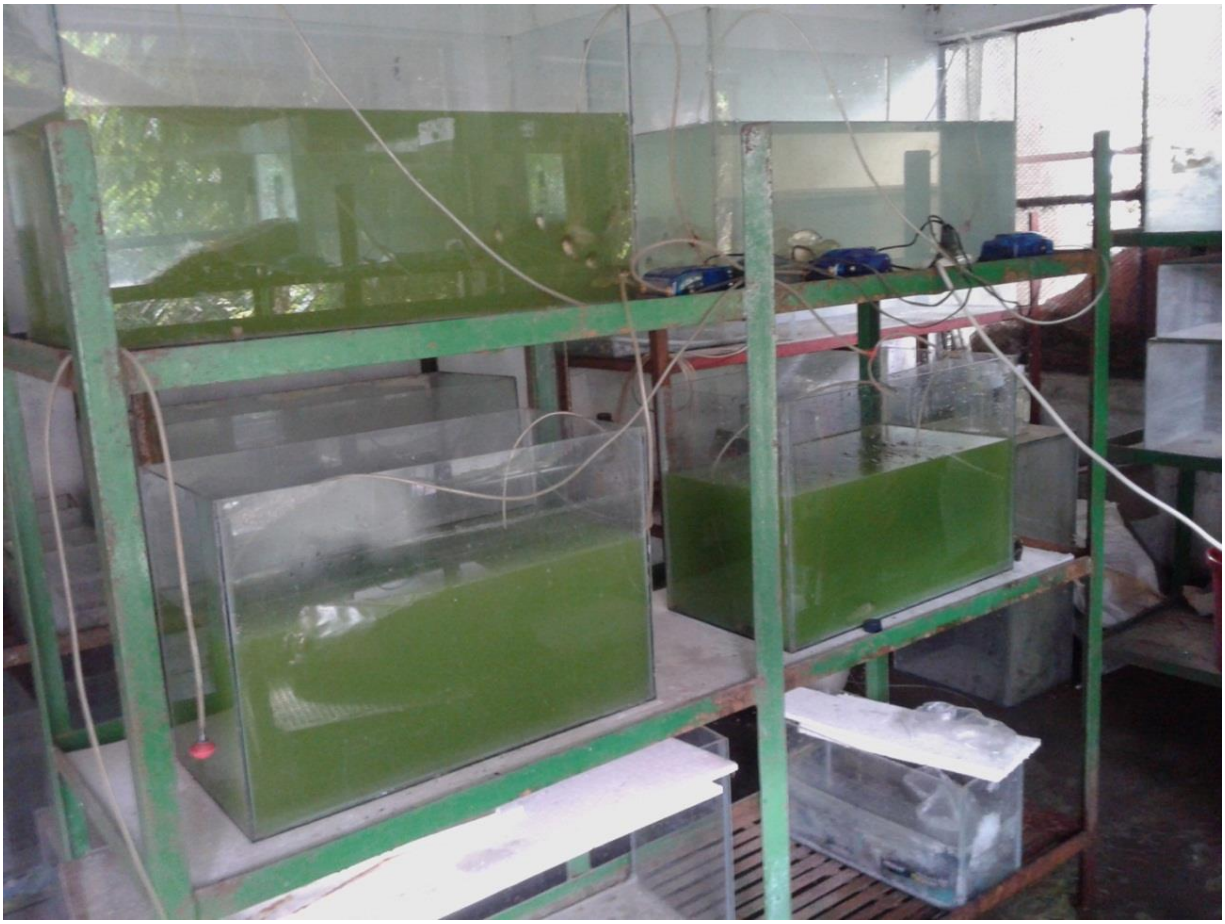


Fig. 6.1. Experimental setup(A1, upper-right; A2, upper- left;, A3- lower-left; A4- lowerright)

Fig. 6.2. Treated Fish (*Oreochromis niloticus*)

Fig. 6.3. Stomach of fish (left side, treated fish stomach; right side, control fish stomach)

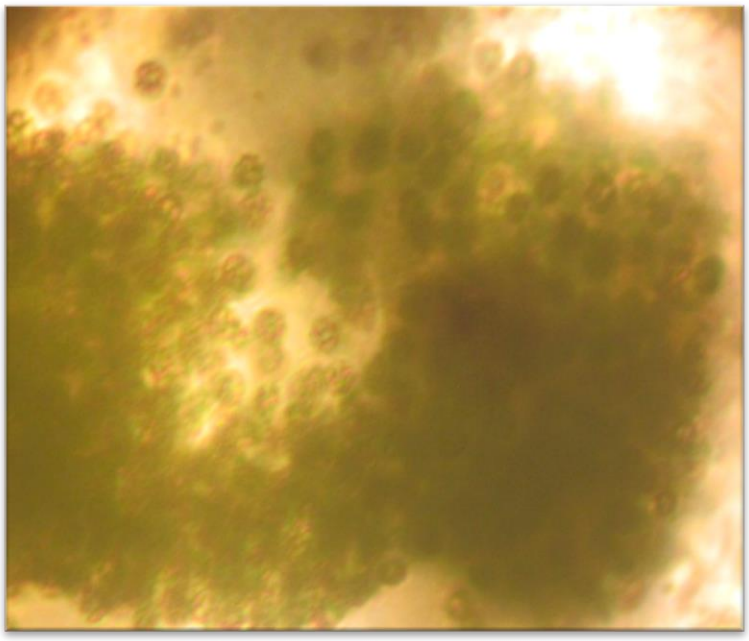


Fig. 6.4. Stomach residue of treated fish showing *Microcystis* sp.

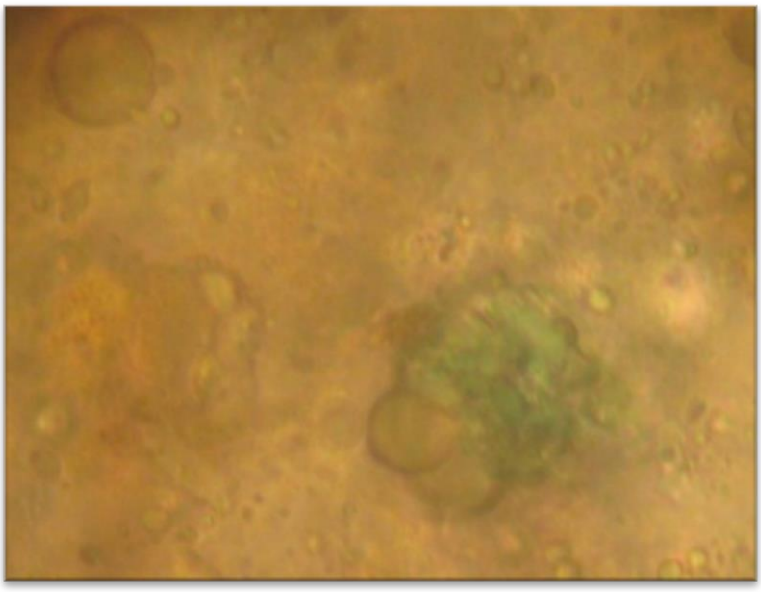


Fig. 6.5. Stomach residue of control fish showing artificial food.

6.3 Results and discussions

For *Oreochromis niloticus*

6.3.1. Microcystis toxins characterization

HPLC analysis of *M. aeruginosa* extract showed six peaks, the retention time of which agreed well with standard MC-RR, MC-YR, MC-LR, MC-LA, MC-WR, dm-MC-RR (Fig. 6.6.). The results of HPLC-MS revealed the identification of six variants of microcystins, according to their corresponding molecular weight MC-LR (at m/z 950.0 M $+H)^{+}$), MC-RR (at m/z 519.5 (M $+2H)^{2+}$), MC-YR (at m/z 1045.0 (M $+H)^{+}$), MC-LA,MC-WR, dm-MC-RR. In *M. aeruginosa* sample, the concentration of MC-RR was the highest (240 µg/g) followed by MC-YR (5.6 µg/g), MC-LR (30nµg/g), MC-LA (12 µg/g), MC-WA (16 µg/g) and dm-MC-RR (15 µg/g; Table 6.1.).

Table 6.1. Characterization and concentration of microcystins in *M. aeruginosa* bloom sample.

	Level of microcystin (µg/g)												
Types of Micro-cystins	MC - RR	MC - YR	MC - LR	MC - LW	MC - LF	MC - LA	Nodul a-rine	MC - WR	dm MC-RR	dm MC-LR	MC-Hty R	MC - LY	Total
Total	**240**	**5.6**	**30**	**<0.6**	**<1.9**	**12**	**<0.2**	**16**	**15**	**<1.3**	**<1.7**	**<1.3**	**318.6**

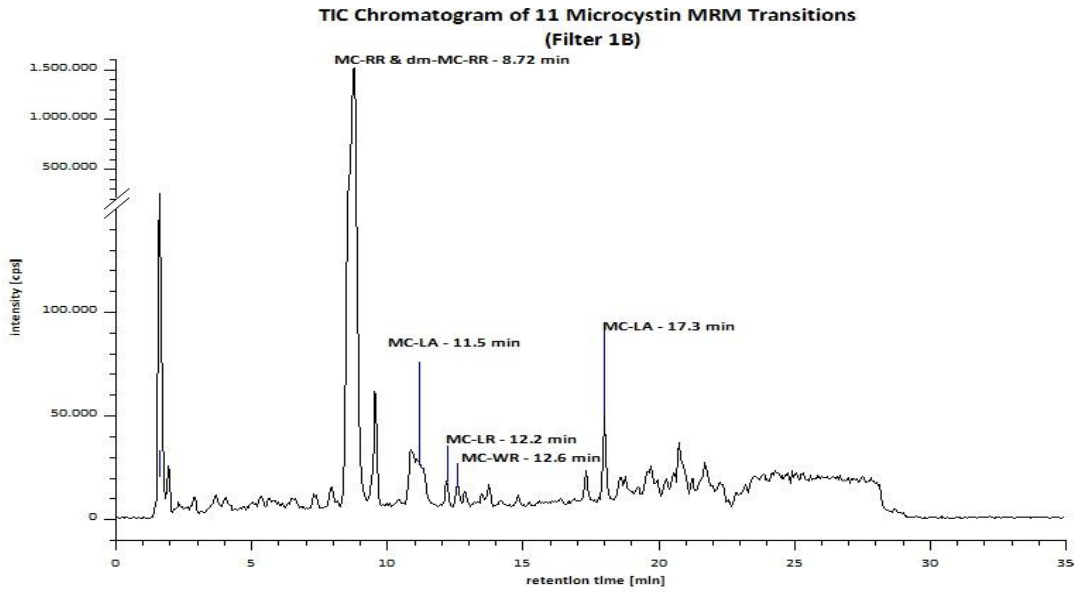


Fig. 6.6. TIC Chromatogram of Microcystins MRM Transition (bloom filter)

6.3.2. Control fishes

The liver of fish comprises a continuous cells mass of large hexagonal hepatic cells (hepatic parenchyma). Hepatic cells are of polygonal shape containing more or less spherical nucleus with a single prominent nucleolus. They are located among sinusoids forming cord like structures known as hepatic cords. Bile canaliculus is centrally located in each cord. There is no clear division of hepatic cells into lobules (Brusle and Anadon 1996; Fig. 6.7.a). No abnormality was observed in the liver cell in controlled fish.

6.3.2.1. Five days of exposure

After five days of exposure in low concentration bloom (35×10^2 colony/mL), cells were normal, no structural change was observed. In moderate concentration of bloom (72×10^2

colony /mL), cells showed changes in the structural organization. Accumulation of bile in the lining of endothelium cells of liver was seen. Accumulation of fat in the cells was also seen. Liver of fish exposed in higher concentration of bloom (149×10^2 colony/mL) was swollen and cytoplasm granular. Vascular proliferation was developed with the comparison of control fish. Occasional bile stasis and mild focal necrosis was found.

6.3.2.2. Ten days exposure

After ten days of exposure, more advanced tissue abnormalities were detected. Bile stasis, fatty change (Fig. 6.7.d), vascular proliferation showed in larger area of fish liver exposed in moderate (72×10^2 colony/mL; Fig. 6.7.c) and high concentration (149×10^2 colony/mL) of bloom. Cell transition is normal in low concentration (35×10^2 colony/mL).

6.3.2.3. Fifteen days exposure

Tissue damage was highest in fish when exposed over 15-day duration in different concentrations of bloom. Moderate vascular proliferation and cellular bile stasis was developed in the intra sinusoidal space of liver exposed in lower concentration of bloom (35×10^2 colony/mL). Bile stasis in endothelial cells, fatty liver was observed in fish exposed in moderate bloom concentration (72×10^2 colony/mL). In the higher bloom concentration (149×10^2 colony/mL) hepatocytes were markedly swollen with granular appearance of the cytoplasm (Fig. 6.7.b). Highest vascular proliferation was observed with huge bile accumulation (Fig. 6.7.e). Cells showed minimal hepatic necrosis (Fig. 6.7.f).

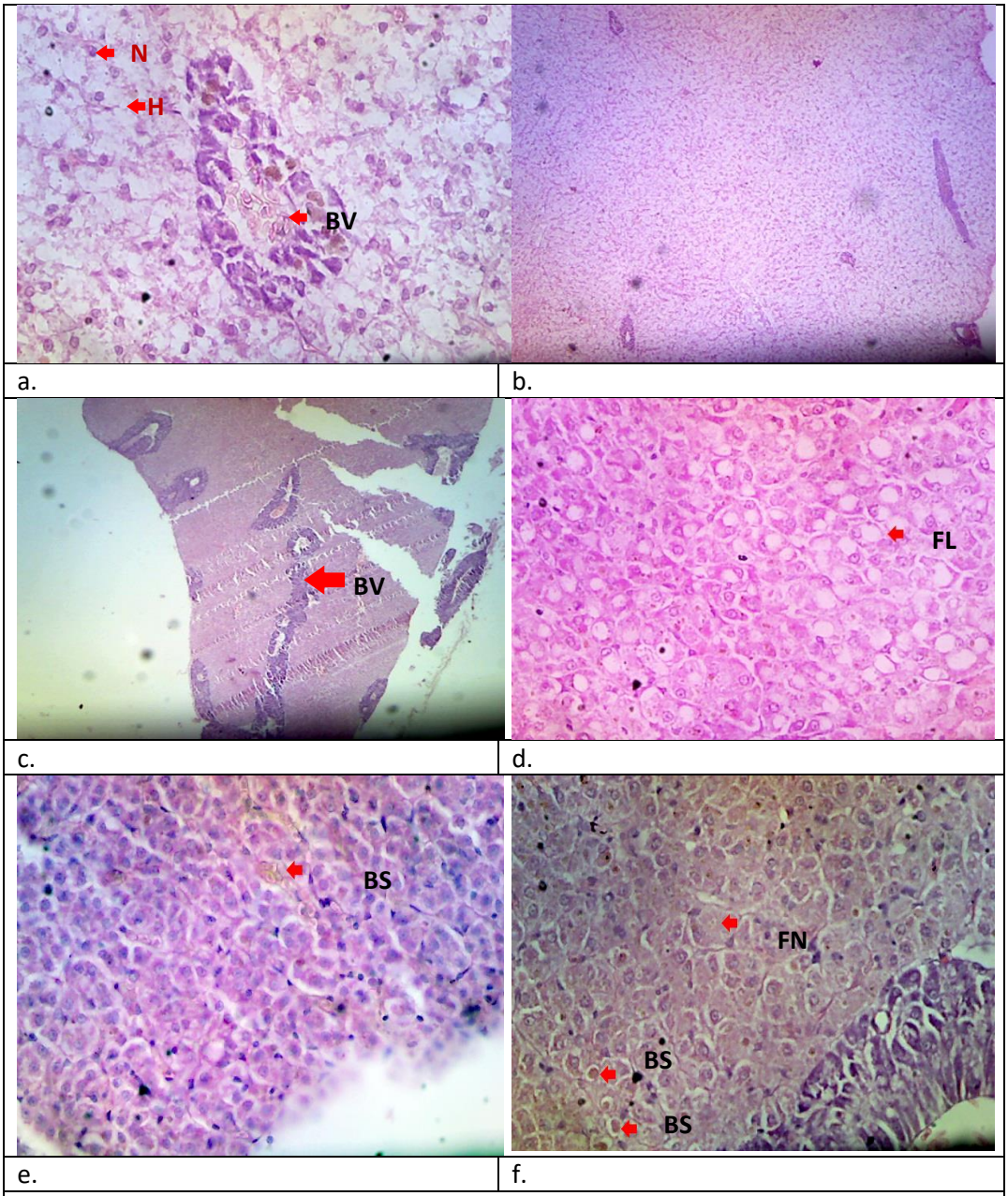


Fig. 6.7. Section of liver of *Oreochromis niloticus*. a. control group showing normal nucleus and hypatocytes. H, hepatocytes; N, nucleus; BV, blood vessel; 10×45 (H&E stain) b. Experimented group (149 × 10^2 colony/ml: 5 days after treatment) showing condense cytoplasm and swelling in hepatocytes; 10×10 (H&E stain). c. Experimented group (149 × 10^2 colony/mL: 15 days after treatment) showing vascular proliferation. BV, Blood vessels; 10×10 (H&E stain). d. Experimented group (72 × 10^2 colony/mL: 10 days after treatment) showing fatty liver; 10×45 (H&E stain) e. Experimented group (149 × 10^2 colony/mL: 15 days after treatment) showing accumulation of bile in the endothelial cell; 10×45 (H&E stain) f. Experimented group (149 × 10^2 colony/mL: 15 days after treatment) showing focal necrosis and bile stasis; BS, bile stasis; FN, focal necrosis.

The cells have been found swollen and cytoplasm was cloudy and granular in this study. At the dose of 1000 µg MC-LR/kg, hepatocytes have been found with condensed cytoplasm and lost their granular appearance with chromatin clumping and condensation (Li and Xie, 2009). Similar observation was reported by Gupta and Guha (2006) that histopathological changes like hepatocytes swelling, dissociation of hepatocytes in liver of *Heteropneustes fossilis* (0.1 mL MCs extracted from natural bloom: 24 h after treatment).

Formation of new blood vessels is known as angiogenesis (Brem, 1976). Angiogenesis is essential for tumor growth, invasion and metastatic spread (Stefansson et al., 2006). The rapid growth of tumor explants is dependent on the development of new blood vessels (Algire et al., 1945) and the growth of malignant tumors depends on the process of angiogenesis (Folkman, 1971). There are several angiogenic markers for assessing metestic spread and prognosis in malignant tumor. And vascular proliferation is a meaningful variable in assessing the angiogenic phenotype of endometric carcinoma (Stefansson et al., 2006). Vascular proliferation has seen in the liver of treated fish, so it could be predicted that cell showed primary response to develop tumor.

Bile stasis is a condition where bile cannot flow from the liver to the duodenum which was also observed in this study. Under a microscope, the individual hepatocytes will have a brownish-green or yellow-brown granules within the cells representing bile that cannot get out of the cell (Pacheco and Santos, 2002). This accumulation of bile indicates possible damage to the hepatic metabolism (Fanta et al., 2003).

Fatty degeneration is the excessive accumulation of fat in the cytoplasm and is often accompanied by nuclear atrophy. In mammals, fatty liver (hepatic lipidosis, hepatic steatosis, lipoid liver disease, fatty degeneration of the liver) is the term to describe liver that contain more visible lipid in hepatocytes than one expects to see in that organ (Kelly 1993). Accumulation of fat can result in of either toxic exposure or nutritionally induced. Japanese medaka, *Oryzias latipes* (Wester and Canton, 1987) and rats (Krajnc et al.,

1984) were exposed to organotines such as bis (tri-n-butyltin) oxide (TBTO), di-n-butyltindichloride (DBTC), and tri-n-butylin chloride (TBTC). Increased hepatic glycogen have been demonstrated histologically, histochemically, and biochemically in medaka and guppies (*Poecilia reticulata*) that were exposed to TBTO or DBTC. Microvesicular steatosis has also been reported in tilapia exposed in MC-LR (Atencio L et al., 2008).

Response of the fish liver to toxins is hepatocyte necrosis. The most characteristic reaction to toxicity is an apoptotic type of single cell death (Boorman et al., 1997). Focal necrosis has been developed in this study. Pathological change includes necrosis and apoptosis has been observed in liver of rainbow trout, *Oncorhynchus mykiss* induced in Microcystin-LR (Fisher et al., 2000). In both mammals and fish, microcystins can cause damage to cytoskeletal elements of hepatocytes, possibly via inhibition of protein phosphatases (Tencalla and Dietrich, 1997). It is assumed that focal necrosis is the primitive stage of cell destruction followed by severe necrosis, hemorrhagic shock resulting total loss of architecture of organ causing death of animal (Kotak et al., 1996).

Hypopthalmicthyes molithrix

Within five days all the experimented fishes were died except control fishes. It may be *Hypopthalmicthyes molithrix* could not tolerate adverse environment like bloom. Accumulation of Microcystins beyond the limit. It may be due to time of exposure. No histopathological change has observed except swelling of cell.

7.1. Summery

A study was conducted on cyanobacterial bloom dynamics, identification of toxic components of bloom, accumulation of this toxic component on liver tissue of fish (*Oreochromis niloticus*) and their toxic effects on it. For this study an extensive monitoring survey was commended concurrently in the four sampling sites in Mymensingh Sadar Upazila, viz., Anandomohon College campus, Bidyamoyei school campus and ponds of Kaowatkhali area. The sample was collected on monthly basis considering four distinct seasons [December-February: winter, March-May: pre monsoon (summer), June-September: monsoon (Rainy), October-November: post monsoon (autumn)] from January 2012 to December 2013.

However, the study is specified into seven distinct chapters. The chapter I instigates with a general introduction presenting cyanobacteria, factors influencing its dominance, cyanotoxins and its toxicology, detection and analysis methods, treatment and control measure and risk assessment. At the end of this chapter, a general view of cyanobacteria in Bangladesh and justification of this research has been clearly mentioned.

The relevant literature regarding exposure of cyanobacteria both for humans and animals have reviewed widely in the chapter II.

Chapter III denotes the phytoplankton species diversity and physicochemical properties of four studies ponds. From there, Bloom species especially cyanobacteria have identified. Relationship between physicochemical properties and phytoplankton have studies. Reason behind cyanobacterial succession or which properties responsible for cyanobacterial growth have explored. Summarized environmental parameters were ranged of Alkalinity (92-197) P1, (40-159) P2, (92-229) P3, (87-315) P4 mg/L; Acidity (23-90) P1, (10-64) P2, (5-63) P3, (17-125) P4 mg/L; Hardness (63-171) P1, (44-141) P2, (67-180) P3, (85-179) P4 mg/L; pH (7.1-9) P1, (6.9-9.5) P2, (7-10) P3, (7.4-9) P4; DO (2-9.83) P1, (4-10.3) P2, (4-11) P3, (5.89-11) P4 mg/L; Free CO_2 (5-51.8) P1, (5-20) P2, (5-27) P3, (15.2-54) P4 mg/L; NO_3-N (0-0.2) P1, (0-0.17) P2, (0-0.1) P3, (0.01-0.55) P4

mg/L; PO_4-P (0.11-0.9) P1, (0.22-0.9) P2, (0.6-0.9) P3, (0.38-0.9) P4 mg/L; NH_3 (0.2-2.4) P1, (0.1-0.85) P2, (0.4-11) P3, (0.6-5) P4 mg/L; BOD (31-36) P1, (29-47) P2, (38-48) P3, (40-190) P4 mg/L; Conductivity (445-544) P1, (245-511) P2, (600-911) P3, (623-1290) P4 µs/cm; Turbidity (1-28) P1, (2-28) P2, (8-107) P3, (60-188) P4 FAU; Temperature of water (19-31) P1, (19.4-31) P2, (20-39) P3, (20-33) P4 ˚C; Temperature of air (16.35-30) ˚C; Rainfall (0-409) mm.

A total of 22 plankton genera, representative of six families, were identified during the study periods. Planktons genera are *Anabaena* sp., *Anabaenopsis* sp., *Chroococcus* sp., *Merismopedia* sp., *Microcystis* sp., *Spirulina* sp., *Euglena* sp., *Lepocinclis* sp., *Phacus* sp., *Trachelomonas* sp. , *Actinastrum* sp., *Coelastrum* sp., *Crucigenia* sp., *Dictyospharium* sp., *Pandorina* sp., *Pediastrum* sp., *Senedesmus* sp., *Cyclotella* sp., *Syndra* sp., *Navicula* sp., *Ceratium* sp., *Peridinium* sp., *Arcella* sp..

In case of P1, among plankton families Ceratiacea (81.36%) represent the highest number followed by Euglenophyceae (11.76%), Chlorophyceae (5.14%), Peridiniaceae (0.63%), Bacillariophyceae (0.40%) and Cyanophyceae (0.06%). For P2, Peridiniaceae (28.67%) represent the highest number followed by Ceratiacea (26.67%), Euglenophyceae (25.67%), Cyanophyceae (5.31%), Chlorophyceae (1.36%) and Bacillariophyceae (0.40%). For P3, Cyanophyceae (72.74%) represent the highest number followed by Euglenophyceae (17.43%), Chlorophyceae (5.32%), Peridiniaceae (0.63%) Bacillariophyceae (0.18%) and Ceratiacea (0.18%). In P4, only three families found, Euglenophyceae (47.23%) represent the highest number followed by Cyanophyceae (45.04%) and Chlorophyceae (7.38%).

It is well known that nitrogen and phosphorus are the most important inorganic elements for plankton production. During study, range of nitrite nitrogen for P1, P2, P3 and P4 were 0-0.2, 0-0.17, 0-0.1, and 0.1-0.55. A successful *Microcystis* sp. summer bloom had developed in the scarcity of nitrogen (0.00) mg/L whereas ammonia concentration was 0.6 mg/L in P3. Different bloom was recorded during study period. In P1 *Ceratium*

bloom was found in March and April 2013, where cell concentration was 9000 cells/l and 8000 cells/L respectively. In P3, *Microcystis* sp. bloom was found in May and June 2013 and cell concentration was 15000 colony/L and 11000 colony/L separately. In P4, mixed algal bloom was found from September 2012 to July 2013. *Spirulina* sp. (5500 cells/L) and *Lepicinclis* sp. (81000 cells/L) were appeared in September 2012. *Microcystis* sp. (9000 cells/L), *Spirulina* sp. (4000 cells/L) and *Trachelomonas* sp.(70050 cells/L) were found in December 2012. Only *Microcystis* sp. bloom was found in June 13 where cell concentration was 30,000 colony/L.

Different similarity analysis has done to find the leading phytoplankton of the community and their status. ANOSIM showed significance difference (5%) in assemblage structure among ponds (R= 0.05955; P=0.001) and months (R= 0.2149; P=0.001). Among the seasons winter-summer and winter-rainy season showed significance difference among them, whereas other season shows similarity among them. According to similarity percentage (SIMPER) overall 94.52% dissimilarity was found among ponds and major contributory species were *Microcystis* sp. (18.52%), *Trachelomonas* sp. (8.88%), *Ceratium* sp. (8.82%), *Lepocinclis* sp. (8.72%), *Spirulina* sp. (8.21%), *Padiastrum* sp. (5.91%), *Arcella* sp. (5.49%), *Merismopedia* sp. (5.44%), *Euglena* sp. (5.37%) and *Peridinium* sp. (5.15%). On the other hand, 93.24% dissimilarity were observed among seasons and major contributing species were *Microcystis* sp. (17.31%), *Ceratium* (9.55%), *Trachelomonas* sp. (8.78%), *Lepocinclis* sp. (8.41%), *Spirulina* sp. (8.05%), *Pediastrum* sp. (5.92%), *Euglena* sp. (5.61%), *Merismopedia* sp. (5.57%). At the level of 6% similarity for ponds, plankton was classified by cluster analysis (Fig. 3.9.) No marked separation of the genera was observed except *Ceratium* sp. and *Chroococcus* sp. At the similarity of 6%, three groups were attained while two genera (*Ceretium* sp., *Chroococcus sp.*) were remained isolated. Among the three groups, second group contains *Microcystis* sp., *Spirulina* sp., *Lepocisclis* sp., *Phacus* sp., *Pandorina* sp. and *Tracheolomonas* sp. where first three genera found bloom forming species in the study or most contributing species in the SIMPER analysis. For the analysis of relationship

between the phytoplankton and environmental parameters, pearson correlation and CCA (Canonical Correspondence Analysis) has done. NO_3-N, BOD, ammonium and turbidity showed positive significance relationship (Pearson Correlation) with phytoplankton. With the CCA analysis, Conductivity, Ammonium, DO, BOD, NO_3-N had positive correlation with axis one. Several phytoplankton species such as *Microcystis* sp., *Spirulina*, *Lepocinclis* sp., *Merismopodium*, *Anabaenopsis* (Cyanophyceae) and *Senedesmus* sp., *Phacus* sp., *Pandorina* sp. (Chlorophyceae), which distributed at the right side of Axis1, were positively related correlated with the content of nutrients such as ammonium, NO_3-N, PO_4. *Ceratium* sp., *Cyclotella* sp., *Euglena* sp. *Pediastrum* sp., *Peridinium* sp., *Trachelomonas* sp. negatively related with the nutrient.

Chapter IV reveals the cyanobacterial bloom and its toxic components. *M. aeruginosa* bloom cell (Sample A) was collected from P3 during May 2013 for HPLC analysis and the concentration of MC-RR was the highest (5.4µg/L) followed by MC-YR (1.14 µg/L), MC-LR (2.0 µg/L), MC-WR (0.46 µg/L) and dm-MC-RR (0.36 µg/L). Besides *M. aeruginosa* bloom cell (Sample B) was collected from P4 during June'2013 and the concentration of MC-RR was the highest (1.0 mg/l) followed by and dm-MC-RR (0.04 µg/l).

Chapter V explore the accumulation of toxic components on fish (*O. niloticus*) tissue. A *M. aeruginosa* bloom sample was collected for further studies from Nazira Bazar Pond, Old Dhaka city (23˚43'26"N and 90˚24'24"E). Characterization of bloom sample was MC-RR (240 µg/L), MC-YR (5.6 µg/L), MC-LR (30 µg/L), MC-LA (12 µg/L), MC-WR (16 µg/L), dm-MC-RR (15 µg/L). Fish (*O. niloticus*) samples were also collected from this pond and fishes were dissected. Livers, stomachs and muscles were analyzed for microcystins accumulation. Microcystin- RR was only found on liver and concentration was 0.049 µg/g.

Chapter VI deals about the effects of toxic components on fish (*O. niloticus*) liver tissue. Four aquarium of 100L each designated as A_1, A_2, A_3, A_4 (Fig. 6.1.) were setup for

experiments. Fish were acclimated for 7 days prior to experiment. Fishes of A_1 treated as control group and reared by artificial feed. In the three treatments A_2, A_3 and A_4, the *M. aeruginosa* cell concentration were 35×10^2 colony/ml, 72×10^2 colony/ml, 149×10^2 colony/ml respectively. After five days of exposure in low concentration bloom (35×10^2 colony/mL), cells were normal, no structural change was observed. In moderate concentration of bloom (72×10^2 colony /mL), cells showed changes in the structural organization. Accumulation of bile in the lining of endothelium cells of liver was seen. Accumulation of fat in the cells was also seen. Liver of fish exposed in higher concentration of bloom (149×10^2 colony/mL) was swollen and cytoplasm granular. Vascular proliferation was developed with the comparison of control fish. Occasional bile stasis and mild focal necrosis was found.

After ten days of exposure, more advanced tissue abnormalities were detected. Bile stasis, fatty change, vascular proliferation showed in larger area of fish liver exposed in moderate (72×10^2 colony/mL) and high concentration (149×10^2 colony/mL) of bloom. Cell transition is normal in low concentration (35×10^2 colony/mL).

Tissue damage was highest in fish when exposed over 15-day duration in different concentrations of bloom. Moderate vascular proliferation and cellular bile stasis was developed in the intra sinusoidal space of liver exposed in lower concentration of bloom (35×10^2 colony/mL). Bile stasis in endothelial cells, fatty liver was observed in fish exposed in moderate bloom concentration (72×10^2 colony/mL). In the higher bloom concentration (149×10^2 colony/mL) hepatocytes were markedly swollen with granular appearance of the cytoplasm. Highest vascular proliferation was observed with huge bile accumulation. Cells showed minimal hepatic necrosis.

7.2. Conclusions

Cyanobacterial blooms occur in the study ponds and the bloom has the seasonal periodicity. Cyanobacterial bloom dynamics was enhanced by Phosphate-phosphorus and nitrate nitrogen contents in water. Beside cyanobacterial bloom was also found low nitrogen, high phosphate- phosphorus and high ammonium content. Five types of microcystins (MC-RR, MC-LR, MC-YR, MC-WR & dm-MC-RR) were isolated from *Microcystis aeruginosa* bloom. Microcystin-LR content in bloom sample was detected as 2.0µg/l which is much higher than the WHO provisional guide value (1 µg/L) for drinking water. Other microcystins are MC-RR (5.4 µg/L), MC-YR (1.14 µg/L), MC-WR (0.46 µg/L), dm- MC-RR (0.36 µg/L). Total Microcystin-LR found in liver is 0.049 µg/g which is much higher than provisional guideline of WHO 0.04 µg/kg TDI (tolerance daily intake). Liver cell damage was observed exposed with *Microcystis aeruginosa* bloom in both in situ and vitro experiment. In exposed fish, histopathological alternations were characterized by swollen and granular cytoplasm, vascular proliferation, bile stasis, fatty change and focal necrosis.

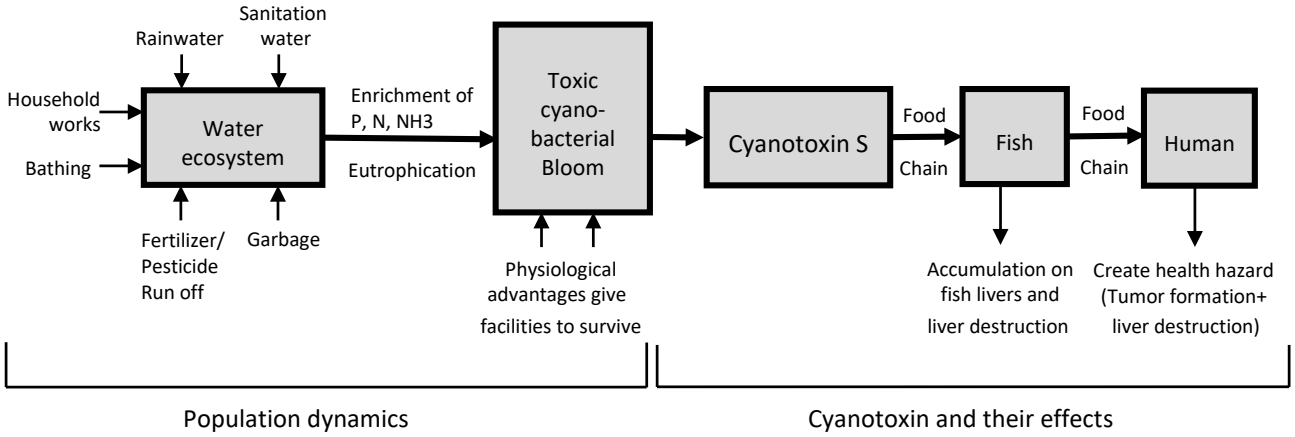


Fig: Possible health hazard for human in Bangladesh- A conceptual frame work

7.3. Recommendations

Microcystins were first time detected from fish liver tissue in Bangladesh, which might have alarming for public health safety as the toxins find their way through food chain. Microcystins are heat stable product. When it dissolved in water it cannot be filtered and boiled. Recently one of the study showed that eating soup of Microcystins contaminated fish also possess more toxins by realizing phosphate-bound microcystins through boiling. Regular monitoring system particularly fish culture and domestic used pond specially drinking water ponds should introduce. Create public awareness on toxic effects of Microcystins and advise them not to consume fish/ drinking water during bloom outbreak. So integrated monitoring of the water body is strongly recommended for public health safety.

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Table 1: Physico-chemical parameters of P1 (2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 12
Alk (mg/L)	162	182	156	153	136	136	104	162	120	136	144	180
ACI (mg/L)	30	34	32	36	28	30	88	61	23	34	59	25
HAR (mg/L)	105	156	171	171	163	162	104	94	130	12O	109	150
pH	7.6	8.1	7.5	7.5	7.6	8	8.2	7.1	9	8.7	8.4	8.4
DO (mg/L)	3.5	4	2	2	2	7.3	4.3	4	8.2	4.3	7.03	7
CO_2 (mg/L)	10	5	20	25	30	50	48	12	51.8	48.8	50	40
NO_3-N (mg/L)	0.01	0.01	0.01	0.01	0.02	0.01	0.03	0.03	0.02	0.02	0.03	0.03
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.12	0.45
AMM (mg/L)	0.4	0.5	0.4	0.4	0.4	0.6	1.2	1	2.1	0.5	0.6	0.6
BOD (mg/L)	32	32	36	34	32	32	32	36	34	36	34	34
TOW (˚C)	19.3	20	25	28	30.2	30	26	26	27	25	22	20
TOAma (˚C)	27.2	31.3	34.8	35.6	36.2	35.2	34.5	37	34.5	31.8	28	28
TOAmi (˚C)	8	9.4	14.5	18.2	20.6	25.5	25	24.5	17.8	12	9.5	8
CON (µs/cm)	445	500	449	490	448	512	500	540	500	448	500	512
TUR (FAU)	2	4	9	2	2	1	8	12	9	11	8	10
RAI (mm)	18	0	1	202	85	241	409	238	221	45	19	0

Table 2: Physico-chemical parameters of P1 (2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Alk (mg/L)		192	197	116	110	92	100	160	141	140	120	140
ACI (mg/L)	69	70	64	25	85	60	63	51	90	80	33	30
HAR (mg/L)	121	171	139	103	63	90	96	123	77	78	95	90
pH	8.4	8.2	8.2	8	8.2	8.5	8.3	8	7.8	8	8	8.2
DO (mg/L)	5.5	5.6	5	6.3	7.23	7.08	7	9.83	2.98	4.02	6.07	7
CO_2 (mg/L)	20	35	20	19	31	14	15	17	34	30	26	20
NO_3-N (mg/L)	0	0.02	0.06	0.03	0.03	0.02	0.2	0.2	0.02	0.01	0.01	0.02
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.36	0.27	0.11	0.9	0.9	0.9	0.9
AMM (mg/L)	0.6	1	24	0.2	0.3	0.4	0.4	1	1.2	0.4	0.4	0.4
BOD (mg/L)	31	32	31	33	36	31	34	32	33	32	32	33
TOW (°C)	19	23	29	31	30	29	29	31	29.5	29	26	30
TOAma (°C)	28	32.5	35	35.6	36.5	36.4	34.3	34.8	36.5	34.2	30.8	36.5
TOAmi (°C)	4.7	10.4	15.2	18.4	18.8	24	25	24.7	23.8	20.7	11.8	8.8
CON (µs/cm)	530	544	541	530	500	494	500	530	498	490	476	500
TUR (FAU)	11	10	14	8	6	1	11	28	7	10	27	22
RAI (mm)	0	18	21	69	308	267	318	343	132	263	0	0

Table 3: Physico-chemical parameters of P2 (2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec'l 2
Alk (mg/L)	96	108	159	120	107	109	94	100	70	98	114	120
ACI (mg/L)	10	20	32	23	22	21	28	40	34	40	50	42
HAR (mg/L)	90	120	141	123	60	60	71	82	63	62	60	63
pH	8.7	8.4	8.4	8.6	8.4	8.2	7.4	7.6	8.1	8.3	8.2	8.4
DO (mg/L)	4.7	5.8	4	5	4.8	6	5.9	4.3	10.3	5.8	5.9	5
CO_2 (mg/L)	5	10	15	15	10	10	16	20	15	10	7	10
NO_3-N (mg/L)	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.10	0.03	0.1	0.17	0.17
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.4	0.45	0.9	0.9	0.9	0.9	0.9	0.9
AMM (mg/L)	0.5	0.6	0.53	0.5	0.54	0.6	0.7	0.5	0.85	0.5	0.5	0.8
BOD (mg/L)	30	32	31	29	32	32	40	41	35	36	36	40
TOW (°C)	19.4	21	25	28	31	30	26.5	26	27	25	22	20
TOAm (°C)	27.2	31.3	34.8	35.6	36.2	35.2	34.5	37	34.5	31.8	28	28
TOAmi (°C)	8	9.4	14.5	18.2	20.6	25.5	25	24.5	17.8	12	9.5	8
CON (µs/cm)	300	245	316	300	295	311	300	405	450	440	460	500
TUR (FAU)	8	2	10	20	12	8	8	28	25	20	11	12
RAI (mm)	18	0	1	202	85	241	409	238	221	45	19	0

Table 4: Physico-chemical parameters of P2 (2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Alk (mg/L)	110	108	117	116	59	40	57	86	60	60	57	58
ACI (mg/L)	40	41	64	10	28	42	50	53	29	28	15	25
HAR (mg/L)	70	129	94	92	54	44	46	63	48	47	45	48
pH	8.2	8.4	9.4	9.5	7.4	8.4	7.9	7.8	6.9	7	7	7.4
DO (mg/L)	5.6	6.6	10	4.9	4.96	7.1	7	6.9	4.2	7	7.5	6
CO_2 (mg/L)	8	12	11	6	18	17	16	9	14	16	20	15
NO_3-N (mg/L)	0.1	0	0.02	0.01	0.04	0.03	0.04	0.02	0.02	0.02	0.05	0.02
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.42	0.26	0.22	0.9	0.9	0.9	0.9
AMM (mg/L)	0.5	0.6	0.5	0.2	0.6	0.1	0.2	0.2	0.4	0.2	0.2	0.2
BOD (mg/L)	39	40	29	47	30	36	38	40	35	36	32	35
TOW (°C)	19.5	24	29	31	29.7	30	30	30.5	29	29	28	22
TOAm (°C)	28	32.5	35	35.6	36.5	36.4	34.3	34.8	36.5	34.2	30.8	36.5
TOAmi (°C)	4.7	10.4	15.2	18.4	18.8	24	25	24.7	23.8	20.7	11.8	8.8
CON (µs/cm)	501	511	507	453	400	378	371	312	316	311	293	299
TUR (FAU)	10	11	25	28	10	10	11	11	8	12	20	19
RAI (mm)	0	18	21	69	308	267	318	343	132	263	0	0

Table 5: Physico-chemical parameters of P3 (2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 12
Alk (mg/L)	207	200	210	210	175	175	154	204	164	160	229	204
ACI (mg/L)	10	10	5	15	20	25	31	32	63	61	30	32
HAR (mg/L)	180	160	171	150	122	122	120	130	137	140	160	160
pH	8.6	8.1	8.7	8.8	8.7	7.8	7.8	8.5	7	7.7	7.7	8
DO (mg/L)	5.5	4	6	5	6	7	6.3	7	7	5.5	7.3	6
CO_2 (mg/L)	5	10	15	15	10	10	16	20	22	23	27	27
NO_3-N (mg/L)	0.01	0.01	0.02	0.02	0.01	0.02	0.02	0.02	0.01	0.02	0.03	0.01
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.7	0.9	0.9
AMM (mg/L)	0.5	0.6	0.6	0.6	0.5	0.5	0.6	0.54	0.4	0.6	0.6	0.6
BOD (mg/L)	41	40	42	40	48	48	42	40	42	41	40	46
TOW (°C)	20.4	20.5	25	28	30	30	26	39	31	26	20	20
TOAma (°C)	27.2	31.3	34.8	35.6	36.2	35.2	34.5	37	34.5	31.8	28	28
TOAmi (°C)	8	9.4	14.5	18.2	20.6	25.5	25	24.5	17.8	12	9.5	8
CON (µs/cm)	611	621	600	630	900	911	616	600	640	685	678	650
TUR (FAU)	58	50	8	34	36	51	20	20	22	85	93	30
RAI (mm)	18	0	1	202	85	241	409	238	221	45	19	0

Table 6: Physico-chemical parameters of P3 (2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Alk (mg/L)	180	175	130	120	117	157	92	130	176	170	176	180
ACI (mg/L)	41	43	31	42	36	54	42	26	43	35	31	40
HAR (mg/L)	110	96	67	90	87	100	86	97	95	94	97	110
pH	8.5	8.5	8.5	8.7	8.5	8.7	8.5	10	9.5	7.8	8.5	8.5
DO (mg/L)	7.5	9	8.3	6.3	8.3	5.9	9.6	9.9	8.3	8	11	7.5
CO_2 (mg/L)	24	26	20	10	10	20	17	15	27	22	20	25
NO_3-N (mg/L)	0.02	0.04	0.04	0	0	0.1	0.02	0.02	0.04	0.02	0	0.01
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.6	0.7	0.9	0.9
AMM (mg/L)	0.6	0.8	0.8	0.8	0.6	0.4	0.4	1.1	0.8	0.4	0.4	0.6
BOD (mg/L)	38	40	44	42	48	45	41	42	40	41	40	40
TOW (°C)	20	22	31	30	29	29	29	32	28	28	27	22
TOAma (°C)	28	32.5	35	35.6	36.5	36.4	34.3	34.8	36.5	34.2	30.8	36.5
TOAmi (°C)	4.7	10.4	15.2	18.4	18.8	24	25	24.7	23.8	20.7	11.8	8.8
CON (µs/cm)	705	660	756	898	910	906	713	640	660	634	622	620
TUR (FAU)	22	20	90	58	34	38	58	81	28	103	107	98
RAI (mm)	0	18	21	69	308	267	318	343	132	263	0	0

Table 7: Physico-chemical parameters of P4 (2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 12
Alk (mg/L)	220	240	258	136	159	136	130	159	200	220	315	220
ACI (mg/L)	60	60	61	37	93	37	30	60	61	93	95	90
HAR (mg/L)	170	175	179	178	111	170	112	170	170	170	161	160
pH	8.7	8.4	8.4	9	7.4	7.9	8.5	8.9	8.3	8.5	8.5	8.7
DO (mg/L)	8.1	8	8.12	7	10	8	8	8	8.1	8.4	9.7	9
CO_2 (mg/L)	24.2	27	32.8	26	30	15.2	25	35.2	30.1	50	49.5	48
NO_3-N (mg/L)	0.2	0.3	0.37	0.01	0.02	0.02	0.01	0.01	0.4	0.5	0.55	0.4
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.9	0.9	0.9
AMM (mg/L)	5	5	5	3	3	3	3	2.1	3	5	5	5
BOD (mg/L)	57	55	57	36	57	58	58	57	58	57	58	190
TOW (°C)	20	21	31	32	29	30	31	30	27	25	22	20
TOAma (°C)	27.2	31.3	34.8	35.6	36.2	35.2	34.5	37	34.5	31.8	28	28
TOAmi (°C)	8	9.4	14.5	18.2	20.6	25.5	25	24.5	17.8	12	9.5	8
CON (µs/cm)	680	623	785	900	914	827	1100	980	1200	1100	1190	1198
TUR (FAU)	60	66	67	78	182	68	181	178	120	150	180	187
RAI (mm)	18	0	1	202	85	241	409	238	221	45	19	0

Table 8: Physico-chemical parameters of P4 (2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Alk (mg/L)	230	250	258	136	156	87	163	170	216	210	266	250
ACI (mg/L)	80	65	61	37	92	36	79	17	87	84	125	122
HAR (mg/L)	170	171	179	178	111	85	109	110	130	130	169	170
pH	9	8.7	8.4	9	8.4	8.3	9	8.9	8	8	8	8.5
DO (mg/L)	8.1	8	6.6	10	11	7	8.2	10	10	6	5.89	7.23
CO_2 (mg/L)	25.2	36.8	48.2	25.2	32.8	27.2	24.4	15.8	36.6	40.2	53	54
NO_3-N (mg/L)	0.3	0.35	0.37	0.01	0.02	0.04	0.02	0.01	0.12	0.1	0.02	0.02
PO_4-P (mg/L)	0.9	0.9	0.9	0.9	0.9	0.9	0.45	0.38	0.9	0.6	0.5	0.9
AMM (mg/L)	5	5	5	5	2.1	3	0.6	2	2.2	3	5	5
BOD (mg/L)	54	55	58	53	57	58	58	40	40	41	43	51
TOW (°C)	20	22	31	32	29	29	28.5	33	28.5	28	27	22
TOAma (°C)	28	32.5	35	35.6	36.5	36.4	34.3	34.8	36.5	34.2	30.8	36.5
TOAmi (°C)	4.7	10.4	15.2	18.4	18.8	24	25	24.7	23.8	20.7	11.8	8.8
CON (µs/cm)	1210	1250	1290	1093	908	623	748	690	827	900	914	912
TUR (FAU)	180	126	126	188	182	85	129	68	66	66	67	66
RAI (mm)	0	18	21	69	308	267	318	343	132	263	0	0

Table 9: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P1 (Jan'2012-Dec'2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 12
Ana	0	0	0	0	0	0	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	0	0	0	0	50	0	0	0	0	0
Mer	0	0	0	0	0	0	0	0	0	0	0	0
Mic	0	0	0	0	0	0	0	0	0	0	0	0
Spi	0	0	0	0	0	0	0	0	0	0	0	0
Eug	250	0	0	100	0	0	0	0	50	0	0	10
Lep	0	0	0	0	0	0	0	0	0	0	0	50
Pha	0	0	50	50	0	0	0	0	0	100	50	0
Tra	0	0	0	600	150	50	0	21600	0	200	100	50
Act	0	0	0	0	0	0	0	0	0	0	0	0
Coe	0	0	0	0	0	0	0	0	0	8200	0	0
Cru	0	0	0	0	0	0	0	0	0	0	0	0
Dic	0	50	0	0	0	0	50	0	0	0	0	0
Pan	0	0	0	0	0	200	250	1300	0	0	0	0
Ped	100	100	150	250	40	0	0	0	1000	0	0	0
Sen	100	0	0	0	0	0	0	0	350	0	0	0
Cyc	0	0	0	0	150	50	0	0	0	0	0	0
Dia	0	0	0	0	0	0	0	0	0	0	0	0
Nav	100	0	0	0	0	0	0	0	500	0	0	0
Cer	0	0	1100	200	0	0	0	0	0	0	0	0
Per	0	0	500	0	950	10	0	0	0	0	0	0
Arc	0	0	100	0	0	0	0	0	1300	0	0	0
Total	550	150	1900	1200	1290	310	350	22900	3200	8500	150	110

Table 10: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P1 (Jan'2013-Dec'2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Ana	0	0	0	0	0	0	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	0	0	0	0	0	0	0	0	0	0
Mer	0	0	0	0	100	0	0	0	0	0	0	0
Mic	0	0	0	0	0	0	0	0	0	0	0	0
Spi	0	0	0	0	0	0	0	0	0	0	0	0
Eug	10	0	0	0	0	0	0	0	0	0	0	0
Lep	0	0	0	0	0	0	0	0	0	0	0	0
Pha	0	0	0	0	0	0	0	0	0	0	0	0
Tra	100	4000	0	0	50	0	0	100	300	0	0	0
Act	0	0	0	0	0	0	0	0	0	0	0	0
Coe	0	0	0	0	0	0	0	0	0	0	0	0
Cru	0	0	0	0	0	0	0	0	0	0	0	0
Dic	0	0	0	0	0	0	0	0	0	0	0	0
Pan	0	0	0	0	50	0	0	0	0	0	0	0
Ped	0	0	0	0	100	0	0	0	0	0	0	0
Sen	0	0	0	0	0	0	0	0	0	0	0	0
Cyc	0	0	0	0	0	0	0	0	0	0	0	0
Dia	0	0	0	0	0	50	50	0	0	50	0	0
Nav	0	0	0	0	0	0	0	0	10	0	0	0
Cer	100	23000	90000	80000	0	0	0	0	0	0	0	50
Per	0	0	0	0	0	50	0	0	0	0	0	0
Arc	0	0	0	0	0	0	0	0	0	10	10	100
Total	210	27000	90000	80000	300	100	50	100	310	60	10	150

Table 11: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P2 (Jan'2012-Dec'2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 12
Ana	0	0	200	50	0	0	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	0	0	0	0	0	0	0	0	0	0
Mer	0	0	50	0	900	200	100	500	50	75	100	100
Mic	0	0	0	0	0	0	0	0	0	0	0	0
Spi	0	0	0	0	0	0	0	0	0	0	0	0
Eug	100	0	1500	3500	0	0	0	0	3750	100	50	40
Lep	0	0	0	0	0	0	0	2250	0	0	0	0
Pha	0	0	50	1100	0	0	0	0	0	0	0	0
Tra	0	0	150	0	0	0	0	0	0	0	50	50
Act	0	0	0	0	0	0	0	0	0	0	0	0
Coe	0	0	0	0	0	0	0	0	0	0	0	0
Cru	0	0	0	0	0	0	0	0	0	0	0	0
Dic	0	0	150	0	0	0	0	0	0	0	50	10
Pan	0	0	0	450	100	100	100	200	0	100	100	100
Ped	100	0	250	150	250	200	400	300	250	50	0	0
Sen	0	0	0	0	0	0	0	0	0	0	0	0
Cyc	0	0	0	0	0	0	0	0	0	0	0	0
Dia	0	0	0	0	0	0	0	0	0	0	0	0
Nav	0	0	0	0	0	0	0	0	0	0	0	0
Cer	0	0	50	0	0	0	0	0	0	0	0	0
Per	0	0	150	1100	0	10	70	1700	0	0	0	0
Arc	500	100	0	0	0	0	0	0	0	0	0	0
Total	700	100	2550	6350	1250	510	670	4950	4050	325	350	300

Table 12: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P2 (Jan'2013-Dec'2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Ana	0	0	0	0	0	0	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	0	0	0	0	0	0	0	0	0	0
Mer	0	0	0	600	0	0	0	0	0	0	0	0
Mic	0	0	0	0	0	0	0	0	0	0	0	0
Spi	100	0	100	50	0	0	0	0	0	100	200	50
Eug	0	0	0	0	0	0	0	0	0	0	0	0
Lep	0	0	0	0	0	0	0	0	0	0	0	0
Pha	0	0	0	0	50	0	0	0	0	0	0	0
Tra	0	0	0	0	0	0	0	0	0	0	0	0
Act	0	0	0	0	0	0	0	0	0	0	0	0
Coe	0	0	0	0	0	0	0	0	0	0	0	0
Cru	0	0	0	0	0	0	0	0	0	0	0	0
Dic	0	0	0	0	0	0	0	0	0	0	0	0
Pan	0	0	0	0	0	0	0	0	0	0	0	0
Ped	0	450	400	400	0	50	800	100	1100	150	100	50
Sen	50	50	0	0	200	50	100	0	0	0	0	0
Cyc	0	0	0	0	0	0	0	0	0	0	0	0
Dia	0	150	0	0	0	0	0	0	0	0	0	0
Nav	0	0	0	0	0	0	0	0	0	0	0	0
Cer	0	10000	3150	1750	0	0	0	0	0	0	0	0
Per	0	6700	3150	1400	1400	50	0	0	0	0	0	50
Arc	0	0	0	0	0	0	0	0	0	0	0	0
Total	150	17350	6750	4200	1650	150	900	100	1100	250	300	150

Table 13: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P3

(Jan'2012-Dec'2012)

	Jan' 12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 12
Ana	0	0	0	0	0	50	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	0	0	0	50	0	0	0	0	150	100
Mer	100	0	0	100	900	100	0	0	200	0	350	300
Mic	0	0	0	0	0	0	0	0	0	0	0	0
Spi	0	0	0	0	0	0	0	0	0	0	0	0
Eug	0	0	0	120	550	300	200	50	250	50	0	0
Lep	0	0	0	50	0	0	10	200	0	0	0	0
Pha	0	0	0	80	200	100	100	0	300	0	0	0
Tra	0	0	50	100	250	110	100	100	100	0	0	0
Act	0	0	0	0	0	0	0	0	0	0	350	100
Coe	0	0	0	0	0	0	0	0	0	0	50	50
Cru	0	0	0	0	0	0	0	0	0	0	0	0
Dic	0	0	0	0	20	0	0	0	0	0	0	0
Pan	0	0	0	0	0	0	0	0	138	0	0	0
Ped	0	0	0	50	100	100	200	50	100	50	150	200
Sen	0	0	0	10	250	200	100	0	350	0	300	100
Cyc	0	0	0	0	0	0	0	0	0	0	0	0
Dia	0	0	0	0	0	0	0	0	0	0	0	0
Nav	0	0	0	0	0	0	0	0	0	0	0	0
Cer	0	0	0	0	0	0	0	0	0	0	0	0
Per	0	0	50	0	50	50	0	150	0	0	0	0
Arc	1500	1050	0	0	0	0	0	0	0	450	0	0
Total	1600	1050	100	510	2320	1060	710	550	1438	550	1350	850

Table 14: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P3 (Jan'2013-Dec'2013)

	Jan' 13	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec' 13
Ana	0	0	0	0	0	0	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	500	200	100
Chr	0	0	0	0	0	0	0	0	0	2100	3000	1000
Mer	1000	0	0	0	0	0	0	0	0	1700	1800	700
Mic	0	0	2000	8000	15000	11000	4000	3300	0	0	0	0
Spi	0	0	1000	24000	0	100	2000	5200	500	0	0	0
Eug	0	0	0	0	0	0	950	150	0	0	0	0
Lep	0	0	0	0	0	0	2450	450	0	1800	2100	2000
Pha	0	0	0	0	0	0	5550	700	0	0	0	0
Tra	0	0	0	0	0	0	2150	750	850	200	100	0
Act	0	0	0	0	0	0	0	0	0	0	0	0
Coe	0	0	0	0	0	0	0	0	0	0	0	0
Cru	0	0	0	0	0	0	0	0	0	100	0	0
Dic	0	0	0	0	0	0	0	0	0	0	0	0
Pan	0	0	0	2500	0	0	750	0	0	0	0	0
Ped	0	0	0	0	0	0	400	100	50	0	0	0
Sen	0	0	0	0	0	0	250	250	0	240	200	0
Cyc	0	0	0	0	0	0	0	0	0	100	50	0
Dia	0	0	0	0	0	0	0	0	0	100	0	0
Nav	0	0	0	0	0	0	0	0	0	0	0	0
Cer	0	0	0	0	0	0	0	0	0	0	0	0
Per	0	0	0	0	0	0	450	100	0	0	0	0
Arc	150	1000	0	0	0	0	100	500	0	0	0	0
Total	1150	1000	3000	34500	15000	11100	19050	11500	1400	6840	7450	3800

Table 15: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P4 (Jan'2012-Dec'2012)

	Jan'12	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec'12
Ana	0	0	0	0	0	0	0	0	0	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	350	0	0	0	85000	0	0	0	0	0
Mer	0	0	0	0	0	0		0	0	0	0	0
Mic	0	0	0	1000	2360	2000	1000	0	1050	2500	10000	9000
Spi	0	0	0	2300	0	300	0	0	55000	3900	6000	4000
Eug	0	0	2600	0	0	0	0	0	0	0	1000	1000
Lep	0	0	8200	0	0	0	0	0	81000	0	0	0
Pha	0	0	0	0	0	0	400	250	0	0	0	0
Tra	2050	0	0	0	0	0	0	0	0	700	1000	70050
Act	0	0	0	0	0	0	0	0	0	0	0	0
Coe	0	0	350	0	0	0	0	0	0	0	0	0
Cru	0	0	0	0	0	0	0	0	0	0	0	0
Dic	0	0	0	0	0	0	0	0	0	0	0	0
Pan	0	0	0	11700	0	0	0	0	0	0	2000	0
Ped	0	0	50	0	0	0	0	0	0	0	0	0
Sen	0	0	50	0	0	0	0	0	0	0	0	0
Cyc	0	0	0	0	0	0	0	0	0	0	0	0
Dia	0	0	0	0	0	0	0	0	0	0	0	0
Nav	0	0	0	0	0	0	0	0	1100	0	0	0
Cer	0	0	0	0	0	0	0	0	0	0	0	0
Per	0	0	0	0	0	0	0	0	2500	0	0	0
Arc	0	0	0	0	0	0	0	0	0	0	0	0
Total	2050	0	11600	15000	2360	2300	86400	250	140650	7100	20000	84050

Table 16: Monthly variations in the abundance (indv/l) of different genus of Phytoplankton in the P4 from (Jan'2013-Dec'2013)

	Jan' 13	Feb	Mar	Apr	Ma y	Jun	Jul	Au g	Sep	Oct	No v	Dec' 13
Ana	0	0	0	0	0	0	0	0	800	0	0	0
Ans	0	0	0	0	0	0	0	0	0	0	0	0
Chr	0	0	0	0	0	0	0	0	0	0	0	0
Mer	0	0	0	0	0	0	0	0	600	200	100	50
Mic	10000	8000	7500	9000	10000	30000	11000	0	100	0	0	0
Spi	8500	1000	1000	500	1000	0	1000	0	0	0	0	0
Eug	0	0	0	0	0	0	0	100	100	600	100	0
Lep	0	0	0	0	0	0	1000	0	7100	7000	2000	0
Pha	1000	1000	950	1000	3000	0	300	300	1500	2000	300	0
Tra	0	0	0	0	0	0	0	0	0	0	0	0
Act	0	0	0	0	0	0	0	0	0	100	100	0
Coe	0	0	0	0	0	0	0	100	400	0	0	0
Cru	0	0	0	0	0	0	0	0		0	0	0
Dic	0	0	0	0	0	0	0	0	500	300	0	0
Pan	2000	1000	1000	3000	0	0	100	0	0	0	0	0
Ped	0	0	0	0	0	0	3100	170	600	100	0	0
Sen	0	0	0	0	0	0	2600	1600	0	0	0	0
Cyc	0	0	0	0	0	0	0	0	0	0	0	0
Dia	0	0	0	0	0	0	0	0	0	0	0	0
Nav	0	0	0	0	0	0	0	0	0	0	0	0
Cer	0	0	0	0	0	0	0	0	0	0	0	0
Per	0	0	0	0	0	0	0	0	0	0	0	0
Arc	0	0	0	0	0	0	0	0	200	0	0	0
Total	21500	11000	10450	13500	14000	30000	19100	2270	11900	10300	2600	50

ANOVA FOR PHISICO-CHEMICAL PARAMETERS AND PONDS

Overall:

summarizeaikaciharph do co2 no3 po4 amm bod tow toa con turrai

Variable	Obs	Mean	Std. Dev.	Min	Max
aik	96	151.5938	54.72372	40	315
aci	96	46.32292	25.30508	5	125
har	96	115.9167	40.77684	44	180
ph	96	8.259375	.5644891	6.9	10
do	96	6.720208	2.001238	2	11
co2	96	23.17708	12.77181	5	54
no3	96	.0683333	.1161185	0	.55
po4	96	.8113541	.2009693	.11	.9
amm	96	1.382917	1.563466	.1	5
bod	96	42.52083	17.64383	29	190
tow	96	26.64063	4.200867	19	39
toa	96	25.09375	4.570508	16.35	30.75
con	96	632.0729	252.7301	245	1290
tur	96	48.77083	53.41939	1	188

rai | 96 134.0833 134.1094 0 409

Pond 1:

. summarize aik aci har ph do co2 no3 po4 amm bod tow toa con tur rai if pond==1

Variable	Obs	Mean	Std. Dev.	Min	Max
aik	24	143.75	28.94861	92	197
aci	24	50	22.44026	23	90
har	24	118.0833	31.51248	63	171
ph	24	8.079167	.4190871	7.1	9
do	24	5.385	2.100077	2	9.83
co2	24	27.98333	14.07754	5	51.8
no3	24	.0354167	.0520851	0	.2
po4	24	.7670833	.2718292	.11	.9
amm	24	.725	.5495057	.2	2.4
bod	24	33.08333	1.639636	31	36
tow	24	26.41667	3.943147	19	31
toa	24	25.09375	4.644429	16.35	30.75
con	24	499.0417	29.51268	445	544

tur | 24 9.708333 7.244063 1 28

rai | 24 134.0833 136.2785 0 409

Pond 2:

. summarize aik aci har ph do co2 no3 po4 amm bod tow toa con tur rai if pond==2

Variable	Obs	Mean	Std. Dev.	Min	Max
aik	24	92.625	28.86974	40	159
aci	24	32.79167	13.80605	10	64
har	24	73.95833	28.89333	44	141
ph	24	8.083333	.6780192	6.9	9.5
do	24	6.0525	1.599802	4	10.3
co2	24	12.70833	4.278146	5	20
no3	24	.0429167	.0486763	0	.17
po4	24	.7854166	.2321727	.22	.9
amm	24	.4591667	.2048311	.1	.85
bod	24	35.45833	4.558596	29	47
tow	24	26.35833	3.900492	19.4	31
toa	24	25.09375	4.644429	16.35	30.75
con	24	373.9167	84.41611	245	511

tur | 24 14.125 7.121996 2 28

rai | 24 134.0833 136.2785 0 409

Pond 3:

Summarize aik aci ha rph do co2 no3 po4 amm bod tow toa con tur rai if pond==3

Variable	Obs	Mean	Std. Dev.	Min	Max
aik	24	170.625	34.10573	92	229
aci	24	33.25	14.86534	5	63
har	24	120.0417	31.05882	67	180
ph	24	8.4	.6143218	7	10
do	24	7.175	1.665311	4	11
co2	24	18.16667	6.545272	5	27
no3	24	.02125	.0202833	0	.1
po4	24	.8666666	.0816496	.6	.9
amm	24	.5975	.1672605	.4	1.1
bod	24	42.125	2.863754	38	48
tow	24	26.7875	4.80365	20	39
toa	24	25.09375	4.644429	16.35	30.75

con | 24 702.75 112.2839 600 911

tur | 24 51.83333 30.84134 8 107

rai | 24 134.0833 136.2785 0 409

Pond 4:

. summarize aik aci har ph do co2 no3 po4 amm bod tow toa con tur rai if pond==4

Variable	Obs	Mean	Std. Dev.	Min	Max
aik	24	199.375	55.51327	87	315
aci	24	69.25	28.24466	17	125
har	24	151.5833	29.80358	85	179
ph	24	8.475	.409931	7.4	9
do	24	8.268333	1.325524	5.89	11
co2	24	33.85	11.44157	15.2	54
no3	24	.17375	.1857023	.01	.55
po4	24	.82625	.1618322	.38	.9
amm	24	3.75	1.375563	.6	5
bod	24	59.41667	28.48022	40	190
tow	24	27	4.331382	20	33

toa | 24 25.09375 4.644429 16.35 30.75

con | 24 952.5833 206.8856 623 1290

tur | 24 119.4167 51.67197 60 188

rai | 24 134.0833 136.2785 0 409

ANOVA FOR PHYSICOCHEMICAL PARAMETERS AND MONTHS

anova alk month

Number of obs = 96 R-squared = 0.2011

Root MSE = 52.0157 Adj R-squared = 0.0965

Source	Partial SS	df	MS	F	Prob> F
Model	57222.0313	11	5202.00284	1.92	0.0475
month	57222.0312	11	5202.00284	1.92	0.0475
Residual	227273.125	84	2705.63244		
Total	284495.156	95	2994.68586		

. anova har month

```
                    Number of obs =      96     R-squared     =  0.1624
                    Root MSE      = 39.6883     Adj R-squared =  0.0527

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  25647.8333    11  2331.62121      1.48     0.1543
                     |
               month |  25647.8333    11  2331.62121      1.48     0.1543
                     |
            Residual |    132313.5    84  1575.16071
           ----------+----------------------------------------------------
               Total |  157961.333    95  1662.75088


. anova ph month

                    Number of obs =      96     R-squared     =  0.1114
                    Root MSE      = .565883     Adj R-squared = -0.0049

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  3.37281194    11  .306619267      0.96     0.4911
                     |
               month |  3.37281194    11  .306619267      0.96     0.4911
```

```
                |
       Residual |  26.8987455    84  .320223161
      -----------+----------------------------------------------------
          Total |  30.2715574    95  .318647973


. anova do month

                          Number of obs =      96     R-squared     =  0.0805
                          Root MSE      = 2.04076     Adj R-squared = -0.0399

                 Source |  Partial SS    df       MS          F     Prob> F
      -----------+----------------------------------------------------
                  Model |  30.6342458    11  2.78493143       0.67     0.7640
                        |
      month |  30.6342458    11  2.78493143       0.67     0.7640
                        |
       Residual |  349.836349    84  4.16471844
      -----------+----------------------------------------------------
          Total |  380.470595    95  4.00495363


anova co2 month

                          Number of obs =      96     R-squared     =  0.1731
```

```
                    Root MSE      = 12.3512     Adj R-squared =  0.0648

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  2681.95202    11   243.81382      1.60     0.1141
                     |
               month |  2681.95202    11   243.81382      1.60     0.1141
                     |
            Residual |  12814.3775    84  152.552113
           ----------+----------------------------------------------------
               Total |  15496.3295    95  163.119258


. 
. anova no3 month

                    Number of obs =      96     R-squared     =  0.0837
                    Root MSE      = .118208     Adj R-squared = -0.0363

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  .107183336    11   .00974394      0.70     0.7376
                     |
               month |  .107183336    11   .00974394      0.70     0.7376
```

```
                |
       Residual |  1.17375004    84  .013973215
----------+----------------------------------------------------
          Total |  1.28093337    95  .013483509


.
. anova amm month

                          Number of obs =      96     R-squared     =  0.0410
                          Root MSE      = 1.62826     Adj R-squared = -0.0846

          Source |  Partial SS    df       MS          F     Prob> F
----------+----------------------------------------------------
           Model |   9.5165585    11  .865141682       0.33     0.9779
                 |
month |   9.5165585    11  .865141682       0.33     0.9779
                 |
       Residual |  222.704024    84  2.65123838
----------+----------------------------------------------------
          Total |  232.220582    95  2.44442718


.
. anova bod month
```

```
                  Number of obs =     96     R-squared     =  0.0805
                  Root MSE      = 17.9924     Adj R-squared = -0.0399

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
              Model |  2380.95833    11  216.450758       0.67     0.7640
                    |
              month |  2380.95833    11  216.450758       0.67     0.7640
                    |
           Residual |      27193    84   323.72619
           -----------+----------------------------------------------------
              Total |  29573.9583    95  311.304825


.
. anova tow month

                  Number of obs =     96     R-squared     =  0.7465
                  Root MSE      = 2.24916     Adj R-squared =  0.7133

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
              Model |  1251.56034    11  113.778213      22.49     0.0000
```

```
                |
month |  1251.56034    11  113.778213     22.49     0.0000
                |
           Residual |  424.931251    84  5.05870537
          -----------+----------------------------------------------------
              Total |  1676.49159    95  17.6472799


.
. anova toa month

                      Number of obs =     96     R-squared     =  0.9199
                      Root MSE      = 1.37588     Adj R-squared =  0.9094

             Source |  Partial SS    df       MS          F     Prob> F
          -----------+----------------------------------------------------
                Model |  1825.49131    11  165.953755     87.67     0.0000
                |
month |  1825.49131    11  165.953755     87.67     0.0000
                |
           Residual |  159.015028    84  1.89303604
          -----------+----------------------------------------------------
              Total |  1984.50633    95  20.8895404
```

```
.
. anova con month

                   Number of obs =     96     R-squared     =  0.0075
                   Root MSE      = 267.758     Adj R-squared = -0.1225

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  45561.1146    11  4141.91951      0.06     1.0000
                     |
               month |  45561.1146    11  4141.91951      0.06     1.0000
                     |
            Residual |  6022325.38    84  71694.3497
           ----------+----------------------------------------------------
               Total |  6067886.49    95  63872.4894

.
. anova tur month

                   Number of obs =     96     R-squared     =  0.0332
                   Root MSE      = 55.8574     Adj R-squared = -0.0934

              Source |  Partial SS    df       MS          F     Prob> F
```

```
-----------+----------------------------------------------------
     Model |  9011.20833    11  819.200758      0.26    0.9909
           |
     month |  9011.20833    11  819.200758      0.26    0.9909
           |
  Residual |   262083.75    84  3120.04464
-----------+----------------------------------------------------
     Total |  271094.958    95  2853.63114
```

```
. anova po4 month

                   Number of obs =     96    R-squared     =  0.1834
                   Root MSE      = .193134   Adj R-squared =  0.0765

  Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
     Model |  .703661414    11  .063969219      1.71    0.0839
           |
     month |  .703661414    11  .063969219      1.71    0.0839
           |
  Residual |  3.13326223    84  .037300741
-----------+----------------------------------------------------
```

```
       Total |  3.83692364    95   .04038867
```

. anova rai month

```
                   Number of obs =     96     R-squared     =  0.8311
                   Root MSE      = 58.6056     Adj R-squared =  0.8090

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  1420099.33    11  129099.939     37.59     0.0000
                     |
           month |  1420099.33    11  129099.939     37.59     0.0000
                     |
            Residual |     288508    84  3434.61905
           ----------+----------------------------------------------------
               Total |  1708607.33    95  17985.3404
```

ANOVA FOR PHYSICOCHEMICAL PARAMETERS AND PONDS.

. anova alk pond

```
                   Number of obs =     96     R-squared     =  0.5217
                   Root MSE      = 38.4591     Adj R-squared =  0.5061
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |  148417.781     3  49472.5938      33.45     0.0000
             |
        pond |  148417.781     3  49472.5938      33.45     0.0000
             |
    Residual |  136077.375    92   1479.1019
-------------+----------------------------------------------------
       Total |  284495.156    95  2994.68586


. anova aci pond

                           Number of obs =      96     R-squared     =  0.3524
                           Root MSE      = 20.6937     Adj R-squared =  0.3313

      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |  21436.0313     3  7145.34375      16.69     0.0000
             |
        pond |  21436.0313     3  7145.34375      16.69     0.0000
             |
    Residual |  39396.9583    92  428.227808
```

```
-----------+----------------------------------------------------
     Total |  60832.9896    95  640.347259
```

```
. anova har pond
```

```
                   Number of obs =     96     R-squared     =  0.4641
                   Root MSE      = 30.3347     Adj R-squared =  0.4466

            Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
             Model |    73303.75     3  24434.5833      26.55     0.0000
                   |
pond |    73303.75     3  24434.5833      26.55     0.0000
                   |
          Residual |  84657.5833    92  920.191123
-----------+----------------------------------------------------
             Total |  157961.333    95  1662.75088
```

```
.  anova ph pond
```

```
                   Number of obs =     96     R-squared     =  0.1029
                   Root MSE      = .543318     Adj R-squared =  0.0736
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |  3.11364628     3  1.03788209      3.52     0.0182
             |
        pond |  3.11364628     3  1.03788209      3.52     0.0182
             |
    Residual |  27.1579112    92  .295194687
-------------+------------------------------------------------------
       Total |  30.2715574    95  .318647973


. anova do pond

                           Number of obs =      96     R-squared     =  0.3048
                           Root MSE      = 1.69558     Adj R-squared =  0.2821

      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |  115.971411     3   38.657137      13.45     0.0000
             |
        pond |  115.971411     3   38.657137      13.45     0.0000
             |
    Residual |  264.499184    92  2.87499113
-------------+------------------------------------------------------
```

```
       Total |  380.470595    95  4.00495363

. anova co2 pond

                  Number of obs =     96     R-squared     =  0.4208
                  Root MSE      = 9.87712     Adj R-squared =  0.4019

        Source |  Partial SS    df       MS          F     Prob> F
      -----------+----------------------------------------------------
         Model |   6521.0446     3  2173.68153      22.28     0.0000
               |
pond |   6521.0446     3  2173.68153      22.28     0.0000
               |
      Residual |  8975.28491    92  97.5574447
      -----------+----------------------------------------------------
         Total |  15496.3295    95  163.119258

. anova no3 pond

                  Number of obs =     96     R-squared     =  0.2822
                  Root MSE      = .099974     Adj R-squared =  0.2587

        Source |  Partial SS    df       MS          F     Prob> F
```

```
-----------+----------------------------------------------------
     Model |  .361416678     3  .120472226      12.05     0.0000
           |
pond |  .361416678     3  .120472226      12.05     0.0000
           |
  Residual |  .919516694    92  .009994747
-----------+----------------------------------------------------
     Total |  1.28093337    95  .013483509
```

```
. anova po4 pond

                   Number of obs =      96     R-squared     =  0.0370
                   Root MSE      = .200407     Adj R-squared =  0.0056

            Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
     Model |  .141936458     3  .047312153       1.18     0.3225
           |
pond |  .141936458     3  .047312153       1.18     0.3225
           |
  Residual |  3.69498719    92  .040162904
-----------+----------------------------------------------------
     Total |  3.83692364    95   .04038867
```

anova amm pond

```
                  Number of obs =     96     R-squared     =  0.7758
                  Root MSE      =  .75234     Adj R-squared =  0.7684

            Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
             Model |  180.147148     3  60.0490494     106.09     0.0000
                   |
pond |  180.147148     3  60.0490494     106.09     0.0000
                   |
          Residual |  52.0734338    92  .566015585
           ----------+----------------------------------------------------
             Total |  232.220582    95  2.44442718
```

anova bod pond

```
                  Number of obs =     96     R-squared     =  0.3446
                  Root MSE      = 14.5155     Adj R-squared =  0.3232

            Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
             Model |  10189.7083     3  3396.56944      16.12     0.0000
```

```
                |
pond |  10189.7083     3  3396.56944      16.12     0.0000
                |
           Residual |    19384.25    92   210.69837
          -----------+----------------------------------------------------
              Total |  29573.9583    95  311.304825


. anova tow pond

                      Number of obs =      96     R-squared     =  0.0040
                      Root MSE      = 4.26023     Adj R-squared = -0.0285

             Source |  Partial SS    df       MS          F     Prob> F
          -----------+----------------------------------------------------
              Model |  6.73364551     3   2.2445485      0.12     0.9459
                |
pond |  6.73364551     3   2.2445485      0.12     0.9459
                |
           Residual |  1669.75795    92  18.1495429
          -----------+----------------------------------------------------
              Total |  1676.49159    95  17.6472799


.
```

anova toa pond

```
                   Number of obs =     96     R-squared     =  0.0000
                   Root MSE      = 4.64443     Adj R-squared = -0.0326

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |         0     3         0       0.00     1.0000
                     |
pond |         0     3         0       0.00     1.0000
                     |
            Residual |  1984.50633    92   21.570721
           ----------+----------------------------------------------------
               Total |  1984.50633    95  20.8895404
```

. anova con pond

```
                   Number of obs =     96     R-squared     =  0.7597
                   Root MSE      = 125.903     Adj R-squared =  0.7518

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  4609539.36     3  1536513.12      96.93     0.0000
```

```
                |
pond |  4609539.36     3  1536513.12      96.93     0.0000
                |
           Residual |  1458347.13    92  15851.5992
          -----------+----------------------------------------------------
              Total |  6067886.49    95  63872.4894


.
. anova tur pond

                      Number of obs =      96     R-squared     =  0.6840
                      Root MSE      = 30.5139     Adj R-squared =  0.6737

             Source |  Partial SS    df       MS          F     Prob> F
          -----------+----------------------------------------------------
                Model |  185434.208     3  61811.4028      66.39     0.0000
                |
pond |  185434.208     3  61811.4028      66.39     0.0000
                |
           Residual |    85660.75    92  931.095109
          -----------+----------------------------------------------------
              Total |  271094.958    95  2853.63114
```

.

anova rai pond

```
                   Number of obs =     96     R-squared     =  0.0000
                   Root MSE      = 136.278     Adj R-squared = -0.0326

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |          0     3          0       0.00     1.0000
                     |
pond |  7.9164e-26     3  2.6388e-26       0.00     1.0000
                     |
            Residual |  1708607.33    92  18571.8188
           ----------+----------------------------------------------------
               Total |  1708607.33    95  17985.3404
```

. pwcorraikaciharph do co2 no3 po4 amm bod tow toa con turrai, star(.01)

```
           |     aikaciharph      do     co2     no3
-----------+------------------------------------------------------------
aik |   1.0000
aci |   0.4101*  1.0000
```

har | 0.7803* 0.1973 1.0000

ph | 0.2343 -0.0049 0.2282 1.0000

do | 0.1608 0.2257 0.0335 0.3745* 1.0000

co2 | 0.4695* 0.6059* 0.4303* 0.0715 0.1974 1.0000

no3 | 0.5059* 0.4132* 0.3387* 0.1332 0.2669* 0.3709* 1.0000

po4 | 0.1480 -0.0997 0.2035 -0.0208 -0.1274 0.0041 0.0309

amm | 0.6216* 0.5543* 0.5855* 0.2417 0.3720* 0.5657* 0.6701*

bod | 0.3451* 0.3072* 0.3248* 0.2038 0.3305* 0.3102* 0.4849*

tow | -0.2789* -0.0616 -0.1982 0.0177 0.1791 -0.0513 -0.1980

toa | -0.3625* -0.0307 -0.2590 -0.0892 0.0662 -0.0803 -0.2588

con | 0.6515* 0.4629* 0.5690* 0.3335* 0.4688* 0.4516* 0.5420*

tur | 0.4611* 0.3851* 0.4057* 0.2849* 0.5771* 0.3654* 0.4562*

rai | -0.3899* 0.0053 -0.3044* -0.0912 0.1470 -0.0753 -0.1975

| po4amm bod tow toa con tur

---

po4 | 1.0000

amm | 0.1317 1.0000

bod | 0.1264 0.5561* 1.0000

tow | -0.1919 -0.0841 -0.0974 1.0000

toa | -0.2170 -0.1696 -0.1129 0.8369* 1.0000

con | 0.1788 0.7309* 0.5945* 0.0379 -0.0510 1.0000

tur | 0.1427 0.6775* 0.6209* 0.0367 -0.0433 0.8170* 1.0000

rai | -0.2933* -0.1838 -0.0630 0.5885* 0.7932* -0.0520 -0.0225

| rai

---

rai | 1.0000

. pwcorraikaciharph do co2 no3 po4 amm bod tow toa con turrai, sig

| aikaciharph do co2 no3

---

aik | 1.0000

|

|

aci | 0.4101 1.0000

| 0.0000

|

har | 0.7803 0.1973 1.0000

| 0.0000 0.0541

|

ph | 0.2343 -0.0049 0.2282 1.0000

| 0.0216 0.9623 0.0253

|

do | 0.1608 0.2257 0.0335 0.3745 1.0000

| 0.1176 0.0271 0.7456 0.0002

|

co2 | 0.4695 0.6059 0.4303 0.0715 0.1974 1.0000

| 0.0000 0.0000 0.0000 0.4886 0.0539

|

no3 | 0.5059 0.4132 0.3387 0.1332 0.2669 0.3709 1.0000

| 0.0000 0.0000 0.0007 0.1957 0.0086 0.0002

|

po4 | 0.1480 -0.0997 0.2035 -0.0208 -0.1274 0.0041 0.0309

| 0.1502 0.3337 0.0467 0.8409 0.2159 0.9680 0.7650

|

amm | 0.6216 0.5543 0.5855 0.2417 0.3720 0.5657 0.6701

| 0.0000 0.0000 0.0000 0.0177 0.0002 0.0000 0.0000

|

bod | 0.3451 0.3072 0.3248 0.2038 0.3305 0.3102 0.4849

| 0.0006 0.0023 0.0012 0.0464 0.0010 0.0021 0.0000

|

tow | -0.2789 -0.0616 -0.1982 0.0177 0.1791 -0.0513 -0.1980

| 0.0059 0.5510 0.0529 0.8637 0.0808 0.6196 0.0531

|

toa | -0.3625 -0.0307 -0.2590 -0.0892 0.0662 -0.0803 -0.2588

| 0.0003 0.7669 0.0108 0.3875 0.5218 0.4370 0.0109

```
         |
con |   0.6515   0.4629   0.5690   0.3335   0.4688   0.4516   0.5420
         |   0.0000   0.0000   0.0000   0.0009   0.0000   0.0000   0.0000
         |
tur |   0.4611   0.3851   0.4057   0.2849   0.5771   0.3654   0.4562
         |   0.0000   0.0001   0.0000   0.0049   0.0000   0.0003   0.0000
         |
rai |  -0.3899   0.0053  -0.3044  -0.0912   0.1470  -0.0753  -0.1975
         |   0.0001   0.9592   0.0026   0.3770   0.1531   0.4657   0.0538
         |


         |     po4amm      bod      tow      toa      con      tur
-------------+------------------------------------------------------------------
       po4 |   1.0000
         |
         |
amm |   0.1317   1.0000
         |   0.2009
         |
bod |   0.1264   0.5561   1.0000
         |   0.2196   0.0000
         |
tow |  -0.1919  -0.0841  -0.0974   1.0000
```

| 0.0610 0.4151 0.3453

|

toa | -0.2170 -0.1696 -0.1129 0.8369 1.0000

| 0.0337 0.0986 0.2733 0.0000

|

con | 0.1788 0.7309 0.5945 0.0379 -0.0510 1.0000

| 0.0813 0.0000 0.0000 0.7138 0.6218

|

tur | 0.1427 0.6775 0.6209 0.0367 -0.0433 0.8170 1.0000

| 0.1653 0.0000 0.0000 0.7229 0.6751 0.0000

|

rai | -0.2933 -0.1838 -0.0630 0.5885 0.7932 -0.0520 -0.0225

| 0.0037 0.0731 0.5423 0.0000 0.0000 0.6152 0.8277

|

| rai

-------------+---------

rai | 1.0000

|

ANOVA FOR POND AND PLANKTON DATA:

.

anova ana pon

Number of obs = 96 R-squared = 0.0249

```
                    Root MSE      = 84.4178     Adj R-squared = -0.0069

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  16770.8333     3  5590.27778      0.78     0.5056
                     |
pond |  16770.8333     3  5590.27778      0.78     0.5056
                     |
            Residual |     655625    92   7126.3587
           ----------+----------------------------------------------------
               Total |  672395.833    95  7077.85088


.

. anova ans pond

                    Number of obs =      96     R-squared     =  0.0682
                    Root MSE      =  54.507Adj R-squared =  0.0378

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |      20000     3  6666.66667      2.24     0.0884
                     |
pond |      20000     3  6666.66667      2.24     0.0884
```

```
                |
       Residual |  273333.333    92  2971.01449
----------+-----------------------------------------------------
          Total |  293333.333    95   3087.7193


.
. anova chr pond

                  Number of obs =      96     R-squared     =  0.0304
                  Root MSE      = 8681.78     Adj R-squared = -0.0012

          Source |  Partial SS    df       MS          F     Prob> F
----------+-----------------------------------------------------
           Model |   217448958     3  72482986.1      0.96     0.4144
                |
pond |   217448958     3  72482986.1      0.96     0.4144
                |
       Residual |  6.9343e+09    92  75373231.4
----------+-----------------------------------------------------
          Total |  7.1518e+09    95  75281960.5


.
. anova mer pond
```

```
                Number of obs =     97     R-squared     =  0.1356
                Root MSE      = 295.315     Adj R-squared =  0.1077

          Source |  Partial SS    df       MS          F     Prob> F
        -----------+----------------------------------------------------
           Model |  1271836.34     3  423945.447      4.86     0.0035
                 |
pond |  1271836.34     3  423945.447      4.86     0.0035
                 |
        Residual |     8110625    93  87211.0215
        -----------+----------------------------------------------------
           Total |  9382461.34    96  97733.9723

. anova mic pond

                Number of obs =     96     R-squared     =  0.2024
                Root MSE      = 3954.35     Adj R-squared =  0.1764

          Source |  Partial SS    df       MS          F     Prob> F
        -----------+----------------------------------------------------
           Model |   365059628     3   121686543      7.78     0.0001
                 |
```

```
pond |   365059628     3   121686543      7.78     0.0001
                |
    Residual |  1.4386e+09    92  15636911.4
-------------+----------------------------------------------------
       Total |  1.8037e+09    95  18985847.1


.
. anova spi pond

                          Number of obs =      96     R-squared     =  0.0543
                          Root MSE      = 6114.94     Adj R-squared =  0.0235

                 Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
                  Model |   197556146     3  65852048.6      1.76     0.1601
                        |
pond |   197556146     3  65852048.6      1.76     0.1601
                        |
                Residual |  3.4401e+09    92  37392531.7
-------------+----------------------------------------------------
                   Total |  3.6377e+09    95  38291253.3


.
```

```
. anova eug pond

                   Number of obs =     97     R-squared     =  0.0464
                   Root MSE      = 607.886     Adj R-squared =  0.0157

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
              Model |  1672868.36     3  557622.788      1.51     0.2173
                    |
pond |  1672868.36     3  557622.788      1.51     0.2173
                    |
            Residual |  34365832.7    93  369525.082
           -----------+----------------------------------------------------
              Total |    36038701    96  375403.136

.
. anova lep pond

                   Number of obs =     96     R-squared     =  0.0500
                   Root MSE      = 8262.78     Adj R-squared =  0.0190

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
```

```
            Model |  330244571     3   110081524      1.61    0.1919
                  |
pond |  330244571     3   110081524      1.61    0.1919
                  |
         Residual |  6.2812e+09    92    68273489
-----------+----------------------------------------------------
            Total |  6.6114e+09    95  69593742.8


. anova pha pond

                        Number of obs =      96     R-squared     =  0.0783
                        Root MSE      =  693.17Adj R-squared =  0.0482

           Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
            Model |  3752741.67     3  1250913.89      2.60    0.0566
                  |
pond |  3752741.67     3  1250913.89      2.60    0.0566
                  |
         Residual |  44204591.7    92  480484.692
-----------+----------------------------------------------------
            Total |  47957333.3    95  504814.035
```

```
.
. anova tra pond

                   Number of obs =     96     R-squared     =  0.0268
                   Root MSE      = 7476.73     Adj R-squared = -0.0050

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |   141469628     3  47156542.7       0.84     0.4735
                     |
pond |   141469628     3  47156542.7       0.84     0.4735
                     |
            Residual |  5.1429e+09    92  55901430.4
           ----------+----------------------------------------------------
               Total |  5.2844e+09    95    55625276

. anova act pond

                   Number of obs =     96     R-squared     =  0.0385
                   Root MSE      = 39.3418     Adj R-squared =  0.0072

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
```

```
            Model |    5703.125     3  1901.04167      1.23     0.3040
                  |
pond |    5703.125     3  1901.04167      1.23     0.3040
                  |
         Residual |  142395.833    92   1547.7808
-----------+----------------------------------------------------
            Total |  148098.958    95   1558.9364


. anova coe pond

                  Number of obs =      96     R-squared     =  0.0294
                  Root MSE      = 838.641     Adj R-squared = -0.0022

           Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
            Model |  1960078.13     3  653359.375      0.93     0.4301
                  |
pond |  1960078.13     3  653359.375      0.93     0.4301
                  |
         Residual |  64705312.5    92  703318.614
-----------+----------------------------------------------------
            Total |  66665390.6    95  701740.954
```

. anova cru pond

```
                   Number of obs =     96     R-squared     =  0.0316
                   Root MSE      = 10.2062     Adj R-squared =  0.0000

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
              Model |       312.5     3  104.166667       1.00     0.3966
                    |
pond |       312.5     3  104.166667       1.00     0.3966
                    |
            Residual |  9583.33333    92  104.166667
           -----------+----------------------------------------------------
              Total |  9895.83333    95  104.166667
```

. anova dic pond

```
                   Number of obs =     96     R-squared     =  0.0438
                   Root MSE      = 60.9314     Adj R-squared =  0.0126

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
              Model |  15636.4583     3  5212.15278       1.40     0.2467
```

```
                |
pond |  15636.4583     3  5212.15278      1.40     0.2467
                |
           Residual |    341562.5    92  3712.63587
          -----------+----------------------------------------------------
              Total |  357198.958    95  3759.98904


. anova pan pond

                          Number of obs =      96     R-squared     =  0.0564
                          Root MSE      = 1257.89     Adj R-squared =  0.0257

             Source |  Partial SS    df       MS          F     Prob> F
          -----------+----------------------------------------------------
              Model |  8708570.12     3  2902856.71      1.83     0.1464
                |
pond |  8708570.12     3  2902856.71      1.83     0.1464
                |
           Residual |   145570251    92  1582285.33
          -----------+----------------------------------------------------
              Total |   154278821    95  1623987.59


.
```

```
anova ped pond

 Number of obs =      97     R-squared     =  0.0344
                   Root MSE      = 362.511     Adj R-squared =  0.0033

            Source |  Partial SS    df       MS          F     Prob> F
          -----------+----------------------------------------------------
             Model |  435776.332     3  145258.777      1.11     0.3510
                   |
pond |  435776.332     3  145258.777      1.11     0.3510
                   |
          Residual |  12221495.8    93  131413.934
          -----------+----------------------------------------------------
             Total |  12657272.2    96  131846.585


. anovasen pond

                   Number of obs =      96     R-squared     =  0.0432
                   Root MSE      = 314.328     Adj R-squared =  0.0120

            Source |  Partial SS    df       MS          F     Prob> F
          -----------+----------------------------------------------------
             Model |      410000     3  136666.667      1.38     0.2529
                   |
```

```
pond |     410000     3  136666.667      1.38     0.2529
                |
    Residual |  9089783.33    92  98801.9928
-----------+----------------------------------------------------
       Total |  9499783.33    95  99997.7193


. anova cyc pond

                  Number of obs =     96     R-squared     =  0.0367
                  Root MSE      = 19.4757     Adj R-squared =  0.0053

      Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
       Model |    1328.125     3  442.708333      1.17     0.3266
                |
pond |    1328.125     3  442.708333      1.17     0.3266
                |
    Residual |  34895.8333    92  379.302536
-----------+----------------------------------------------------
       Total |  36223.9583    95  381.304825
anovadia pond

                  Number of obs =     96     R-squared     =  0.0175
```

```
                Root MSE      = 20.2375     Adj R-squared = -0.0146

------------------------------------------------------------------------------
      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------------------
       Model |  669.791667     3  223.263889       0.55     0.6527
             |
        pond |  669.791667     3  223.263889       0.55     0.6527
             |
    Residual |  37679.1667    92  409.556159
-------------+----------------------------------------------------------------
       Total |  38348.9583    95  403.673246


. anova nav pond

                Number of obs =      96     R-squared     =  0.0245
                Root MSE      = 123.561     Adj R-squared = -0.0073

------------------------------------------------------------------------------
      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------------------
       Model |     35312.5     3  11770.8333       0.77     0.5132
             |
        pond |     35312.5     3  11770.8333       0.77     0.5132
             |
```

```
    Residual |  1404583.33    92  15267.2101
-------------+------------------------------------------------------
       Total |  1439895.83    95  15156.7982


. anova cer pond

                          Number of obs =      96    R-squared     =  0.0768
                          Root MSE      = 12139.8    Adj R-squared =  0.0467

    Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |  1.1280e+09     3   376002986       2.55     0.0604
             |
pond |  1.1280e+09     3   376002986       2.55     0.0604
             |
    Residual |  1.3558e+10    92   147374862
-------------+------------------------------------------------------
       Total |  1.4686e+10    95   154594697


. anova per pond

                          Number of obs =      96    R-squared     =  0.0955
                          Root MSE      = 804.234    Adj R-squared =  0.0660
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |  6279103.12     3  2093034.37      3.24     0.0258
             |
        pond |  6279103.13     3  2093034.38      3.24     0.0258
             |
    Residual |  59504870.8    92  646792.074
-------------+----------------------------------------------------
       Total |    65783974    95  692462.884


. anova arc pond

                          Number of obs =      96     R-squared     =  0.0841
                          Root MSE      = 250.969     Adj R-squared =  0.0543

      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |  532361.458     3  177453.819      2.82     0.0434
             |
        pond |  532361.458     3  177453.819      2.82     0.0434
             |
    Residual |   5794662.5    92   62985.462
```

```
-----------+----------------------------------------------------
     Total |  6327023.96    95  66600.2522
```

ANOVA for season and Plankton data:

```
. anova ana season

                   Number of obs =     96     R-squared     =  0.0187
                   Root MSE      = 84.6873     Adj R-squared = -0.0133

             Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
              Model |   12578.125     3  4192.70833      0.58     0.6266
                    |
             season |   12578.125     3  4192.70833      0.58     0.6266
                    |
           Residual |  659817.708    92  7171.93161
-----------+----------------------------------------------------
              Total |  672395.833    95  7077.85088

. anova ans season

                   Number of obs =     96     R-squared     =  0.0831
```

```
                    Root MSE      =  54.069Adj R-squared =  0.0532

              Source |  Partial SS    df       MS         F     Prob> F
           ----------+----------------------------------------------------
               Model |      24375     3       8125      2.78     0.0455
                     |
season |      24375     3       8125      2.78     0.0455
                     |
            Residual |  268958.333    92  2923.46014
           ----------+----------------------------------------------------
               Total |  293333.333    95   3087.7193

. anova chr season

                    Number of obs =      96     R-squared     =  0.0196
                    Root MSE      = 8729.93     Adj R-squared = -0.0124

              Source |  Partial SS    df       MS         F     Prob> F
           ----------+----------------------------------------------------
               Model |   140307240     3  46769079.9      0.61     0.6078
                     |
season |   140307240     3  46769079.9      0.61     0.6078
                     |
```

```
    Residual |  7.0115e+09    92  76211728.4
-------------+------------------------------------------------------
       Total |  7.1518e+09    95  75281960.5


. anova mer season

                          Number of obs =      96     R-squared     =  0.0548
                          Root MSE      = 310.457     Adj R-squared =  0.0239

                Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
                 Model |  513652.344     3  171217.448       1.78     0.1572
                       |
season |  513652.344     3  171217.448       1.78     0.1572
                       |
                Residual |  8867278.65    92  96383.4635
-------------+------------------------------------------------------
                 Total |  9380930.99    95   98746.642


.
. anovamic season

                          Number of obs =      96     R-squared     =  0.0177
```

```
                    Root MSE      = 4388.38     Adj R-squared = -0.0143

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  31934268.8     3  10644756.3       0.55     0.6476
                     |
season |  31934268.8     3  10644756.3       0.55     0.6476
                     |
            Residual |  1.7717e+09    92  19257839.2
           ----------+----------------------------------------------------
               Total |  1.8037e+09    95  18985847.1

. anova spi season

                    Number of obs =      96     R-squared     =  0.0097
                    Root MSE      = 6257.54     Adj R-squared = -0.0226

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  35245416.7     3  11748472.2       0.30     0.8253
                     |
season |  35245416.7     3  11748472.2       0.30     0.8253
                     |
```

```
    Residual |  3.6024e+09    92  39156778.8
-------------+------------------------------------------------------
       Total |  3.6377e+09    95  38291253.3


. anova eug season

                          Number of obs =      96     R-squared     =  0.0304
                          Root MSE      = 616.229     Adj R-squared = -0.0012

      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |     1095975     3     365325       0.96     0.4142
             |
      season |     1095975     3     365325       0.96     0.4142
             |
    Residual |  34935887.5    92  379737.908
-------------+------------------------------------------------------
       Total |  36031862.5    95  379282.763


.  anova lep season

                          Number of obs =      96     R-squared     =  0.0224
                          Root MSE      = 8381.77     Adj R-squared = -0.0095
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |   148038742     3  49346247.2       0.70     0.5530
             |
      season |   148038742     3  49346247.2       0.70     0.5530
             |
    Residual |  6.4634e+09    92  70253987.2
-------------+------------------------------------------------------
       Total |  6.6114e+09    95  69593742.8
```

. anova pha season

```
                  Number of obs =      96     R-squared     =  0.0161
                  Root MSE      = 716.176     Adj R-squared = -0.0160
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |  769772.917     3  256590.972       0.50     0.6830
             |
      season |  769772.917     3  256590.972       0.50     0.6830
             |
    Residual |  47187560.4    92  512908.265
```

```
-----------+----------------------------------------------------
     Total |  47957333.3    95  504814.035
```

```
. anova tra season
```

```
                   Number of obs =     96     R-squared     =  0.0278
                   Root MSE      = 7472.74     Adj R-squared = -0.0039

              Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
               Model |   146953263     3  48984420.8      0.88     0.4560
                     |
season |   146953263     3  48984420.8      0.88     0.4560
                     |
            Residual |  5.1374e+09    92  55841825.7
-----------+----------------------------------------------------
               Total |  5.2844e+09    95    55625276
```

```
. anova act season
```

```
                   Number of obs =     96     R-squared     =  0.1008
                   Root MSE      =  38.047Adj R-squared =  0.0714
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |   14921.875     3  4973.95833      3.44     0.0201
             |
      season |   14921.875     3  4973.95833      3.44     0.0201
             |
    Residual |  133177.083    92  1447.57699
-------------+----------------------------------------------------
       Total |  148098.958    95   1558.9364


. anova coe season

                          Number of obs =      96     R-squared     =  0.0509
                          Root MSE      = 829.291     Adj R-squared =  0.0200

      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |  3394817.71     3   1131605.9      1.65     0.1843
             |
      season |  3394817.71     3   1131605.9      1.65     0.1843
             |
    Residual |  63270572.9    92  687723.619
-------------+----------------------------------------------------
```

```
            Total |  66665390.6    95  701740.954
```

anova cru season

```
                  Number of obs =     96     R-squared     =  0.0526
                  Root MSE      = 10.0947     Adj R-squared =  0.0217

             Source |  Partial SS    df       MS          F     Prob> F
           -----------+----------------------------------------------------
              Model |  520.833333     3  173.611111      1.70     0.1717
                    |
season |  520.833333     3  173.611111      1.70     0.1717
                    |
           Residual |       9375    92  101.902174
           -----------+----------------------------------------------------
              Total |  9895.83333    95  104.166667
```

. anova dic season

```
                  Number of obs =     96     R-squared     =  0.0145
                  Root MSE      = 61.8586     Adj R-squared = -0.0177

             Source |  Partial SS    df       MS          F     Prob> F
```

```
-----------+----------------------------------------------------
     Model |     5162.5     3  1720.83333      0.45     0.7181
           |
season |     5162.5     3  1720.83333      0.45     0.7181
           |
  Residual |  352036.458    92  3826.48324
-----------+----------------------------------------------------
     Total |  357198.958    95  3759.98904

. anova pan season

                  Number of obs =      96     R-squared     =  0.0574
                  Root MSE      = 1257.22     Adj R-squared =  0.0267

             Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
     Model |  8862363.42     3  2954121.14      1.87     0.1403
           |
season |  8862363.42     3  2954121.14      1.87     0.1403
           |
  Residual |   145416457    92  1580613.67
-----------+----------------------------------------------------
     Total |   154278821    95  1623987.59
```

```
.
. anova ped season

                   Number of obs =     96     R-squared     =  0.0879
                   Root MSE      =  354.14Adj R-squared =  0.0582

              Source |  Partial SS    df       MS          F     Prob> F
           ----------+----------------------------------------------------
               Model |  1112078.13     3  370692.708      2.96     0.0366
                     |
season |  1112078.13     3  370692.708      2.96     0.0366
                     |
            Residual |  11538217.7    92   125415.41
           ----------+----------------------------------------------------
               Total |  12650295.8    95  133161.009


.
. anova sen season

                   Number of obs =     96     R-squared     =  0.0577
                   Root MSE      = 311.935     Adj R-squared =  0.0269

              Source |  Partial SS    df       MS          F     Prob> F
```

```
-----------+----------------------------------------------------
     Model |  547848.958     3  182616.319      1.88    0.1390
           |
season |  547848.958     3  182616.319      1.88    0.1390
           |
  Residual |  8951934.38    92  97303.6345
-----------+----------------------------------------------------
     Total |  9499783.33    95  99997.7193


.
. anova cyc season

                   Number of obs =      96     R-squared     =  0.0316
                   Root MSE      = 19.5265     Adj R-squared =  0.0001

              Source |  Partial SS    df       MS          F     Prob> F
-----------+----------------------------------------------------
     Model |  1145.83333     3  381.944444      1.00    0.3958
           |
season |  1145.83333     3  381.944444      1.00    0.3958
           |
  Residual |   35078.125    92  381.283967
-----------+----------------------------------------------------
```

```
       Total |  36223.9583    95  381.304825

.
. anova dia season

                  Number of obs =      96     R-squared     =  0.0253
                  Root MSE      = 20.1565     Adj R-squared = -0.0065

      Source |  Partial SS    df       MS          F     Prob> F
-------------+----------------------------------------------------
       Model |  970.833333     3  323.611111      0.80     0.4989
             |
      season |  970.833333     3  323.611111      0.80     0.4989
             |
    Residual |   37378.125    92  406.283967
-------------+----------------------------------------------------
       Total |  38348.9583    95  403.673246

.
. anovanav season

                  Number of obs =      96     R-squared     =  0.0349
                  Root MSE      = 122.899     Adj R-squared =  0.0035
```

```
      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |     50312.5     3  16770.8333      1.11     0.3491
             |
      season |     50312.5     3  16770.8333      1.11     0.3491
             |
    Residual |  1389583.33    92  15104.1667
-------------+------------------------------------------------------
       Total |  1439895.83    95  15156.7982


.
. anova cer season

                  Number of obs =      96     R-squared     =  0.0601
                  Root MSE      = 12248.8     Adj R-squared =  0.0295

      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |   883370625     3   294456875      1.96     0.1251
             |
      season |   883370625     3   294456875      1.96     0.1251
             |
```

```
    Residual |  1.3803e+10    92   150033974
-------------+------------------------------------------------------
       Total |  1.4686e+10    95   154594697


. anova per season

                          Number of obs =      96     R-squared     =  0.0222
                          Root MSE      = 836.155     Adj R-squared = -0.0097

      Source |  Partial SS    df       MS          F     Prob> F
-------------+------------------------------------------------------
       Model |  1461673.96     3  487224.653      0.70     0.5563
             |
      season |  1461673.96     3  487224.653      0.70     0.5563
             |
    Residual |    64322300    92  699155.435
-------------+------------------------------------------------------
       Total |    65783974    95  692462.884


. anova arc season

                          Number of obs =      96     R-squared     =  0.0692
                          Root MSE      = 253.004     Adj R-squared =  0.0389
```

Source	Partial SS	df	MS	F	Prob> F
Model	438026.042	3	146008.681	2.28	0.0845
season	438026.042	3	146008.681	2.28	0.0845
Residual	5888997.92	92	64010.8469		
Total	6327023.96	95	66600.2522		