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Degradation of microcystin-LR by aquatic bacteria

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M.Sc. **2007**

Degradation of microcystin-LR by aquatic bacteria

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(B.Sc., M.Sc. Microbiology)

**A thesis submitted in partial fulfilment of the requirements of
The Robert Gordon University
for the degree of Master of Science by research**

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Declaration

I declare that this thesis has been composed by myself and has not been previously submitted for any degree. Sources of information are referenced and help provided by other people throughout this research have been duly acknowledged.

Buddhi Sagar Ghimire

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Abstract

Microcystins are cyclic heptapeptide toxins produced by cyanobacteria. They are potentially a threat to human and domestic animals so are of worldwide interest. Microcystin-LR is stable in various environmental conditions. It is important to know the factors affecting microcystin degradation which can be utilised in water treatment methods. This research is focused on activity of indigenous microflora in biolog MT2 plates and use of active isolates for degradation study. The growth and degradation patterns were compared with the known microcystin-LR degrading bacteria *Paucibacter toxinivorans (DSMZ-16998)* as a positive control. A total of 18 bacteria were isolated from three different sources and screened for the degradation study. Individual isolates and *P. toxinivorans* were exposed to microcystin-LR in a range of different media namely, physiological saline, R2A medium, nutrient broth and water at different concentrations of 0.50 µg/ml and 10.00 µg/ml of microcystin-LR. Degradation of microcystin-LR content was analysed by HPLC. Bacteria in mixed culture i.e. natural water samples were able to degrade microcystin-LR in low concentration within a week but were unable to degrade in high concentration until 30 days at room temperature. Degradation of microcystin-LR by cell extract of active isolates and *P. toxinivorans* was observed. Percentage loss of microcystin content by enzymatic degradation was observed from 22-42 percent in tested samples.

Key words: Cyanobacteria, Microcystin-LR, Toxin, Degradation, Biolog MT2 plate, HPLC.

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List of abbreviation

Adda	3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid
AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
DIC	dissolved inorganic carbon
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
GAC	granular activated carbon
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
LD	lethal dose
LPS	lipopolysaccharide
MC-LR	microcystin-LR
Mdha	N-methyldehydroalanine
Mdhb	N-methyldehydrobutyrine
PAC	powdered activated carbon
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
TFA	trifluroacetic acid
UV	ultraviolet
WHO	world health organisation

Chapter 1

Introduction

1. Introduction

1.1 Cyanobacteria

Cyanobacteria, also called blue-green algae, are a common and naturally occurring component of most aquatic ecosystem and contaminate natural and man-made bodies of water. They also occur on rocks and soil, and in symbiosis with plant and fungi (Whitton *et al*., 2000). Cyanobacteria also possess a photosynthetic apparatus enabling them to perform photosynthesis as in algae and higher plants. Unicellular and filamentous forms are commonly found amongst the cyanobacteria. Several filamentous genera also produce other differentiated cells termed as akinetes which permit them to survive during adverse conditions such as cold and drought. Individual cells are quite small and can be present in a water body without being visible. One feature of cyanobacteria is their frequent formation of mass growth known as blooms in water bodies creating aesthetic, operational and health problems (Lawton *et al*., 1991). They can affect water making it unappealing for recreational activities i.e. swimming, boating, or fishing. Water affected by a bloom is also unsuitable for agricultural use. Cyanobacterial blooms may be visible as thick mats or scum on the surface of water and particularly when the scum begins to decay it can be smelly. It can be bluish-green to red in colour and might look like paint on the surface of the water. When environmental conditions cause them to bloom, they can threaten the quality of

drinking water supplies. Formation of blooms in water reservoirs or dams can produce chemicals which cause musty or earthy taste and odour in drinking water which requires extensive treatment of water for human consumption. Some species produce toxins that can be dangerous to animals and human if they are consumed, inhaled or in contact with the skin. While other cyanobacteria create problems in water treatment plants by clogging of filters, developing deposits in pipe lines, forming toxic chlorination by products. Fluctuation in water quality such as oxygen levels and increase in turbidity associated with blooms can harm aquatic organisms. In most cases cyanobacteria need warm temperature, light, phosphorus and nitrogen to reproduce. Phosphorus and nitrogen are commonly found in animal and human waste and in fertilizers. Some common ways for phosphorus and nitrogen to enter lakes and streams are from agricultural and lawn runoff and improperly functioning septic system. Cyanobacteria are cosmopolitan and possess high potential for adoption to diverse environmental factors.

1.2 Ecology of cyanobacteria and bloom formation

It is widely accepted that phosphorus and nitrogen supplies are the most important factors enhancing the growth of cyanobacteria (Chorus *et al*., 1999). Eutrophication is the enhancement of the natural process of biological production in River lakes and reservoirs caused by increasing levels of nutrients usually phosphorus and

nitrogen. Eutrophic and hypertrophic condition favours the mass development and cyanobacterial blooms worldwide (Pearl, 1996). In the tropics cyanobacterial blooms can occur at almost any time if temperature, light, nutrient content and water current is favorable for their growth (Lungayia *et al*., 2000). The decay of organic matter when the bloom dies may lead to the depletion of dissolved oxygen in the water which in turn can cause secondary problems such as fish mortality from lack of oxygen. A field study in South Africa by Wicks *et al*. (1990) has shown that certain environmental factors are associated with the amount of toxins found in cyanobacterial blooms. The effect of environmental factors on toxin production by cyanobacteria has also been shown in laboratory studies (Sivonnen, 1990). Cyanobacterial blooms persist in water supplies that contain adequate level of essential inorganic nutrients such as nitrogen and phosphorus, suitable water temperature and pH. The amount of light needed to optimize growth depends on the species. Turbulence and high water flows are unfavorable to the growth of cyanobacteria as they interfere with the organisms' ability to maintain a position in the water column and due to their slow growth rates. Heavy rain storms can increase runoff and nutrient level in the water which encourages the formation of blooms. Grazing, competition, parasitism and microbial interaction are biological factors influencing the growth of cyanobacteria (Lindel *et al*., 2005). The heterotrophic bacteria, protozoa and viruses have

been shown to share complex interactions and the effects of all of these on the growth of cyanobacteria and fluxes of organic matter within microbial loop are being studied intensively by Hoppe *et al*. (2002).

1.3 Economic importance of cyanobacteria

Several genera of cyanobacteria are capable of fixing atmospheric nitrogen either as free living microorganisms or in symbiosis with other living organisms (Sinha *et al*., 1997). They use the enzyme nitrogenase to reduce atmospheric nitrogen into ammonium ion NH_4^+ which can be utilized by other organisms. Not all cyanobacteria are problematic; some of them are beneficial to humans and the environment. Applied biotechnology has proved their contribution towards food supply. They are valuable in food production as fertilizer of soils and rice paddies. Sinha *et al*. (1997) has shown that the introduction of cyanobacteria to saline and alkaline soil improve the content of nitrogen and organic matter and also their capacity for holding water. Thomas *et al*. (1992) found that introduction of *Tolypothrix tenuis* resulted in increase of rice crops. They also increased the available nitrogen status of the soil in rice culture. Significant increase in the growth and yield upon inoculation of these mutants into rice culture was observed in comparison with chemical nitrogen fertilizer urea. Booth *et al*. (1941) showed by experiments that a coating of cyanobacteria on

prairie soil binds the particles of the soil to their mucilage coating increasing water content and reducing erosion. Cyanobacteria are rich in protein content. *Spirulina* is a staple food in most parts of Mexico and the African continent. In China, Taiwan, and Japan several species of cyanobacteria are served as a side dish or delicacy. In North America certain cyanobacteria are processed for various foods and medicinal products such as vitamins, drug components and growth factors.

Cyanobacteria have also been recognized as an excellent source of vitamins and proteins, and found in health food stores through out the world. They are also reported as sources of fine chemicals and renewable fuels. The role of bioactive compounds such as antiviral, antitumor, antibacterial and food additives have been well established (Singh *et al*., 2005). Scientists are engaged in the production of antiviral products against HIV causative agent of Acquired Immunodeficiency Syndrome (AIDS) (Jaspars *et al*., 1998). Cyanobacteria produce bioactive compounds including plant growth regulators (Zaccaro *et al*., 2006). Along with many bioactive compounds that can be exploited for their positive benefits, cyanobacteria also produce a range of harmful toxic metabolites referred to as cyanotoxins which are studied in detail.

1.4 Toxins from cyanobacteria

Out of more than 150 genera of cyanobacteria few of them are known to produce toxins in addition to cell wall LPS endotoxins.

Production of toxin is highly variable both within and between blooms and production and potency can vary over time for an individual bloom. Cyanotoxins are usually secondary metabolites and are grouped according to the physiological system, organ, tissues or cell which are primarily affected (Wiegand *et al*., 2005) as described in table 1.

Table -1 Common cyanobacteria and toxins produced by them.

Cyanotoxin	**Produced by**
Neurotoxin	
Anatoxin-a, Homoantoxin-s	*Anabaena, Aphanizomenon, Oscillatoria*
Anatoxin-a(s)	*Anabaena, Oscillatoria (planktothrix)*
Saxitoxins	*Anabaena, Aphanizomenon, Lygbya, Cylindrospermopsis*
Hepatotoxins	
Cylindrospermopsis	*Aphanizomenon, Umezakia, Cylindrospermopsis, Rophidiopsis*
Microcystins	*Anabaena, Aphanocapsa, Nostoc, Hapalosiphon, Microcystis, Oscillatoria, Planktothrix*
Nodularins	*Nodularia*
Dermal toxins/irritants	
Debromoaplysiatoxin, Lyngbyatoxin	*Lyngbya*
Aplysiatoxin	*Schizothrix*

Modified from Carmichael, 1994

1.4.1 Dermatotoxin

Marine cyanobacteria such as *Lyngbya* and *Schizothrix* may produce toxins that can cause severe dermatitis among swimmers and

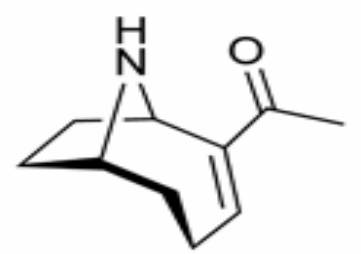
others that come in contact with them. Freshwater species of *Lyngbya* can also produce dermal toxins. Symptoms include burning sensation, erythema, blistering and deep desquamation (Moore, 1984). The toxin responsible for these effects have been identified as aplysiatoxin and debromoaplysiatoxin (Moore *et al*., 1996)

1.4.2 Cytotoxins

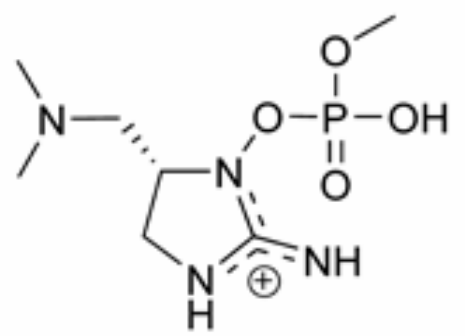
The term cytotoxin is used to refer to compounds that have the potential to affect cells of most tissues. *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum* and *Umezakia natans* have the potential to produce the toxin cylindrospermopsin which affect a number of different organs or tissues; it is regarded as a cytotoxic cyanotoxin. Although it is often classified along with the hepatotoxins since one of its main effect is on the liver. Cylindrospermopsin (fig-1) is one of the most recently characterized cyanotoxins and is causing increasing concern in water supplies.

1.4.3 Neurotoxin

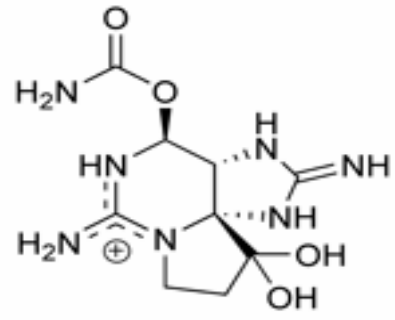
There are three type of cyanobacterial neurotoxins, Anatoxin-a, Anatoxin-a(s) and the saxitoxins also called paralytic shellfish poison (PSP) toxins.



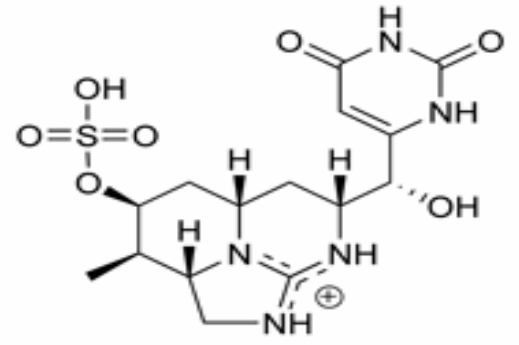
Anatoxin -a



Anatoxin-a(s)



Saxitoxin



Cylindrospermopsin

Figure–1 Cytotoxin and neurotoxins produced by cyanobacteria

Saxitoxin (fig-1) includes a number of structurally similar toxins including saxitoxin, neosaxitoxin, C-toxin and gonyoutoxins. A number of genera of cyanobacteria can produce neurotoxins. Neurotoxic cyanotoxins affect nerve conduction in various ways and ingestion of these toxins can cause paralysis of skeletal and

respiratory muscles leading to respiratory failure within a few minutes to a few hours of exposure depending on the species, the amount of toxin ingested, and the amount of food in the stomach (Carmichael, 1992).Mode of action of saxitoxins regarding toxicity in vertebrates is via the blockage of voltage-gated sodium channels resulting in paralysis and in acute cases death.

The suffix (s) of anatoxin-a(s) indicates that as with synthetic organophosphorus pesticides one of the symptoms of intoxication is hypersalivation. Anatoxin-a(s) (fig-1) has been found in some *Anebaena* species *(*Sivonen *et al*., *1999).* Anatoxin-a(s) is an anticholinesterase inhibitor with intraperitoneal LD_{50} in mice of 20 µg/kg.

Anatoxin–a (fig-1) is a secondary amine and its molecular mode of toxic activity is as a post synaptic acetylcholine antagonist resulting in paralysis, asphyxiation and death. Five naturally occurring structural variants including homoanatoxin-a are known, with some variants thought to be degradation products of the parent toxin. Neurotoxins are not considered to be as widespread in water supplies and they do not appear to pose the same degree of risk from chronic exposure as microcystins (Fawell *et al*., 1993). Dog poisonings in Scotland were reported to be due to consumption of *Oscillatoria* containing anatoxin-a (Codd *et al*., 1992) suggesting the main risks are to pets and livestock or where recreational water is affected.

1.4.4 Lipopolysacharide endotoxins

The LPS component of cyanobacterial cells has been implicated as the potential causative agent of skin irritations, gastrointestinal disorder and respiratory allergy among people who have had recreational contact with water affected by cyanobacterial bloom. Cyanobacterial LPS has been demonstrated to affect the phase II microcystin detoxication mechanism in zebra fish (*Danio revio*). LPS preparations from cyanobacteria significantly reduce activity of both the soluble and the microsomal glutathione S-transferases (Wang *et al*., 1994). Cyanobacterial blooms always contain a cocktail of different cyanotoxins like lipopolysachharides and microcystins. The dermal route of uptake and allergic/irritant reactions probably caused by LPS may pose a hazard during the recreational use of water bodies contaminated with cyanobacterial bloom (Falconer, 1996). LPS is heat stable and toxic to mammals. LPS molecules consist of 3 main parts; antigens, core polysaccharides and lipid a moieties. The lipid a region is responsible for biological responses and symptoms of LPS exposure including fever, diarrhea, vomiting and hypertension. Fever is generally caused by the release of pyrogenic compounds by the host body in response to LPS ingestion with haemodialysis water and aerosolized LPS being important potential source of exposure.

1.4.5 Hepatotoxins

Globally the most frequently found cyanotoxins in blooms are the hepatotoxic microcystins and nodularin. The most common microcystin variant is microcystin-LR which has a LD_{50} value of 50.0 µg/kg in mice by intraperitoneal injection (Carmichael, 1998). It is 200 times more toxic than cyanide. The two letter suffixes are derived from the variability of the molecule as a result of amino acid substitutions at position 2 and 4 of the heptapeptide ring with L-leucine and R-arginine occupying these positions in this particular microcystin variant (fig-2). Seventy-five structural variants of this toxin are currently known which represent a range of amino acid substitutions along with other alterations such as methylation and demethylation. The general structure of microcystin is characterized as cyclo (D-Ala^1-X^2-D-$MeAsp^3$-Z^4-$Adda^5$-D-glu^6-$Mdha^7$) in which X, and Z is variable L-amino acids, D-MeAsp is D-erythro-B-methylaspartic acid. Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 6-trimethyl-10-phenyl-deca-4, 6-dienoic acid and Mdha is N-methyldehydroalanine (Rapala *et. al*., 1994).

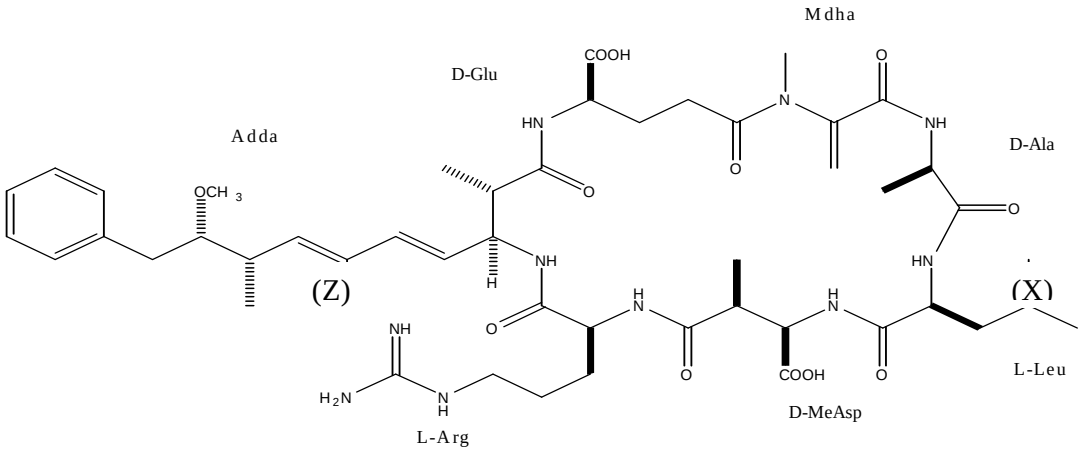


Figure -2 Structure of microcystin-LR in which X and Z are variable of L amino acids.

Cyclic peptides are small molecules with the molecular weight ranging from over 800-1000 Da (Botes *et al*., 1982). Most congeners are hydrophilic and generally not able to penetrate vertebrate cell membranes and therefore require uptake via adenosine triphosphate (ATP) dependent transporters (Fisher *et al*., 2000).

Microcystin is composed of five amino acids and two variable L-amino acids e.g. MC-LR contains leucine and arginine while nodularin only five amino acids. Nodularin differs from microcystin in that it is missing D-ala and the X variable amino acid and substitution of N-methyldehydrobutyrine (Mdhb) residue for N-methyldehydroalanine (Mdha) residue. Toxins of this class disrupt

the integrity of liver cells leading to cell death and depending on the extent of tissue damage, bleeding within the liver may also occur. They are tumour promoter (Falconer *et al*., 1996) and carcinogens (Ito *et al*., 1997). Microcystins are produced by various species within the genera *Microcystis, Anabaena, Oscillatoria (Plankohrix), Nodularia, Nostoc, Umezakia, Cylindrospermopsis* (Fawell *et al*., 1993). An unidentified multi specific organic anion transporter / bile acid transporter has been described as the carrier of these cyclic peptides in rat liver (Runnergor *et al*., 1991; Erikson *et al*., 1990). Microcystins and nodularin distort liver cells by acting on the cytoskeleton, the grid work of protein strands that among other functions give shape to cells. The cytoskeleton components most affected by the toxins are the protein polymers. As a cytoskeleton shrinks the finger like projection through which hepatocytes interact with neighboring cells withdraw breaking the cell contact with other hepatocytes and sinusoidal capillaries.

It is supposed that one possible mechanism for cancer promotion by microcystin-LR is the inhibition of protein phosphatase PP1 and PP2A which leads to hyperphosphorylation of some cellular proteins and results in uncontrolled cell proliferation. It is also believed that microcystin-LR may directly induce gene mutations. The toxic effects are largely due to inhibition of phosphatase enzymes acting to regulate protein phosphorylation. The consequences include structural damage, apoptosis and at lower concentration cell cycle

effects and tumour promotion. Death of vertebrate animals is mostly the consequence of severe liver damage which starts with cytoskeletal disorganization and can include cell bleeding, cellular disruption, and lipid peroxidation, loss of membrane integrity, DNA damage, apoptosis, necrosis, intrahepatic bleeding and ultimately death by hemorrhagic shock (Wickstron *et al*., 1996).

1.5 Major health issues caused by cyanotoxins

Human health is of concern to each of us having a direct impact on our lives through our well being and that of others. In the face of the widespread occurrence of microcystins the World Health Organization has published preliminary guideline value of 1 µg/l of microcystin-LR in drinking water. Cyanobacterial growth leading to blooms along with toxin formation and their fatalities to livestock, pets, wild animals, aquatic animals, birds and human are known worldwide but there are no reliable figures for the number of people affected except in the Brazil outbreak. Health effects can occur when surface scums or water containing high level of microcystin are swallowed, through contact with skin while swimming, wading or showering or by airborne droplets while staying near by pond or reservoirs contaminated with toxins. Some major incidences are highlighted here.

1959 Canada - 13 people who were exposed to recreational water contaminated with cyanobacteria became ill with symptoms of head

ache, nausea, muscular pain and pain full diarrhea (Dillenberg *et al*., 1996) where *Microcystis* and *Anabaena sp* were identified.

1979 Australia - There was a major outbreak of hepatoenteritis among the children of Queensland Australia (Byth, 1980) altogether 140 children and 10 adult required treatments. The causative organism was identified as *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* (Terao *et al*., 1994). It has since been speculated that the causative toxin was cylindrospermopsin which was uncharacterized at the time of the event. Elsaadi *et al*. (1993) reported on 26 cases with skin diseases and multiple symptoms associated with exposure to River water in Australian during 1991-1992. The isolates were identified as *Anabaena sp*.

1988 Brazil - A severe gastro-enteritis epidemic in Brazil where some 2000 gastroenteritis cases and 88 of which resulted in death recorded where toxin produced by cyanobacteria was confirmed as causative agent.

1989 - United Kingdom 20 army recruits showed symptoms indicating intoxication after swimming and canoe training in water with a dense bloom of *Microcystis sp* (Turner *et al*., 1990).

Primary liver cancer is one of the most common cancers in China. In 1994 and 1995 it accounted for 24 mortalities per 100000 populations in some rural counties and cities. Recently an epidemiological survey in Haimen city and Fisui County in China found a close relationship between the incidence of primary liver

cancer and the use drinking water from ponds and ditches (Ueno *et al*., 1996).

1996 Brazil - An outbreak of severe hepatitis occurred at Brazilian haemodialysis centre where over hundred patients developed acute liver failure and fifty of them died (Jochimsen *et al*., 1998).

1.6 Synthesis and release of cyanotoxins

The factors inducing toxin production by cyanobacterial bloom are not well known so far. Laboratory studies demonstrate that temperature, light, nitrogen concentration, carbon availability, phosphate concentration and pH play an important role. As toxin production varies greatly among different strains of the same species, genetic difference and metabolic processes may also be important in the production of these secondary metabolites. As per cited literature, the ability to produce toxins can vary temporally and spatially at a particular site (Ressom *et al*., 1994).

Cyanobacterial toxins are associated with cyanobacterial cells and may be membrane bound or occur free within the cells. In laboratory studies most of the toxin release occurs as cells age and die and passively leak their cellular contents. Sometime, active release of toxins can also occur from young growing cells (Carmichael *et al*., 1992)

Microcystins are synthesized non-ribosomally by a multi-enzyme complex, comprised of polyketide synthases and polypeptide

synthetases (Meissner *et al.,* 1996). The ability of a cyanobacterial strain to produce toxins may thus depend primarily on the possession of these genes and on their expression under certain environmental conditions. The huge enzymes (200-2000 kDa) are composed of modules. The modules (mcyA-mcyJ) are coded in one gene-cluster with ten open reading frames. Each is responsible for one synthesis step (Tillet *et al*., 2000)

Toxic cyanobacteria release their toxins to the surrounding water after a momentary collapse caused by unfavorable conditions (Berg *et al*., 1987), by disintegration over longer times (Watanabe *et al*., 1992) or by lysis following algicide treatments to remove nuisance algae (Jones *et al*.,1994). Cyanobacterial cells are also lysed by different chemicals including reglone A, potassium permanganate, copper sulphate, chlorine, ozone, simazine. Use of such algicides led to several intoxications of livestock with cyanobacterial toxins due to release of cyanotoxins (Carmichael *et al*., 1995). Several cases of severe intoxication of humans have also been reported after treatment of a drinking water dam with copper sulphate. In addition, lysis of cyanobacterial cells has been observed as a result of passage through a cooling system in the nuclear power plant in Illinois USA (Landgraf *et al*., 1978).

1.7 Stability and degradation of microcystin-LR

Microcystins are chemically stable in water (Jones *et al*., 1994). Dissolved microcystin has been documented to be resistant to conventional water treatment processes (Hoffman, 1976). Cousins *et al*. (1996) found that microcystin is stable over 27 days in deionized water and over 12 days in sterilized reservoir water. Microcystins tolerate heating up to 100°C, chlorination and other treatment used for removal of unwanted compounds (Nicholson *et al*., 1994). Microcystins are relatively persistent in the aquatic environment. Studies in different parts of the world have shown that microcystin-LR was present up to 21 days following treatment of a *M. aeruginosa* bloom with an algicide (Jones *et al*., 1994). Research conducted in the United Kingdom indicated that five days were required for the destruction of 50% of the toxin (Fawell *et al*., 1998). Biodegradation and photolysis are means by which released microcystin-LR can naturally decrease in concentration (Kenefick *et al*., 1993; Tsuji *et al*., 1994). Cousins *et al*. (1996) demonstrated that the primary biodegradation of microcystin–LR in reservoir water had a half-life of approximately four days. It was noted that the half-life of this toxin in natural water would likely vary considerably with changes in water temperature and the size of the microbial population but was generally unstable after one week in reservoir raw water due to biodegradation. The mechanism of inactivation is probably by modification of the Adda side chain

(Cousins *et al*., 1996) which is known to be essential for toxicity. Purified microcystins are stable under irradiation by sunlight although sunlight irradiation with pigments contained in cyanobacteria significantly decomposed the toxins by isomerization of the double bond in the Adda side chain the half-life for this process was observed to be about 10 days. Much more rapid degradation of microcystin-LR by additional isomerization mechanism was observed when the toxins were exposed to UV light at wavelength around their absorption maxima (238-254 nm) (Tsuji *et al*., 1995).

Microcystins are resistant to chemical hydrolysis or oxidation. For example complete chemical hydrolysis is achieved only by 6 M hydrochloric acid treatment at high temperature (Harada *et al*., 1996). Furthermore they are not enzymatically cleaved by common proteases and peptidases.

1.8 Microcystin and existing water treatment techniques

Detoxification of microcystin can be achieved by dilution, adsorption, thermal decomposition aided by temperature and pH, photolysis and biological degradation (Tsuji *et al*., 1994). The destruction of cyanobacterial cells by chemical or mechanical means may enhance the release of toxins contained within the cells and thus mandates further treatment of the water for drinking purposes. Rapid filtration and slow sand filtration are also not efficient in

removing cyanobacterial cells and in the event of cell lysis on the filter may lead to release of toxins in to the water. Adsorption via granular activated carbon (GAC) or powdered activated carbon (PAC) can be an efficient method. Granular activated carbon has been successful in removing microcystin (Falconer *et al.,* 1989; Lahti *et al*., 1989; Jones *et al*., 1993).

Chlorination which might generate chlorination by-products has been shown in several studies to be inefficient in removing cyanobacterial toxins. But higher concentration with at least 30 minutes contact time can be effective. Combination of titanium dioxide ultraviolet light and hydrogen peroxide has been proved as potentially viable technique for water works faced with microcystin contamination in raw water (Cornish *et al*., 2000). Ozonation has been shown to be very effective method for destroying microcystin and nodularin. A single step is not sufficient as combination of ozone and organic material may result in depletion of ozone resulting in incomplete oxidation of toxin. However pure microcystin and nodularin can be oxidized within seconds to minutes (Rositano *et al*., 1998). Biological filtration techniques are becoming more attractive for water authorities as they incorporate natural biodegradation principle and do not produce deleterious by-products. The majority of researchers have used sand media for biological filtration studies (Sherman *et al*., 1995; Grutzmacher *et al*., 2002).

1.8.1 Chlorination

Chlorination is not always an effective process in destroying cyanotoxins (Hoffmann, 1976; Rositano *et al*., 1994). The efficiency of chlorination seems to depend largely on the chloride compounds and the concentration used. Aqueous chlorine and calcium hypochlorite at <1 mg/L remove more than 95% of microcystins or nodularin while sodium hypochlorite at the same dose or chloramines achieve 40-80% removal at the most (Rositano *et al.,* 1994). A chlorine residue of at least 0.5 mg/l should be present after 30 min contact time in order to destroy microcystin completely.

The toxins are easily decomposed by chlorination with sodium hypochlorite and the decomposition depends on the free chlorine dose (Tsuji *et al*., 1997). In this process many reaction products were formed one of which was determined to be dihydroxy-microcystin formed through the addition of a chlorine ion at the conjugated diene of Adda followed by hydrolysis. Although this result suggested that chlorination at an adequate chlorine dose is very effective for the removal of microcystin in raw water, peroxidation of the cell itself with chlorine must be avoided because it frequently causes toxin release from cyanobacteria and produce trihalomethanes (THM) which are known to be harmful to human health.

1.8.2 Photochemical degradation

Microcystins are very stable under natural sunlight (Tsuji *et al*., 1994) where as ultraviolet (UV) light around the absorption maxima of 238 nm rapidly decomposed the toxins. The removal of number of microcystin variants including microcystin-LR, -RR and -YR have been demonstrated using titanium dioxide (TiO_2) with UV activation in a number of different reactor designs (e.g. batch powder reactor, and falling film reactor) in which an oxygen purge, UV radiation and semi conductor titanium dioxide (TiO_2) catalyst and H_2O_2 were used to decompose the microcystin pollutants (Shepherd *et al*., 1998, 2002; Lee *et al*., 2004; Lawton *et al*., 2003, 2004; Babica *et al*., 2005; Cornish *et al*.,1999). Fe (VI) assisted photocatalytic degradation of microcystin-LR using titanium dioxide was well documented by Yuan *et al*. (2005). During their study they focused on enhancing photocatalytic degradation of microcystin-LR following the addition of ferrate as the indirect electron acceptor to the process. Robertson *et al*. (1998) investigated and examined the mechanism of photocatalytic destruction of microcystin-LR at the photocalyst surface. The result indicated that the destruction process occurred by the attack of hydroxyl radical generated on the surface of the photocatalyst. Only few studies on other microcystin variant are available. Photocatalytic destruction of the cyanotoxin nodularin using TiO_2 was investigated by Liu *et al*. (2005). They used TiO_2 photocatalysis to initiate the destruction of nodularin. The

intermediate products were monitored by liquid chromatography-mass spectrometry and reduction of toxicity following photocatalytic treatment was evaluated by using protein phosphatase inhibition assay.

1.8.3 Bacterial degradation

Biodegradation is considered as the most important mechanism for microcystin breakdown in natural conditions (Welker *et al*., 2001). Studies have shown that in bacteria there exists an enzymatic pathway

for the degradation of microcystin via three enzymes of which the first has been termed microcytinase followed by bacterial serine protease and metaloprotease (Bourne *et al*., 1996). Microcystinase is possibly for hydrolytic cleavage of the ring structure of microcystin-LR at the Adda-arginine peptide bond. This has a huge environmental significance as

toxicity data has shown that the linear (acyclo) form of microcystin-LR is considerably less toxic than the conventional microcystin-LR cyclic form (Bourne *et al*., 1996). Bacterial serine protease is responsible for breaking down the linearised microcystin-LR into tetra-peptide (more specifically NH_2-Adda-isoGlu-Mdha-Ala-OH). Metaloprotease then breaks down the tetra-peptide in to smaller peptides and amino acids. Similarly Takenaka *et al*. (1997) isolated an alkaline protease enzyme from *Pseudomonas aeruginosa* that

has been shown to be enzymes, pyochelin and pyocyanin which could generate superoxide radicals implicated in the nucleopholic hydrolysis of microcystin. Bacteria, Fungi, Protozoa as well as algae often accumulate in a polysaccharide matrix, cover submerged surfaces and constitute structurally and functionally complex communities commonly named biofilms (Wimpenny *et al*., 2001). These microbial assemblages are crucial components for assimilation, retention and transformation of dissolved and particulate organic material in the aquatic environment (Pusch *et al*., 1998). It is also shown that biofilms are able to affect the fate of environmental pollutants by their sorption, accumulation and biodegradation (Takada *et al.,* 1994; Lee *et al.,* 1997). Cyanobacteriologists working in this field have reported degradation of microcystin by mixed bacterial populations in laboratory scale batch experiments (Watanabe *et al*., 1992; Jones *et al*., 1994; Rapala *et al*., 1995; Cousins *et al*., 1996; Tsuji *et al*., 1996; Cristoffersen *et al*., 2002). Watanabe *et al*. (1992) employed a specific growth medium in their batch experiments whereas Lam *et al*. (1995) conducted their experiments using sewage effluent from a waste treatment plant as their growth medium. Miller *et al*. (2001) reported biological degradation of microcystin via batch scale bank filtration experiments using soil/water matrices. A study by Saito *et al*. (2002) showed that a biofilm grown on a nonadsorbing honeycomb tube made of vinyl chloride was capable

of degrading microcystin-LR. In that study they scraped off the surface of the tube and used it as an inoculum in laboratory batch experiments. Holst *et al.* (2003) has documented biological degradation of microcystin under anaerobic conditions and confirmed microcystin degradation with ^{14}C-microcystin-LR. Ho *et al. (2005)* carried out investigations where biological sand filtration was assessed in laboratory column experiment for its ability to remove microcystins. No traces of microcystin were found in effluent after 4 days.

1.9 Aim and objective of the study

The aim of the present investigation is to further elucidate the microbial degradation of microcystin-LR with the view to isolate stains that may be exploited in future water treatment which will be achieved by

- the study of degradation characteristics of microbial populations from a range of natural fresh water sources.
- isolation and identification of microorganisms from different lakes and their response in biolog MT2 plates while using microcystin as sole source of carbon and energy
- effect of concentration and nutrient content in microcystin degradation in laboratory studies.

Chapter 2

Materials and methods

2. Materials and Methods

2.1 Water sampling:

Samples were collected in sterile glass bottles (500 ml, 1000 ml) and ten liter plastic carbouys during winter season (November) of 2005. They were transferred to the laboratory with in three hours of collection and stored at 4-8°C until required. Sampling sites were Forfar Loch (NO 293458 Angus, Scotland), River Carron (NO 877857 Aberdeenshire, Scotland) and Loch Rescobie (NO 52505159 in Angus, Scotland). Temperature and pH were recorded on the site using calibrated portable instruments.

2.2 Preparation of samples for degradation study

2.2.1 Degradation of microcystin-LR at low concentration

Water samples (350 ml) were placed in sterile 500 ml conical flasks (four flasks per water source). Each flask was spiked with filter sterilized microcystin-LR to a final concentration of 0.50 µg/ml. Flasks were placed in a water bath to maintain constant temperature (25°C) and sparged continually with sterile air. Identical flasks were prepared with sterile water from each site to confirm that any loss of compound was due to microbial activity. Microcystin-LR was obtained from the phytotoxin research laboratory The Robert Gordon University, Scotland. All samples were monitored regularly for air supply and temperature. Samples were taken 2, 6, 9, 12, 15, 18, 21 day of degradation.

2.2.2 Degradation of microcystin-LR at high concentration

To establish the effect at higher concentration of microcystin-LR on degradation rate it was necessary to use smaller volume of water (i.e. to limit amount of toxin used). Water was placed in sterile flask (50 ml with 10 µg/ml microcystin-LR and placed in orbital shaker at 100 rpm at 29°C. Samples were collected and analyzed on 0, 3, 6, 9 12, 15, 18, 21, 24, 27, and 30 day of degradation.

2.3 Sampling for HPLC analysis

Two millilitre samples was collected from each flask (control and experimental) in 4 ml capacity glass vials. Samples were transferred to freezer at minus 20ºC. Lid of those frozen samples were removed, replaced by Parafilm (Pechiney plastic packaging) which punctured with needle then samples were freeze dried.

2.3.1 Processing of samples for HPLC

Freeze dried samples were reconstituted with 200 µl of 80% methanol (Ruthburn Chemicals Ltd Scotland) and vortexed for one minute. Two hundred micro liter of each sample was transferred to microcentrifuge tubes (1.5 ml) and centrifuged for 10 min (Eppendorf centrifuge 5410) then 100 µl of supernatant was transferred to HPLC vials for analysis. HPLC analysis was performed as described in Lawton *et al.* (1994) using the following system: 600E powerline gradient Module pump, a 2996 photodiode array

detector and a millennium 2010 chromatography manager (Water Ltd). Separation was achieved over linear gradient at 1 ml/ml using mobile phase consisted of milliQ (Millipore) water and acetonitrile (Ruthburn Chemical Ltd Scotland) both containing 0.05% trifluroacetic acid (TFA) (Fisher Scientific). The initial starting condition was 305 B which was increased to 35% over 10 minute followed by an increase to 70% B over the subsequent 30 minutes. The HPLC column used throughout was a Water C18 Symmetry column (4.6 X 250 mm) with the column temperature maintained at 40ºC. The limit of quantification using this method was determined to be 0.005 µg microcystin-LR on the column. The chromatogram was monitored at 238 nm and peak areas were used to determine concentration for each sample. Toxin was identified by their retention time and characteristic absorption spectra with a maximum of 238nm. Quantitative analysis of toxins was carried out using calibration curve (fig-3) based on peak area measurement for standard solution. Data were collected and processed using Waters Millennium software.

Table-2 HPLC Linear Gradient condition at 1 ml/min used in HPLC of microcystin. Solvent A= water-0.5% TFA and B= Acetonitrile-0.5% TFA.

Time /Min							
Mobile Phase	0	10	40	42	44	46	50
Solvent A (%)	70	65	30	0	0	70	70
Solvent B (%)	30	35	70	100	100	30	30

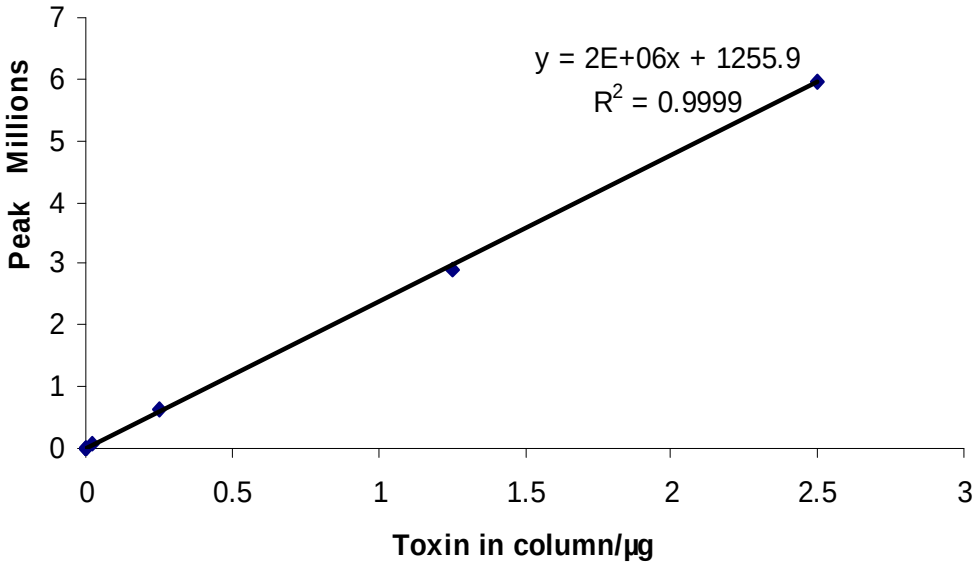


Figure-3 Linear calibration graph for ■ Microcystin-LR by HPLC

2.4 Physico-chemical analysis of water sample

Nitrogen in the form of nitrate(NO_3-N), nitrogen in the form of ammonia(NH_4-N), total nitrogen-N, organic nitrogen (Org-N), nitrogen in the form of nitrite(NO_2-N), phosphorus in the form of phosphate(PO_4-P), total phosphorus (Tot-P), organic phosphorus (Org-P), dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) were analyzed at Macaulay Institute Aberdeen.

2.5 Isolation and cultivation of bacteria

After 18 days of degradation, 1 ml of water sample was taken aseptically from each flask treated with microcystin-LR. Total of 12 samples were processed for microbiological analysis.

Plate count agar (Oxiod Ltd, UK) was used throughout the project for the isolation of bacteria from different sites. Ringer's solution (Oxoid ltd, UK) was used to make serial dilutions of water samples. Serially diluted samples (up to 10^{-5}) were spread over the agar surface and incubated at 25°C for 96 h. Colonies developed on the agar surface were counted and recorded. Single colonies from these plates were transferred to nutrient agar (NA medium) to obtain pure culture. A total of 18 isolates were sub cultured and kept in agar slants and nutrient broth for further study and characterization.

2.5.1 Positive control

Paucibacter toxinivorans (DSMZ-16998) isolated and identified by Rapala *et al*. (2005) from DSMZ Braunschweig Germany was used as positive control. The *P. toxinivorans* is a Gram negative rod shaped bacterium motile by means of single polar flagellum. It forms grayish colonies on R2A (Oxoid) plates. After incubation for 65 h at 20±2°C on R2A agar media colonies are ≤ 1mm in diameter. Cell size is 0.5-0.7 X 1.3-5.0 µm. It fails to grow at high temperature and in rich media but grows well at 20-30°C. It is oxidase positive, catalase positive, positive for acid and alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, and phosphodiesterase, chymotrypsin, esterase, lipaseesterase and leucine arylamidase. Most of the strains are positive for cysteine and valine arylamidase. It degrades microcystin and nodularin.

2.6 Morphology and biochemical characteristic of isolates

2.6.1 Gram staining

Grease-free slides were taken and smears of each isolate was prepared, air dried and heat fixed. Smears were flooded with crystal violet solution (BDH Laboratory Supplies) and left for one min. Slides were washed with distilled water and stained with Grams iodine (BDH Chemical Supplies) for one minute. Slides were decolorized with 95% ethanol (Ruthburn Chemicals Scotland) until no more stain comes away. Again the slide was washed with water

and counter stained with safranin (BDH Chemical Supplies) for two min. Slides were dried and observed under oil immersion. Bacteria with violet colored cells were noted as Gram positive and with red coloured cell were noted as Gram negative.

2.6.2 Catalase test

Three percent hydrogen peroxide, H_2O_2 (Sigma Chemical Co) was poured into the screw caped bottle containing the slant culture and the cap loosely replaced. Effervescence of oxygen (air bubbles) from bacterial growth was marked as a positive catalase test.

2.6.3 Oxidase test (cytochrome oxidase test)

Filter paper soaked with 1% aqueous tetramethyl-p-phenylenediaamine dihydrochloride (Sigma Chemical Co) was prepared. Fresh colonies were scraped from an agar plate with a clean glass rod and rubbed on the filter paper. Isolates giving a blue colour within ten second were considered as oxidase positive.

2.6.4 Motility test

The motility of bacterial isolates was checked by the hanging drop method. A small drop of diluted bacterial culture was placed in the centre of a 16 mm square number one cover slide with the aid of an inoculating loop. Drops of water or grease were placed at each corner of the cover slide. A cavity slide was inverted over the cover

glass. When cavity slide was inverted over the cover glass the hanging drop was suspended in the well. The hanging drop was observed under microscope.

2.7 Screening of bacteria using Biolog MT2 plates

2.7.1 Preparation of inoculums

Isolates were inoculated on to plate count agar. One to two colonies of 48 h culture were removed from the agar plate and suspended in 10 ml sterilized saline (0.85% W/V NaCl, Fisher scientific). The cultures were incubated at 30°C for 24 h to exhaust residual carbon nutrient content. Turbidity of cell suspension was equalized (A_{590}= 0.35) using spectrophotometer (Pharmacia biotech Nova Spec II). Adjustment was made by addition of colonies or saline solution.

2.7.2 Inoculation and incubation of Biolog MT2 microplates

Three different concentrations of microcystin-LR 160 µg/ml, 16 µg/ml and 1.6 µg/ml were prepared. Three wells with 10 µl of 160 µg/ml, three wells with 10 µl of 16 µg/ml and three wells with 10µl of 1.6 µg/ml were added in to MT2 wells before inoculation of cell suspension. A cell suspension of 150 µl was inoculated into the wells of the MT2 plate to make final microcystin-LR concentration of 10, 1 and 0.1 µg/ml in those wells. Three wells of MT2 plates (Biolog USA) were inoculated with saline and culture suspension as controls. Each isolate was tested against microcystin-LR at the three

different concentrations and saline as a control. All wells were prepared in triplicate. Plates were incubated at 25°C in plastic containers with moist paper towels to maintain humidity. Absorbance was recorded using microplate reader at 595 nm wavelengths (Dynex technologies) immediately after inoculation which is considered as zero hours then at time intervals of 4, 8, 12, 24, 48 hours.

2.8 Degradation of microcystin in growth media

From the Biolog results six isolates that are active in utilizing microcystin-LR were selected. Their ability to degrade microcystin-LR as a sole source (i.e. in saline) and in the presence of other nutrients (10 % nutrient broth) was monitored. Eighteen flasks with normal saline (50 ml) and 18 flasks with 10% nutrient broth (50 ml) were inoculated with six different isolates in triplicate. Turbidity of the culture was maintained as A_{590}=0.35 in both set. Turbidity reading of the content of the flask with medium and culture was recorded at start of the experiment i.e. 0 hour, and during every sampling to determine the increase in biomass during degradation. Sterile microcystin-LR was added to each flask at a final concentration of 5.0 µg/ml. Triplicate sterile controls were also prepared. Flasks were incubated on a rotary orbital shaker (100 rpm) at 25° C and 3 ml samples were removed from each flask at 0, 1, 3, 5, 8, 24, 48, and 72 hour intervals. Samples from nutrient

broth were tested after 15 days. Samples were processed for HPLC analysis (2.3.1).

2.9 Degradation of microcystin-LR by *Paucibacter toxinivorans*

Degradation of microcystin-LR was studied using different growth media and concentration at 10 to 20 µg/ml to compare the degradation pattern and results shown by other isolates. Three set of conical flask (total 12) each set containing 4 flasks with water (35 ml), 4 flasks with 10% nutrient broth (35 ml), 4 flasks with 10% R2A medium (35 ml) were sterilized. Three flasks with sterilized water without culture were kept as controls. Turbidity of the culture was maintained at A_{590}=0.35. Microcystin-LR was added to a final concentration 10 µg/ml level in to three flask of each set leaving one as control. These conical flasks (50 ml capacity) were incubated in shaker at 25°C. Samples were collected and analysed by HPLC (2.3.1) at 0h, 72h and 144 h.

The same experimental set up was repeated with high concentration of microcystin-LR at concentration 20 µg/ml. Sampling (3 ml) from each flask was done at 0, 1, 3, 5, 8, 24, 48, and 72 h interval. Samples were processed for HPLC analysis (2.3.1).

2.10 Enzymatic degradation of microcystin

Active isolates screened from Biolog MT2 plates were selected for enzymatic study. A bacterial strain was inoculated into peptone-yeast extract agar and incubated at 27°C. The culture was harvested after 48 h of incubation and centrifuged to yield a pellet, the supernatant was decanted, and an equal volume of 0.05 M potassium phosphate buffer (pH 6.90) was added. The cells were re-suspended and re-pelleted and the supernatant was discarded. The cells were washed with buffer in this manner three times. The re-suspended cells were sonicated (Status 200) at 4°C with MS 73 probe using 40% power cycle (Output, 150 W). The cell debris was pelleted by centrifugation at 7000 X g (ALC 4237R refrigerated centrifuge) for 20 minutes. The cell extract was decanted and used for the enzyme activity analysis. The enzyme assays were done by using 0.25 ml of cell extract and microcystin-LR at final concentration of 1 µg/ml. Phosphate buffer was added to make the assay volume up to 1.5 ml. All assay mixture were prepared in 1.5 ml eppendrof tubes (on ice), vortexed and incubated at 30°C. Sample (200 µl) were removed periodically, centrifuged at 15000 X g for 5 min and then analyzed by high performance liquid chromatography (HPLC) as described in 2.3.1.

Chapter 3

Results and discussion

3. Results and discussion

3.1 Chemistry and microbiology of fresh water

In order to obtain suitable bacteria capable of degrading microcystin-LR, water samples from three different sources after 18 day of primary degradation study (i.e. enrichment culture) were serially diluted. Diluted samples were spread on nutrient agar plates. Different types of colonies were isolated from the Petri dish on the basis of size, colour and morphology and used for subsequent experiments. Morphology and some biochemical characteristics of the 18 different isolates studied which are shown in table–3. All 18 isolates were used in degradation studies of microcystin-LR with different growth media at different microcystin-LR concentrations.

All three source water was found to be heavily populated with bacteria and suspended particles from different sources like plant debris, animal and human waste. Soil bacteria are also found in fresh water as they are in close contact with soil. Some of these bacteria are ubiquitous and can proliferate in the most diverse habitats including water. According to the kind of aquatic habitat the composition of the bacterial flora differs widely depend on the water's content of organic and inorganic materials, its pH, turbidity and temperature, sources from where organisms can enter in to the water and time of sampling. The physico-chemical

Table -3 Morphology and biochemical characteristics of isolates

Isolate	Gram's reaction	Catalase	Oxidase	Motility	Colony Characteristics
F1	Negative Rod	Positive	Positive	M	Creamy white, convex, spreading small
F2	Negative Rod	Negative	Positive	M	Slightly Colored, smooth, entire, soft
F3	Negative Rod	Positive	Positive	M	White, soft, smooth , entire, convex
F4	Negative Rod	Positive	Negative	M	Small, smooth, white, round and entire
F5	Negative Rod	Positive	Positive	M	Creamy white, convex, spreading small
F6	Negative Cocci	Positive	Positive	M	Tiny, white, soft, slightly elevated
C1	Positive Cocci	Positive	Negative	M	Round , soft, white, entire
C2	Positive Rod	Positive	Negative	M	Soft , white, spreading
C3	Negative Rod	Positive	Positive	M	Entire margin, soft and creamy white, flat
C4	Negative Cocci in cluster	Negative	Negative	M	Yellowish, smooth and spreading
C5	Negative Cocci	Positive	Positive	NM	Small yellowish, entire, convex
C6	Positive Cocci	Positive	Negative	M	Creamy white, spreading, rough
R1	Positive Rod	Positive	Negative	M	Tiny creamy white, entire
R2	Negative Rod	Positive	Positive	M	Pinkish, small, entire, smooth, soft
R3	Positive Cocci in cluster	Positive	Negative	NM	Tiny yellowish, entire and convex
R4	Negative Rod	Positive	Negative	NM	Smooth, soft, small, convex, white
R5	Negative Cocci in chain	Positive	Negative	M	Slightly yellowish, small, entire, regular
R6	Positive Cocci	Positive	Negative	M	Tiny, creamy white, regular, elevated

M= Motile, NM= Non motile

Table – 4 physicochemical parameters of water sample

Sites	Physico-chemical parameters											
	NH_4-N	NO_3-N	Total-N	Org-N	NO_2-N	PO_4-P	Tot-P	Org-P	DOC	DIC	Temp	pH
	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	µg/ml	°C	
Carron	0.025	6.469	7.077	0.582	0.006	0.047	0.065	0.018	5.935	10.100	8.00	7.17
Forfar	0.574	2.428	3.479	0.476	0.056	0.362	0.798	0.435	4.001	37.450	8.10	7.19
Rescobie	0.112	8.084	8.815	0.620	0.041	0.139	0.162	0.023	7.005	17.650	7.90	7.00

Parameters of the water samples are presented in table–4. The majority of aquatic bacteria are heterotrophic. Morphologically most aquatic bacteria are spherical, rod shaped, commas and spirals. Majority are motile by means of flagella. Genuine aquatic bacteria are distinguished by their ability to utilize very small concentrations of nutrients. Twelve isolates were found to be Gram negative and other characteristics were similar to *Pseudomonas sp* and remaining 6 isolates showed similar characteristics of *Bacillus sp*. Full microbial identification was not performed at this stage as many of the rapid systems e.g. API are focused mainly on industrial and medical microorganisms hence are known to give limited information of the relevance to these diverse aquatic bacteria. It was decided to elucidate all isolates for their ability to degrade microcystin-LR before any detailed classification was made.

3.2 Utilization of microcystin-LR by bacteria in Biolog MT2 microplates

A total 18 bacterial isolates (labeled as C1, C2, C3, C4, C5, C6, F1, F2, F3, F4, F5, F6, R1, R2, R3, R4, R5, and R6 according to their origin) and *Paucibacter toxinivorans* were selected and used for respiratory activity and utilization of microcystin-LR as sole source of carbon and energy in biolog MT2 microplates. The plates were read between 0 to 48 hours following inoculation with pre-grown isolate. The Biolog identification system (Biolog Inc., Hayward, CA,

USA) is a bacterial identification method that establishes identifications based on the exchange of electrons generated during respiration leading subsequently to tetrazolium redox dye based colour changes. This system tests the ability of microorganisms to oxidize a panel of 95 different carbon sources. Information on functional diversity (metabolic potential) is essential for understanding the role of microbial communities in different environments. Variations of the commercially available Biolog bacterial identification system plates are now widely used to assess functional diversity of microorganism from environmental samples based on utilization patterns of a wide range of (up to 95) of the single carbon sources. Metabolism of the substrate in particular wells results in formazan producing colour change in the tetrazolium dye. MT2 microplates contain the redox chemicals without any substrate which

allowed using a specific substrate i.e. microcystin-LR. Most bacterial Biolog investigations have used fixed incubation temperature between 15-30 °C (Campbell *et al*., 1997; Glimm *et al*., 1997). The colour development in individual wells of the plates was read rapidly by employing microtitre plate reader with an appropriate filter (590 nm). Data are expressed as individual well optical densities (Garland *et al*., 1991) which were corrected against the initial reading at time zero. Out of 19 bacteria, F1, F2, F4, C2, C3 and R1 and *P. toxinivorans* were found

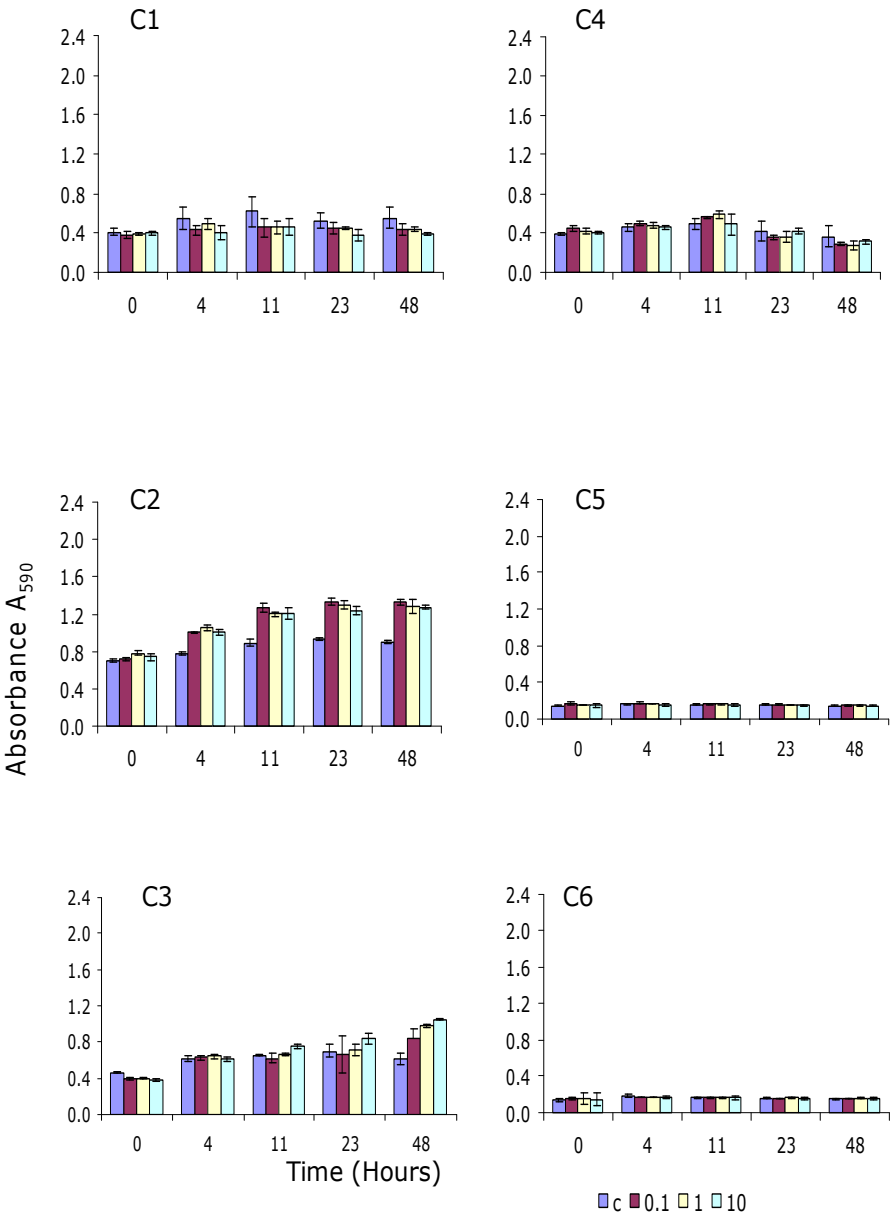


Figure-4 Metabolism of microcystin-LR by bacterial isolates from River Carron as indicated by colour development in Biolog MT2 test at range of conncentration (control , 0.1, 1.0 10.0 µg/ml) and at time intervals as shown. Data are mean± 1SD of three replicates.

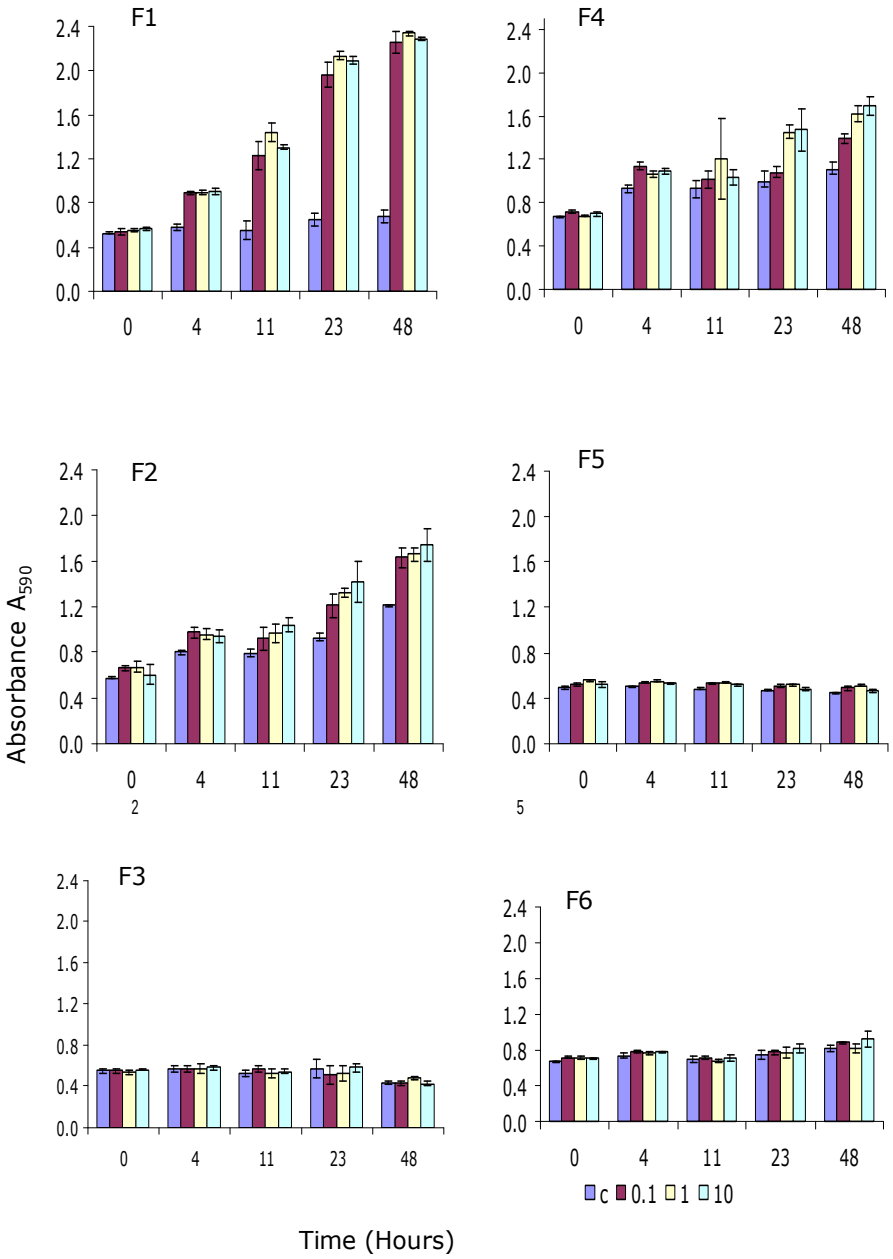


Figure- 5 Metabolism of microcystin-LR by bacterial isolates from Forfar Loch as indicated by colour development in Biolog MT2 test at range of concentration (control , 0.1, 1.0 10.0 µg/ml and at time intervals as shown. Data are mean± 1SD of three replicates.

to be very active in utilization of microcystin-LR on the basis of colour development up to 48 hours of incubation (fig 4-7). Isolates responded differently at a range of concentration and at different time intervals. There are different factors affecting colour development which are inoculum size, growth activity of isolate, oxygen concentration, substrate concentration and incubation time and incubation temperature. When the isolates from River Carron were tested in the MT2 plates with microcystin-LR the general metabolic response was low however isolate C2 and C3 (fig-4) both initiated an increase in metabolism when provided with microcystin-LR as their sole carbon source. In contrast, 3 isolates from Forfar Loch (fig-5) were active in this test with isolate F1 giving a very pronounced response clearly utilizing a high level of microcystin metabolism. Interestingly the bacterial isolates from Loch Rescorbie which has a long history of the occurrence of microcystin-producing blooms gave little to no metabolic increase with the exception of R1 (fig-6). In fact some of the isolates may actively have experience inhibition of metabolism in the presence of microcystin-LR namely R5 and R6. This observation is worthy of further investigation. It would be interesting to observe inhibition of responses with alternative carbon sources present. This may account for later observations where high level of microcystin-LR inhibits degradation (fig-8).

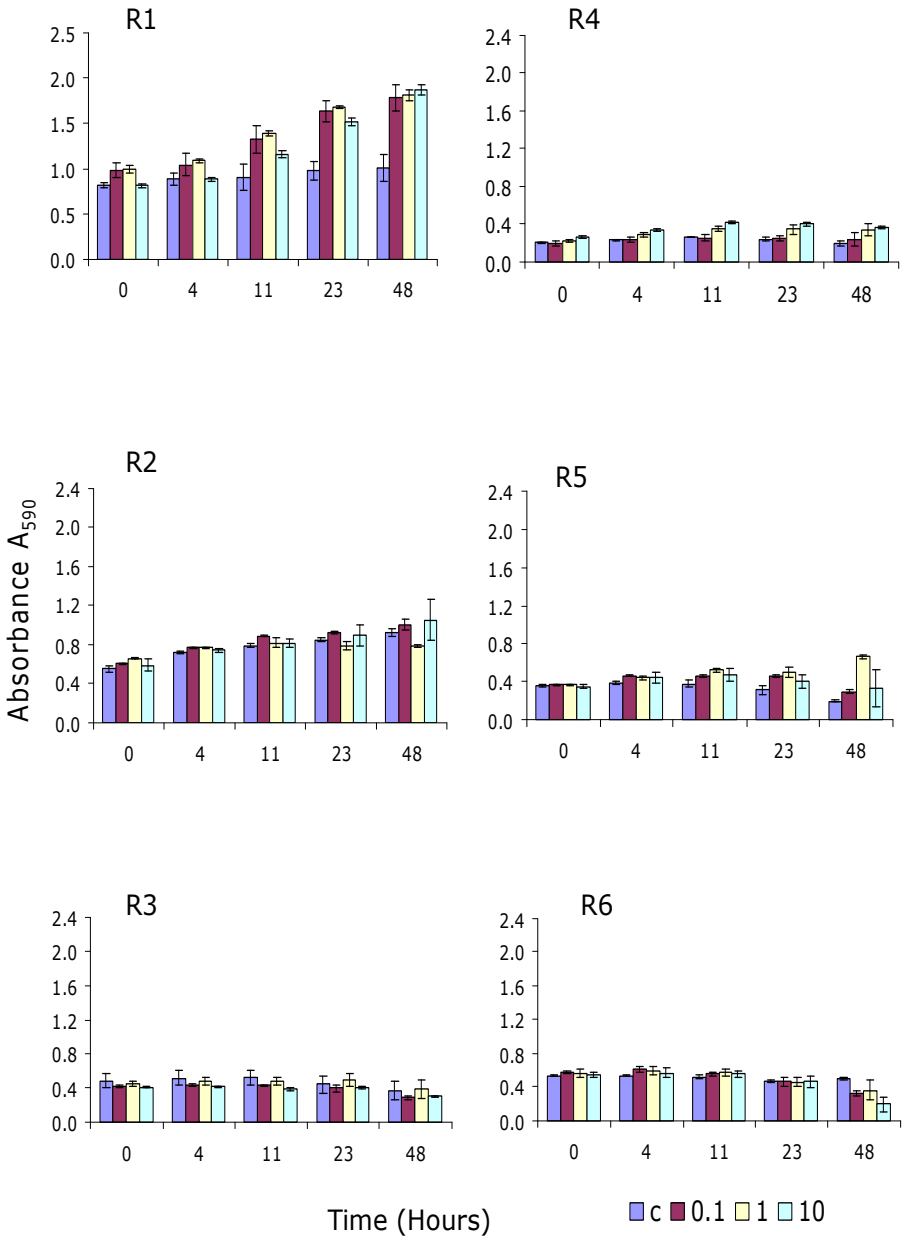


Figure-6 Metabolism of microcystin-LR by bacterial isolates from Loch Rescorbie as indicated by colour development in Biolog MT2 test at range of concentration (control , 0.1, 1.0 10.0 µg/ml and at time intervals as shown. Data are mean± 1SD of three replicates.

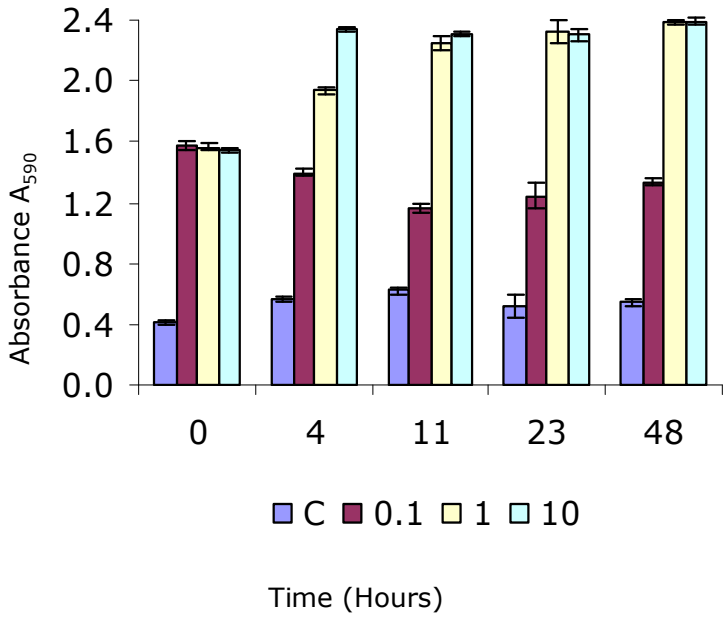


Figure- 7 Metabolism of microcystin-LR by *Paucibacter toxinivorans* (DSMZ-16998) as indicated by colour development in Biolog MT2 test at range of concentration (control , 0.1, 1.0 10.0 µg/ml and at time intervals as shown. Data are mean± 1SD of three replicates.

Incubation of P.toxinivorans with a range of microcystin-LR concentrations in the Biolog MT2 plate clearly demonstrated its active metabolism of the toxin (fig-7). At four hours, a clear dose response relationship was observed after which time the high concentration become saturated. It is interesting to note that at time 0 a colour change has already been observed indicating that the onset of

microcystin-LR metabolism is extremely rapid. Time 0 does include a short period after addition of the isolate and before the plate is read which typically takes 5-10 minutes.

To date over a hundred scientific papers have been published on the use of Biolog method for characterizing bacterial communities from a range of environments. However the length of incubation time before reading is made can markedly affect results due to several causes (Garland *et al*., 1991; Haack *et al*., 1995; Garland *et al*., 1996). If time before reading is too short colour development in some wells may be missed but if time is too long saturation levels will be reached in some wells.

Colour development time relationships may vary between wells there may not be a single optimum time for reaching between the extremes of too short and too long hence repeated monitoring is essential. Growth rates may affect colour production such that the initial population size together with the specific growth rate (doubling time) high concentration of the carbon sources in biolog

plates may inhibit some species or result in substrate accelerated cell death (Konopka *et al*., 1998) in such cases high nutrient tolerant and fast growing species produces colour quickly. Species which are unable to use some carbon sources as sole carbon source are eliminated.

The Biolog approach is sensitive to O_2 concentration (Winding *et al*., 1997; Garland *et al*., 1996). This is indicated by the fact that under anaerobic conditions no formazan is produced (Winding *et al*., 1997). And the diversity of actively respiring cells is better correlated with rate of colour development then total cell density indicating that the physiological state of cell i.e. rate of O_2 consumption can influence rate of colour development (Garland *et al.,* 1996). In some cases tetrazolium dye immediately introduces some bias since not all bacteria are able to reduce this dye hence the plates do not necessarily give a complete picture. (Winding *et al*., 1997). Thus even distribution of O_2 throughout the wells is important to limit errors. It has been suggested that due to the dependent of colour change on the production of reducing power only microbial energy releasing reactions other than those ascribed to ATP production cell growth and maintenance may result in rate of colour production on the maximum value obtained greater than is directly connected to microbial growth (Lindstrom *et al*., 1998)

3.3 Degradation of microcystin-LR under different conditions

The primary degradation study was carried out with raw water from River Carron, Forfar Loch and Loch Rescorbie. Water with indigenous microflora from three different sampling sites were exposed to microcystin-LR concentrations at 0.50 µg/ml and 10 µg /ml. Sterilized water with same concentration level was used as a control in each experiment. Figure 8a and fig 8b show the result of primary degradation studies. Fig-8a indicates activity of indigenous microflora of different samples at 0.50 µg/ml concentration of microcystin-LR. There is no drop of microcystin concentration in the control sample i.e. sterilized water sample. The Loch Rescorbie sample showed no lag phase and complete degradation of microcystin-LR after 9 days of incubation however the microcystin-LR concentration in water from Forfar Loch showed no drop in concentration after 6 days of incubation but complete degradation after 9 days. Degradation in the Carron sample was very slow and observed 15 days lag phase with complete degradation after 18 days of incubation under set experimental conditions. It is clear that the raw water contains high numbers of soil and aquatic organisms and provides an excellent inoculum for biodegradation. Biodegradation was observed in all three samples from three different sampling sites with different lag phase varying between 3 to 15 days. Indigenous microflora, when exposed to high concentrations i.e.10 µg/ml (fig-8b) showed no remarkable change

in concentration until 24 day of incubation for all samples. A very slightly drop in concentration was detected after 24th day of degradation until 30 day of degradation. This finding does suggest the microbial activity is inhibited by high concentrations. Several authors in the past have in fact explored the use of bacteria as a bioassay organism for the detection of microcystins. This includes published studies on the Microtox® system which observes the drop in bioluminescence as an indication of inhibition in microbial respiration of *Vibrio fisherii* (Microbics, 1982). It was found with this system that only high concentrations of microcystin gave reliable results hence it was not adopted as a routine assay (Lawton *et al*., (1990).

Activity of *P*. *toxinivorans* - a known microcystin degrading bacterium, was also used as positive control for degradation studies under the same experimental condition. Fig-9a and 9b shows activity of *P. toxinivorans* with different growth media and water at the concentration of 10 µg/ml and 20 µg/ml respectively. At low concentration, degradation was very fast where complete degradation was observed with in 72 hours tap water and after 144 hours with both 10% nutrient broth and in media enriched with 10% R2A. At high concentration i.e. 20 µg/ml, very slow degradation was observed after 48 h. Most rapid degradation was found when the isolate was incubated with microcystin-LR and water alone.

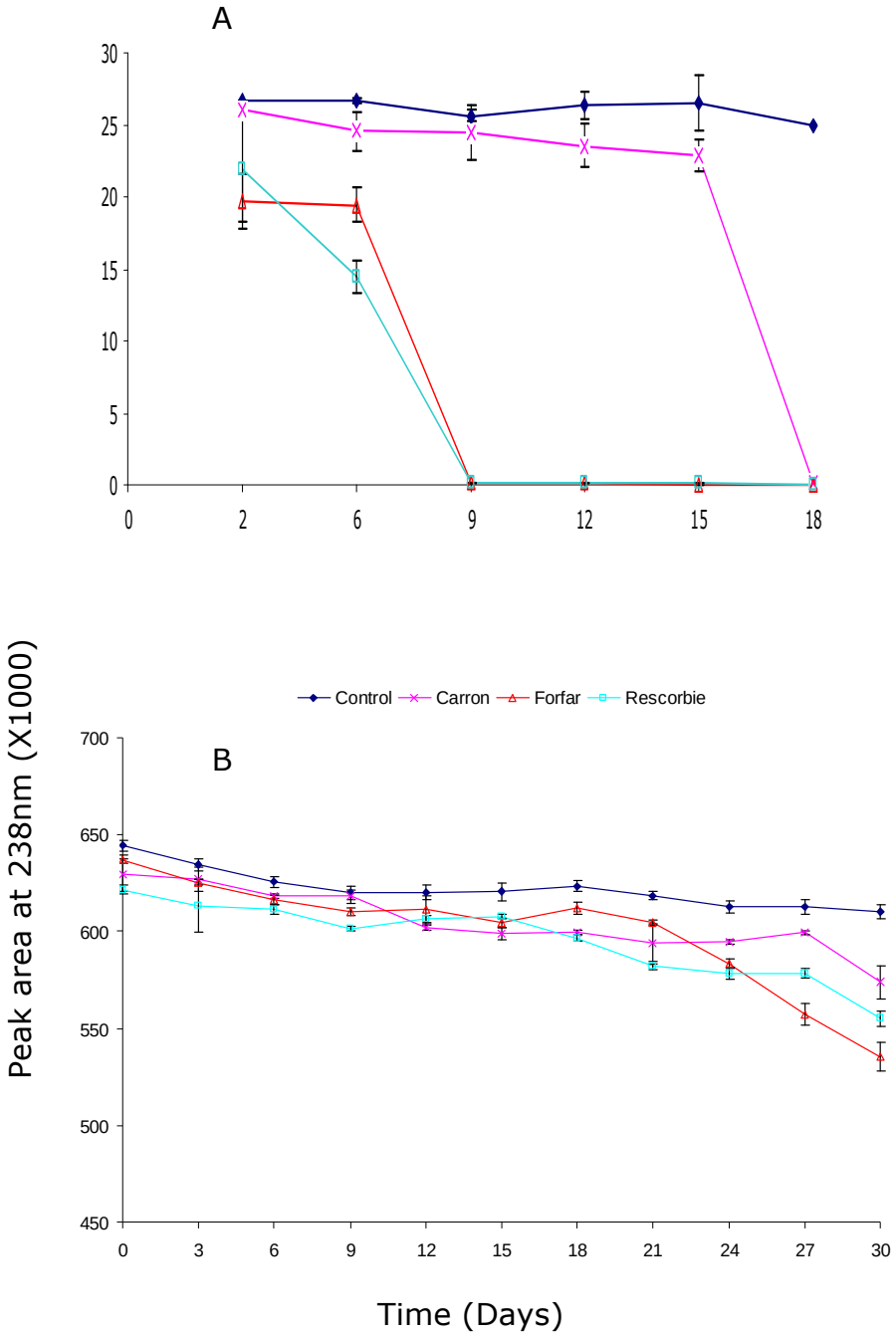


Fig- 8 Degradation of microcystin-LR by natural microflora from different sources at different concentration. A- 0.50 µg/ml, B- 10 µg/ml. Data are mean ±1SD of three replicates.

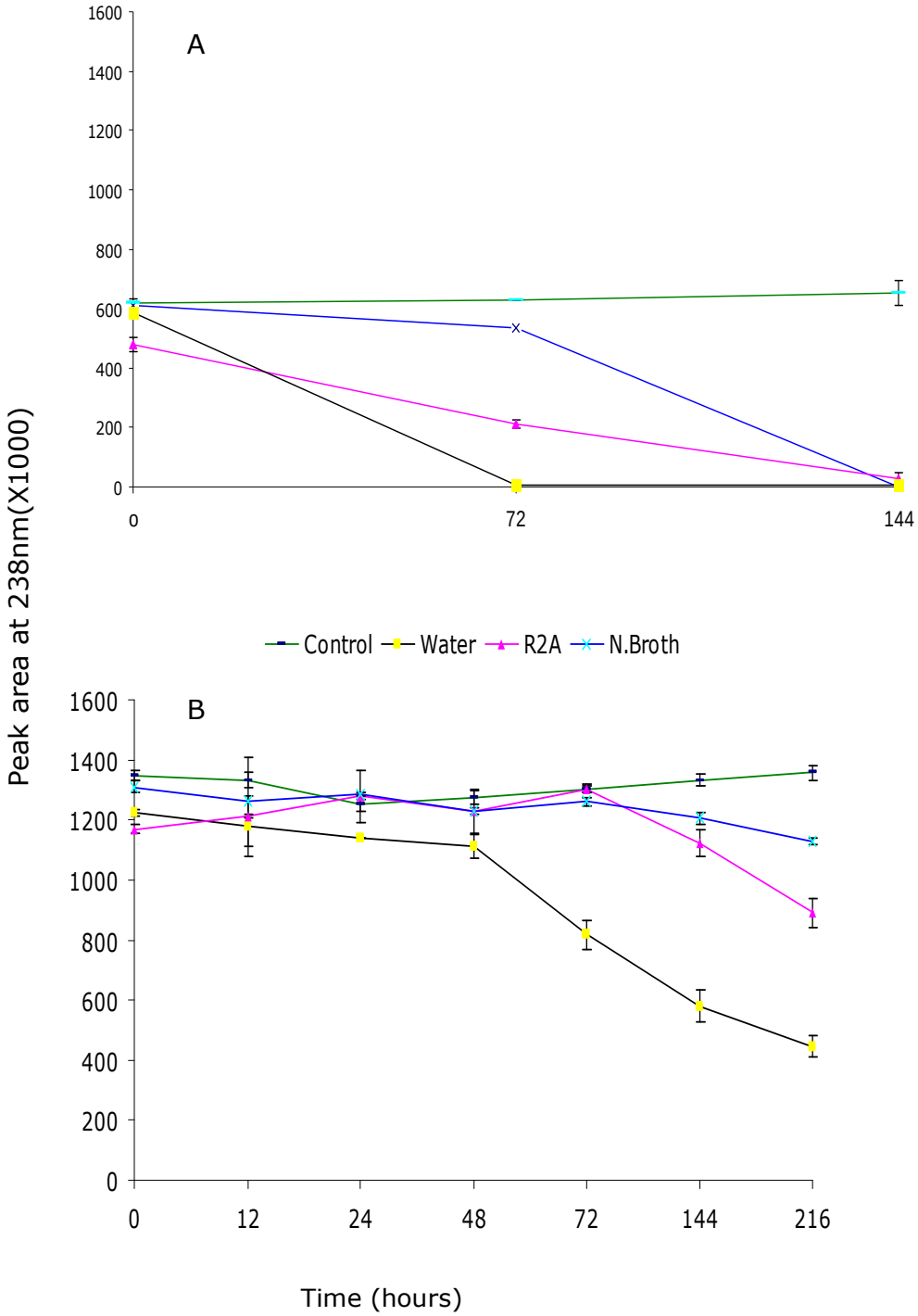


Figure- 9 Microcystin-LR degradation by *Paucibacter toxinivorans* at different concentration A- 10 µg/ml B- 20µg/ml in different growth media. Data are mean ±SD of three replicates.

When the isolate was incubated in the presence of toxin plus R2A media degradation began after 72 h but proceeded slowly and no degradation was observed in the nutrient broth flask. At high concentration i.e. 20 µg/ml very slow degradation was marked after 48 hours and remarkable drop in concentration in water and decreased in concentration after 72 hour and continue dropping until 216 hours of incubation was seen in R2A enriched growth media but no significant change in concentration in nutrient broth enriched media and control sample.

At high concentration there was an initial slow removal of microcystin-LR before more rapid degradation commenced which is comparable with trichlorophenol degradation studied by Modsen *et al*. (1992). No reason was concluded but it might be due to non specific absorption of microcystin-LR in to the periplasm by fast growing non-microcystin degrading bacteria (Jones *et al*., 1995). The lag phase observed in our experiment was up to 15 days with residual microcystin-LR being present after 30 days incubation in some cases (fig-8). The same results of microcystin persistence were observed by other researchers (Kenefic *et al*., 1992; Jones *et al*., 1992). Water temperature, pH, organic carbon content may also influence the length of such lag phase. Similar progression of microcystin degradation i.e. a lag phase of

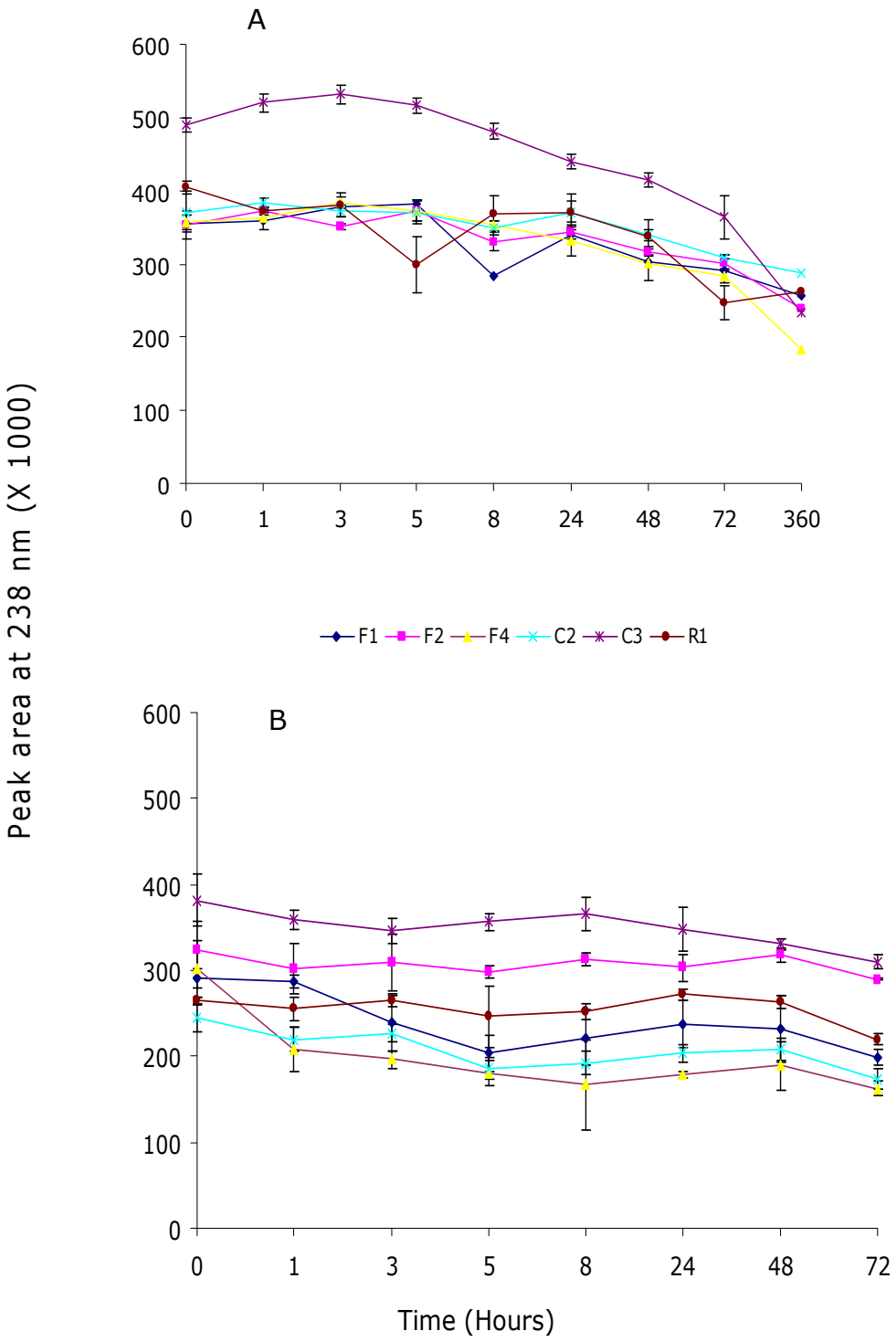


Figure-10 Degradation of microcystin-LR by different isolates at different growth media. A= nutrient broth B=normal saline at 5.0 µg/ml of concentration. Data are mean ±1SD of three replicates.

varying length then relatively rapid degradation has been shown previously by many other researchers (Cousins *et al*., 1996; Rapala *et al*., 1994; Lam *et al*., 1995; Jones *et al*., 1994; Takenaka *et al*., 1997; Watanabe *et al*., 1992; Ishi *et al*., 2000; Park *et al*., 2001; Kiviranta *et al*., 1991).

It is well known that the metabolic activity of heterotropic bacteria depends upon available concentration of both organic and inorganic nutrient and the degradation capacity can be induced by external additions of organic substances (fig-10). Our experiment is aimed to investigate the possible role of different nutrient substances on the degradation potential of aquatic bacteria. Jones *et al*. (1994) demonstrated a reduced rate of microcystin degradation after addition of acetate or glucose into the River water. On the other hand both microcystin containing as well as microcystin free complex cyanobactrial extract were shown to induce bacterial microcystin degradation (Welker *et al*., 2001).

Growth of the microbial isolates was monitored during the degradation studies and it was found that of the isolates examined, their growth curves were relatively similar (fig-11). Interestingly 3 of the bacteria have almost identical growth curves (*P.toxinivorans*, F1, and F2). This suggested a similarity in the identity of these three microbes is unknown but F1 was the isolate that demonstrated the most pronounced effect in the biolog MT2 test

and it has similar morphology and physiology to the *P.toxinivorans* i.e. Gram negative, oxidase and catalase positive and motile.
The origin of microbial flora and physico-chemical conditions during the degradation process seem to be important for an optimal degradation process. Rapala *et al*. (1994) found that the degradation efficiency increased when the microbial inoculums originated from water with previous cyanobacterial bloom. It is found by various researchers that there is a long history of microcystin producing blooms in Loch Rescorbie. Cyanobacterial blooms were noted previously in Forfar Loch but no microcystins have been found. The ability to degrade microcystin may be more widespread among naturally occurring bacteria than previously found. Most reports which identified the microorganisms responsible for degradation was found to be *Sphingomonas sp.* Only a few researchers have isolated and used new bacteria like *P. toxinivorans, Pseudomonas sp and Sphingosinicella sp for degradation studies.*

3.4 Enzymatic degradation of microcystin-LR

Enzymatic activity of the cell extract of 12 bacteria was studied. Isolates were selected on the basis of results of Biolog MT2 plate and primary degradation studies. Results are shown in fig-12. There was decrease in microcystin concentration after 12 hour to 37% by indigenous

microflora from the Loch Rescorbie water sample and dropped to 39% after 36 hours. The Individual isolate labeled as R1was found active where decrease in concentration was 40% after 36 hour of incubation.

The range of microcystin-LR concentration remaining in experimental samples was 58- 78%. It was found by previous researchers that indigenous aquatic bacteria required several days of lag time to begin degrading microcystin-LR in laboratory experiments. However after acclimatization with microcystin-LR the indigenous bacteria did not require lag time to start degrading microcystin-LR (Saito *et al*., 2003). This suggests that minor succession occurred in indigenous mixed population to a microcystin degrader possessing specific enzyme activities.

Further more microcystin-LR is stable against several existing protease, trypsin, chymotripsin, elastase, thrombin, papain, collagenase, corboxypeptidase and pepsin. So far, relatively few microcystin degrading bacteria isolated from different areas have been reported. Bourne *et al*. (2001) performed cloning and gene library screening of the *Sphingomonas sp* strain and detected the microcystin degrading gene cluster mlrA B, C, and D.

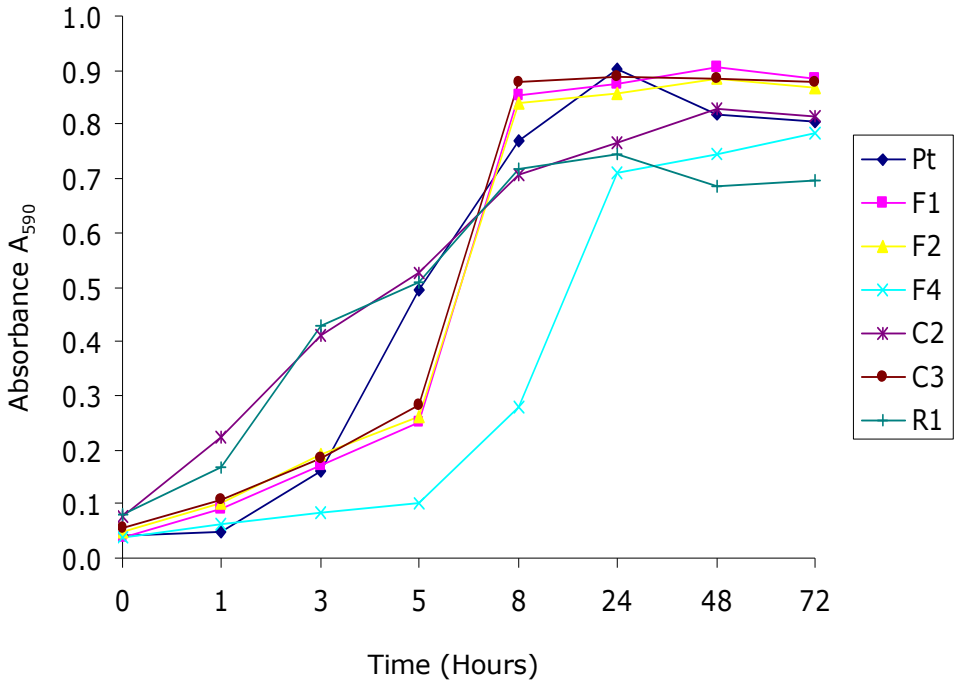


Figure-11 Growth curves of active isolates during microcystin-LR degradation in nutrient broth.

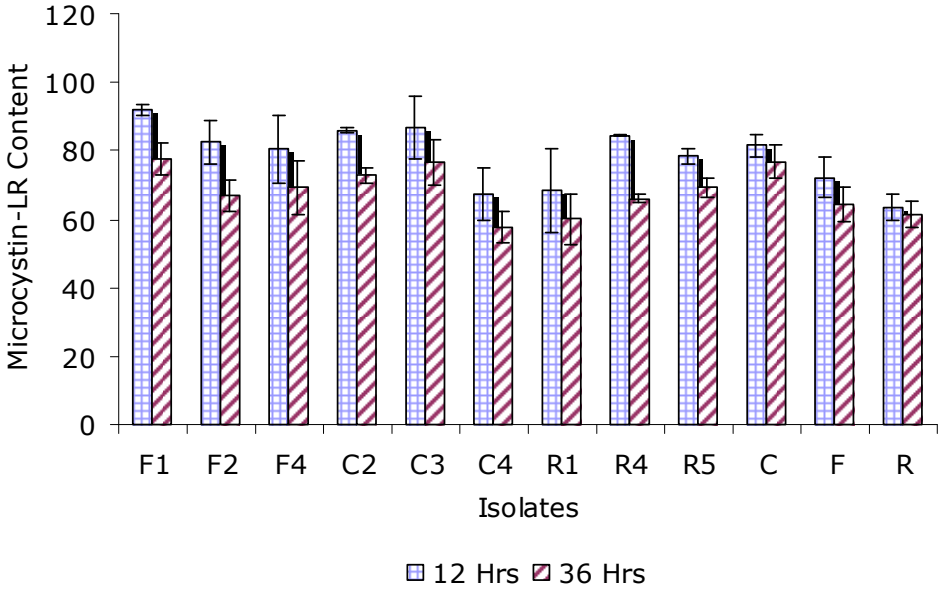


Figure-12 Percentage remaining of microcystin-LR by cell extract of different isolates. Data are mean ± 1SD of three replicates.

According to the available literature it is believed that the enzyme microcystinase mlrA cleaves the ADDA-Arg peptide bond in microcystin-LR and opens the cyclic structure. After opening the cyclic structure, linear microcystin-LR is degraded by the peptidase ecoded by mlrB and mlrC and divided into each amino acid. The enzyme mlrD encoded the transport protein that allows the uptake of microcystin in to the cell (Saito *et al*., 2003; Bourne *et al*., 1996). Further work is now required to confirm identification specifically of isolate F1 and some of the other isolates. Also the condition of enzymic degradation required optimizing and charactering.

Chapter 4

Conclusions

4. Conclusions

The presence of cyanobacteria throughout the world has been well recognized as a major problem in water treatment and risk to microalgae, zooplankton, aquatic and terrestrial plants, fish, terrestrial insects, birds and animals including crop plant, mussels, crayfish, and fish used for human consumption. Although a large variety of toxins have been identified their function, toxicity and stability is still unclear and remain to be determined.

It is very clear from the experiments reported here that Biolog MT2 plates are useful as a quick and easy method for screening active isolates utilizing microcystin-LR as sole source of carbon or energy. The use of Biolog MT2 plates provides the capability of selecting microbial system adapted to metabolite conscripted chemicals as their sole carbon and or energy source. Several concentrations of the target chemical may be added to the same plate so that a range of concentrations can be tested. Simultaneously this result indicated that Biolog MT2 plates are useful tools for screening bacterial isolates and consortia for their ability to survive, metabolize and potentially degrade selected organic chemicals.

It is very sensitive towards many parameters like oxygen, inoculums size, growth rate, incubation temperature, concentration of compounds to be tested and pH. It is also considered more rapid then conventional growth tests. The findings of this project have clearly shown that various genera of bacteria are able to degrade

microcystin-LR. It also demonstrated that degradation can be altered by addition of other nutrients. In order to elucidate the microbial degradation of microcystin-LR in water we focused on the microflora in natural water in their synergistic activities and their individual activities. We found that the degradation was fast by mixed culture with low microcystin-LR concentration and slow when exposed to microcystin–LR in individual isolates with high concentration. It is concluded that complete degradation of microcystin-LR was observed by natural microflora from the aquatic environment which implies that degradation of microcystin-LR is possible in natural water environment. So far most of the research centered on only few bacteria such as *Sphigomonas, Pseudomonas, Paucibacter and Sphingosinicella.* But there are many possible bacteria which can degrade faster than those studied. Due to limitation of time we could not complete identification and complete degradation by isolated bacteria. Several factors which affect microcystin-LR degradation by various bacterial species from different sources should be explored in future. Biodegradation is the most effective and inexpensive way of destruction of microcystin-LR in water so operational and viable approaches should be exercised to minimize risk with cyanotoxins.

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