INVESTIGATION OF NOVEL METHODS TO REMOVE THE CYANOTOXIN, CYLINDROSPERMOPSIN, FROM DRINKING WATER

By

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A thesis submitted to partial fulfilment for the degree of Doctor of Philosophy

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DECLARATION

I declare that the work presented in this thesis is my own, except where otherwise acknowledged, and has not been submitted in any form for another degree or qualification at any other academic institution.

Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Vijith Cholakkal Parambil Chandu

DEDICATION

TO

GOD

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

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ABBREVIATIONS

BET	Brunauer, Emmett, and Teller
BG 11	Blue green algae media
CO_2	Carbon dioxide
CYN	Cylindrospermopsin
cm	centimetre
ELISA	Enzyme linked immunosorbent assay
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
h	Hours
LC_{50}	Lethal Concentration$_{50}$
LED	Light emitting diode
L	Litre
MeOH	Methanol
MB	Methelyne blue
MS	Mass spectrometry
min	Minutes
mm	millimetre
OD	Optical density
PBS	Phosphate buffered saline
RP	Reversed phase
rpm	Revolutions per minute
UMP	Uridine mono phosphate

Abstract

The widespread distribution of cyanobacteria and their toxic effects on humans has become a major concern for researchers. Cyanobacteria are a group of oxygenic phototrophic bacteria that exhibit an enormous diversity in shapes and arrangements and occupy widespread habitats that include brackish, marine and fresh water. The major concern among these organisms is not so much the cyanobacteria themselves but their toxin production. Under suitable circumstances, cyanobacteria can reach high biomass levels and form toxic algal blooms. These toxins, known as cyanotoxins, are produced as secondary metabolites by several cyanobacterial species. Cyanotoxins affect not only aquatic ecosystems but also impact on human health. The current work focuses on the cyanotoxin cylindrospermopsin that was originally known to be produced by the cyanobacterium species *Cylindrospermopsis raciborskii* but has since been identified in a number of other cyanobacterial species. Cylindrospermopsin is hepatotoxic and acts as a strong inhibitor of protein synthesis. The increasing occurrence of toxic cyanobacterial blooms is of major concern, particularly within drinking water supply systems. Therefore, the investigation of more effective water treatment technologies is of great importance in order to ensure the removal of these potent toxins. There are many techniques that have been used so far, but the need to use an effective and efficient method to remove the toxicity is paramount. Removal of the toxin by the use of microbial degradation has been evaluated. Experiments with different strains of bacterial isolates showed positive signs in the removal of toxin by Biolog MT2 assay. In addition to that shake flask culture experiments were carried out and did not show any significant removal of the toxin. Studies with natural water sources showed some pronounced effect on the removal of CYN. The use of TiO2 photocatalysis as another potential water treatment strategy was also evaluated. Current study successfully demonstrated the potential degradation of purified cylindrospermopsin using the titanium dioxide (TiO_2) photocatalysis treatment method. TiO_2 photocatalysis was performed by using a powder form (Degussa P25) catalyst effectively removing the toxin; however, the powder is difficult to remove from the treated water. TiO_2 pellets (Hombikat K01/C) were found to be slower in degradation although they allowed for an easier adaption to a continuous treatment system. As an alternative Photospheres™ (buoyant glass beads coated in TiO_2) was evaluated and showed the same efficacy as that of Degussa P 25. Studies were extended to investigate the application of UV-LEDs in the photocatalysis reaction to show better efficiency.

CHAPTER 1

GENERAL INTRODUCTION

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1. INTRODUCTION

1.1. General overview of Cyanobacteria – Origin and distribution.

Cyanobacteria are a group of phototrophic, prokaryotic organisms, ubiquitous in nature. So far over 2500 species have been identified, found in nearly all environments such as surface waters, marine and fresh water (Cavalier-Smith, 2002; Oren, 2011). They can be found in extreme environments such as Antarctic waters and hot springs where few other photoautotrophs (e.g. algae) can survive. They have been referred to by a variety of names including cyanobacteria, blue-green algae, myxophyceans, cyanophytes, cyanophyceans and cyanoprokaryotes. At present they are commonly known as either cyanobacteria or blue-green algae. They are unique in some aspects which include their capacity to reduce both nitrogen and oxygen, and they also differ from other groups of bacteria in that they contain pigments (chlorophyll) which helps them produce energy via photosynthesis. As a group of organisms, cyanobacteria have some interesting potential applications and are currently being researched in a number of scientific fields. These include photoproduction of bio-fuels and ammonia, consumption of atmospheric CO_2 and production of various secondary metabolites. All cyanobacteria possess the pigments: chlorophyll a and phycocyanin, whilst others present include allophycocyanin, carotenoids and phycoerythrin.

A number of environmental factors are known to affect cyanobacterial growth. The major factors required for their survival include nutrient availability, especially nitrogen (N) or phosphorous (P), which are the major limiting nutrients in most ecosystems. Also important are appropriate levels of light and

CO_2 together with optimum temperatures. Trace metals including Fe, Mn, Co, Cu, Zn, are also necessary for cyanobacterial growth (Howarth, 1988). Surface water temperatures above 20 ºC are optimal for cyanobacterial growth; however, maximum growth rates are attained by most cyanobacteria at temperatures above 25 ºC, which are higher than the equivalent temperatures for green algae and diatoms (Chorus and Bartram, 1999). In addition, cyanobacteria can be regarded as a low quality food source for zooplankton as it lacks sterol (Lambert, 1987)

Eutrophication has a pivotal role in excessive cyanobacterial growth. Rast and Thornton (1996) state that: "eutrophication is the natural ageing process of lakes while others suggest that it is the enhancement of the natural process of biological production caused by nutrient enrichment". In other words, it is a process of excessive nutrient enrichment of waters that typically results in problems associated with macrophyte, algal or cyanobacterial growth. The occurrence of additional nutrients from human sources can arise from agricultural run-offs or storm water, sewage discharges and industrial wastes, which are recognised as one of the main reasons for the excessive growth of cyanobacteria in bodies of water. Eutrophication provides the perfect conditions for cyanobacterial growth and leads to toxin production. Secondary metabolites and toxins produced by the cyanobacteria may then enter the natural water environment, hence polluting the aquatic system. It is well known that these toxins can result in both animal and human fatality.

1.2. Occurrence of cyanobacterial blooms

Algal blooms that adversely affect environment, animal health or plants are referred to as harmful algal blooms (HABs) (Backer, 2002). These blooms commonly occur in brackish, marine, fresh water lakes, recreational and drinking water bodies. The more nutrient rich the water body, the more likely to sustain surface cyanobacterial blooms. The major hazard is the production of toxins in cyanobacterial bloom. This results in the intoxication or death of livestock, wildlife and pets worldwide. Recent reports indicate that these toxins are a major threat to human life as well. Because of the health issues these blooms create, it has attracted the worldwide attention of both the public and scientists. Cyanobacteria are unique organisms that can synthesise their food by means of photosynthesis. Many of the cyanobacterial strains have specialised nitrogen-fixing 'heterocyst'. These are typically distinguishable from vegetative cells by their somewhat larger and circular in shape and are specialised cells for nitrogen fixation. Several strains also possess gas vesicles, which regulate the buoyancy of cells in water bodies.

Approximately 150 cyanobacterial genera (eg. *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia, Planktothrix* and *Oscillatoria*) are able to produce 'cyanotoxins' (Apeldoorn *et al*., 2007). Because animals have more opportunity to consume water that may be contaminated by a cyanobacterial bloom, they are more at risk than humans of being poisoned from toxins. Humans, in the main, can only be exposed to toxin poison through direct contact, or accidental intake.

Freshwater cyanobacterial blooms have been reported in over 45 countries and in every continent (Table 1.1). Cyanobacteria with the ability to

generate toxins are a major public health threat. The most widely reported tragedy occurred in Brazil, in 1996 when the deaths of 76 patients undergoing dialysis were attributed to microcystin contamination in the clinic's water supply (Carmichael *et al*., 2001). There are also many reports of cyanobacterial toxins exposure to farm animals such as cattle (Saker *et al*., 1999).

Table.1.1. Countries with reported cyanobacterial blooms (Metcalf and Codd, 2004)

Europe	Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Ukraine, United Kingdom
America	Argentina, Bermuda, Brazil, Canada, Chile, Mexico, USA (at least 27 states), Venezuela
Middle East and Asia	Bangladesh, India, Israel, Japan, Jordan, Malaysia, Nepal, Peoples Republic of China, Philippines, Saudi Arabia, Sri Lanka, South Korea, Thailand, Turkey, Vietnam
Australia	Australia (New South Wales, Queensland, South Australia, Tasmania, Victoria, Western Australia), New Caledonia, New Zealand
Africa	Botswana, Egypt, Ethiopia, Kenya, Morocco, South Africa, Zimbabwe
Marine	Baltic Sea, Caribbean Sea, Atlantic, Indian and Pacific Ocean
Antarctica	McMurdo Ice Shelf

1.3. *Cylindrospermopsis raciborskii* and its morphology.

Cylindrospermopsis raciborskii is now recognised as a toxin producing cyanobacterium that is hazardous for human health due to production of the potent cytotoxins cylindrospermopsin and saxitoxin. It was first identified in Java, Indonesia (Woloszynka, 1912) and its worldwide occurrence has since been reported. Researchers proposed that the wide spread nature of this species might be due to physiological tolerance to high light intensity and high temperature hence their occurrence being favoured by higher water temperatures promoted by climate warming (Briand *et al*., 2004).

This particular organism normally possesses 2-3 µm wide filaments also called trichomes, which are irregular in length 10-120 µm long (Fig.1.1; St. Amand, 2002). Individual cells are often difficult to differentiate because they are infrequently constricted at the cell walls. Cell length ranges from 3 – 10 µm long (Table 1.2). Differences in cell size are a well known feature common to natural *C. raciborskii* populations (Hawkins *et al*., 2001). Due to the small size of *C. raciborskii*, they are often missed in typical tow net plankton samples as they can easily pass through the mesh.

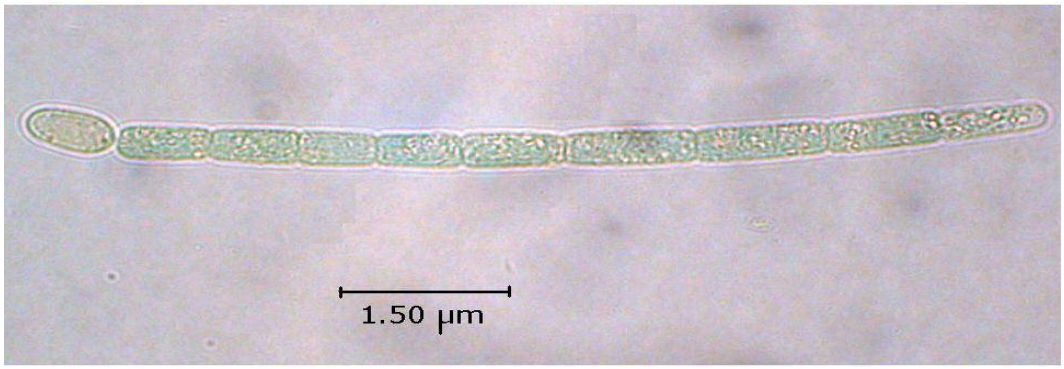

Fig.1.1 *Cylindrospermopsis raciborskii*

Table 1.2. Varying World Wide Morphologies of *C. raciborskii*

Country	Morphology	Cell Width (µm)	Cell Length (µm)	References
Australia	straight, coiled, sigmoid	2-2.3 2.5-3 1.5-1.8	4.5 – 6 6-7 5-7	McGregor and Fabbro, 2000
France	straight	1.5	11.5	Briand *et al*., 2002
Japan	straight, coiled	1.9-2.3	3.0-18.5	Chonudomkul *et al*., 2004

The cyanotoxins produced by *C. raciborskii* include cylindrospermopsin, saxitoxin, and anatoxin-a. Anatoxin-a and saxitoxin are neurotoxins whereas CYN belongs to the hepatotoxin, cytotoxin category (Chorus and Bartram, 1999). Not all strains of *C. raciborskii* produce cylindrospermopsin, however, it is not possible to differentiate toxic from non-toxic isolates by their morphology. Well known features, familiar to natural *C. raciborskii* populations are their cell size and capacity to vary morphology from straight to coiled filaments. Heterocysts, the site of nitrogen fixation, can be seen as large refractive cells on the trichome. Earlier studies reported that when grown under laboratory conditions, *C. raciborskii* trichome lengths decreased as cell density increased. This was believed to be an adaptation to reduce entanglement or allow trichome to move freely through the water to capture light more efficiently. (Hawkins *et al*., 2001).

The occurrence of cyanotoxin cylindrospermopsin (CYN) has been attributed to the water bodies with blooms of cyanobacterial species *Cylindrospermopsis raciborskii* (majority), *Aphanizomenon ovalisporum* and *Umezakia natans*. *C raciborskii* occurs worldwide in freshwater ponds, rivers, reservoirs and eutrophic lakes. In comparison to microcystin producing cyanobacteria, CYN- producing cyanobacteria generally occur in tropical and sub-tropical regions (Duy *et al*., 2000; Hawkins *et al*., 1997; Carmichael and Falconer, 1993). Apart from its occurrence in the tropics, *C. raciborskii* is also found in water bodies of warmer temperate regions (eg. in Australia; Baker and Humpage, 1994; Thomas *et al*., 1998); in warmer summer months of temperate region (eg. Florida, USA; Williams *et al*., 2000; Burns 2000; Chapman and Schelske, 1997) and temperate climates (eg. New Zealand, Stirling and Quilliam, 2001).

C. *raciborskii* have some particular characteristics in their growth and nutritional requirements. They inhabit freshwater lakes and as previously highlighted are capable of fixing atmospheric nitrogen. A number of reports suggested that *C. raciborskii* was not particularly dependent on nutrient status although it has been observed that the growth of this bacterium was positively related with ammonium concentration, while the toxins it produces, cylindrospermopsin, were negatively correlated (Hawkins *et al*, 2001). On the other hand, another study reported that this bacterium could be less dependent on nutrients and also had a greater affinity and storage capacity for phosphorous than some other bacteria. In the case of laboratory grown cultures of *C. raciborskii,* faster growth has been demonstrated with nitrate or ammonium

when compared to nitrogen fixation. Therefore, this species did not seem to be highly dependent on nitrogen fixation (Bouvy *et al*., 2001).

1.4. Cyanotoxins

Cyanotoxins, toxins produced by cyanobacteria, can be classified as dermato-, cyto-, neuro-, and hepatotoxins (Pearson *et al*., 2010) depending on the target of action to the animal organs that they affect and their chemical structures. There are neurotoxins, which affect the nervous system, hepatotoxins block protein synthesis, promote chromosome breakage, and may even cause tumours, and dermatoxins which irritate skin and mucous membranes. The three most common cyanotoxins are described chemically as cyclic peptides, alkaloids, and lipopolysaccharides (Mur and Skulberg, 1999). Microcystins and nodularins are the members of a small molecular weight cyclic peptide group. Alkaloid toxins comprised of both neurotoxic and cytotoxic toxins. The major neurotoxic alkaloids include anatoxin and saxitoxin, while cylindrospermopsin (CYN) is often classified as hepatotoxic, although, since it is now known to affect many organisms, it is said to be cytotoxic. Lipopolysaccharides (components of the Gram negative cell wall) include endotoxins and are often referred to as "irritant toxins" (Sivonen and Jones, 1999). CYN imposes increasing concern as it has been reported in a significant number of countries, however, relatively little work has been carried out on this toxin compared to other groups of cyanotoxins. While many of the cyanotoxins are named after the genera they were first characterised from, other organisms are now known to produce them (Table.1.3).

Table 1.3. Main classes of cyanotoxins: Referenced from Chorus and Bartram, 1999; Chorus *et al*., 2000; Haider *et al*., 2003; Metcalf and Codd, 2004.

Toxin	Organisms	Nature of toxicity	Class of toxin	Mechanism of toxin action
Anatoxin a	*A. flos aquae, A.lemmermanii, Anabaena, Planktothrix, Aphanizomenon.*	Neurotoxicity	Alkaloid	Blocks post-synaptix depolarisation, mimics acetylcholine
Anatoxin-a(s)	*A. flos aquae*	Neurotoxicity	Alkaloid	Anticholinesterase
Saxitoxin, neosaxitoxin	*Anabaena, A. flos aquae, Lyngbya, Cylindrospermopsis*	Neurotoxicity	Alkaloid	blocks sodium channels, most commonly known for dinoflagellate marine Red tides also in Paralytic Shellfish Poisoning (PSP)
Cylindrospermopsin	*Cylindrospermopsis, Aphanizomenon,* U*. natans,* R*. curvata.*	Hepatoxicity	Alkaloid	Inhibition of protein synthesis, cumulative toxicity affecting kidneys, intestines, and lungs. The liver is the main organ affected.
Microcystins	*Microcystis, Anabaena, Planktothrix, Nostoc, O. Hepalosiphon Anabaenopsis.*	Hepatoxicity	Peptide	Alterations of actin microfilaments, destruction of parenchymal cells, lethal hemorrhage or hepatic insufficiency, inhibition of protein phosphatases, tumor-promoting activity, liver hemorrhage
Nodularins	*Nodularia spumigena*	Hepatoxicity	Peptide	Inhibition of protein phosphatases, tumor promoting activity, liver hemorrhage.

1.4.1. Cylindrospermopsin (CYN)

Cylindrospermopsin (CYN) is the primary toxin produced by a tropical cyanobacterium *Cylindrospermopsis raciborskii*. (St. Amand, 2002; Bownik, 2010). It is an alkaloid hepatotoxin and is a strong inhibitor of protein synthesis. Thirty years ago, there was a significant incident in Palm Island, Australia, where human poisoning occurred due to consumption of water affected by a cyanobacterial bloom. About 149 people were hospitalised with symptoms of gastroenteritis (Griffiths and Saker, 2003). The causative agent was unknown at the time of incident, however, in the following years CYN was identified (Bourke *et al*., 1983, Ohtani *et al*., 1992). The same toxins have been found to be produced by different cyanobacterial species other than the *C. raciborskii*. This includes *Anabaena bergii, Aphanizomenon ovalisporum, Raphidiopsis curvata*, and *Umezakia natans* (Banker *et al*., 1997; Sivonen and Jones 1999; Li *et al*., 2001). Toxin production and toxicity level of a particular cyanobacterium can vary from isolate to isolate. In the case of CYN production, it is very particular and has been established as strain specific rather than species specific.

CYN is a tricyclic alkaloid comprising a tricyclic guanidine moiety combined with hydroxymethyl uracyl (Fig.1.2, Ohtani *et al*., 1992). in which the hydroxyl group and uracil moiety are believed to be crucial for CYN toxicity (Banker *et al*., 2000). Studies reported that the uracil side chain of CYN inhibits protein translation, which then binds to DNA, causing strand breakage and promoting toxicity (hepato-, cyto-, and geno-.) (Westrick *et al*., 2010). These molecules are zwitterionic, highly water soluble in nature and have a relatively low molecular weight of 415 Da (Sivonen and Jones, 1999). In comparison with other

cyanotoxins, only two naturally occurring analogues of cylindrospermopsin have been identified to date, namely 7-deoxy-cylindrospermopsin (lacking the hydroxyl group) which is one tenth as toxic as cylindrospermopsin (Codd *et al*., 2001) and 7-epi-cylindrospermopsin (an epimer at the hydroxyl bridge) which appears to have similar toxicity to cylindrospermopsin (Banker *et al*., 2001).

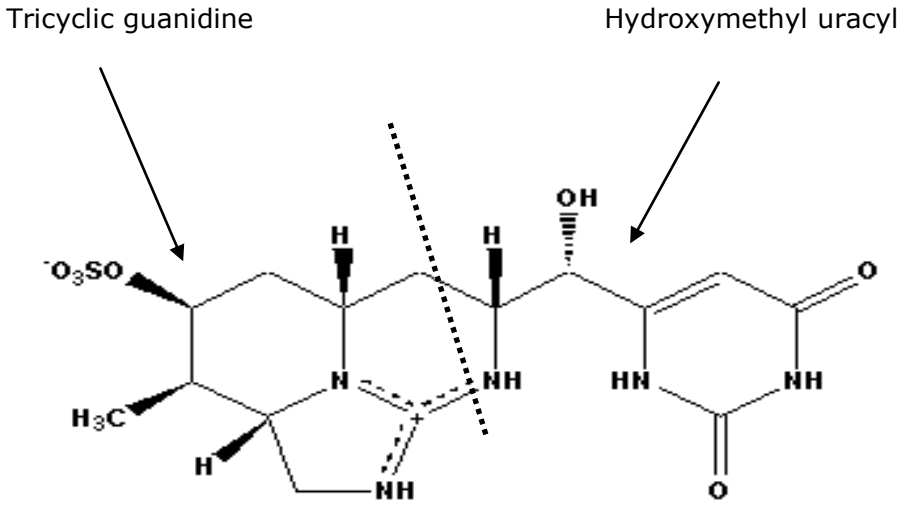

Fig.1.2. Chemical structure of Cylindrospermopsin (CYN)

There are several factors that influence the toxin production and concentration, including environmental pressures such as nutrient availability and temperature. In *C. raciborskii* cultures, a negative correlation between temperature and cellular CYN concentration has been recorded. Another study showed that CYN production was higher in cultures lacking a source of fixed nitrogen when compared to those supplied with ammonium (Ohtani *et al*., 1992). In addition to environmental pressures, genetic variability may also

influence the varying concentration of CYN. Researchers observed that different concentrations of CYN content were exhibited in multiple isolates of *C. raciborskii,* varying up to several orders of magnitude (Saker and Griffiths, 2000).

When considering effective toxin removal, it is important to know whether the toxin is intracellular or extracellular. Intracellular toxins are found inside the bacterial cell so they could be less problematic for human health unless they are released. Toxin release could occur when the bacteria are undergoing stress, as a result of unfavourable environmental conditions, or after cell death and lyses. It was reported that even when the *C. raciborskii* blooms dropped below detectable levels, CYN remains above the detectable concentrations threshold for up to 6 weeks (McGregor and Fabbro, 2000). For this reason, it is ineffective to use cyanobacterial cells as indicators of CYN levels in a water body, as their absence does not necessarily correlate with an absence of toxin.

Toxic effects of cylindrospermospin were reported in both animals and humans worldwide. *C. raciborskii has* also been identified as the cause in cattle mortality (Hawkins *et al*., 1997). Reports show CYN accumulation in crayfish (Saker and Eaglesham, 1999), mussels (Saker *et al*., 2004), and daphnids (Nogueira *et al*., 2004). This toxin has also been shown to elicit irritant and hypersensitivity reaction (Stewart *et al*., 2006). There are also studies which report the accumulation of CYN in plants that recover a few nanograms from the plant tissues (Kinnear *et al*., 2007)

The toxicity of cylindrospermopsin has been studied and seems to be much lower than saxitoxin and anatoxin. Even the toxin production seems to be

several orders lower when compared to *Microcystis* sp. and *Anabaena sp*. Anatoxin-a illustrate a reported LD_{50} of 20 µg/kg, while that of saxitoxin was 10-30 µg/kg (Chorus and Bartram, 1999). However, CYN has shown to be toxic only at around 2100 µg/kg (LD_{50}, 24 hrs) and 200 µg/kg (LD_{50}, 5-6 days) (Ohtani *et al*., 1992). Organisms like, *Microcystis* sp. and *Anabaena* sp. were known to attain high cell densities and hence produce high concentrations of toxins. *Microcystis* sp. is capable of producing 25000 µg/L of MC-LR (Griffiths and Saker, 2003) while *Anabaena* sp. was known to produce up to 3300 µg/L of anatoxin-a (Griffiths and Saker, 2003). In comparison, *C. raciborskii* has been reported to accumulate only up to 589 µg/L of CYN (Saker and Eaglesham, 1999).

Studies have reported a large variation within *C. raciborskii* both in the amount and quality of toxin produced. The variation could be due to factors such as morphotype and environmental conditions. Earlier studies reported that blooms with curled morphs produced a lower CYN to cells ratio (McGregor *et al*., 2000). One study suggests that there are variations in the toxic properties of CYN; the Australian variant produced a protein synthesis inhibiting CYN, whereas the American variant had a neurotoxic effect (Bernard *et al*., 2003).

1.4.2. Microcystins

Microcystins are the most widely known hepatotoxin, with over 70 variants (Edwards and Lawton, 2009). Microcystin-LR is the most common variant with a LD_{50} value 50.0 µg/kg in mice by intraperitoneal injection. It is 200 times more toxic than cyanide. The two letter suffixes represents 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 in this particular microcystin

variant (Fig.1.3). The structural variants of this toxin include amino acid substitutions and alterations such as methylation and demethylation. The general structure of microcystin is characterised 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-Bmethylaspartic 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).

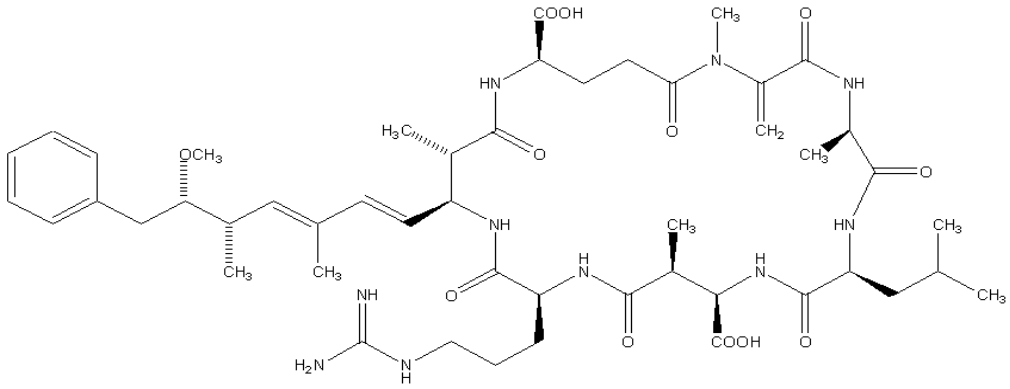

Fig.1.3. Microcystin-LR

1.4.3. Nodularin

Nodularin, a cyclic pentapeptide hepatotoxin with chemical structure as cyclo-(D-MeAsp^1-L-arginine^2-Adda^3-D-glutamate^4-Mdhb^5), in which Mdhb is (methylamino)-2-dehydrobutyric acid. Nodularin is structurally similar to Microcystins but differs due to the absence of D-ala and the X variable amino acid and substitution of N-methyldehydrobutyrine (Mdhb) residue for N-methyldehydroalanine (Mdha) residue (Fig.1.4)

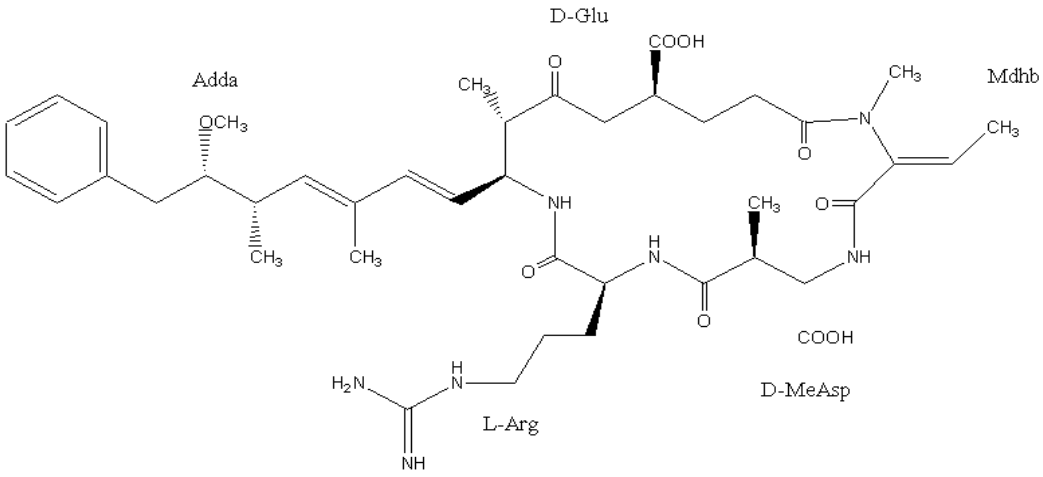

Fig.1.4. Nodularin

1.4.4 Anatoxin-a and Anatoxin-a(s)

Anatoxin–a (Fig.1.5) is an alkaloid toxin with a molecular weight of 165kDa. In Anatoxin a(s), the suffix (s) implies that one of the symptoms of intoxication is hyper salivation, similar to the symptoms of synthetic organophosphorous pesticides. (Fig.1.6)

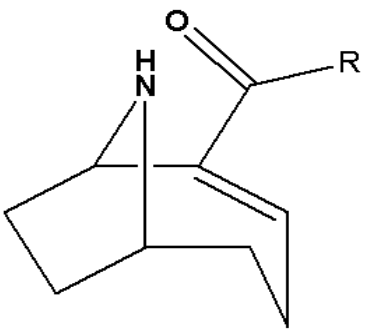

Fig.1.5. Anatoxin-a

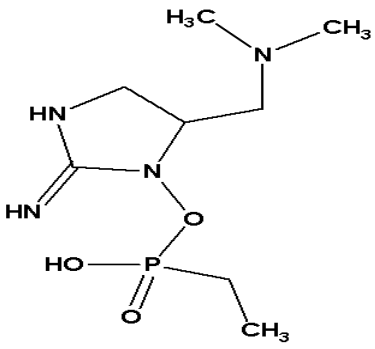

Fig.1.6. Anatoxin–a(s)

The compound (Anatoxin-a) is a secondary amine and it acts 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 sparingly occur in water supplies and hence pose a lesser risk compared to microcystins (Fawell *et al*., 1993). Some cases of poisonings have been reported due to consumption of water containing anatoxin-a. This suggests that the main risks are to pets and livestock or where recreational water is utilised.

1.4.5. Lipopolysacharide (LPS) endotoxins

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 has been related to LPS endotoxins (Falconer, 1996). It has been demonstrated to affect the phase II microcystin detoxication mechanism in zebra fish (*Danio revio*). LPS is heat stable and toxic to mammals and has been shown to significantly reduce activity of both the soluble and the microsomal glutathione S-transferases (Wang *et al*., 1994). LPS molecules consist of 3 main parts; antigens, core polysaccharides and lipid a moieties. The lipid a region of LPS triggers the biological responses and symptoms including fever, diarrhoea, vomiting and hypertension. Fever is generally caused due to the release of pyrogenic compounds by the host body in response to LPS ingestion with haemodialysis water and aerosolized LPS being an important potential source of exposure.

1.5. Method of Analysis

Cylindrospermopsin was initially detected by Harada et al., 1994 and Hawkins *et al*., 1997 by chemical methods. Later, the HPLC-PDA method of detection was demonstrated by Welker *et al*., in 2002. This method was seen to be very effective and could detect CYN even in lyophilized samples (Törökné *et al*., 2004). This method, however, was ineffective for detecting CYN in environmental samples as false signals from the matrix interfered with the toxin signals (Welker *et al*., 2002). Also, the presence of other unknown compounds from environmental samples greatly masked toxin signals (Spoof *et al*., 2006). One of the reasons for the false signals could be contaminants co-eluting during the extraction procedures. This problem has been overcome by the use of solid phase extraction cartridges (SPE) (Metcalf *et al.*, 2002).

Several other methods have been developed for the detection of CYN. In 1994, Harada et al demonstrated the use of HPLC as an efficient purification method, wherein CYN was separated over a silica gel column. In 1999, Eaglesham et al demonstrated the use of HPLC MS/MS as an ideal CYN detection method in water samples. This method was rapid and could analyse the samples in 2 hours' time, however, one of the drawbacks of this method was that other compounds also did co-elute. In 2004, a more advanced technique was demonstrated by Dell' Aversano *et al*., where they used Hydrophilic Interaction Chromatography coupled with Mass spectrometry (HILIC-MS). Later, in 2008 a method was developed by Haande *et al* where they used UPLC MS/MS for the detection of CYN and Deoxy CYN.

Two other, rapid detection methods have also been studied recently. ELISA is one of the methods that are rapid and highly sensitive for the detection

of CYN. Apart from detecting toxins, ELISA can also be used to detect other cyanobacterial strains that produce toxins. The protein phosphatase inhibition assay could also be an effective alternative method for detection. The detection by this method could be as low as 200 ng/ml (Kinnear, 2010)

To confirm the effective removal of CYN from treated water, the use of toxicity assessment is required. Mouse bioassays were the standard techniques for sometime, however, due to ethical concerns mouse bioassays were discouraged. In the current research, the brine shrimp (*Artemia salina*) assay was used. This was found to be a reliable and cost effective alternative to the mouse assay (Akin-Oriola and Lawton, 2006). *A. salina* is available in frozen cysts form and is readily available in aquarium stores and through biological suppliers. The cysts last for several years and can be hatched without special equipment, brine shrimp medium was used and the assay was performed in a 96 well plate. In order to check the toxicity of a samples, they were added to wells containing brine shrimp. Mortality was recorded on a 24 h and 48 h basis.

1.6. Water treatment and its utility in controlling cyanobacterial toxins

The basic principle behind removal of cyanotoxins from water is by removal of cells and by adsorption and destruction of toxins present. The flocculation method described by Rositano and Nicholson (1994) is effective in removing suspended cells. In addition, flocculants like alum and calcium hydroxide coagulate cells with little or no release of cyanotoxins (Kenefick *et al*., 1993; Lam *et al*., 1995).

Water treatment methods are inadequate in removing cyanobacterial biomass and sometimes leach cyanotoxins into the product water. This is

because the chlorination step leads to cell lysis which releases cyanotoxins into the water (Falconer, 1998). The subsequent processes such as flocculation, sand filtration and chlorination do little to remove the cyanotoxins (Hoffman, 1976; Donati *et al*., 1994). The efficiency of such processes is about 11 to 18 % (Duy *et al.*, 2000). Also, some chemicals such as copper sulphate or the algicide simazine may also release dissolved organic compounds from the biomass (Kenefick *et al*., 1993; Sukenik., 1998). Physical factors such as prolonged storage for distribution and negative pressure on the storage containers may also result in lysis of cyanobacterial cells.

Increasing occurrence of cyanobacterial blooms in drinking water bodies throughout the world is a major health concern for research authorities. It is therefore necessary to generate an efficient and sustainable method for water treatment. Many techniques have been investigated for the treatment and removal of cyanotoxins during water purification. The methods used so far have been shown to be effective on a toxin specific basis. There has been little research on the removal of CYN from drinking water, with findings suggesting that some treatment methods are ineffective. The methods that have been used so far have been found to be not particularly efficient in CYN removal and few treatment methods showed some disadvantages including hazardous by-products formation (eg. Tri Chloro Methane).

Slow sand filtration is one of the methods used for intracellular toxin removal but it could only be used for small scale water treatment. The only benefit from this method is the gentle removal of cells that minimise the release of extra cellular toxins. However, blocking caused by overloading can limit the effectiveness of sand filtration (Chorus and Bartram, 1999). Membrane filtration

is the physical removal of materials through a semi-permeable membrane. In fact, there is some evidence regarding this that might damage the small portions of cells and by using this technique no significant increase in toxins has been observed (Chorus and Bartram, 1999).

Coagulation and dissolved air flotation are the other current methods used for the toxin removal. Coagulations, the process promotes aggregation of small or dispersed particles into larger particles, which can be separated by sedimentation, filtration, or flotation. The most commonly used chemicals for this process includes aluminium or ferric chloride. More recently some synthetic organic polymers gained some approval. It is very effective at removing intact cyanobacterial cells, but not at removing toxins. Rositano and Nicholson (1994) demonstrated this expectation by evaluating removal of purified, soluble microcystins by three coagulants: ferric sulphate, alum and polyaluminium chloride. The effectiveness all depends on doses, and there are reports of high doses of alum promoting cell lysis (Chorus and Bartram, 1999; Haider *et al*., 2003).

Ozonolysis is one of the chemical oxidation methods which are used in water treatment. The process has generally two mechanisms of oxidation, ozone and hydroxyl radical. Ozone is a strong oxidising agent and this is extremely effective in destroying many pollutants including microcystins, nodularin and anatoxin-a. Dissolved organic carbon and alkalinity have been shown to reduce the efficacy of ozone treatment and it also releases toxins and causes cell lysis, so it is recommended that it also be used in combination with primary treatments (Hoeger *et al*., 2002; Svrcek and Smith, 2004). Studies revealed

that ozone is capable of deactivating CYN through the oxidation of the double bond and amino moieties of its structure (Onstad *et al*., 2007).

Another commonly used alternative oxidant in water treatment process is potassium permanganate ($KMnO_4$). Studies revealed that potassium permanganate is not very reactive with cylindrospermopsin (Banker *et al*., 2001; Rodriguez *et al*., 2007). In contrast, reports also suggest that $KMnO_4$ can inactivate *Cylindrospermopsis* cells with no release of toxin (Chen *et al*., 2009).

It has been demonstrated that chlorination removes toxins such as microcystin, nodularin, saxitoxins and cylindrospermopsin at varying pH ranges, but not anatoxin-a. However, very few studies have been done to characterise the chlorination by-products which may also be toxic (Svrcek and Smith, 2004). Degradation of CYN by chlorination results show that relatively low doses of chlorine are sufficient for degradation of CYN, when the dissolved organic content is low. However, if organic matter other than CYN is present in the solution, the effectiveness of chlorine for CYN degradation is reduced as other organic matter present consumes chlorine. pH studies suggest that CYN is more stable to chlorine degradation at lower pH. However, in normal water treatment this is not relevant since the pH is consistently higher than 6. Application of chlorination resulted in the inactivation of CYN between pH 6 and 9. The same study identified two chlorinated by-products including chlorouracil, 5-chloro-cylindrospermopsin (Senogles *et al*., 2000) and a carboxylic acid derivative (Banker *et al.*, 2001). These chlorinated by-products produced fatty vacuolations on the liver in mice (Senogles *et al*., 2003). A more recent study revealed that maximum rate of inactivation of CYN was found to be at pH 7 (Rodriguez *et al*., 2007).

Activated carbon is used to absorb soluble organic materials including cyanotoxins. Two main forms of activated carbon exist. Powdered activated carbon (PAC) is used for targeted treatments while granular activated carbon (GAC) can be used continuously. It has been proven that activated carbon is effective to treat microcystin and saxitoxins (Svrcek and Smith, 2004), but tests have shown that chlorine is more effective than PAC at removing CYN (Griffiths and Saker, 2003).

Besides the method mentioned there are several other methods that have used advanced water treatment methods. Membrane filtration is one of these, in which the use of a semi-permeable membrane to separate a water stream into two parts called a permeate (particle are passes through) and a retentate (particles are retained) (Screck and smith, 2004). There are generally four types of membrane filtrations used in water treatment plants, such as microfiltration (MF), Ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (Zhou and smith., 2001). MF and UF are highly effective at removing intact cyanobacterial cells. These processes apply low pressure to pass the water stream through. While NF and reverse osmosis apply high pressure and can be applied to filter out the dissolved metabolites. One study that reported the removal of CYN by NF in two different water bodies was between 90 and 100% for the three different NF membranes used such as NF90, NF270 and DK membranes (Dixon *et al.*, 2011).

Advanced oxidation processes (AOPs) are those which generate and use the hydroxyl radicals and are relatively non-selective oxidants. The hydroxyl radical generally reacts with cyanotoxins in the order microcystin ($1.1x10^{10}$ $M^{-1}S^{-1}$) > CYN ($5.5x10^{9}$ $M^{-1}S^{-1}$) > anatoxin-a ($3.0x10^{9}$ $M^{-1}S^{-1}$) (Onstad *et al*., 2007).

Hydroxyl radical ($HO^{\bullet}$) are a powerful oxidant and can lead to the degradation of a range of organic toxins and pollutants (Antoniou *et al*., 2008; Song *et al*., 2009; Deng *et al*., 2009). Hydroxyl radicals can also play a vital role in the environmental transformation of organic substrates. Since CYN is highly resistant to biological degradation, photochemically assisted transformation has the potential to have greater impact on reducing CYN concentration.

Significant interest has been shown towards the application of microbes for the removal of cyanobacterial toxin from the water bodies. It could certainly provide an alternate option against chemical methods used in this industry. A recent study investigated the degradation of CYN by naturally occurring bacterial populations that revealed no degradation after 40 days of experiment (Wormer *et al*., 2008). Interestingly, a study conducted on water bodies that have had a history of pre exposure to *C. raciborskii* blooms showed degradation of CYN. However, the degradation was found to be concentration dependent suggesting the optimum toxin concentration is needed to induce the degradation (Smith *et al*., 2008). One study suggests that grazers may be an effective control against CYN producing cyanobacterial strains (Fabbro *et al*., 2001). Another study conducted with pro-biotic bacteria, in which a most efficient strain, *Bifidobacterium longum* 46; being capable of removing 31.6% of CYN over 24 h periods has been recently investigated (Nybom *et al*., 2007). A further study on degradation of CYN in sediments revealed that the compound is highly mobile during sediment passage and its sorption depends on organic carbon availability (Klitzke *et al*., 2010). Klitzke and Fastner, 2012 carried out a recent investigation into the degradation of CYN in surface waters and found that it is facilitated by oxic conditions and inhibited in anoxic environment. CYN

degradation by *Bacillus* strain (AMRI-03) isolated from cyanobacterial bloom has also been investigated recently. Results revealed that complete degradation is dependant on initial concentration and occurred in 6 days with a highest concentration of 300 µg/L tested (Mohamed and Alamri, 2012).

1.7. Research Aims.

In recent years there have been increasing reports of not only cyanobacterial blooms, but also, the occurrence of cyanotoxins. The presence of CYN has been reported in water bodies in a number of countries and concerns about its impact on human health have been expressed. It is therefore important to explore methods that could reliably destroy CYN and remove its associated toxicity. As part of the work, alternative removal techniques including bioremediation of the cyanotoxin will be studied. This will be achieved by:

- Evaluation of cylindrospermopsin utilisation by bacterial isolates using Biolog MT2 plate.

The Biolog plate was first used as a screening method to compare metabolic potential of heterotrophic microbial communities from different habitats such as water, soil and wheat rhizosphere (Garland and Mills, 1991). Generally it is a redox reaction, while utilising carbon resources (here CYN), microbes reduce a colourless dye to violet formazan. Current research examines the utilisation of CYN with isolated bacteria from natural water sources.

- Examination of cylindrospermosin degradation by isolated bacteria in batch experiments.

Cylindrospermopsin in general has been shown to be highly resistant to bio degradation. In comparison, studies with 10 isolated bacteria have showed successfully degraded microcystin in short period and found to be cost effective and reliable (Manage *et al.*, 2009). This study investigates the utilisation of CYN by incubating the isolated bacterial culture for a longer period. Further study examines the utilisation of CYN by collecting natural water samples (eg. River Carron, Forfar Loch, Balgavies, Rescobie, Leven). Experiments were carried out in small scale (with bacterial isolates in bijou bottles) and large scale (natural water samples in 1 L Conical flasks) for long time incubation.

Titanium dioxide assisted photocatalysis was shown to be successful for the destruction of other cyanotoxins especially microcystins, this technology will be investigated as an alternative to biodegradation methods for its potential in the elimination of CYN. This will be achieved by:

- Evaluating the efficacy of a range of TiO_2 photocatalysts for removal of CYN.

Applications of photocatalysis have made a huge impact in water treatment after it was successfully applied on several organic pollutants and potent toxins (Fujishima *et al*., 2000). Photocatalytic degradation of cyanotoxins microcystin and nodularin, treated with Degussa P25 showed successful removal (Lawton and Robertson, 1999). CYN has been previously described to be degraded by UV irradiation (Senogles *et al*., 2000). The effect of UV irradiation was enhanced with addition of TiO_2 materials such as Degussa P25, Hombikat UV-100 were successfully used in the removal of CYN (Senogles *et al*., 2001) but

by-products formation, removal of the powder form after the treatment never mentioned. In the current study the intention is to examine the effectiveness of three brands of titanium dioxide catalyst for the removal of CYN.

- Optimisation of the factors influencing the degradation process, e.g. catalyst load, light intensity and water quality.

There are various parameters that influence the photocatalytic activity such as pH, temperature, toxin concentration, light intensity and dissolved organic compound. The current research examines the different catalyst load, light intensity and water quality.

- Construction of pilot LED photo reactor to test photospheres in the photocatalytic degradation of CYN.

The Recent availability of UV-LEDs has prompted research into their application in photocatalysis. Advantages of LEDs include high energy efficiency (little heating) long life span, and the availability of monochromatic irradiation (Wang *et al*., 2011). The compact size of LEDs and the utilisation of DC power supply offer greater flexibility for field purpose. There are studies that have recently used UV LEDs the photocatalytic degradation of aqueous pollutants such as perchloroethylene (PCE) (Chen *et al*., 2005) reactive red (Wang and Ku, 2006) o-cresol (Chen *et al*., 2007) and methylene blue (Tayade *et al*., 2009). In the current research the efficacy of photosphere on the degradation of CYN with a novel LED photo reactor has been examined.

- Evaluation of CYN degradation by HPLC and brine shrimp bioassay.

Traditional bioassay used for the cyanotoxin detection was mouse bioassay. Difficulties in time constraints, data interpretation and the ethical considerations all led to the investigation of alternative assays. This study examines a rapid and cost effective bioassay using brine shrimps

- Hydrophilic interaction liquid chromatography as an alternative method to the analysis of CYN samples.

HPLC-PDA (reversed phase, Atlantis C_{18}) has been used as the basic detection method for the cyanotoxin. However, results from the environmental samples have been shown poor in detection, leading to investigate an alternative method of detection. Previous studies on HILIC-MS have been shown to be an efficient and effective method for CYN detection. This study investigates an optimised method for CYN analysis by HILIC.

- Application of SPE in testing of biodegradation samples prior to HPLC analysis.

Difficulties suffered during the environmental sample analysis by HPLC have led to the investigation of an alternate option in the form of HILIC. It was suggested that the extraction procedure could play a vital role in reducing the contaminant peak, and allowing the detection of much lower concentration than was generally observed by HPLC. A solid phase extraction step was developed to allow sample clean up and concentration. In this study the use of SPE prior to HPLC analysis was investigated.

CHAPTER 2

Cylindrospermopsin extraction, purification and method developments.

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2.1 Introduction

Cyanobacteria are phototrophic organisms that produce various secondary metabolites (Moore, 1996; Burja *et al*., 2001). Secondary metabolites includes toxins although their natural function has not been clearly described yet (Lawton and Edwards, 2001; Weidner *et al*., 2002). These toxins includes microcystin and nodularin which have been widely studied due to their diversity of different analogues, acute and chronic toxicity on humans and animals, worldwide distribution and long persistence in the environment. On the other hand, cylindrospermopsin (CYN), another secondary metabolite produced by several cyanobacteria species has received less attention. Its potential effect on human health includes liver, kidneys, lungs, spleen and intestine injuries. Studies revealed that uracil moiety and the hydroxyl group are believed to be crucial for CYN toxicity (Banker *et al*., 2001) although the mechanism by which CYN enters intracellular environment is not yet understood. The potential risk to human and animal health highlights the importance of environmental monitoring and toxicological studies of CYN.

Rising attention of secondary metabolites in toxicology and pharmacology studies has increased the demand of purified cyanobacterial secondary metabolites (Lawton and Edwards, 2001). To support this, harvesting of cyanobacterial secondary metabolites has increased amongst the researchers in this field of study. Generally, Cyanobacteria are extracted either directly from collected blooms or laboratory grown cultures. Harvesting cyanotoxins directly from the bloom field may be cost effective due to no extra investment in culturing and maintenance. In order to do this, large quantities of cyanobacterial

cells can be collected from surface waters along the shoreline. However, in the majority of cases cyanobacteria remain in the water column. In that case, additional resources are required for collection (eg. Plankton net, boat; Lawton and Edwards, 2001) and that too can become a time consuming process. In addition, samples containing more than a single strain of cyanobacteria require characterisation to identify the particular organism responsible for the toxin production (Lawton and Edwards 2001). In comparison to the sampling from field, culturing and processing of cyanobacteria in the laboratory has been shown to be an efficient and reliable alternative. Laboratory cultures are found to be well characterised with recognised toxin and can be maintained as single strain cultures. This in turn helps a reliable investigation of toxin production and the development of consistent analysis of toxin produced at a specific time period. Laboratory cultured cyanobacteria are highly reproducible, less complex for harvesting and provide easier purification of cyanotoxins (Lawton *et al*., 1999).

Cyanotoxins are generally extracted as a mixture of chemically different components. In order to use them as analytical standards or in some assays (biochemical) high quality compounds are required. This can only be attained by extracting cyanotoxins from cyanobacterial cells followed by multiple purification steps (Lawton and Edwards, 2001; Edwards and Lawton, 2010). A study revealed that a combination of high performance liquid chromatography (HPLC) coupled to a tandem mass spectrometer (MS/MS) resulted in an assay which is efficient for monitoring low CYN concentration in water samples without sample concentration (Eaglesham *et al*., 1999). However, intact algal cells in the water samples require lysis which can be achieved by freeze-thaw cycle. The growth of *Cylindrospermopsis raciborskii* was cultured to provide purified CYN to facilitate

studies into detection and removal. Analytical protocols were also evaluated to select those most suitable for use in monitoring microbial and photocatalytic removal.

2.2. Materials and Methods

2.2.1. Chemicals

Chemicals were of analytical-reagent grade unless stated otherwise and acquired from Fisher Scientific, Leicestershire, UK. HPLC-grade methanol and acetonitrile were obtained from Rathburn, Walkerburn, UK. Milli-Q Water was obtained from a Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK). Methylene blue was received from PHIOBIO, England.

2.2.2. Laboratory scale culturing of cyanobacteria

Cylindrospermopsis raciborskii (originally isolated by M. Saker from an Australian lake) cultures were maintained on a regular basis and sub culturing was carried out from stock cultures in exponential growth phase to fresh sterile media containing BG-11 minus nitrate media (Stanier 1971, Table 2.1).

Table 2.1 Composition of the BG-11 media (Stanier *et al*., 1971).

Component	BG-11 (g/L)
K_2HPO_4	0.040
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Na_2CO_3	0.020
Citric Acid	0.006
$FeSo_4.7H_2O$	0.006
EDTA (disodium)	0.001
Trace element solution*	1ml/L

*Trace Element Solution:

Component	g/L
H_3BO_3	2.680
$MnCl_2.4H_2O$	1.810
$NaMoO_4.2H_2O$	0.390
$ZnSO_4.7H_2O$	0.222
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H_2O$	0.049

When large quantities of biomass was required for the cyanotoxin purification, cultures were grown in 10 L vessels by placing a volume of 1 L cyanobacterial culture in stationary phase inoculated in 9 L sterile freshly prepared BG-11 minus nitrate media. BG-11 minus nitrate media were sterilised by taking in polycarbonate containers and autoclaved at 121 °C, 15 psi for 50 min. Autoclaved media were then transferred to the large vessel containing 1 L stationary phase cultures. Ten litre vessels were also sterilised with Presept and

rinsed with sterile media as they could not be sterilised in an autoclave due to their size and material they were made from. Cultures were exposed to continuous illumination generated by cool white OSRAM fluorescent tubes (58 W) 20 µmol/m^2 s^{-1} (Li-Cor intelligent light meter LT-250) in a temperature controlled room (22 °C ± 2).

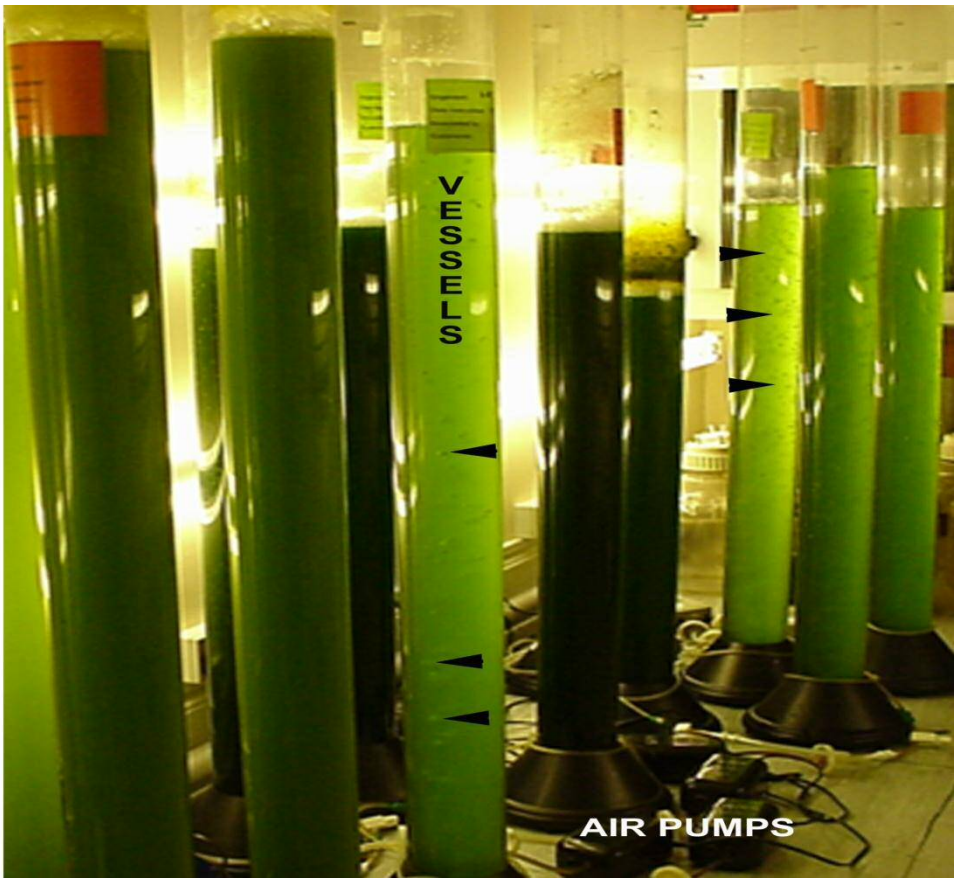


Fig. 2.1. *Cylindrospermopsis raciborskii* grown in 10 L culture vessels under continuous illumination sparging with sterile air.

2.2.3 Harvesting and extraction of the cultures

Cyanobacterial cultures growing in vessels were harvested by centrifugation (1500 x g, 30 min) and stored at –20 °C prior to being thawed for extraction. One litre of Milli-Q water was added and allowed extraction for 1 h

and the extract was filtered by tangential flow filtration. Filtration was performed with Schleicher and Schuell, ULTRAN® Ultra using microfiltration membrane cassettes with a pore size of 0.2 µm. The filtrate containing the toxin was collected until approximately 500 ml remained in the retentate container. In tangential flow filtration, a medium is used to flush the filtrate along the filter tangentially. Three sequential extracts were performed producing 1.5 L extract for each extraction. HPLC was used to analyse the amount of CYN in this crude extract in order to allow the efficiency of the purification to be monitored.

2.2.4 Concentration and clean up: Flash Chromatography

Concentration and clean up of CYN was achieved by using a Horizon flash chromatography system (Biotage Inc., Cardiff, UK). The instrument has a pneumatic pump, and is equipped with a UV detector and a fraction collector. Preconditioned (20 ml Methanol: 20 ml Water) ENV+ cartridge was used. Reverse phase solid extraction was performed using mobile phase A: Milli-Q water and B: Methanol. Aqueous extracts of CYN were loaded at 40 ml/min and was eluted using a stepwise gradient from 10% to 100% B (i.e. increasing percentage of methanol).

Ten fractions of 40 ml were collected and the quality and quantity of the eluted CYN in each fraction was determined by analysing sample by HPLC-PDA. The selected fractions were concentrated and finally purified by preparative HPLC (Waters PrepLC 4000 System, Millipore).

2.2.5 Desalting and drying of the purified CYN

Preparative HPLC fractions assure acceptable purity (≥95%) were pooled and diluted 1:5 with Milli-Q water. The diluted samples were then injected onto a preparative HPLC cartridge followed by Milli-Q wash step. CYN retained in the cartridges were eluted with 100% methanol (Edwards *et al*., 1996). Eluted CYN were transferred into dry vials (pre-weight determined), and then dried under gaseous nitrogen and stored at –20 °C.

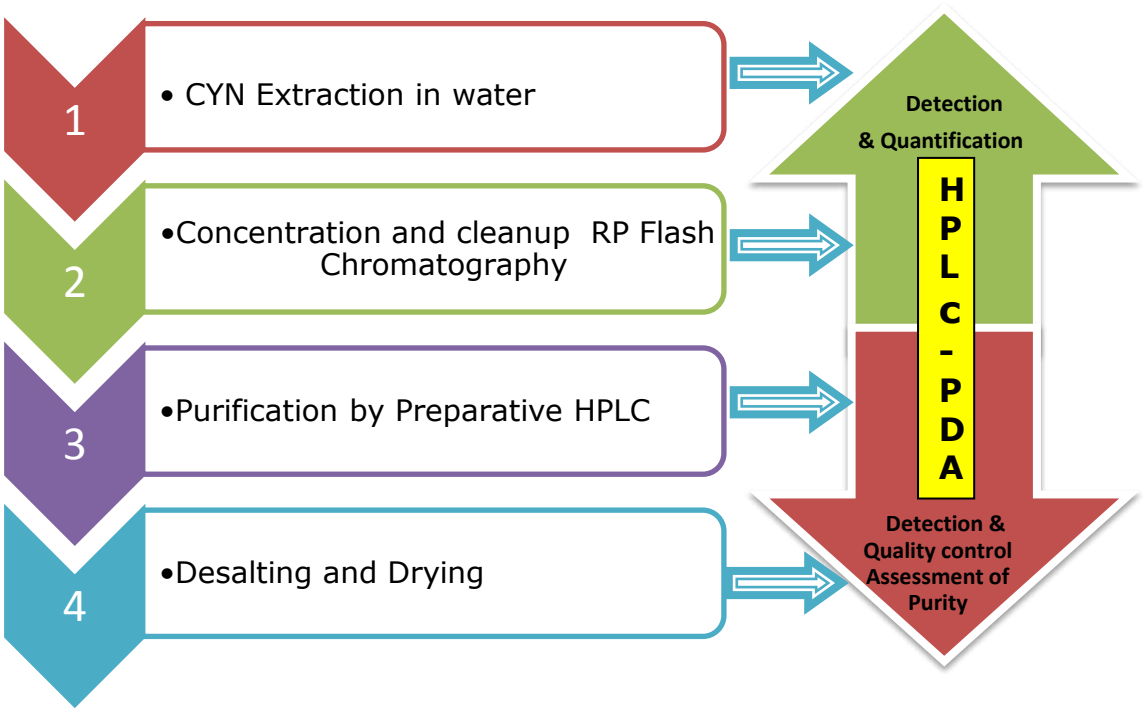


Fig. 2.2. Schematic diagram of the HPLC based purification of cyanotoxins (Edwards *et al*., 1996).

2.2.6. Analysis of CYN (HPLC)

Analysis of CYN was carried out using the waters HPLC system consisting of a solvent delivery system Alliance 2695 model and detection by photodiode array (PDA) model 2996. The wavelength in PDA was set from 200 to 400 nm. The separation was obtained through using Atlantis dC_{18} column (2.1 mm ID X 150 mm long; 5 µm particles size) which was maintained at 40 °C. The mobile phases were Milli-Q water (A) and methanol (B) respectively. Purified CYN were identified on the basis of both retention time and characteristic UV spectra (262 nm) by correlating previously validated CYN standards. The gradient used for the current study by HPLC method shown in Table.2.2.

Table 2.2. The solvent gradient used in the HPLC analysis of cylindrospermopsin by using C_{18}. Solvent A: Milli-Q Water; Solvent B: Methanol.

TIME	%A	%B	Curve
0	100.0	0.0	-
25	90.0	10.0	6
26	0.0	100.0	1
27	0.0	100.0	1
28	100.0	0.0	1
35	100.0	0.0	6

2.2.7. Preparation of standard calibration curves for CYN

Standard stock solutions of purified CYN were prepared by dissolving known amounts of the dry sample in Milli-Q. Samples were always made by dilution of the stock solution in Milli-Q water for both the biodegradation and photocatalysis experiments. A standard curve was prepared by analysing the following range of CYN concentration 1.00, 0.50, 0.10, 0.05, 0.01 µg/ml. Triplicates of the samples were analysed by HPLC-PDA as mentioned in section 2.2.6. The correlation coefficient was determined from the plotted graphs of concentration against peak area of the UV at 262 nm.

2.2.8. An alternative approach for CYN analysis: Hydrophilic Interaction Liquid Chromatography (HILIC)

Being one of the recently identified cyanotoxins there are relatively few analytical methods to detect CYN reported in the scientific literature compared to other cyanotoxins. The most widely used method for detecting CYN is high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS) and/or photo diode array detection (PDA). Cell and invertebrate assays have been used to detect CYN, but these were non-specific and insensitive methods and were useful only to confirm residual toxicity. So a sensitive, selective rapid screening method was essential for detecting CYN (Lawton and Edwards, 2008).

The HPLC technique has improved in recent years with the use of HPLC columns having better capacity to retain polar components, which were poorly retained by reversed-phase C_{18} Column. The CYN has a characteristic UV spectrum with λ max of 262 nm. This can be useful along with retention time, when confirming the identity of CYN. The retention and separation of polar analytes is an on-going challenge for many chromatographers. Polar analytes were traditionally retained by ion exchange or ion pairing, mobile-phase pH manipulation, and reversed-phase chromatography with specially designed columns. However, each of these techniques had certain disadvantages. Ion exchange or ion pairing work well only if the analytes of interest are ionisable, also because of signal suppression, ion pairing is difficult to use with mass spectrometry (MS) (Gustavsson *et al*., 2007). The precision and accuracy of

quantitative bio analytical methods can be affected adversely by these suppression effects.

Reverse phase chromatography has been widely used for a diverse range of application because of its versatility although it is not always ideal for very polar compounds. The retention of polar analytes often requires a highly aqueous mobile phase, which can cause dewetting of the stationary phase. Dewetting is a major problem especially in reversed-phase causing chromatography problems. There are newer solutions to address this issue of phase dewetting by introducing stationary phases that contain an embedded polar group. These packing materials offer unique selectivity and solve the issue of phase dewetting but often show decreased retention. Because of the problems related to using reversed-phase packing material an alternative solid phase was investigated.

HILIC is a mode of chromatography that can address these issues. The term HILIC was suggested by Alpert in 1990 (McCalley, 2007) to describe a chromatographic technique employing a hydrophilic stationary phase and a relatively hydrophobic eluent in which water is the strongest eluting molecule. This is a variant of normal phase chromatography, where the retention mechanism is believed to be a partitioning of the analyte between the water-enriched layer of semi immobilised eluent on a hydrophilic stationary phase and a relatively hydrophobic bulk eluent, usually consisting of 5-40% water in acetonitrile (Alpert, 1990).

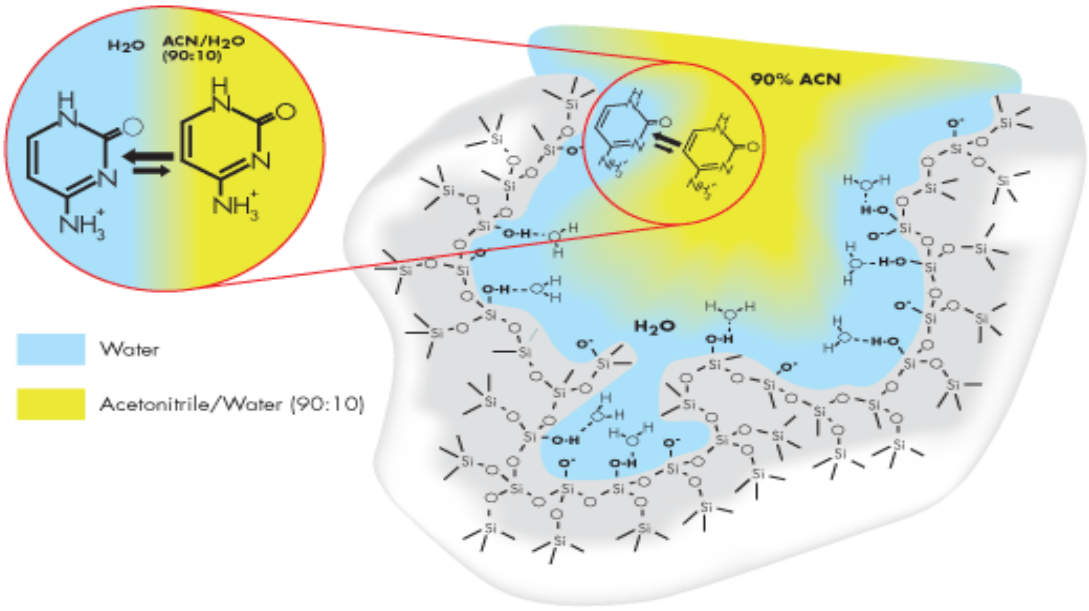


Fig.2.3. Retention mechanism on silica – adapted from Waters website

HILIC, being a more reproducible technique has a number of advantages compared to conventional normal phase chromatography. The eluent preparation is simpler since there is no need for absolute control over lower water content in the solvents. The elution order in HILIC is more or less orthogonal to that seen in reversed-phase separations, which means that HILIC works best for solutes that are the most problematic in reversed-phase chromatography (Alpert, 1990). That the typical eluent is high in acetonitrile, also gives it two additional advantages over RP-LC; higher sensitivity in electrospray ionisation mass spectrometry and faster separation due to the lower viscosity (Shou *et al*., 2005).

The development of an analytical method was highly desirable to provide simultaneous detection and unambiguous identification of different cyanobacterial toxins. Liquid chromatography-mass spectrometry (LC-MS) with

electrospray ionisation has proven to be a powerful tool for the analysis of toxin at trace levels. Improved design and lower costs of LC-MS instruments are making this technique a viable tool in many laboratories involved in both toxin monitoring and research.

The HPLC, LC-MS methods that have been reported on cyanotoxins are usually based on reversed phase liquid chromatography separations. It is always difficult to resolve all of the toxins in one analysis due to the wide range of structures and charge state of most cyanobacterial toxins (Poon *et al*., 1993).

A study of separation of acidic, neutral and particularly basic solutes was investigated using a bare silica column by HILIC conditions (McCalley, 2007) with water concentrations of >2.5% and with >70% acetonitrile (ACN) showing acidic solutes with low retention or exclusion in ammonium formate buffers but which were strongly retained when using trifluoroacetic acid (TFA) buffers. Due to low viscosity of mobile phases with high ACN content, HILIC separation was ideal for fast analysis of ionised bases. HILIC retention seemed to be a major factor contributing to retention with >70% ACN in the mobile phase, although ionic retention was also suggested. Despite the complication of the mechanism the technique has shown some good responses including the reasonable peak shapes obtained for the base samples tested, the order of elution of solutes is generally the opposite of that found in RP separations, giving useful alternative selectivity, good retention of polar compound is obtained in HILIC, whereas very poor retention is obtained in RP- HPLC (McCalley, 2007).

HILIC coupled with electrospray mass spectrometry (HILIC-MS) for the analysis of cyanobacterial toxins has also been investigated. Toxins examined included saxitoxin and its various analogues, cylindrospermopsin,

deoxycylindrospermopsin, and microcystins–LR and RR. The column used for the analysis was TSK gel Amide -80 column eluted with 65% B, where eluent A is water and B is 95% acetonitrile/water solution, both containing 2.0 mM ammonium formate and 3.6 mM formic acid. The analysis of all other toxins was performed under 75% B isocratic. HILIC proved to be suitable for the analysis of microcystins, but peak shape was not symmetric and it was concluded that these compounds are best analysed by existing reversed-phase methods. The analysis of CYN and doCYN with 65% B eluted too quickly. A higher percentage of solvent B was required for effective retention of these toxins. In particular, with 75% B both toxins were eluted 7.1, 6.2 min respectively. This study also proposed the algal samples tested did not require any sample clean up or pre- concentration step and proved to be quite robust. (Dell' Aversano *et al*., 2004).

The HILIC separation of anatoxin-a(s) was carried out using a bare silica column (250 mm x 2.0 mm, 5 µm, Phenomenex) under isocratic conditions using a mixture of methanol/water containing 5.0 mM Ammonium formate and 0.01% formic acid as mobile phase with a flow rate 0.2 ml/min (Dorr *et al*., 2010), alternatively, a ZIC-HILIC Column was employed. Separation was achieved under gradient elution at 0.7 ml/min where eluent A was acetonitrile/water (80:20 v/v) containing 5 mM ammonium formate and 2 mM formic acid and eluent B was water containing 10 mM ammonium formate and 10 mM formic acid (Dorr *et al*., 2010). Considering the polar nature of anatoxin-a(s), HILIC was found to be a well-suited separation technique. Indeed, very good retention was obtained with a bare silica column with an aqueous-organic mobile phase. Using ZIC-HILIC column was tested; good peak profile was obtained with relatively short retention time.

As a comparison of sensitivity between silica and C_{18} column, in the first one low organic content mobile phase was used to retain polar analytes. On the Silica column, high organic content mobile phases were used, resulting in higher sensitivity and high retention. The advantage of using HILIC Silica column, is that a high flow rate is achievable because of the very low backpressure. A common perception regarding silica columns is that they are not stable when used for biological sample analysis, as polar ionic endogenous compounds are strongly retained and eventually deteriorate the column. However, it was found that LC-MS/MS with aqueous organic mobile phase on a silica column was compatible with the common sample extraction procedures (Naidong, 2003).

The current study examines the improved analysis of cylindrospermopsin by using HILIC method. Regarding CYN as a neutral compound, there is a need for optimisation of conditions in order to produce a new method. The method could perform well in aspects of good separation, good peak shape and high retention time.

2.2.9. HILIC Method optimisation

In order to investigate an optimised HILIC analysis for CYN, several methods have been performed. The first attempt was with eluents water (A) and Acetonitrile-water (95:5, B), both eluents containing 2.00 mM Ammonium formate and 3.6 mM formic acid. Elution was carried out with 75% B isocraic. Second method tested was with a mobile phase composed by 0.1% aqueous formic acid (A) and acetonitrile (B), and the following gradient 0-0.5 min at 70% B, decreased to 30% B in 3 min and held at 30% B for 7 min, then increased to 70% B in 8 min and held till 14 min. Another method performed was with a

mobile phase Water (A) and (B) aqueous 5.0 mM formic acid and 2.0 mM ammonium formate, 5% - 45% B at 20 min, then to 75% at 21 min. Third gradient from B 75% to 5% at 22 min, 95% A to 5% B at 30 min and the column then equilibrated for 5 min. Section 2.2.10 explains the method selected for the CYN analysis which was found to perform best out of those evaluated.

Table. 2.3. HILIC Method Optimisation: Method development performed with different solvents and gradients.

METHOD	SOLVENT USED	ELUTION TYPE	Comments
1st	A- Water B- 95:5 acetonitrile/ water solution both eluents containing 2.00 mM Ammonium formate and 3.6 mM formic acid.	75% B isocratic	Poor peak shape
2nd	A- 0.1% aqueous formic acid B- Acetonitrile	gradient 0-0.5 min at 70% B, decreased to 30% B in 3 min and held at 30% B for 7 min, then increased to 70% B in 8 min and held till 14 min	Poor peak shape
3rd	A- Water B- 5.0 mM formic acid 2.0 mM ammonium formate with water	5% to 45% B at 20 min to 75% at 21 min. Third gradient from B 75% to 5% at 22 min , 95% A to 5% B at 30 min and the column then equilibrated for 5 min	Poor peak shape

2.2.10. HILIC Analysis of Cylindrospermopsin

The analysis of cylindrospermopsin was carried out by using Hydrophilic Interaction Liquid Chromatography (HILIC) using a HPLC Waters system. Waters Alliances 2695 separation module with photodiode array (2996 PDA) was used for the experiment. The separations were carried out with HILIC column

(Atlantis® HILIC Silica; 5 µm particle size, 2.1 x 150 mm column) the column was maintained in acetonitrile. The mobile phase used was 100% Acetonitrile (A), 5.0 mM Formic acid and 2.0 mM Ammonium formate (B). The samples were separated using a gradient increasing from 5% to 45% B over 20 min and a further increase to 75% over 21 minutes with an equilibration time of 10 min. The samples were quantified at the standard wavelength of 262 nm. The sample injection was 10 µl with a single injection and the flow rate used was 0.3 ml/min with a runtime of 35 min.

2.2.11. Standard calibration curve

Standard stock solutions of purified CYN were prepared by dissolving known amount of the dry sample in 100% MeOH. Samples were always made by dilution of the stock solution in MeOH for future use and stored at –20 ºC.

Standard calibration curve for cylindrospermopsin was prepared by analysing a range of CYN concentration 100, 50, 5.0 µg/ml by using the HILIC Method. To prepare the dilution series a stock of CYN in methanol was used. The samples from the dilution series were analysed by HILIC as described in section 2.2.10. The correlation coefficient determined at 262 nm using the mean of replicate samples.

2.2.12. Solid phase extraction (SPE).

Solid phase extraction is one of the most powerful and rapid technique currently available for selective sample preparation. This technique can be used for purposes, such as purification, desalting, solvent exchange, derivatisation, trace enrichment and class fractionation. This process offers several advantages

including high recoveries and concentration of the analytes, ability to simultaneously extract analytes of wide polarity range, ease of automation, compatibility with instrumental analysis, and reduction in organic solvent consumption. Solvents can be applied via vacuum applied to the column outlet, pressure applied to the column inlet or centrifugation. Out of these vacuum is the most commonly performed. There is a range of SPE cartridges in use in environmental sampling.

In order to get the effective recovery and the correct quantification of toxin in the field samples, efficient methods of extraction and concentration are required. Concentration and extraction of dissolved CYN from natural samples has not been widely studied or published, while quantification methods are well developed (Eaglesham *et al*., 1999; Torokne *et al*., 2004), A study performed with a range of SPE cartridges showed best results when using graphitised carbon (Norris *et al*., 2001). Another study combined graphitised carbon with C_{18} and produced good recoveries when working with high concentration of CYN. When compared with a wide range of the most common SPE sorbents, an effective SPE method was developed by choosing graphitised carbon as the most suitable compound (Norris *et al*., 2001).

Sample pre-treatment is not often necessary for the ENV+ cartridges due to their high hydrophobic nature. However, the column can be rinsed with miscible solvents (eg. MeOH, Acetone) prior to sample loading to ensure high sensitivity for the analytes needed and also to avoid interference from the sorbent. It's also important to make sure cartridges are rinsed with water to remove the excess solvents. At present, extraction becomes difficult using silica based sorbent cartridges. Also many analytes are highly water soluble where

these cartridges are mainly used. The resin based sorbents are capable of retaining analytes of a wide range of polarities due to high surface area, highly cross linked polystyrene based polymer column. Due to the high surface area of these cartridges non-polar sorbents provides retention of high polar and water soluble analytes.

The retention characteristics of the ENV+ column are based on the hydrophobic interaction between the sorbent and analyte to retain the analyte during loading. On the other hand miscible solvents, such as methanol, can compete for interaction with the surface and elute the analyte.

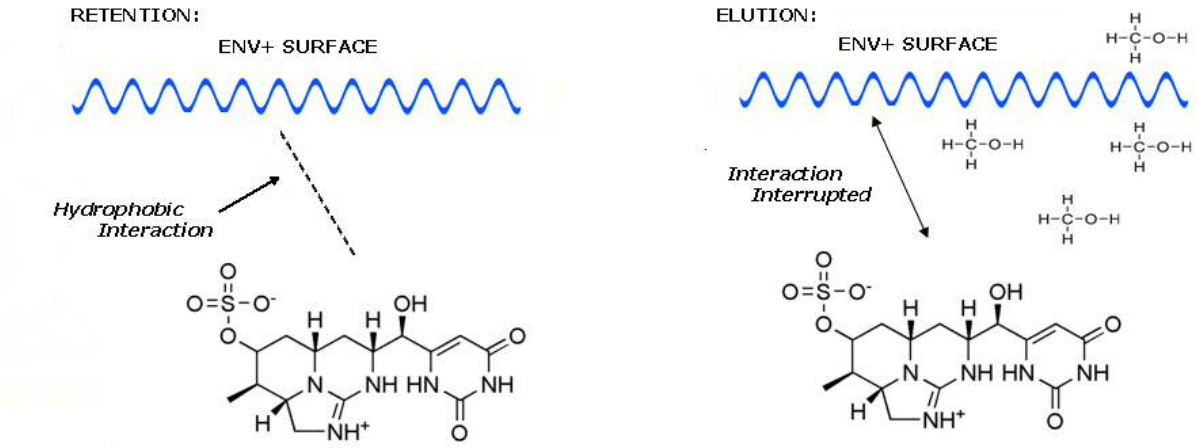


Fig.2.4. Schematic diagram of ISOLUTE ENV+ retention and elution mechanism

This study examines the application of ENV+ cartridges in the pre concentration of CYN from aqueous samples, in order to achieve better concentration prior to the HPLC analysis. ISOLUTE ENV+ cartridges (500 mg bed weight, 3 ml volume and 100 mg bed weight, 1 ml volume, Biotage, Uppsala, Sweden) were used for the experiments. Solvent Methanol was supplied by Rathburn (Walkerburn, Scotland). Milli-Q water obtained from the laboratory

Milli-Q system (purified to 18.2 mΩ, Millipore Watford, UK). Distilled water was received from the laboratory water distillation system.

For advanced elution of multi samples at one time through several cartridges a vacuum manifold system can be used (10 cartridges at a time, Vac master). The experiment was performed with a flow rate of approximately 8 ml/min. Set-up and function can be seen in Fig.2.5.

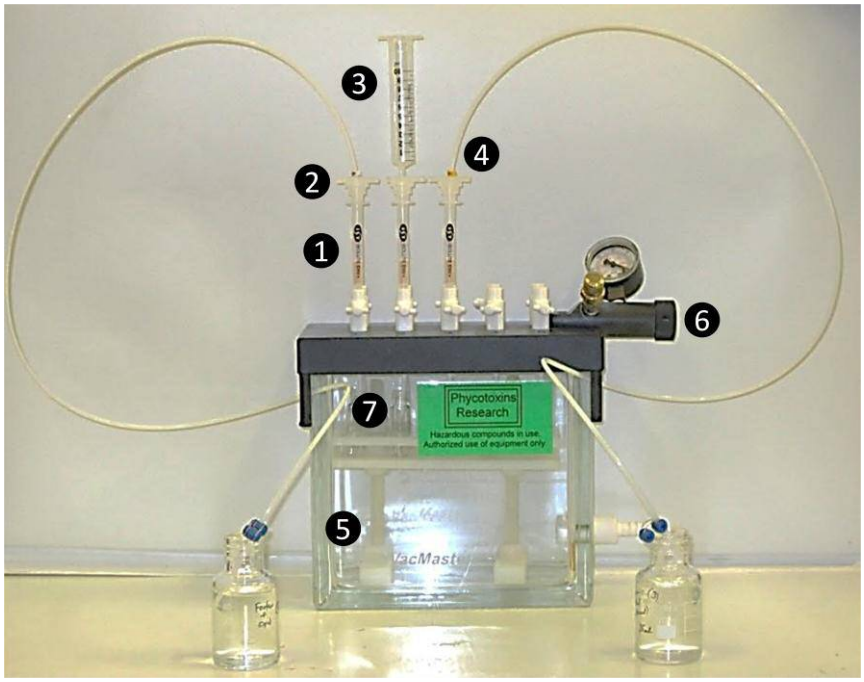


Fig.2.5. Vacuum manifold system. The cartridge ❶ is attached into the tap inlets on the top of the vacuum manifold using an adapter ❷ . A 10 ml syringe ❸ can be fixed on the cartridges is used for the small amounts of sample delivery or a tube ❹ leading to the reservoir for the large sample amount. A rack ❺ (with small glass vials ❼ , 4 ml) placed inside the device helps to collect the samples. The vacuum is produced by the use of a water jet pump and can be adjusted using a release valve ❻

2.2.13. CYN recovery using ISOLUTE ENV+ (500 mg) by SPE.

Recovery of CYN was determined using ISOLUTE ENV+ (500 mg) SPE columns. Conditioning of the cartridges were performed by rinsing 10 ml of 100% MeOH followed by 10 ml of water wash. Triplicates samples of CYN in distilled water with two varying concentration of 10 µg and 1 µg were applied through the cartridges. Thereafter, 10 ml of 10% MeOH was used to wash the cartridges in order to improve the detection of CYN in HPLC analysis. The cartridges were then dried by passing vacuum for 20 min to minimise the amount of water eluted with sample. After drying, 80% of MeOH was employed

to elute the analyte in 4 fractions (4 x 2 ml each). Aliquots were evaporated to dryness under a stream of nitrogen using heat block (Techne Dri Heat Block DB.3, UK) at 45 ºC. Dried samples were then dissolved in 200 µl Milli-Q water and 100 µl used for the HPLC analysis.

2.2.14. CYN recovery from two different water samples using ISOLUTE ENV+ (500 mg) cartridges.

SPE method was carried out in two different water samples (Tap water and Loch Rescobie water) to see the efficiency of the cartridges and the effect of typical matrix interference. The experiment was performed as stated in the section 2.2.13. CYN spike concentrations of 10 µg and 1 µg were evaluated with both waters. A volume of 250 ml of the water samples were applied through the cartridges.

2.2.15. Effect of MeOH washes before the elution step

This study was carried out to examine the effect of 10% MeOH wash before the elution step to assess if it could be used to reduce matrix interference. Four ISOLUTE ENV+ (100 mg) cartridges were used for this experiment. Cartridges were preconditioned by washing them with 10 ml of 100% of MeOH, followed by 10 ml of water rinse, making sure that cartridges were not allowed to dry. Duplicate samples of distilled water containing 10 µg CYN in 50 ml were applied through the cartridge. Cartridges (2n) were then washed with 10 ml of 10% MeOH and the other (2n) with no MeOH wash. Cartridges were then dried by passing air to minimise the amount of water eluted with sample. After drying cartridges were eluted with 80% MeOH in 3

fractions of 1.5 ml (3 x 1.5 ml) eluted in each fraction. Samples were collected in 4 ml glass vials. Samples were then transferred in to micro centrifuge tubes and centrifuged to remove traces of the solid phase. Afterwards, supernatant were pipetted out in to new glass vials. Samples were evaporated to dryness under N_2 a Techne Dri heat block DB.3 (UK) at 45 ºC overnight. Dried samples were dissolved in 200 µl of Milli-Q water from 100 µl for the HPLC analysis.

2.2.16. Breakthrough curve for CYN.

Breakthrough curve was performed by applying aqueous CYN extract through 100 mg ENV+ cartridges with 50 x 10 ml fractions collected and the presence of CYN breakthrough analysed by HPLC by taking a sample of 100µl. Conditioning of the cartridges were carried out as mentioned in the section 2.2.13. ISOLUTE ENV+ (100 mg) cartridges were used for the experiment. Duplicate samples of filtered aqueous CYN extract with a concentration of 1 µg/ml CYN were applied through the cartridges. Sample volume of (500 ml) was prepared by adding 50 ml of the aqueous extract to 450 ml of Milli- Q water. This was passed through the cartridge with every 10 ml of elute collected separately to determine the point at which breakthrough of the analyte (CYN) occurs. Aliquots of 50 samples with 10 ml each fraction were collected to see the breakthrough of CYN. Samples (100 µl) of fraction samples were taken for HPLC analysis. The two cartridges were then eluted with 10 aliquots (10 x 1 ml) of 80% methanol and samples were collected in 4 ml glass vials. Elute (1 ml) was diluted 1:10 with water prior to HPLC analysis as high methanol concentration can influence the chromatography.

2.2.17. Elution solvent optimisation for SPE

The experiment was carried out to determine the optimal percentage of MeOH used as an eluent in SPE methods and also, to find out the highest recovery of CYN by using ENV+ (100 mg) cartridges with lowest elution volume. Cartridges were preconditioned by washing them with 10 ml of 100% of MeOH, followed by 10 ml water rinse, making sure that cartridges were not allowed to dry. Experiments were carried out in duplicate for each percentage of MeOH elution. Samples containing 50 µg CYN in 10 ml water were applied through the cartridge. Cartridges were then dried by passing air to minimise the amount of water eluted with sample. After drying cartridges were eluted in 5 fractions with 0.5 ml (5 x 0.5 ml) eluted in each fraction. Aforementioned elution steps were carried out with a range of MeOH concentration (0%-100%) as elution solvent. Samples were eluted in 4 ml glass vials and were then diluted by adding Milli-Q water (1:10) in microcentrifuge tubes. The samples were centrifuged and aliquots of 100 µl taken for HPLC analysis.

2.2.18. CYN recovery from three water samples by SPE.

To evaluate the maximum recovery of CYN through 100 mg ENV+ cartridges, three water samples with a range of concentration 1 µg/L, 5 µg/L, 10 µg/L CYN and an unspiked solution as control were tested by SPE. Conditioning of the cartridge was carried out as mentioned in the section 2.2.13. ISOLUTE ENV+ (100 mg bed weight, 1 ml volume) cartridges were used for each water sample. Three water samples were tested, distilled water, tap water and River Carron. Different CYN concentrations used to spike the water samples were in the ranges of 1 µg/L, 5 µg/L, and 10 µg/L. Sample volume used to find the

recovery of CYN was 50 ml (50 ng, 250 ng, and 500 ng per 50 ml) and were applied through cartridges. The cartridges were then dried by passing vacuum until dried in order to minimise the amount of water eluted with sample. After drying, the cartridges were each diluted with 100% MeOH. Eluted sample (1.5 ml) was collected in 4 ml glass vials. Samples (1.5 ml) were transferred to a clean microcentrifuge tube centrifuged and evaporated to dryness. Milli-Q water (150 µl) was added to the dried sample and centrifuged prior to analysis by HPLC.

2.3. Results

2.3.1. Standard calibration curve for cylindrospermopsin

Calibration curves for purified CYN were obtained by taking concentration against the peak area responses by HPLC-PDA. The wavelength in PDA was set between 200 and 400 nm and CYN chromatograms were extracted at their highest absorption wave length 262 nm. Purified toxin showed a linear response at 262 nm and retained a correlation coefficient (R^2) greater than 0.99 on their linear regression (Fig.2.7).

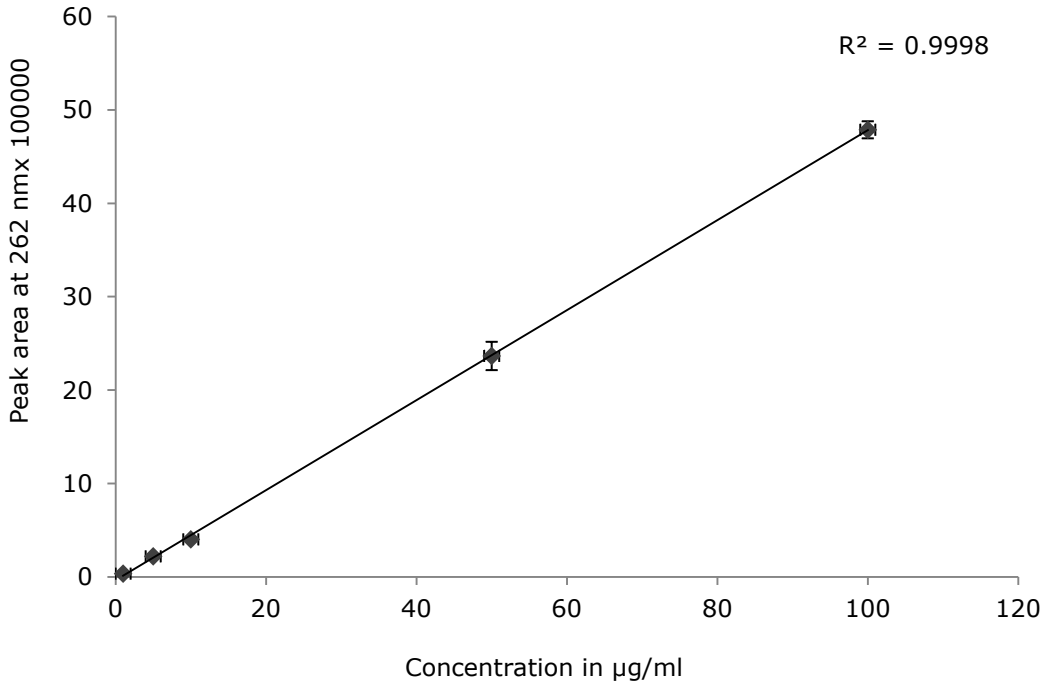


Fig.2.6. The calibration graph for cylindrospermopsin (CYN). Displayed with the graphs are the formula and the correlation coefficient (R^2 – value) determined at 262 nm. Triplicates were used, i.e. n=3. Bars equivalent to 1 standard deviation.

2.3.2. Standard calibration curve for cylindrospermopsin by HILIC.

Standard calibration curve for purified CYN was performed by taking concentration versus peak area responses by HILIC-PDA as described in the section 2.2.6. Chromatograms of the toxins were extracted at their highest absorption wavelength 262 nm. Calibration curve prepared for purified CYN by HILIC method demonstrated a linear response and maintained a correlation coefficient (R^2) greater than 0.99 on their linear regression (Fig.2.7)

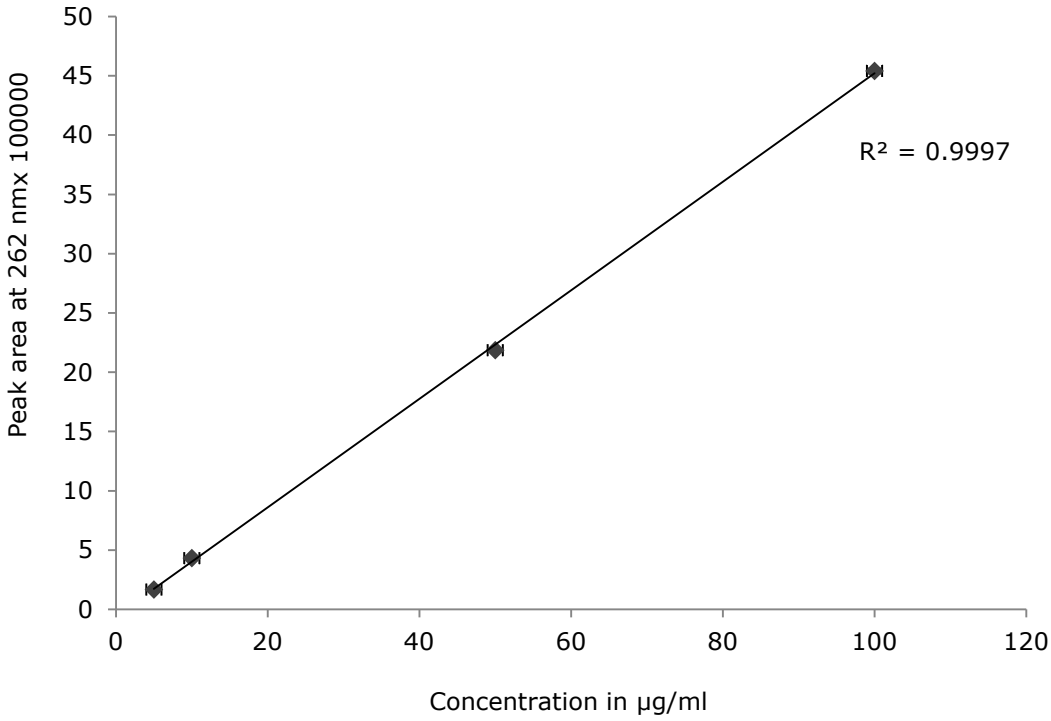


Fig.2.7. The calibration graphs for CYN in methanol displayed with the graphs are the formula and the correlation coefficient (R^2-value) determined at 262 nm. Triplicates were used, i.e. n=3. Bars equivalent to 1 standard deviation.

2.3.3. HILIC analysis of cylindrospermopsin

In order to optimise a method for the HILIC analysis of CYN, the study attempted different methods. First method was performed with a 75% B isocratic elution with eluents water (A) and Acetonitrile-water (95:5, B), both eluents containing 2.00 mM Ammonium formate and 3.6 mM formic acid. The method resulted in the chromatogram with no peaks identified. This method was tested in both crude and pure CYN samples (Fig.2.8 A & B).

The second method performed was with a mobile phase composed by 0.1% aqueous formic acid (A) and acetonitrile (B), and the following gradient 0-0.5 min at 70% B, decreased to 30% B in 3 min and held at 30% B for 7 min, then increased to 70% B in 8 min and held until 14 min. This method resulted in the chromatogram with CYN peaks. Both crude and pure CYN samples produced peaks by this method. It was noticed that in crude sample retention time was high (12.4 min) as compared with pure CYN (9.2 min). However, both chromatograms did not show the better peaks. (Fig 2.9 A & B)

As compared to the other method tested, the selected method resulted in chromatograms with CYN peaks. The peak shape was not satisfactory with a widening noticed at the base (Fig. 2.10). However, the compound has retained in the column more than that of RP-HPLC. In comparison to RP-HPLC, HILIC was found to be efficient method for retaining the polar compound. On the other hand better peak shape was observed using C_{18} method (Fig.2.11).

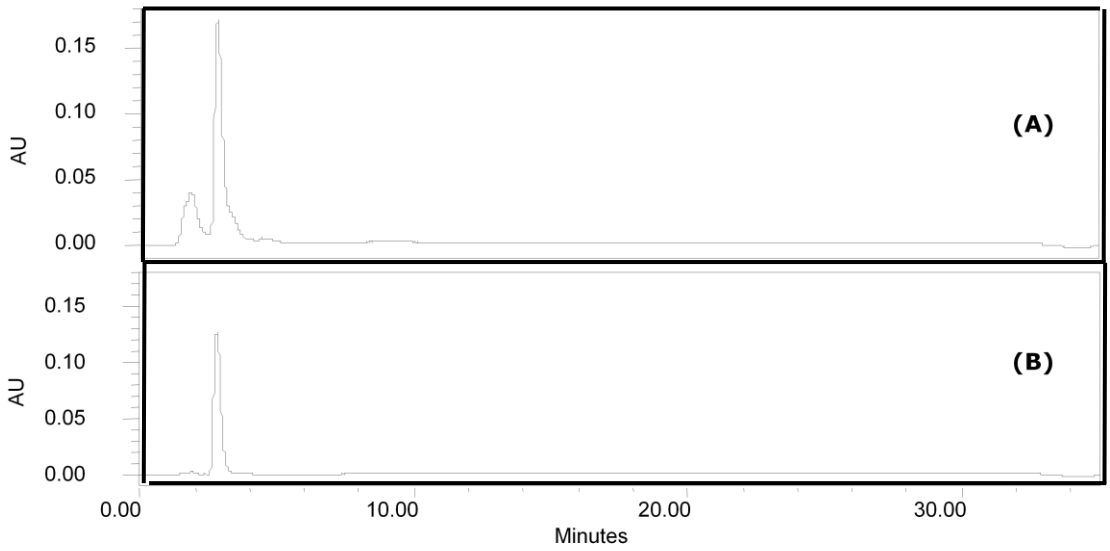


Fig.2.8. HILIC Method development (1): Chromatograms of the CYN samples by HILIC. (A) crude and (B) pure CYN samples (water (A) and acetonitrile-water (95:5, B), both eluents containing 2.00 mM Ammonium formate and 3.6 mM formic acid, B 75% isocratic.

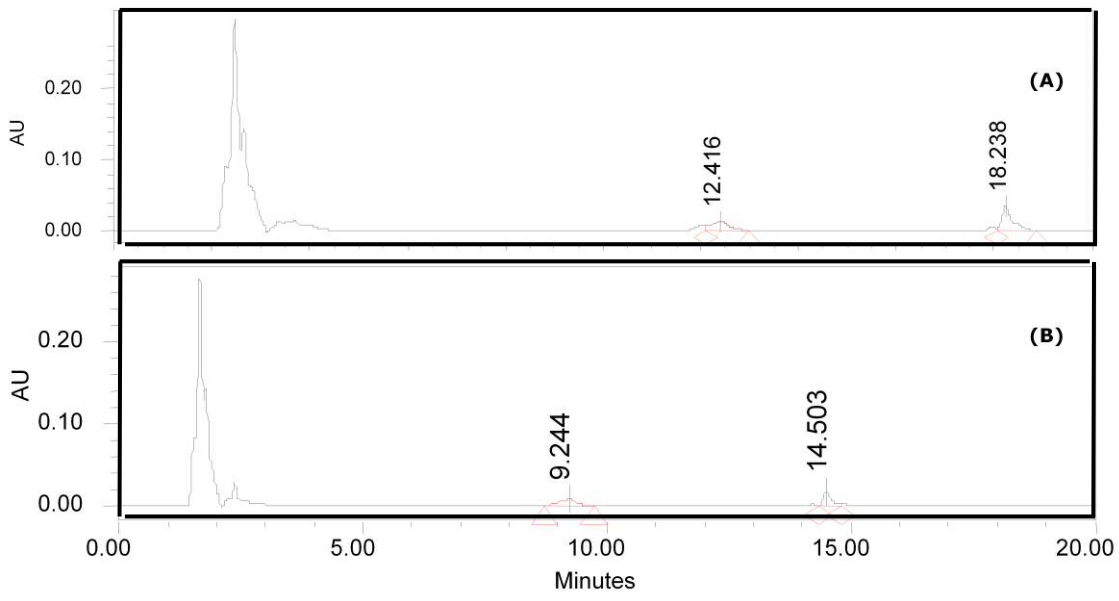


Fig.2.9. HILIC Method development (2): Chromatograms of the CYN samples by HILIC. (A) crude and (B) pure CYN samples (0.1% aqueous formic acid (A) and acetonitrile (B), and the following gradient 0-0.5 min at 70% B, decreased to 30% B in 3 min and held at 30% B for 7 min, then increased to 70% B in 8 min and held till 14 min.

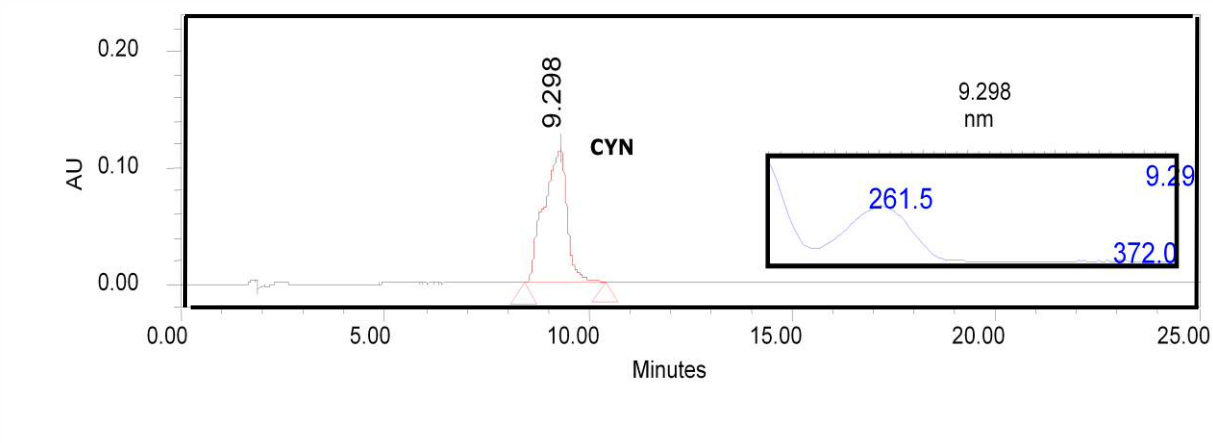


Fig.2.10. HILIC Method development (opted): Chromatograms of the CYN samples by HILIC (pure CYN). The mobile phase used was 100% Acetonitrile (A), 5.0 mM Formic acid and 2.0 mM Ammonium formate (B). The Samples were separated using a gradient increasing from 5% to 45% B over 20 minutes and a further increase to 75% over 21 minutes with an equilibration time of 10 minutes.

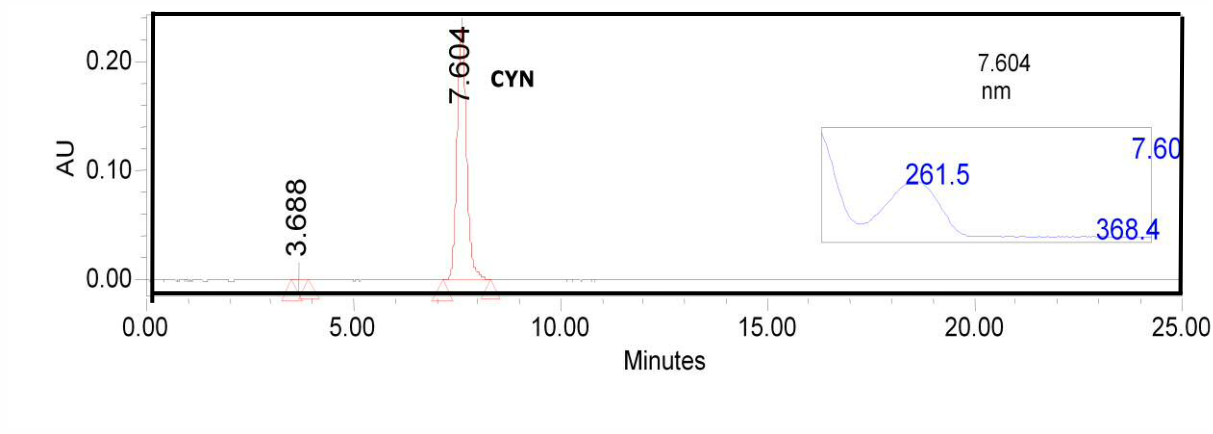


Fig.2.11. HPLC (C_{18}) analysis: Chromatograms of the pure CYN samples. Method as mentioned in section 2.2.6.

2.3.4. CYN recovery using ISOLUTE ENV+ (500 mg) by SPE.

Initial experiments were carried out with ISOLUTE ENV+ (500 mg) SPE cartridges. The aim was to optimise the pre-concentration step for the CYN in water samples prior to the HPLC analysis.

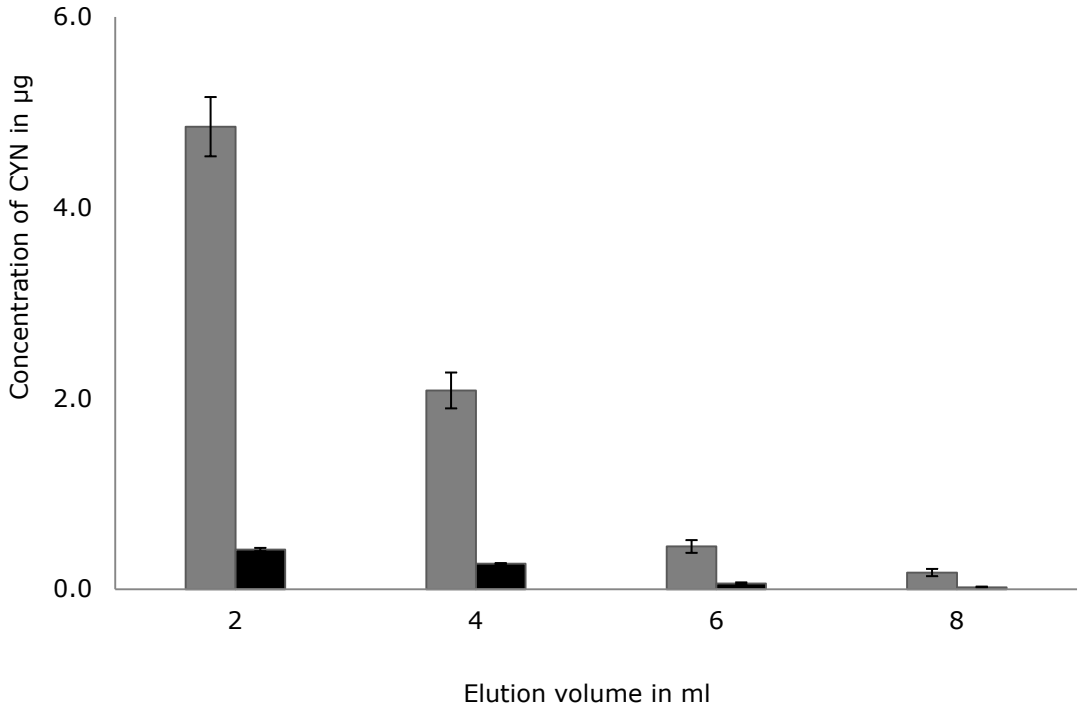


Fig.2.12. HPLC analysis of eluted CYN samples by solid phase extraction evaluation using two quantities of CYN; 10 µg ( ) and 1 µg ( ). Duplicates were used, i.e. n=2. Bars equivalent to 1 standard deviation.

CYN in distilled water samples eluted by using SPE method showed 75.5% (10 µg) and 76.0% (1 µg) of toxin recovery. Total volume of 8 ml samples were eluted through the cartridges and major portion of the analyte eluted in half of the total volume (70% approx), were similar for both concentrations of CYN tested (Fig.2.12).

2.3.5. CYN recovery from two different water samples using ISOLUTE ENV+ (500 mg) cartridges.

In order to investigate the recovery of CYN in different waters this experiment was carried out. Tap water and Loch Rescobie were the two waters tested.

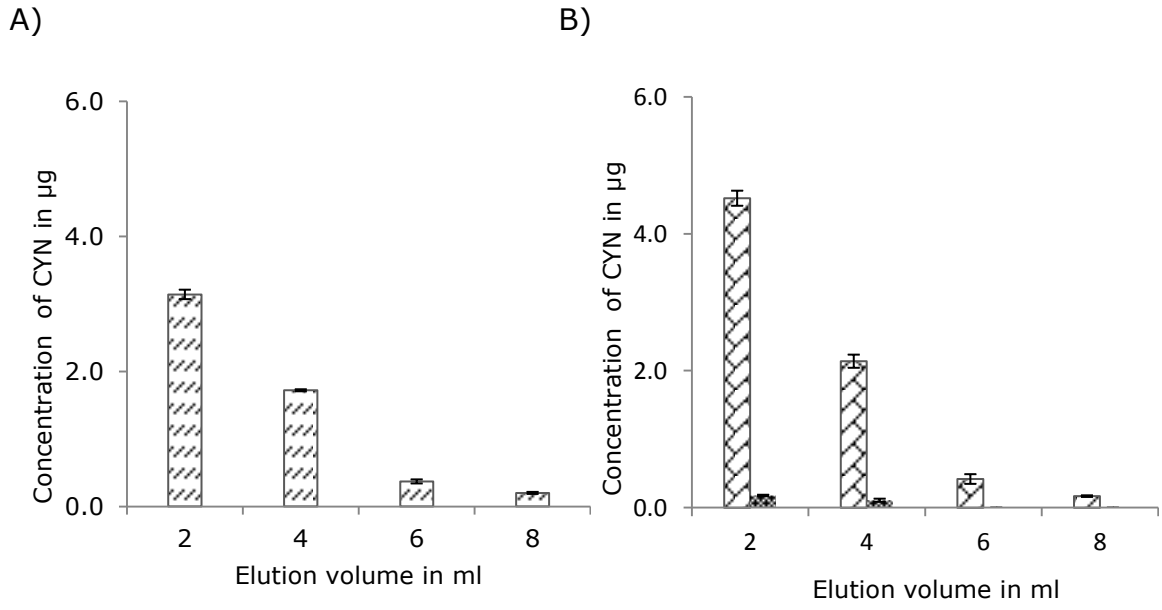


Fig.2.13. HPLC analysis of two different water samples (A – Tap water, B-Loch Rescobie) for the CYN recovery through ENV+ (500 mg) cartridges. Two different concentration 10 µg and 1 µg used in duplicates, i.e. n=2. Bars equivalent to 1 standard deviation.

Maximum recovery was observed in Loch Rescobie water treated (Fig.2.13.B). It was also noticed that, with tap water, at lower concentration no analyte has been eluted (Fig.2.13.A) while at the same time analyte has eluted in Loch Rescobie water at lower concentration.

2.3.6. Effect of MeOH washes before the elution step

This study examines the effect of MeOH wash before the elution step in order to provide better detection (i.e., removal of some matrix interference) of CYN by HPLC analysis. The experiment was carried out to compare the effect of methanol wash or no methanol wash before the elution step.

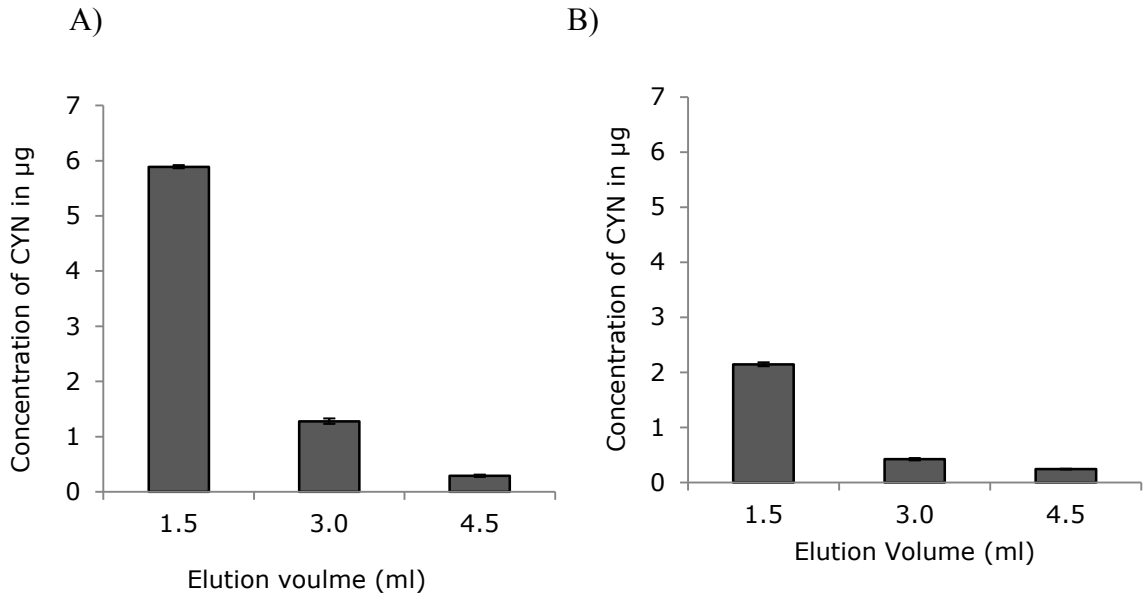


Fig.2.14. SPE processing of CYN. A) 10% methanol wash was excluded before the analyte elution. B) 10% methanol wash was employed before the analyte elution. Duplicates were used, i.e. n=2. Bars equivalent to 1 standard deviation.

Clearly using the 10% methanol wash significantly reduced the amount of CYN recovery. With methanol wash 50% of the analyte was recovered but 75% without.

2.3.7. Breakthrough curve for CYN

CYN was retained until 50 ml of solution was applied, in other words until about 50 µg of cylindrospermopsin was successfully retained by the ENV+ cartridge. Subsequently a constant increase of breakthrough could be detected (Fig.2.15).

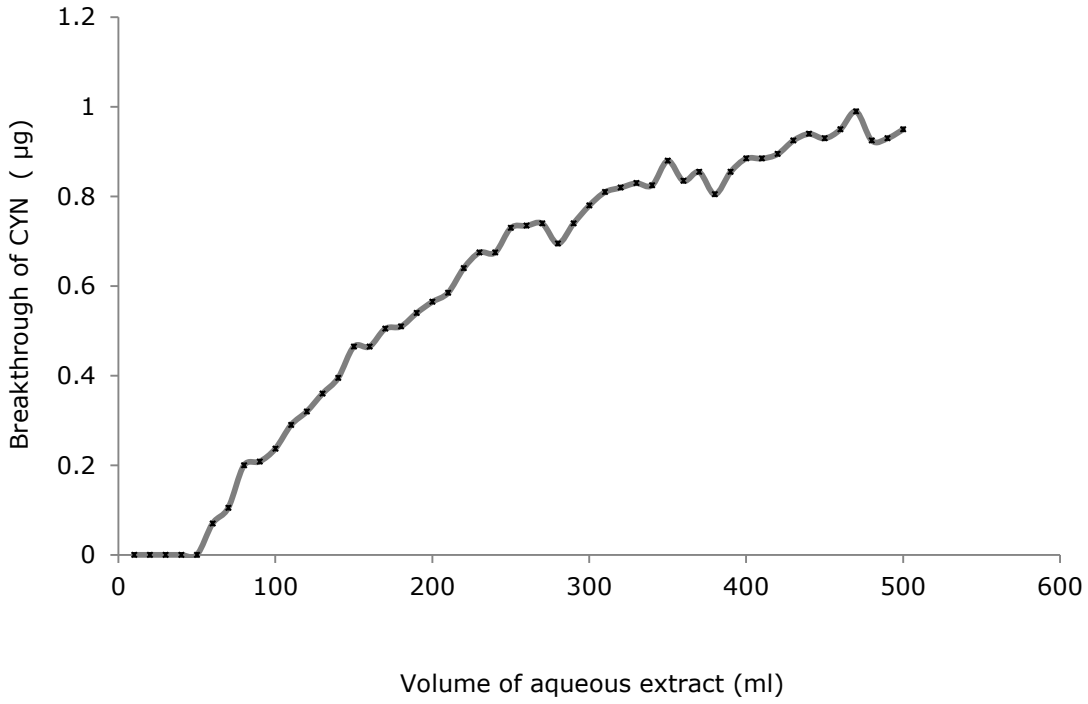


Fig.2.15. Breakthrough of cylindrospermopsin from ENV+ cartridge (100 mg) applying CYN 1.0 µg/ml of extract pointing out a retention capacity of 50 µg.

2.3.8. CYN recovery from the three water samples by SPE

In the current study the efficiency of ENV+ (100 mg) cartridges for the recovery of CYN was investigated. Application of these cartridges in the pre concentration step prior to the HPLC analysis of environmental water samples was a concern. In order to examine the performance of the cartridge, the experiment was carried out with three different water samples.

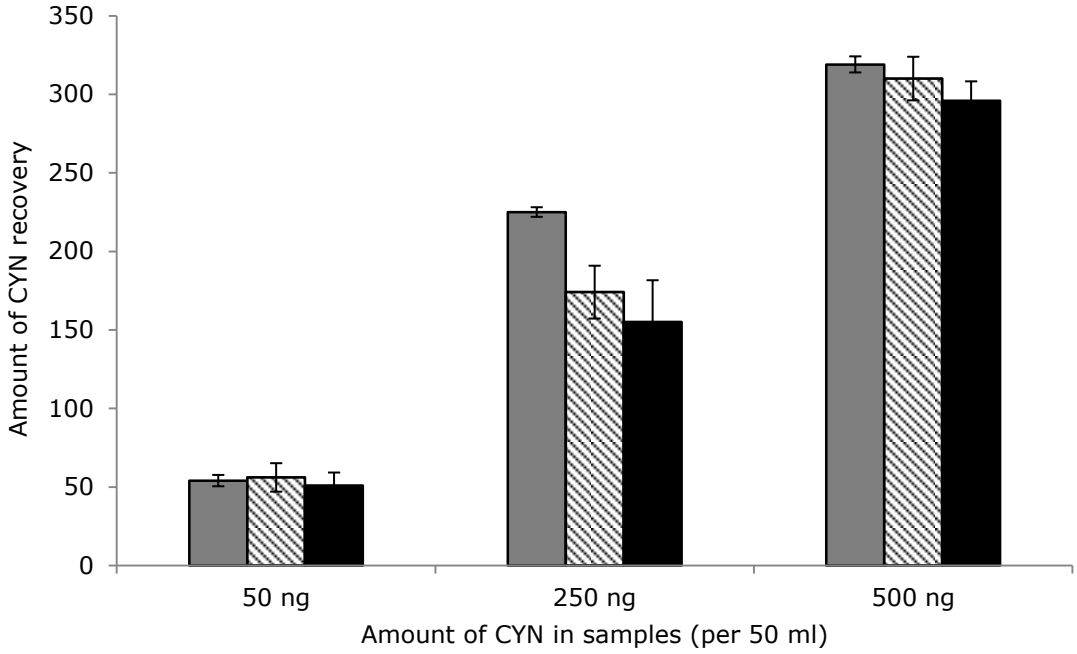


Fig.2.16. Three water samples Distilled water ( ), River Carron ( ) and Tap water ( ) with varying concentrations (50, 250, 500 ng/50 ml) were analysed by SPE. Bars represent standard deviation deduced from triplicates (n=3)

CYN recovery from the three water samples , highest recovery was found in all three water types performed with lowest concentration (50 ng/ 50 ml, Fig. 2.16 and 2.17). HPLC analysis of the SPE samples demonstrated that CYN in distilled water showed highest recovery compared to the other water samples. However, at lowest concentration, all three waters showed 100% recovery.

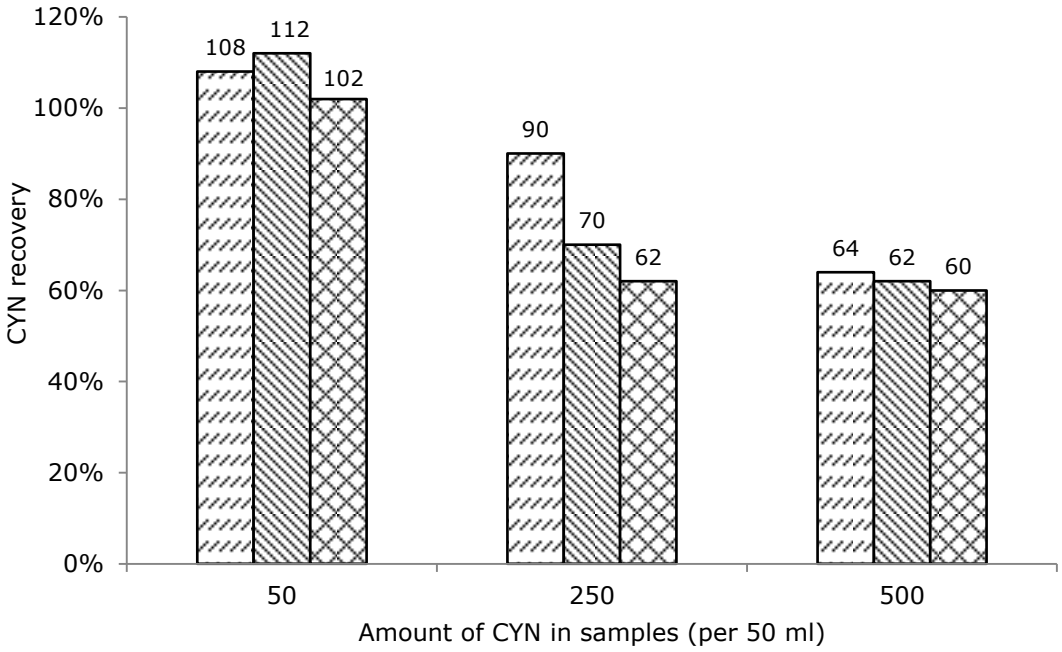


Fig.2.17. Percentage recovery of CYN from three water samples (Distilled water, River Carron and Tap water in varying concentrations (50, 250, 500 ng/50 ml)

At a higher concentration (250 ng/ 50 ml) samples showed 90 % recovery with distilled water, 70% recovery for River Carron and 62% for Tap water (Fig.2.17). The result showed that lowest concentration could be tested using environmental water samples. On the other hand, CYN recovery from highest concentration tested showed lowest recovery 64% (Distilled water) 62% (River Carron) and 60% (Tap water).

2.3.9. Elution solvent optimisation for SPE

This study was to optimise the elution solvent for a better recovery of CYN by SPE. Results demonstrated that methanol tested with high concentration (100% MeOH) showed highest recovery of CYN (Fig.2.18). At higher

concentration ranges (80%-100%), it was observed that the recovery is highest as compared to the lower concentration. Samples were eluted in fractions of 0.5 ml MeOH and observed that the complete recovery was achieved in 1.5 ml of sample volume eluted (Fig.2.18).

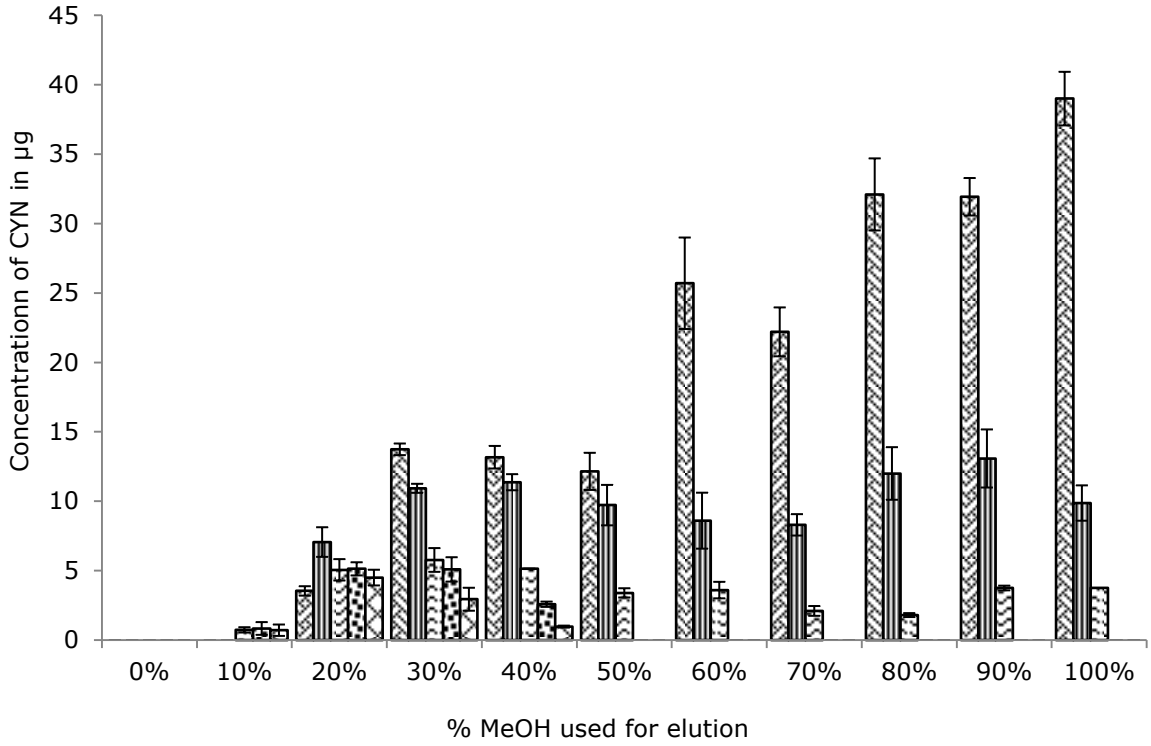


Fig.2.18. Elution profile for CYN recoveries with a range of MeOH concentrations used (0%-100%). Five x 0.5 ml each elution fractions were collected 0.5 ml ▨, 1.0 ml ▥, 1.5 ml ▨, 2.0 ml ▩, 2.5 ml ▨. Duplicates were used, i.e. n=2. Bars equivalent to 1 standard deviation.

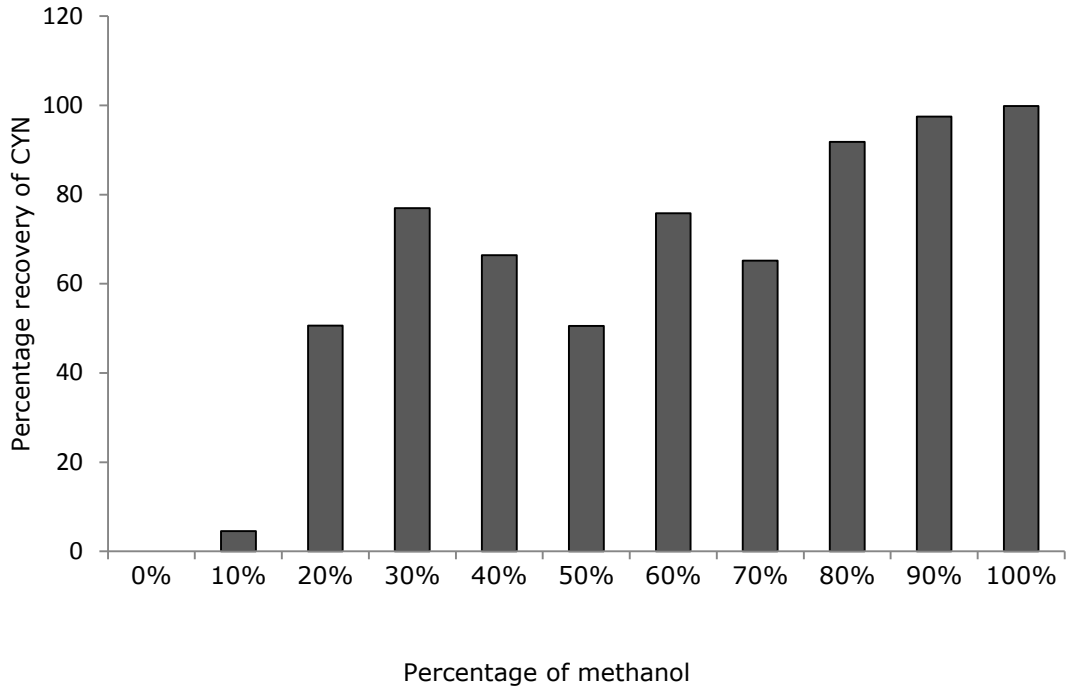


Fig.2.19. Recovery of CYN (%) by SPE with different concentration of MeOH (0-100%) used as an eluent.

Methanol concentration ranges from 50% to 100% showed the complete recovery of CYN in 1.5 ml elution volume. On the other hand, at lower ranges (20% - 40%), complete recovery occurred only in 2.5 ml elution volume. Also noticeable was that the first fraction elutes the major portion of the analyte (CYN) in all the concentration range tested except 20% (Fig.2.19).

2.4. Discussion

This chapter has described culturing of cylindrospermopsin and its purification by reverse phase flash chromatography and HPLC. Step wise methodologies have been described including extraction, concentration and clean up of the extracts. The chapter mainly discusses the two method developments for the pre-concentration and the analysis of cylindrospermopsin. Difficulties observed in the HPLC analysis of CYN containing environmental samples has led the study to investigate some alternatives. Other contaminants present in the environmental samples made the analysis difficult. General presence of CYN in the dissolved fraction demands effective methods of extraction and concentration. In order to find the correct quantification of toxin in field samples or for the purpose of recovery from the spent medium, both methods need to be optimised. Some improvement can be achieved by developing an optimised solid phase extraction method for the desired toxin. Very poor retention often obtained in RP-HPLC (Guo *et al.,* 2005) has turned the attention to investigate alternatives.

The HILIC method development was investigated by testing a known method that has already been used for the detection of cyanotoxins. There has been very few studies carried out on CYN detection by HILIC method. The first attempt was based on the study using TSK gel amide -80 column on the analysis of CYN toxin by HILIC-MS (Dell' Aversano, 2004) but performing the same method showed no peaks with Atlantis HILIC column.

The Second method development was based on a study with Waters Atlantis HILIC Silica Column (100 mm x 2.1 mm, 5 µm) for streptomycin analysis. Using the aforementioned method (section.2.2.9) produced

chromatograms that indicated the HILIC analysis of crude CYN and purified CYN. Both samples tested did not show very good peaks with some tailing (Fig.2.9 A&B). The buffer loading used in this method could have affected and resulted in bad peak shape. The highest concentration of the buffer normally used in Atlantis HILIC is 0.2 % (McCalley, 2007). Buffer loading affects the peak shape in HILIC but the previous studies used a range 0.1%- 0.2 % (Dorr *et al*., 2010). However, it was also noticed that the retention time was high as compared to RP-HPLC.

The current study produced a method development for CYN analysis by HILIC. Study revealed that it is a useful method in application with polar, hydrophilic and ionic analytes (eg. CYN) as classical RPLC analysis may require derivatisation, ion pairing reagents, post column addition of organic solvent for optimal ionisation. Very poor retention time observed in RP-HPLC has been improved by this method. Generally the retention time of CYN in the RP-HPLC column stays between 7-8 min. On the other hand, it stays between 9-10 min. However, the peak shape observed by HILIC analysis (Fig.2.10) did not show satisfactory results as compared to the RP-HPLC (Fig.2.11). Previous studies carried out on cyanotoxin analysis has utilised the involvement of MS based detection since sensitive and specific multi analyte runs often involve this technique. Thus, MS assisted HILIC could be a better analysis option by this method.

The other method development investigated in this chapter was solid phase extraction. Due to the difficulties suffered in the analysis of toxin containing environmental samples, an improvement can be achieved by SPE. Use of ENV+ cartridges in the pre-concentration step of CYN recovery process

not previously reported. There are studies reported with different types of cartridges on CYN extraction and concentration such as C_{18}, anion exchange cartridges, Stacked SPE cartridges with C_{18}-packed material , Hypersep C_{18} SPE cartridge (Norris *et al*., 2001; Metcalf *et al*., 2002; Kikuchi *et al*., 2007). Initial experiment was started with ENV+ (500 mg) cartridges. Conditioning of the cartridges was the first step. Methanol selected as the conditioning solvent and for the elution of CYN. Second conditioning step is required now that the particles have been hydrated. Particles can only react with the sample when the methanol environment gets displaced. In this study water was used to wash off the remaining methanol inside the cartridges. Norris *et al*., 2001 also suggested methanol as the solvent selected for the CYN elution and highlighted the significance of acidification of the solvent although this was not investigated here.

The current study with 500 mg cartridges showed elution volume of methanol was 8 ml (Fig. 2 12). Similarly the work from Norris *et al.,* 2001 and Metcalf *et al*., 2002, reports total recovery was obtained in about 8 ml. According to diverse manufactures, these cartridges allow loadings of more than 1 g analyte and are recommended for large sample volume (100 ml – 1 L). These cartridges can be more suitable for natural samples because of low concentration of the toxin in the field sample requiring high volume to pass through the SPE cartridges. Reducing the amount of packing material from 500 mg to 100 mg allowed elution to be achieved in 1.5 ml which is much more practical as the sample requires drying to remove methanol since methanol dramatically affects the chromatography.

Methanol wash before the elution of analyte was investigated to see whether it affects the analysis or recovery in HPLC. Two water samples tested with methanol wash and no methanol wash resulted in the high recovery of CYN without methanol wash. Methanol wash was employed to remove the interference while not decreasing the recovery of the analyte of interest at the same time. The study suggests that methanol wash did significantly decrease the recovery of analyte hence this was not adopted.

Investigation of the ENV+ cartridge proved that no breakthrough of CYN could be detected until 50 µg were successfully retained from a spiked Milli-Q sample. This is far more than is needed for investigation of environmental samples but could become relevant in the utilisation of culture medium of *C. raciborskii* since it was suggested (Norris *et al*., 2001) that far more toxin is available in solution in the culture media than in the cells collected for extraction.

The elution solvent should be chosen such that the analyte is soluble. It should also be able to overcome primary retention mechanisms. Hence a mixture of solvents offering multiple interactions is usually most effective. For a better final analysis, elution solvent should also be compatible. Generally water miscible elution solvent such as methanol is used to elute analytes and minimise cartridge drying times. Results showed that 100% of methanol was the best eluent, however, at higher concentration above 80% can be used as a better elution solvent. It was reported that, at high solvent concentration precipitation of cylindrospermopsin was not observed (Metcalf *et al.,* 2002).

The investigation to optimise a pre-concentration step has produced an efficient result. This can be used in the environmental samples with low elution

volume. The analysis was found to be effective prior to the HPLC analysis. As a summary, an optimised SPE method is represented below on the Table 2.5.

Table.2.5. Optimised SPE method for the environmental samples prior to HPLC analysis

Sorbent	**100 mg ENV+**
Conditioning	MeOH + H_2O
Elution	1.5 ml MeOH
Processing	Dry and then resuspend in Milli-Q
Analysis	HPLC (C_{18} method)

The optimized method was found to be a successful pre-concentration step prior to HPLC analysis. Matrix interference observed while analysing environmental samples could be removed by this method. It was observed that that conditioning of the cartridges could be carried out best with a combination of MeOH and water. Elution volume has been significantly reduced to 1.5 ml, which is promising with 100 mg ENV+. Processing was carried out by centrifuging the samples before it is taken for drying. The samples were then resuspended in Milli-Q before the HPLC analysis. HPLC Analysis was carried out using C_{18} method.

2.5. Conclusion

This chapter mainly discussed the extraction, concentration, purification and analysis of CYN. The HILIC method has been shown to be sensitive, straightforward and readily automated. It has solved the problem over RP-HPLC holding low retention capacity. Another advantage is that no sample clean up or pre-concentration step was necessary here. However, the current study found that chromatography is better when analysed in RP-HPLC. Generally in HILIC columns prefer organic as diluents where CYN is hydrophilic in nature. The solvent cost is expensive when compared with RP-HPLC. Future study will be using RP-HPLC for the analysis of the desired toxin. As a pre concentration step prior to HPLC analysis, SPE proved an efficient method to concentrate the toxin from the environmental samples. Use of methanol at higher concentration highly recommended as it allows the increased bed weight while guaranteeing optimum recovery in small elution volume. This method can be successfully applied in future works including biodegradation and photocatalysis experiments where the pre concentration of the sample is mandatory before the analysis.

CHAPTER. 3

Novel approach of CYN removal by bacterial degradation

TABLE OF CONTENTS

3.1. Introduction

The occurrence of cyanotoxin cylindrospermopsin (CYN) has been attributed to the water bodies with blooms of cyanobacterial species *Cylindrospermopsis raciborskii* (majority), *Aphanizomenon ovalisporum* and *Umezakia natans*. This species occurs worldwide in freshwater ponds, rivers, reservoirs and eutrophic lakes. In comparison to microcystin producing cyanobacteria, CYN-producing cyanobacteria occur commonly in tropical and sub-tropical regions (Duy *et al.*, 2000; Hawkins *et al.,* 1997; Carmichael and Falconer, 1993). Apart from its occurrence in the tropics, *C. raciborskii* is also now being found in water bodies of warmer temperate regions (e.g. in Australia; Baker and Humpage, 1994; Thomas *et al.,* 1998); in warmer summer months of temperate region (e.g. Florida, USA; Williams *et al.,* 2000; Burns 2000; Chapman and Schelske, 1997) and temperate climates (eg. New Zealand, Stirling and Quilliam, 2001). Unlike *Microcystis* and *Anabaena*, blooms of *C. raciborskii* do not form scum on the water surface, instead they get concentrated several meters below the surface (Falconer, 1997). This poses a serious threat, as water for drinking and other purposes is usually drawn several meters below the water surface.

Various treatment measures have been employed to remove cyanotoxins, most common and recent being coagulation followed by flocculation, rapid sand filtration and chlorination (Donati *et al.,* 1994; Falconer, 1998) or use of algicides chemicals like copper sulfate and simazine (Sukenik *et al*., 1998). Using such treatment methods have been found to be ineffective and inefficient for removing cyanobacterial toxins from water (Duy *et al*., 2000). The most effective way of reducing the risk of toxicity would be therefore, either

elimination of cyanobacterial cells or adsorption and destruction of toxins (ILS report, 2000). Methods for elimination of cyanobacterial cells from water bodies employ treatment of raw water with flocculants like lime or calcium hydroxide (Rositano and Nicholson, 1994; Falconer, 1998) or through membrane filtration. Currently, research is being carried out to determine the amount of flocculants required and type of membrane filters. Aforementioned methods may be efficient in removing cyanobacterial cells but not effective against water soluble toxins especially CYN (Hitzfeld *et al.,* 2000). Treatment procedure involving prechlorination flocculation with alum-sedimentation sand filtration chloramination reduced CYN concentration below the detection limit of HPLC-MS in drinking water (Duy *et al*., 2000). Other methods of oxidation and inactivation of toxins using excess chlorine, potassium permanganate and ozone are presently being tested (Senogles *et al*., 2000; Drikas *et al.*, 1994; Falconer 1998).

Biological processes like denitrification have been used for centuries for the treatment of drinking water and are considered integral processes for the treatment of waste water. Advantages of these low technology processes includes little or no maintenance or running costs, and being a natural treatment e.g. without using any chemicals and producing no harmful by-products.

The most commonly reported of the cyanotoxins is microcystins and they are predominantly produced by *Microcystis*. spp. Although other cyanobacteria including *Anabaena, Nostoc, Hapalosiphon, Anabaenopsis and planktothrix (Oscillatoria)* have been known to produce these toxins (Sivonen and Jones, 1999; Falconer, 2005). Microcystins have been widely used in biodegradation studies. A growing number of isolated organisms are reported to have the ability

to degrade microcystin in water; most of them belong to the family Sphingomonadaceae (Maruyama *et al.,* 2003, 2006; Harada *et al.*, 2004; Bourne *et al.*, 2005).

Other research has identified three isolates, *Arthrobacter* sp*.* *Brevibacterium* sp*.* and *Rhodococcus* sp; as having the capability to degrade microcystin (Manage *et al.,* 2009). While it is clear that microcystin can be degraded by organisms via pathways originally proposed by Bourne *et al.*, (1996, 2001) studies have indicated that there may be some other pathways also involved in the degradation process as the authors were unable to find the appropriate gene (*mlr*) in their isolates (Manage *et al.,* 2009). The biodegradation studies of microcystin have identified that no toxic by-products are produced during the process.

Nodularin, a cyclic pentapeptide hepatotoxin closely resembles microcystin in its structure. A unique feature of NOD is that it can be biodegraded by bacteria and most of them also have the ability to degrade microcystins (Manage *et al.,* 2009). It is believed that this may be due to the enzymes acting similarly for both cyanotoxins by cleaving their cyclic structure at the Adda- Arg peptide bond (Kato *et al.*, 2007, Edwards, 2008).

The aim of the current investigation is to elucidate the biodegradation of cylindrospermopsin. To date, few studies have been conducted on CYN biodegradation. Peer-reviewed literature studies have demonstrated that CYN could be biodegraded in natural water bodies (Senogles *et al.,* 2002). In addition, it was also found that the concentration of CYN influenced biodegradation with a near linear relationship existing between the biodegradation rate and the initial CYN concentration. It was found that at higher

concentration the degradation was rapid while lack of complete degradation was found at the lower CYN levels. It implies an existence of a threshold CYN concentration which is required to induce the CYN degradation (Smith *et al.,* 2008) Biodegradation of CYN can also be affected by temperatures and the presence of copper based algicides. Furthermore, biodegradation of CYN in sediments has been observed, where the pre-conditioning of the sediments resulted in enhanced biodegradation of CYN (Klitzke *et al.,* 2010). No studies to date have isolated any definitive CYN degrading organisms.

Factors affecting the biodegradation of cyanobacterial metabolites have also been well studied. The biodegradation of CYN occurs only in water supplies which had a history of toxic C. *raciborskii* blooms (Smith *et al.,* 2008), although this is in contrast to the study conducted by Wormer, where 40 days of CYN degradation did not observe any degradation of the particular toxin, despite the water body having been exposed to CYN producing cyanobacteria (Wormer *et al.,* 2008). This is an indication that there are other factors which influence biodegradation of cyanobacterial metabolites particularly CYN, including the dependency of the types of degrading microorganisms present and other environmental conditions.

From the limited data available on the effect of temperature on the biodegradation of CYN, the maximum degradation rate appears to occur at approximately 25 ºC, with a much slower rate at 20 ºC (Smith *et al.,* 2008). The presence of aquatic dissolved organic matter yielded higher CYN degradation rates than dissolved organic matter released from lysed cyanobacterial cells (Klitzke *et al.,* 2010). This suggests that the character of organic matter, substrate specificity may influence biodegradation. The presence of other water

constituents such as cadmium, nickel, and zinc have been shown to inhibit the biodegradation of aromatic hydrocarbon (Amor *et al.,* 2001). In this case metals can inhibit the degrading organisms by blocking critical functional groups or interfering with the incorporation of essential metal ions with biological molecules.

Aforementioned studies identified that secondary metabolites and toxins produced by cyanobacteria were proven to be biodegradable. Microcystin and Nodularin, toxins produced by cyanobacteria can be degraded using different strains of bacterial isolates. The interest of this study was to investigate the possibility of biodegradation of CYN, a relatively less studied cyanotoxin using any bacterial isolates.

The present study was undertaken to investigate the biodegradation of CYN using bacterial isolates. Towards this, a series of cost effective and rapid screening methods were performed to identify bacterial communities that have the capability to metabolise CYN. A redox based method to assess utilisation efficiency of carbon substrates by microorganisms (namely, BIOLOG; Garland and Mills, 1991; Stefanowicz, 2009) was employed as an assay to determine the degree of toxin removed from water samples. It is performed on microplates containing colourless dye, tetrazolium which is sensitive to bacterial respiration. When microbes consume the available substrate by an oxidation reaction, this simultaneously reduces the tetrazolium dye to a violet-coloured substrate called formazan, which can be quantified spectrophotometrically (Chang-Ping Yu and Yue–Hwa Yu, 2000). The intensity of coloured formazan is proportional to the rate of utilisation of carbon substrate in the medium. Thus, a pattern of colour development in microplates can be characterised to microbial community known

as their "metabolic fingerprint" or "community-level physiological profile (CLPP)" (Baudoin *et al.*, 2001; Gomez *et al.*, 2004; Grayston *et al*., 2005). Following promising results with isolates further studies were carried out to assess the degradation of CYN by isolates and natural water samples.

3.2. Materials and Method

3.2.1. Chemicals and reagents

Chemicals were of analytical-reagent grade unless stated and obtained from Fisher Scientific, Leicestershire, UK. HPLC-grade methanol and acetonitrile were purchased from Rathburn, Walkerburn, UK. Milli-Q water was obtained from the laboratory Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK). Cylindrospermopsin were dissolved in Milli-Q and sterilised in a passage through 0.22 µm filters (Dynaguard filter, Fisher, UK).

3.2.2. BIOLOG MT2 assay to assess the utilisation of CYN by identified bacterial isolates.

Microbial degradation of cylindrospermopsin was investigated as an alternative/complimentary method for the removal of the potent toxin from the drinking water bodies. Previous studies performed on ten bacterial isolates (Table II.3) from three Scottish water resources on the degradation of microcystins have been successfully demonstrated (Manage *et al.*, 2009). In the present study a Biolog MT2 assay was performed to evaluate whether these bacterial isolates could metabolise CYN with its potential use to test multiple carbon sources against any set of bacteria of interest (Garland *et al.,* 1991).

Table.3.1. Identification and origin of bacterial isolates screened for MC-LR metabolism and degradation (Manage *et al.,* 2009; Lawton *et al*., 2011).

Bacterial isolates	Identification	Origin of Isolation
C1	*Rhodococcus* sp.	Carron river (NO 877 857)
C3	*Rhodococcus* sp.	
C6	*Arthrobacter* spp.	
F3	*Brevibacterium* sp.	Loch Forfar (NO 450 507)
F7	*Arthrobacter* sp.	
F10	*Arthrobacter* sp.	
R1	*Arthrobacter* sp.	Loch Rescobie (NO 525 515)
R4	*Arthrobacter* sp.	
R6	*Arthrobacter* sp.	
R9	*Arthrobacter* sp.	

Identified isolates were transferred into 8 ml liquid nutrient broth and incubated for 24 hrs at 25 ºC (Stuart scientific –Orbital Incubator SI 50). The exponentially growing bacterial cultures were washed three times with 0.01 M Phosphate buffered saline (PBS) followed by centrifugation at 3000 x g for 15 min with re-suspension of the pellet in sterile 0.01 M PBS and cultures were incubated at 25 ºC for 24 hrs to deplete the remaining residual carbon. This

allowed the addition of a single carbon and energy source, in this case CYN. Resulting respiration caused a colour change which could be measured as an indication of CYN utilisation by any chosen bacterium. Selected samples were analysed by HILIC to confirm degradation. CYN was added to the Biolog MT2 plates (Techno path, Limerick, Ireland) in triplicate at final concentration of 0.1, 1.0. 10 µg/ml. Optical density of all the bacterial suspensions in PBS were adjusted to 0.35 by absorbance at 590 nm using spectrophotometer (Pharmacia biotech Nova Spec II) by adding overnight grown culture or by diluting with sterile 0.01 M PBS in triplicate. Colour development in the plate was measured using a Dynex microplate reader (Jencons, Leighton Buzzard, UK) at 595 nm immediately after the incubation of bacteria (0 h) followed by incubations equal to 3, 6, 12, 18, 24 hours. The degradation of the analyte (CYN) was measured indirectly through the development of colour resulting from metabolism of the CYN.

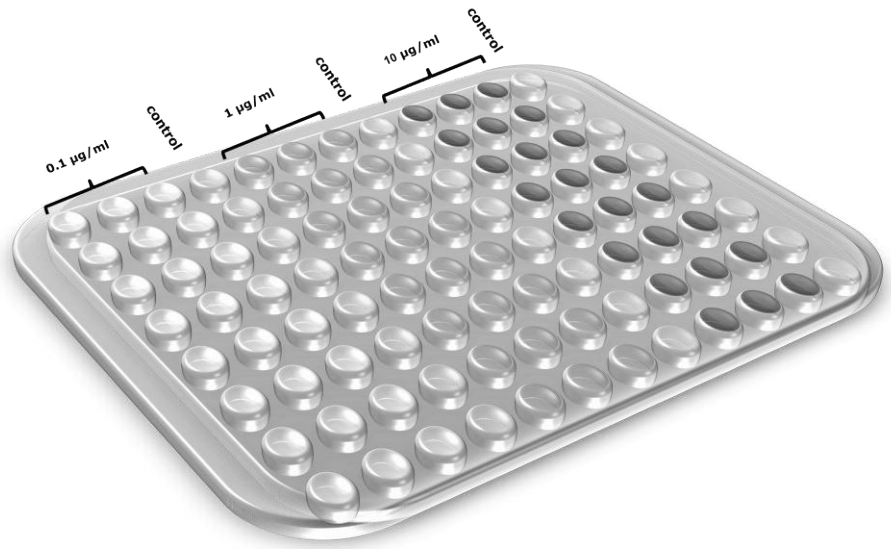


Fig.3.1. Biolog MT2 assay plate model used to assess the utilisation of cylindrospermopsin by identified bacterial isolates. Three different concentration ranges of CYN applied in triplicates and control.

3.2.3. Evaluation of CYN degradation by isolated bacteria in batch experiments.

Biolog MT2 assay revealed the ability of the bacterial isolates to metabolise CYN. To evaluate the metabolism of CYN, six isolates out of ten were selected showing comparatively high metabolic response with CYN representing three different genera and water sources. The selected six bacterial isolates were *Rhodococcus* sp. (C6), *Arthrobacter* spp. (F7 & R1) and *Brevibacterium* spp. (F3). All strains had previously been reported to degrade another group of cyanotoxins, microcystin (Manage *et al*., 2009) bacterial strains of *Paucibacter toxinivorans* DSMZ-16998 (Braunschweig, Germany) and *sphingomonas* have also been evaluated. In order to confirm the degradation of CYN in the water, it was important to examine whether the microbes could degrade the cyanotoxin. In this experiment, bacterial inoculums (P. *toxinivorans*, *Sphingomonas*, R1, C6, F3, and F7) were transferred to an 8 ml nutrient broth and incubated in a shaker at 25 ºC for 24 hr at 150 rpm (Stuart scientific-orbital incubator SI 50). Bacterial cultures at exponential phase were washed three times with 0.01 M Phosphate buffered saline (PBS) followed by centrifugation at 3000 x g for 15 min. Each pellet was resuspended in sterile 0.01 PBS and cultures were incubated for another 24 hr under the same conditions mentioned before, in order to deplete any residual carbon. Turbidity of bacterial suspensions was adjusted to A_{590} = 0.35 and 50 µl added into bijou bottles in triplicates containing 900 µl of 0.2 µm filter sterilised water from their original location. Sterile CYN toxins 50 µl were added to each bijou bottles under aseptic conditions with a final concentration of 10 µg/ml. Three bacterial isolates were inoculated in Loch Rescobie water (P. *toxinivorans*, *Sphingomonas* and R1). Other isolates were inoculated in Loch

Forfar water (F3, F7) and in River Carron water (C6). The sample containing bijou bottles were incubated for 7 days at 25 ºC with 150 rpm. At 24 hr intervals, 0.5 ml aliquots were transferred to microcentrifuge tubes (1.5 ml) under aseptic condition, frozen at –20 ºC immediately and then freeze dried. Samples for the HILIC analysis were prepared from freeze dried samples. In brief, each sample was resuspended in 200 µl of 100% methanol and centrifuged at 15,000 x g for 10 minutes and the supernatants (100 µl) were taken for analysis.

3.2.4. Effect of bacterial cell numbers on the degradation of cylindrospermopsin by using *Rhodococcus* sp. (C1).

Bacterial inoculums (*Rhodococcus* sp., C1) were transferred in tubes containing 8 ml nutrient broth and incubated on a shaker at 25 ºC for 24 hr at 150 rpm (Stuart scientific orbital incubator SI 50). Exponentially growing bacterial cultures were washed three times with 0.01 M Phosphate buffered saline (PBS) followed by centrifugation at 3000 x g for 15 min. Pellets were re-suspended in sterile 0.01 M PBS and cultures were incubated for another 24 hr under the conditions mentioned above, in order to deplete remaining residual carbon. Turbidity of the bacterial suspensions were adjusted to A_{590} = 2.0, 1.0, 0.5, 0.1, 0.05 and added (50 µl) to bijou bottles in triplicate containing 900 µl of 0.2 µm filter sterilised water from their original location (Carron water). Sterile CYN toxin (50 µl) was added to each bijou bottle under aseptic conditions with a final concentration of 10 µg/ml. The bijou bottles were incubated for seven days at 25 ºC at 150 rpm. At 24 hr intervals, aliquots of 0.5 ml of samples were transferred to microcentrifuge tubes (1.5 ml) under aseptic conditions, frozen at

–20 ºC immediately and freeze dried. Samples for the HILIC analysis were prepared from freeze dried samples, resuspended in 200 µl of 100% methanol and centrifuged at 15,000 x g for ten minutes and the supernatant (100 µl) was taken for HILIC analysis.

3.2.5. Evaluation of CYN degradation in natural waters by using River Carron and Cowie Loch.

An investigation was carried out to assess the CYN degradation using natural waters collected from River Carron and Cowie Loch. Sterile controls (50 ml) were autoclaved in 100 ml Erlenmeyer flask at 121 ºC for 15 min. Triplicate samples were prepared for all samples and controls. Samples were incubated at constant temperature (25 ºC) and shaken at 100 rpm. Aliquots were removed from each flask under sterile conditions in alternate days for four weeks and frozen immediately at –20 ºC. The frozen samples were freeze dried and reconstituted with 200 µl of Milli-Q water. The samples with controls were transferred into microcentrifuge tubes and centrifuged at 15,000 x g for 10 min and the supernatant (100 µl) was taken for HPLC analysis.

3.2.6. Evaluation of CYN degradation in five natural water bodies, assessing the influence of nutrients and bacterial population density in the degradation process.

To determine if bacterial density influenced CYN degradation an investigation was performed using natural water collected from five Scottish locations, namely Loch Rescobie, Balgavies Loch, Loch Leven, River Carron, and Forfar Loch. Following collection, water was immediately filtered to concentrate the bacterial population. To achieve this, each natural water sample collected in sterile Duran bottles (1 L) was passed through 0.22 µm sterile filter apparatus, filter was then removed and bacteria resuspended in a final volume of 10 ml source water. The other set of samples were prepared by using water directly collected from the natural sources. The experiments were conducted in 7 ml Bijou bottles and samples taken at 0, 15 and 30 days. Aliquot of 100 µl of sterile CYN was added to 900 µl of water sample in Bijou bottles. Nutrient broth (10 µl) was added to each Bijou bottles in order to introduce an extra nutrient source hence boosting bacterial numbers through growth. The samples were frozen at −20 ºC, freeze dried, and resuspended in 200 µl of Milli-Q water. All samples including controls were transferred to microcentrifuge tubes and centrifuged at 15,000 x g for 10 min. The supernatant was removed and analysed by HPLC.

The plate count method was performed to determine the bacterial numbers in five natural water samples. The experimental procedure involves concentrating bacterial isolates (x100) by filtration and subsequent counting of Colony Forming Units (CFUs) on Nutrient Agar Plates. The samples containing bacteria in suspension underwent a ten fold serial dilution e.g. 1:10, 1:100, 1:1,000, 1:10,000 and 1:1, 00,000 and performed in sterile PBS. After addition

of 10 µl bacterial inoculum to plate, 20 to 30 ml of agar medium is poured into each plate. The plate is gently rotated for thorough distribution of inoculum throughout the medium and kept for setting. Plates were then placed in an incubator at 25 ºC for 24 hr. Plate count was carried out by using the formula, with the number of colonies counted on plate x dilution of samples= number of bacteria per ml.

3.2.7. HPLC analysis of Biolog samples performed by using purified and semi purified CYN.

In order to elucidate the mechanism related to CYN utilisation of the isolated bacteria in Biolog plates, further studies were carried out with purified and semi purified CYN. Two bacterial isolates were assessed, *Paucibacter toxinivorans* and *Rhodococcus* sp. C1. The experiment was performed as mentioned in the section 3.2.2. In addition, samples were analysed by HPLC method.

3.2.8. Evaluation of CYN degradation in two natural waters with extended incubation.

Previous results showed that degradation of CYN in both River Carron and Loch Forfar. In order to confirm the degradation, an experiment was carried out in larger scale using sparged flasks for a longer incubation period. Negative control samples were prepared in 2 L sparge flasks by autoclaving 1 L of both River Carron and Loch Forfar at 121 ºC for 15 min. Samples were in triplicate and the toxin CYN added. All samples were prepared in triplicate in 2 L conical flasks containing 1 L of the appropriate water with 10 µg/L CYN. A parallel set of

identical flasks were prepared with the addition of nutrient broth to stimulate the bacterial growth. All flasks were gently sparged with sterile air and samples removed at 0, 15, 30, 45 and 60 days for toxin analysis. Sampling was achieved using a 50 ml disposable plastic syringe. Prior to SPE processing water was filtered through a GF/C filter. SPE was described as in section 2.2.20 followed by LC-MS analysis.

3.3. Results

3.3.1. Evaluation of cylindrospermopsin utilisation by bacterial isolates using BIOLOG MT2 assy.

The current study was to evaluate the utilisation of CYN in the Biolog plate assay using eleven bacterial isolates which showed degradation of microcystin in previous studies. This study was carried out using Biolog MT2 assay, as it had previously proved to be a rapid screening method to assess the bacterial metabolism (Manage *et al.*, 2009). Initial studies revealed that most of the bacterial isolates demonstrated the capacity to metabolise CYN as it was the only carbon energy source available in the Biolog plates. Three different concentrations of CYN were evaluated. It was noted that maximum metabolism for all isolates was observed between 6 to 12 h, with extended incubation times giving little change in the readings. For all isolates there was greater metabolism in the CYN treated bacterial isolates compared to the control although in a number of isolates this was only a modest increase in respiration Fig (3.2).

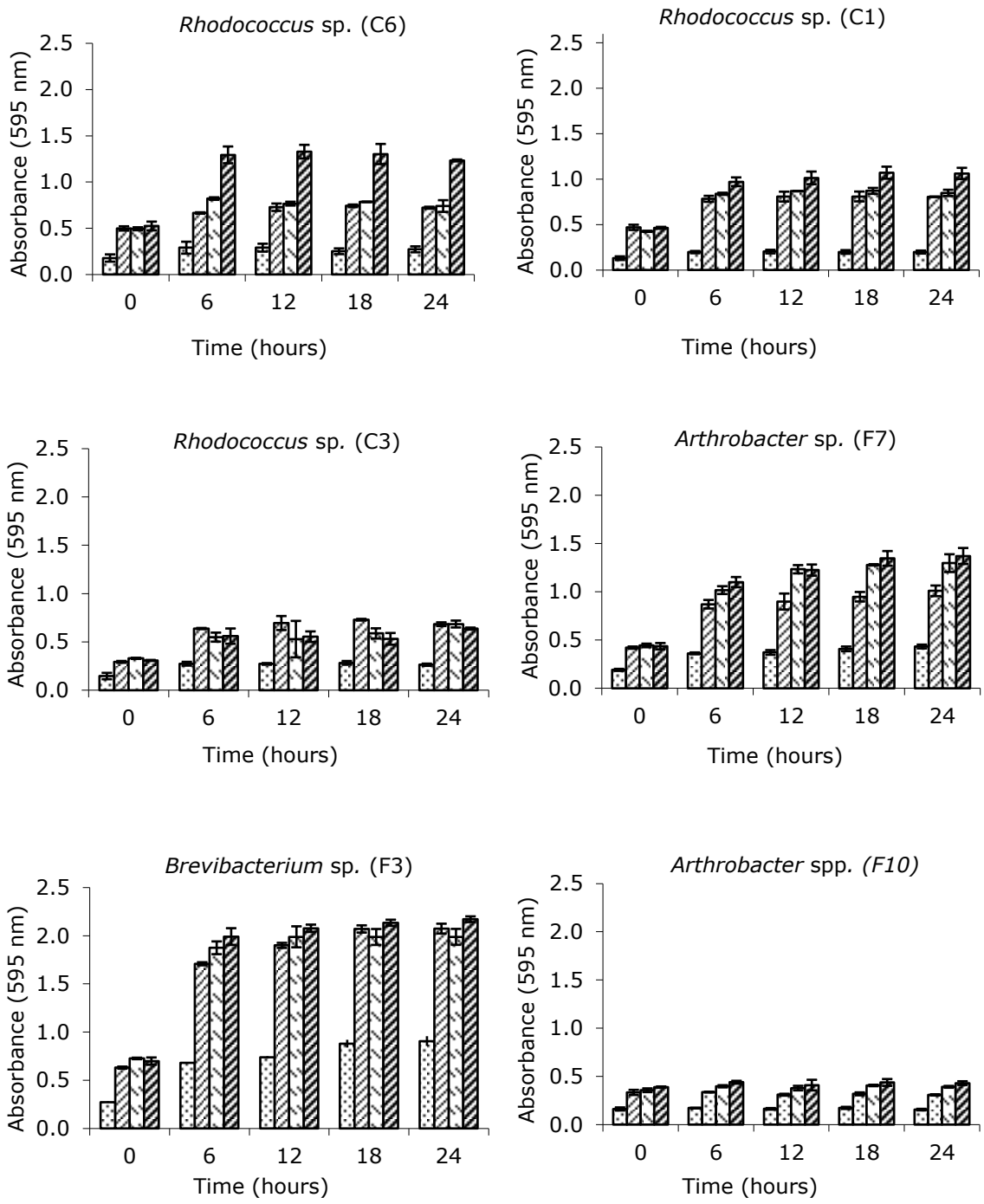


Figure.3.2. Biolog MT2 assay of CYN utilisation by the isolated bacteria (Carron River and Forfar Loch) during 48 h. Control (⬚) contains no added carbon. CYN concentration in wells were 0.1 µg/ml (▨), 1 µg/ml (▧) and 10 µg/ml (▨). Error bars represent one standard deviation (n=3).

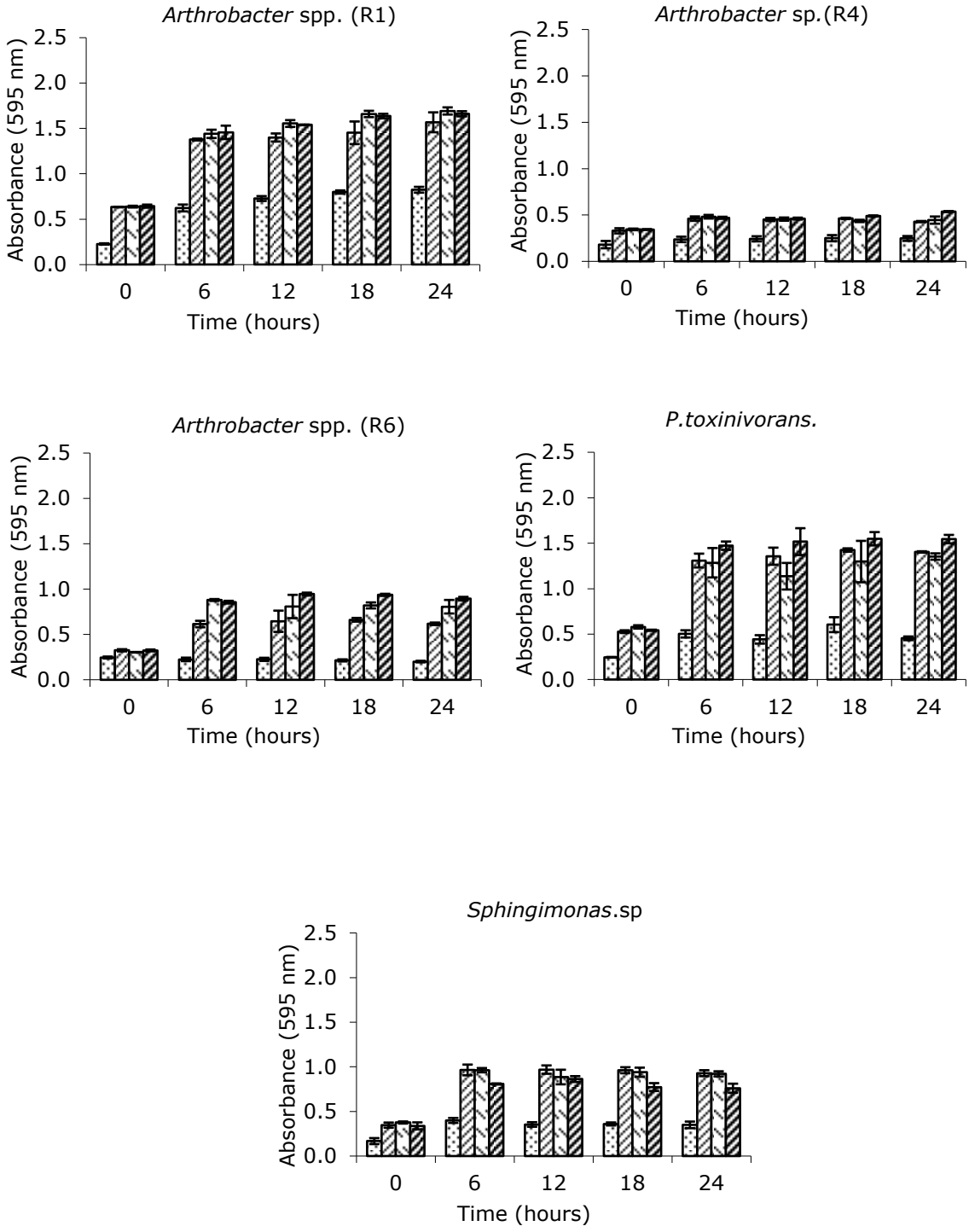


Figure.3.2. Biolog MT2 assay of CYN utilisation by the isolated bacteria (Rescobie Loch) during 48 h. Control (⬚) contains no added carbon. CYN concentration in wells were 0.1 µg/ml (▨), 1 µg/ml (▨) and 10 µg/ml (▨). Error bars represent one standard deviation (n=3).

Out of the eleven bacterial isolates assessed, six of them showed a more pronounced utilisation of CYN in Biolog plates. The isolates including *Arthrobacter.* sp (R4), *Arthrobacter.* spp (F10 and R6) and *Rhodococcus sp.* (C3) showed little variation in absorbance after a time period of 24 hr suggesting that these bacteria are not metabolising CYN. Six isolates in which showed highest absorption value were chosen for the shake flask culture experiments including *Rhodococcus sp.* (C6), *Brevibacterium.* sp (F3), *Arthrobacter.* sp (F7), *P. toxinivorans, Sphingomonas.* sp and *Arthrobacter.*spp (R1). Interestingly, the slow growing bacterial isolate *Brevibacterium sp.* (F3) showed the highest indication of metabolism. Similar levels of metabolisms were found in the isolates R1, F7 and *Paucibacter toxinivorans* DSMZ-16998 (Braunschweig, Germany). In the case of bacterial strain *Sphingomonas* sp there was no pronounced utilisation of CYN shown after 24 h. Although three different concentrations of CYN were tested for most of the isolates this had little effect on the metabolism. However, in *Rhodococcus.* sp (C1) a pronounced increase in metabolism appeared at the 10 µg/ml level, furthermore, *Arthrobacter* sp F7 demonstrated a small dose dependant response. The samples were analysed by HILIC method to confirm the toxin had been removed from the sample. The chromatogram did not identify any CYN peaks which could be attributed to the shake flask experiment.

3.3.2. Evaluation of CYN degradation by isolated bacteria in batch experiments.

Previous studies with Biolog MT2 plate assay demonstrated the utilisation of CYN by different bacterial isolates. The bacterial isolates which showed

highest absorbance variance, were further evaluated to determine CYN degradation in batch culture experiments.

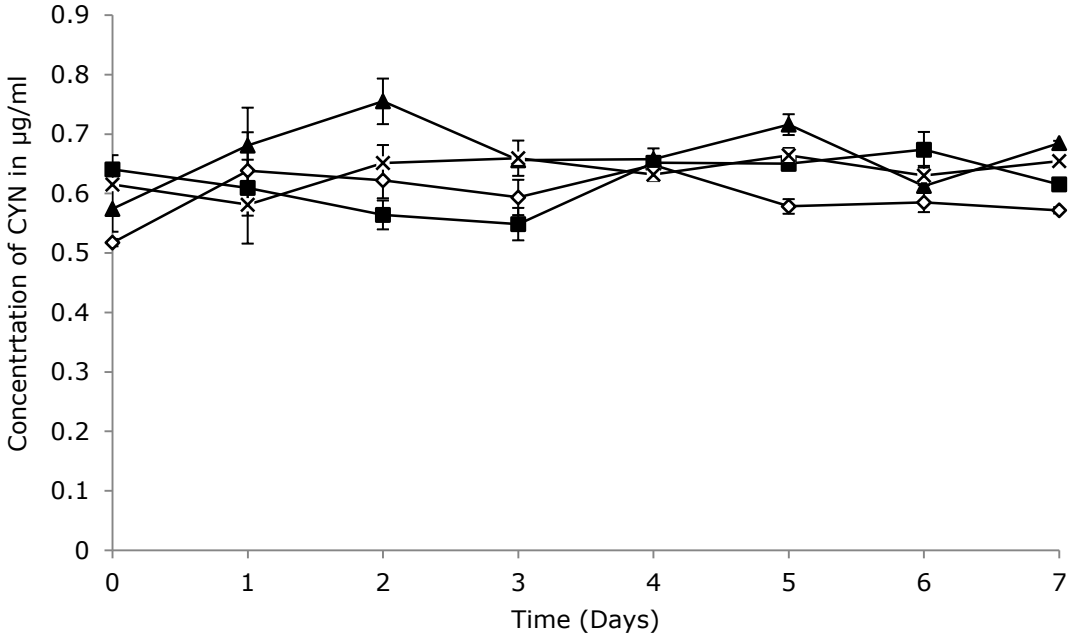


Fig. 3.3. Batch culture CYN degradation. Study using sterile Loch Rescobie water (◇) with *Paucibacter toxinivorans* (■) *Sphingomonas* (▲) and *Arthrobacter*. sp , R1 (×). All incubation at 25 °C at 100 rpm. Error bars represent one standard deviation (n=3)

Bacterial isolates were assessed by adding them into the water source from where they originated, such as *Arthrobacter.sp* (R1) from Loch Rescobie. Two other bacterial strains *Paucibacter toxinivorans* and *Sphingomonas* were assessed in Loch Rescobie water. No degradation of CYN was observed in these cultures with the sterile control also showing that CYN was stable under these conditions. Both *Brevibacterium.* sp (F3) and *Arthrobacter.* sp (F7) demonstrated no observed degradation during a period of seven days sampling.

A slight drop in CYN concentration was initially observed for the *Rhodococcus* sp. C6, However, the detected amount retained was too close to that of the control by the end of the incubation period (i.e. 7 days). The reason for this was not clear but could be due to some non-specific binding.

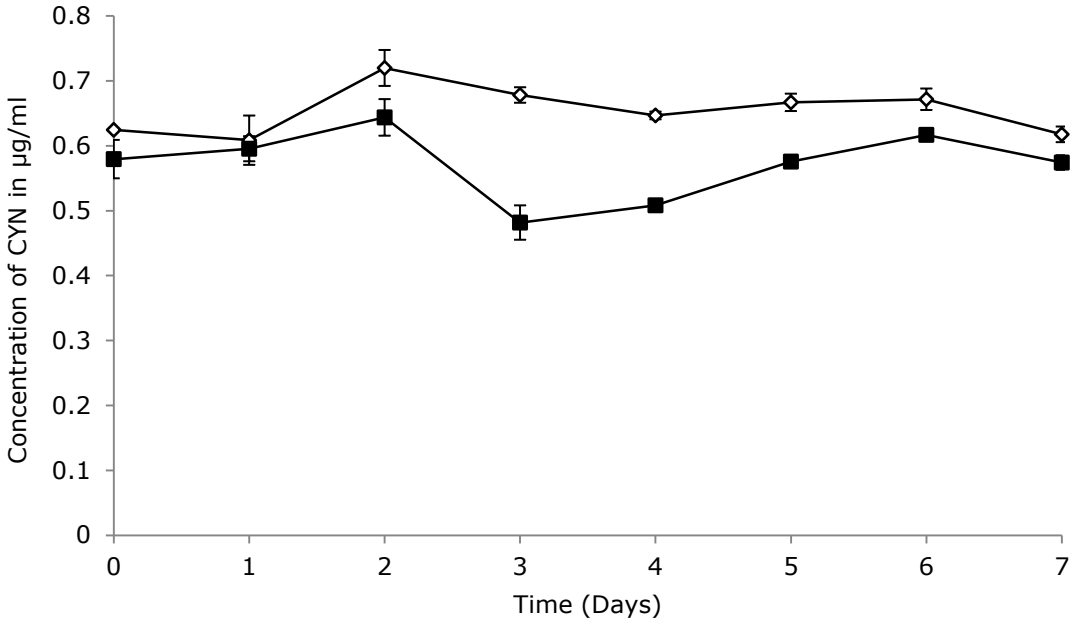


Fig.3.4. Batch culture CYN degradation. Study using sterile Carron water (◇) with a *Rhodococcus* sp. C6. (■). All incubation at 25 °C at 100 rpm. Error bars represent one standard deviation (n=3).

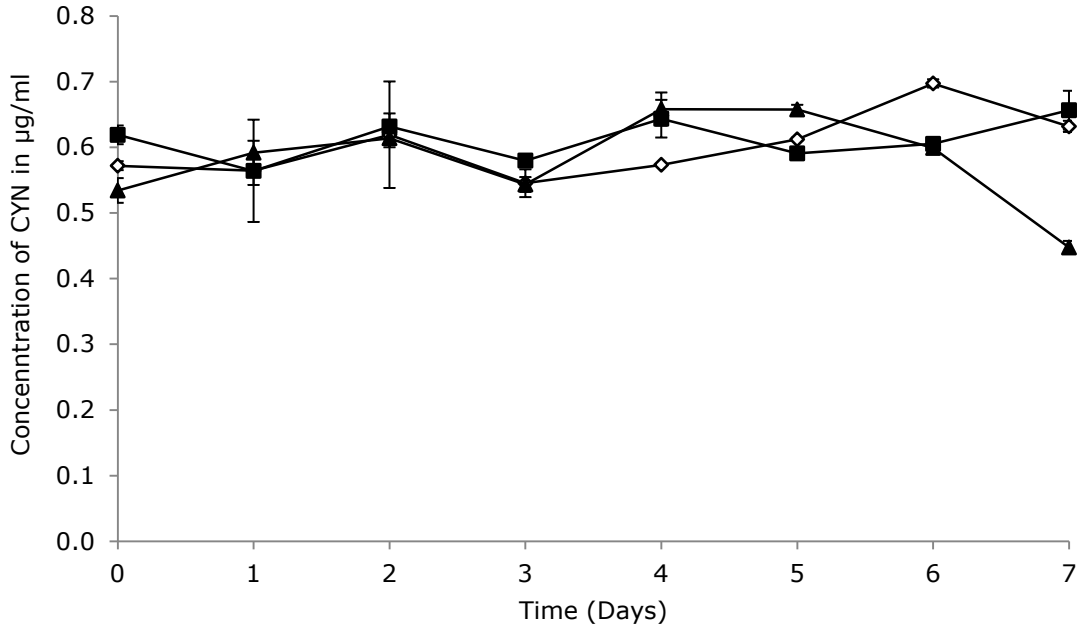


Fig.3.5. Batch culture CYN degradation. Study using sterile Forfar Loch water (◇) with *Brevibacterium.* sp F3, (▲) *Arthrobacter.* sp F7, (■). All incubation at 25 °C at 100 rpm. Error bars represent one standard deviation (n=3).

All isolates assessed failed to demonstrate evidence of biodegradation of CYN in the shake flask experiment despite there being total metabolism in the Biolog MT2 plate experiments. The reason for the difference could be that the number of bacterial cells present in the Biolog assay was significantly higher than in the shake flask experiments. Another reason could be the chemicals present in the Biolog MT2 assay.

3.3.3. Effect of bacterial cell numbers on the degradation of CYN by using *Rhodococcus* sp. C1.

Initial studies with Biolog assay demonstrated the utilisation of CYN, however, the shake flask culture experiment showed no utilisation. It was suggested that bacterial numbers may be the reason for this variation. In order

to examine the effect of bacterial cell numbers on the degradation of CYN, the bacterial isolate *Rhodococcus* sp. (C1) was used.

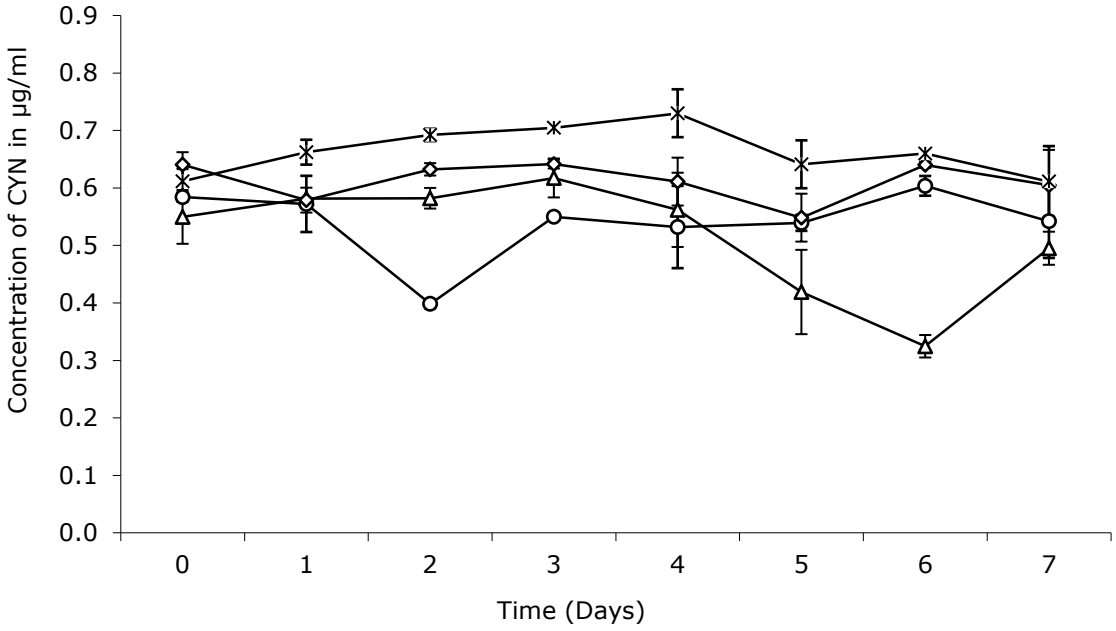


Fig.3.6. CYN degradation by *Rhodococcus.* sp. C1 with a range of optical density assessed O.D =2.0 (△), 1.0 (○), 0.5 (◇) and 0.1 (×). Error bars represent one standard deviation (n=3)

Bacterial cell density was monitored and adjusted spectrophotometrically and the turbidity was adjusted to A_{590} = 2.0, 1.0, 0.5, 0.1, 0.05. It was observed that there was no significant effect of bacterial cell numbers on the degradation of CYN; however, a slight decrease in toxin concentration was noticed with higher optical density performed. It began declining from day 3 to day 6 and showed a toxin concentration increase on day seven.

3.3.4. HPLC analysis of Biolog samples performed by using purified and semi purified CYN.

This experiment was carried out to cross check the results obtained in the Biolog with semi purified CYN. In order to do that, an experiment was carried out with both purified and semi purified CYN against two bacterial isolates. Results demonstrated that both semi purified and purified CYN with two bacterial isolates examined in Biolog assay identified no CYN. This was further analysed by HPLC and the chromatograms reveals no CYN peaks (Fig.3.7 (a-d))

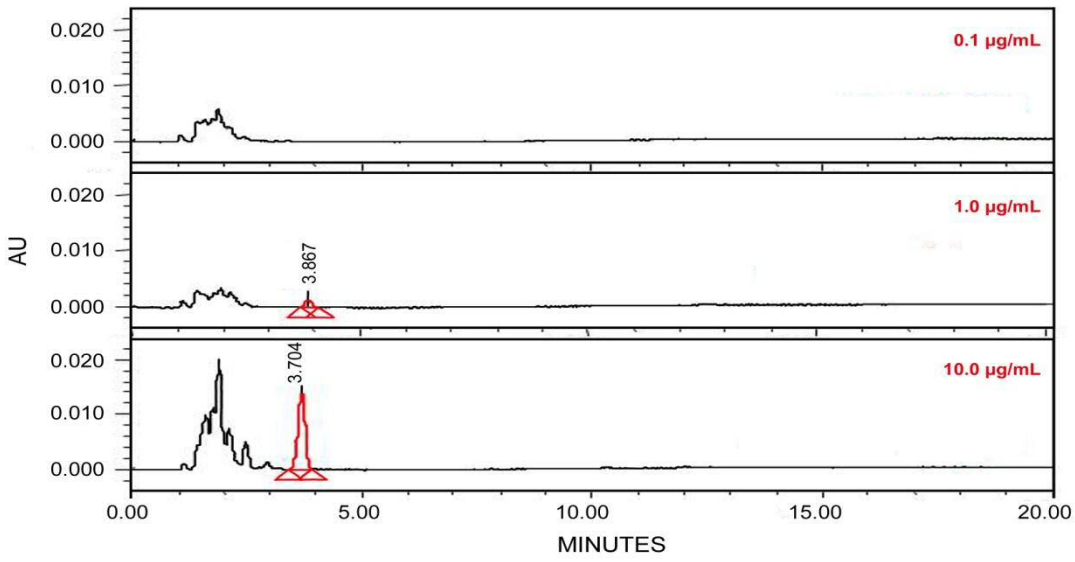


Fig.3.7.(a) HILIC analysis of Biolog samples. Chromatogram represents three concentration range of purified CYN with *Rhodococcus. sp* C1

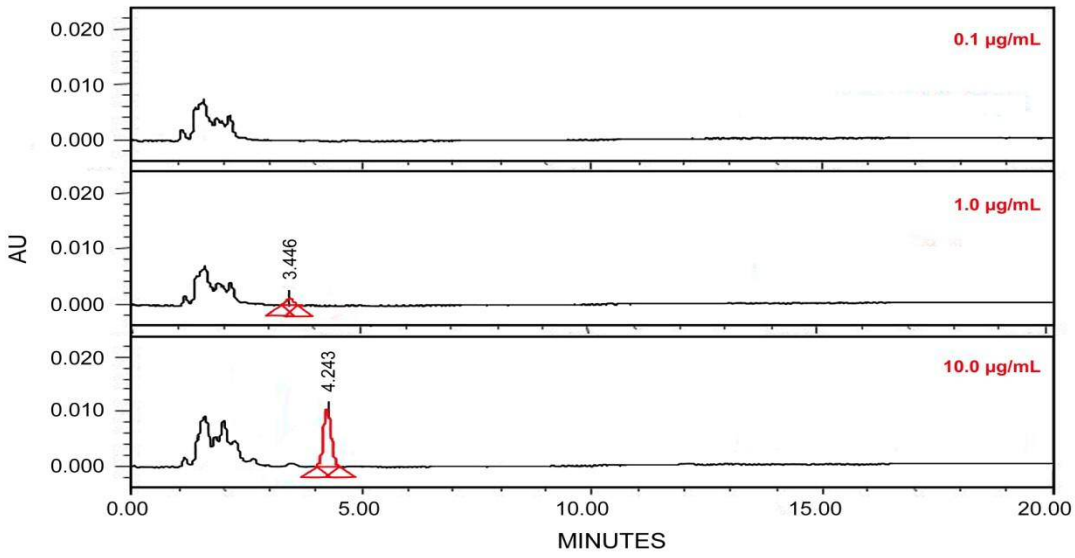


Fig.3.7.(b) HILIC analysis of Biolog samples. Chromatogram represents three concentration range of purified CYN with *P. toxinivorans* **.**

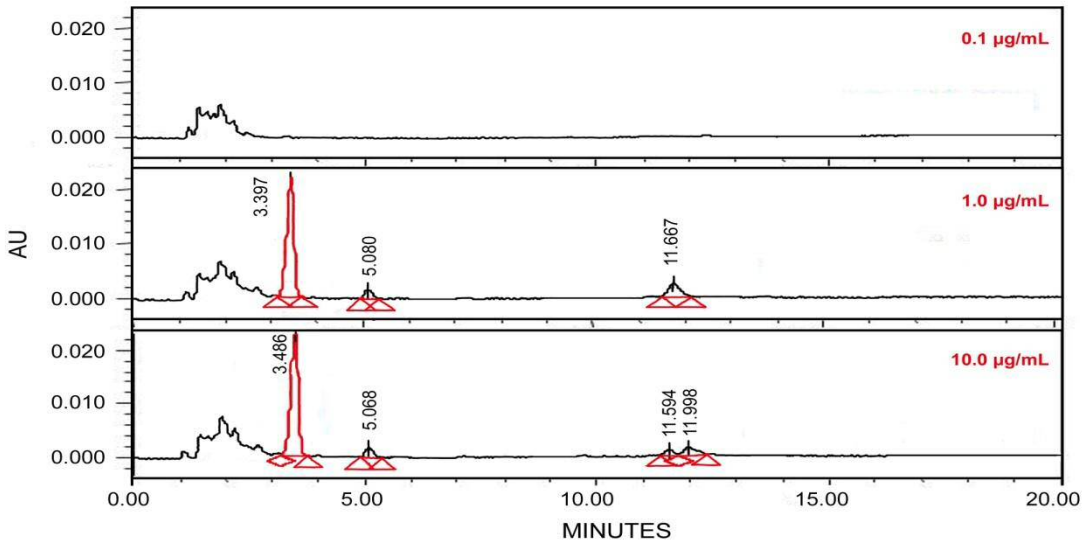


Fig.3.7.(c) HILIC analysis of Biolog samples. Chromatogram represents three concentration range of semi purified CYN with *Rhodococcus. sp* C1

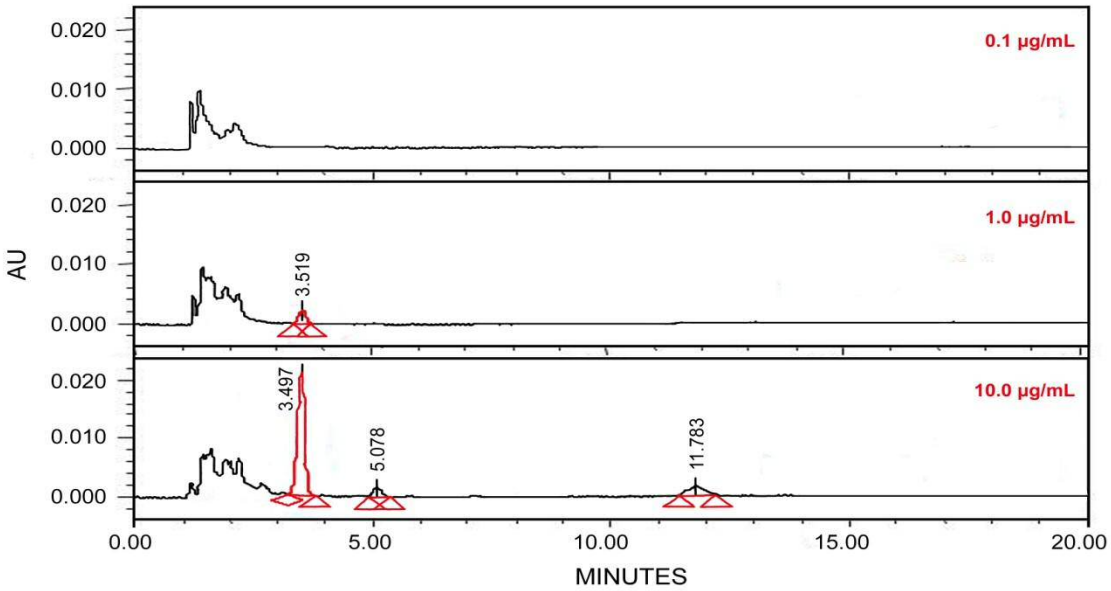


Fig.3.7.(d) HILIC analysis of Biolog samples. Chromatogram represents three concentration range of semi purified CYN with *P. toxinivorans*

3.3.5. Evaluation of CYN degradation in natural waters using River Carron and Cowie Loch.

Previous studies with shake flask experiment showed no degradation. It was suggested that the particular bacteria are not interested in the degradation of CYN. Next was the introduction of natural water to examine any bacterial strains capable of degrading CYN. In order to do the experiment two natural waters collected from River Carron and Cowie Loch were utilised. Sampling was carried out on alternate days for four weeks.

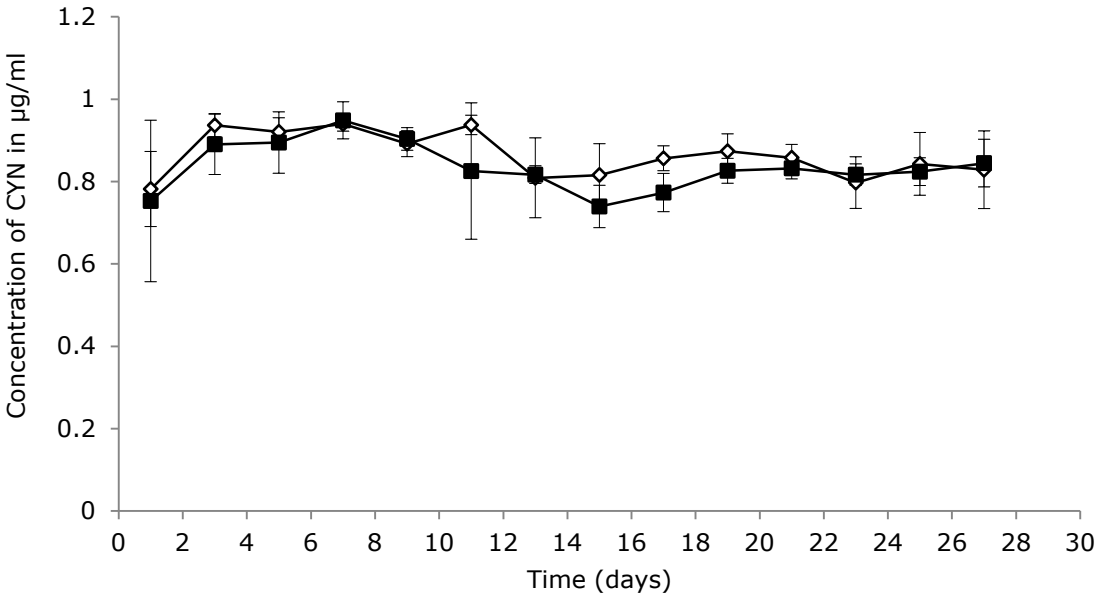


Fig.3.8. Batch culture evaluation of CYN degradation (10 µg/ml) in water from Carron River (■), Sterile control (◇). All incubation at 25 °C at 100 rpm. Error bars represent one standard deviation (n=3).

Results did not show any CYN degradation in the River Carron water. The toxin level was found to be steady over the sampling period. Results revealed there were no CYN degraders present in the River Carron water evaluated.

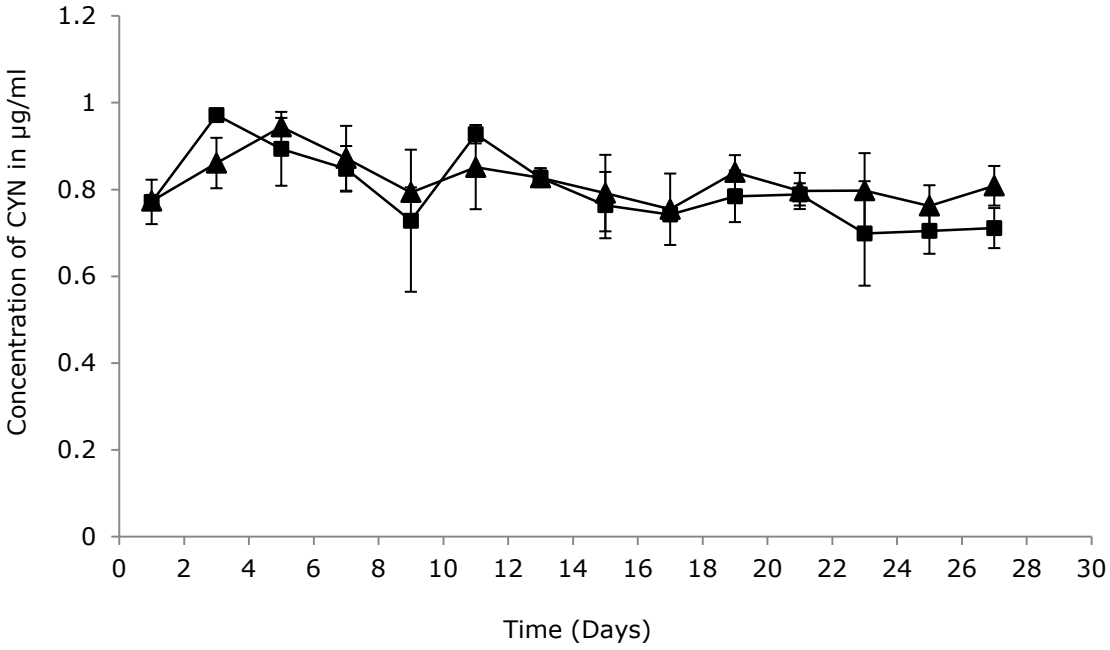


Fig.3.9. Batch culture evaluation of CYN degradation (10 µg/ml) in water from Cowie Loch (■), Sterile control (▲). All incubation at 25 ºC at 100 rpm. Error bars represent one standard deviation (n=3).

Batch culture experiment carried out with Cowie water produced similar results to those observed for the River Carron with no CYN degradation over the period of four weeks examined. It was observed that CYN levels were steady over the period of incubation. From this result, it can be confirmed that there was no CYN degraders present in the Cowie water.

3.3.6. Evaluation of CYN degradation in five natural water bodies and assessing the influence of nutrients in the degradation process.

Current study was to investigate the degradation of CYN in five natural waters. Experiment was carried out with an increased concentration of bacterial numbers by filtering them and applying nutrients. Plate count method was achieved to examine the number of bacterial cell communities. Plate count results showed that higher number of bacterial cells were observed in Loch Leven (1:10 dilution), interestingly none of them capable of degrading cylindrospermopsin. The highest bacterial number was observed in Loch Forfar (1:10 dilution). Interestingly, it was noticed that water collected from River Carron showed no bacterial colony in plate count method.

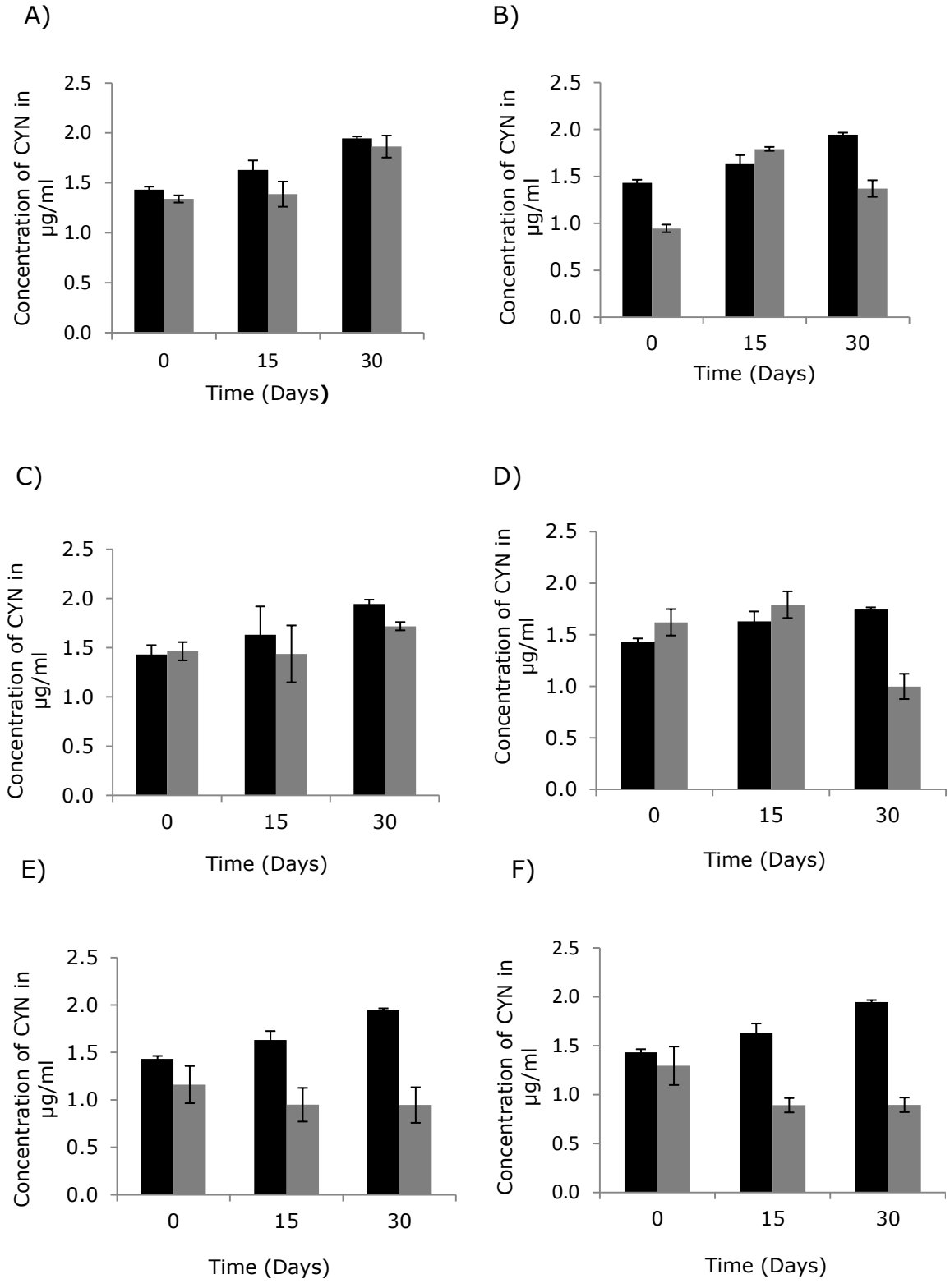


Fig.3.10. Evaluation of CYN degradation in three natural waters. **A)** Rescobie treated water **B)** Rescobie untreated water **C)** Leven treated water **D)** Leven untreated water **E)** Balgavies treated water **F)** Balgavies untreated water. Sterile control (Black) Samples (Grey). Error bars represent one standard deviation (n=3).

Investigation to evaluate the degradation of CYN in three water bodies produced no clear findings. Thirty days sampling showed some degradation in all the waters tested. It was observed that the influence of bacterial cell numbers in treated water showed no positive results. Also noticed was the addition of nutrients which produced no degradation of CYN in the tested waters. It was found that the analysis by HPLC was difficult to produce good chromatograms with natural waters. Degradation studies with two other waters tested shows positive results. Water bodies tested includes River Carron and Loch Forfar for evaluating the removal of CYN in HPLC analysis (Fig.3.10). It was observed that both treated and untreated waters showed the degradation of CYN. Nutrient was a common supplement in the entire sample performed, thus could not really predict the influence. Degradation of CYN was observed in both treated and untreated waters. In the case of River Carron, degradation of CYN was achieved in both treated and untreated water after 15 days. In the case of the water examined at Loch Forfar, the degradation occurred after 15 days in treated water but 30 days in untreated water.

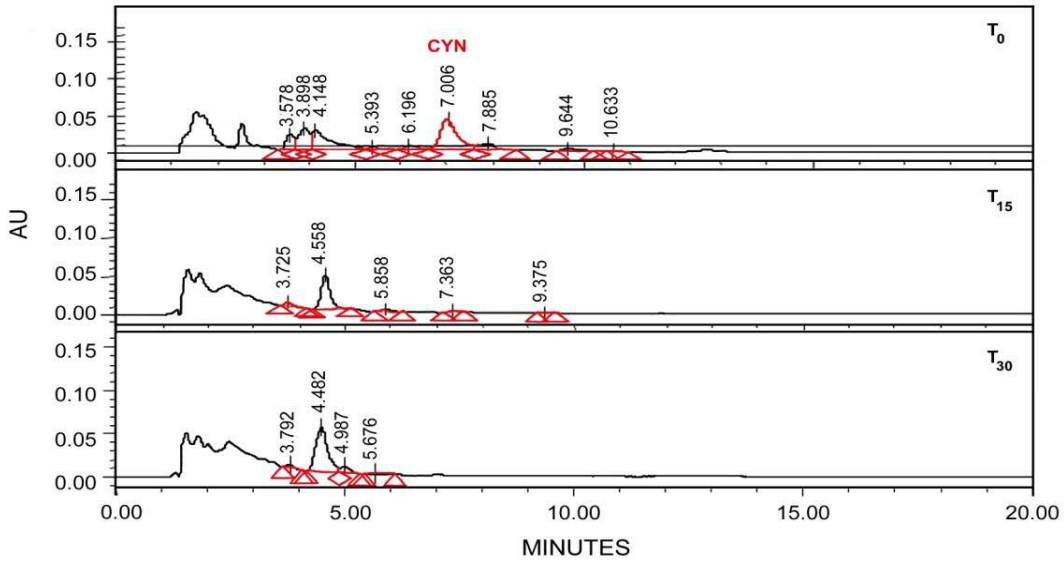


Fig.3.11. Chromatograms of the degradation of CYN over a period of 30 days assessed in treated River Carron water. **A)** T_0 **B)** T_{15} **C)** $T_{30.}$ Error bars represent one standard deviation (n=3).

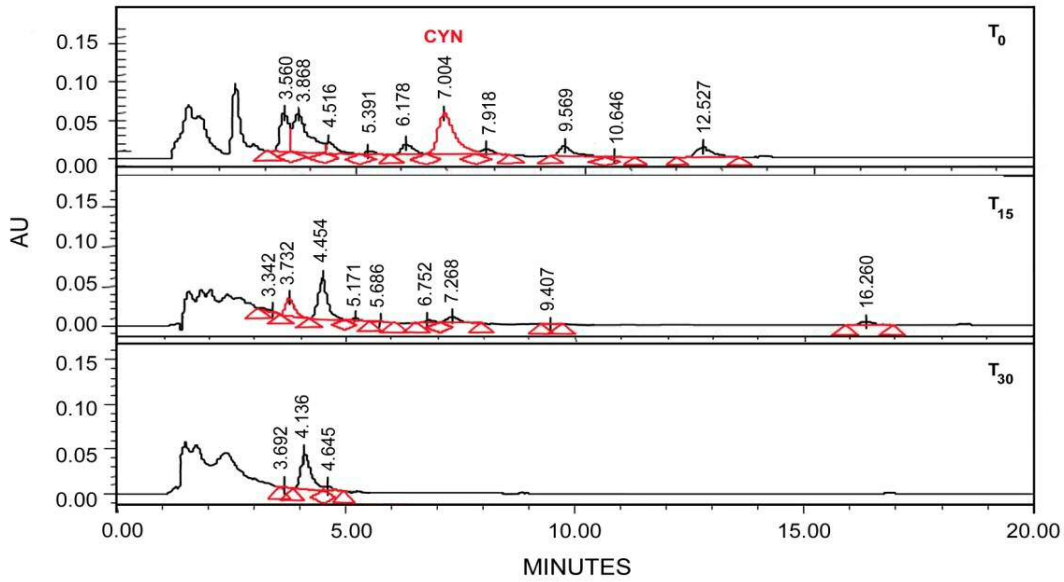


Fig.3.12. Chromatograms of the degradation of CYN over a period of 30 days assessed in non treated River Carron water. **A)** T_0 **B)** T_{15} **C)** $T_{30.}$ Error bars represent one standard deviation (n=3).

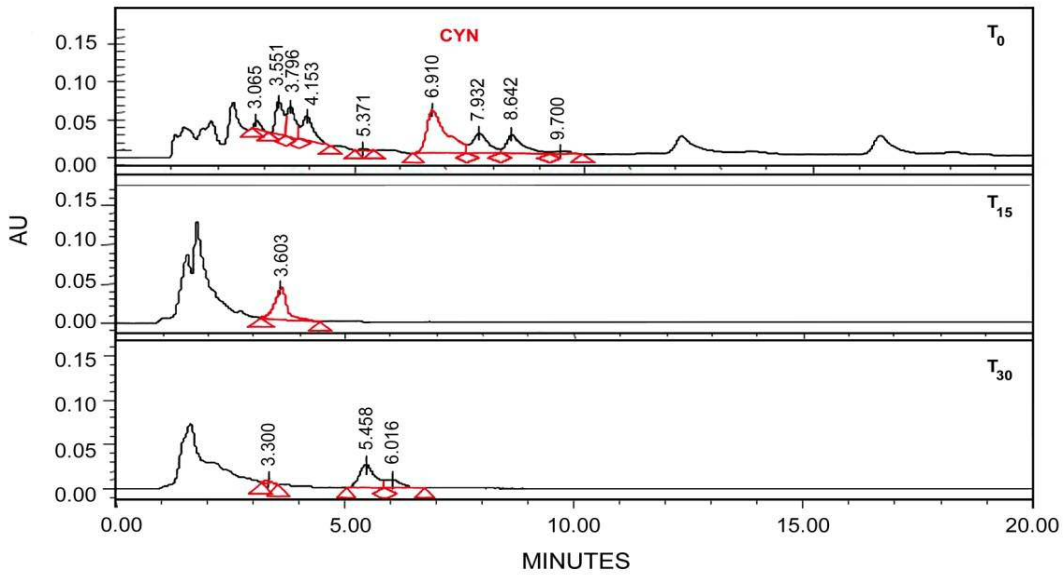


Fig.3.13. Chromatograms of the degradation of CYN over a period of 30 days assessed in treated Loch Forfar water. **A)** T_0 **B)** T_{15} **C)** T_{30}. Error bars represent one standard deviation (n=3).

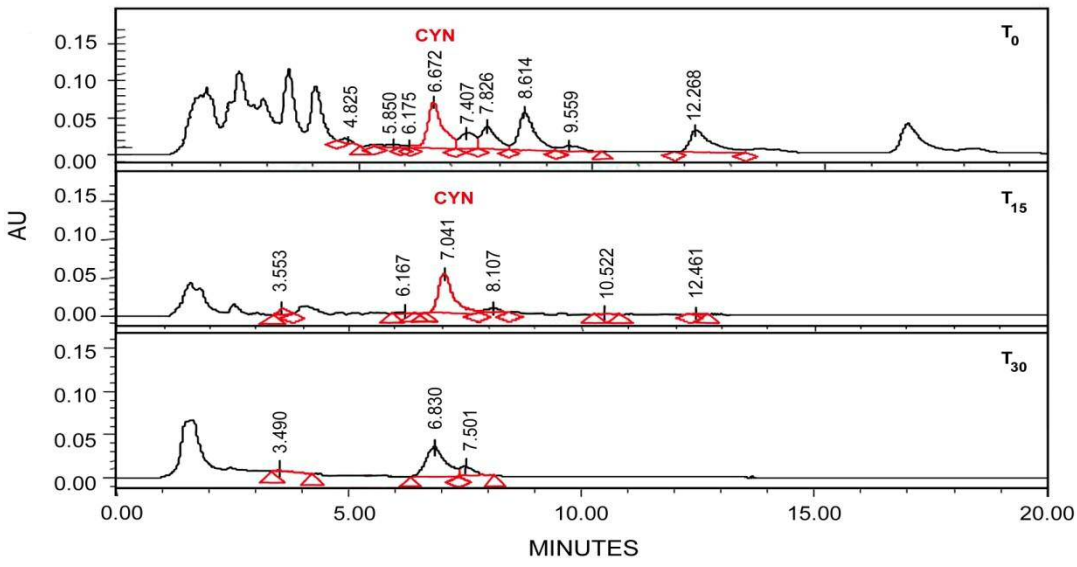


Fig.3.14. Chromatograms of the degradation of CYN over a period of 30 days assessed in untreated Loch Forfar water. **A)** T_0 **B)** T_{15} **C)** T_{30} **.** Error bars represent one standard deviation (n=3).

3.3.7. Evaluation of CYN degradation in two waters with longer term incubation.

Previous studies revealed that CYN degradation was achieved in two water bodies tested. In order to investigate the specific bacterial population that caused the degradation of CYN in two water bodies, a further investigation was carried out in shake flask with longer incubation period. To reduce matrix interference and allow the use of more environmentally relevant concentration, SPE was applied to each sample prior to HPLC analysis.

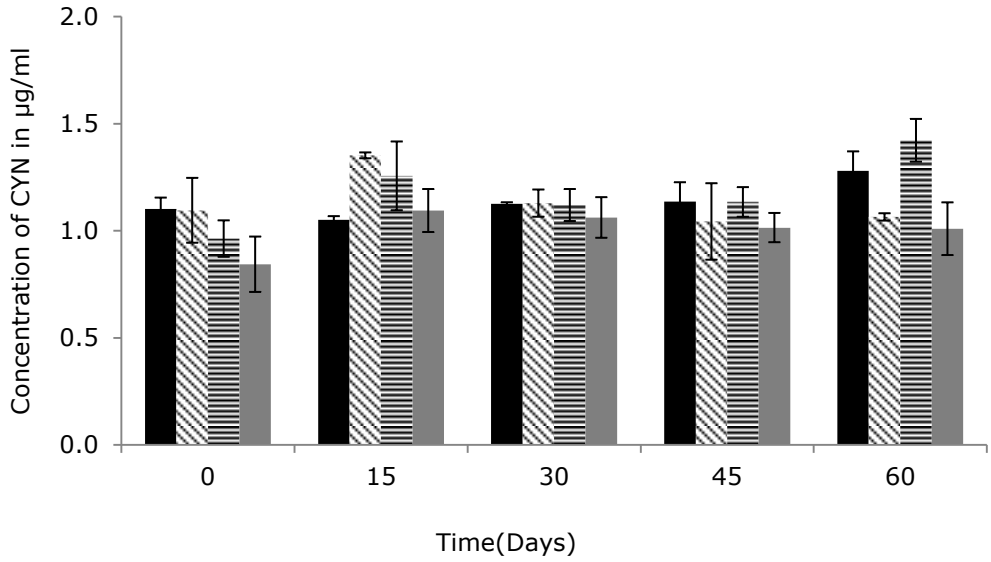


Fig.3.15. Evaluation of CYN degradation in two waters performed over a time period of 60 days. Carron control (■) Carron + CYN (▨) Forfar control (▤) Forfar+CYN (■). Error bars represent one standard deviation (n=3).

The results did not show any degradation in both waters assessed over the longer incubation time. It was observed that the two waters performed over a period of 60 days showed a steady toxin level. It was contradictory to the data obtained in the previous studies. Even such a longer incubation period did not really influence the degradation process.

3.4. Discussion.

Bacterial degradation has the potential to eliminate toxic chemicals both in natural waters and during water treatment processes. Biodegradation has been studied and observed in some of the potent cyanotoxins such as microcystin (Manage *et al*., 2009). In their studies 10 novel bacterial strains capable of degrading microcystin-LR have been identified and isolated from three Scottish waters. Initial experiments were carried out with the utilisation of Biolog MT2 plate assay which was found to be a rapid screening tool to evaluate the bacterial metabolism (Manage *et al*., 2009).

The consequences of cylindrospermopsin and its toxicity in the water bodies have not been well studied or discussed in comparison with Microcystin. Wormer *et al.,* 2007 conducted a 40 day study on biodegradation of cylindrospermopsin produced by *Aphanizomenon ovalisporum* UAM 290 observe the degradation. Another study carried out by Smith *et al*., 2008 reported that biodegradation of CYN was only evident in waters that had a history of toxic *Cylindrospermopsis raciborskii* blooms. A recent and successful study was carried out by Mohamed *et al,* 2012 revealing CYN degradation by microcystin-degrading bacteria isolated from cyanobacterial blooms. A bacillus strain (AMRI-03) isolated from cyanobacterial blooms has been utilised for the degradation studies.

The current study first investigated the degradation of CYN with microcystin degrading bacteria as the genera were know to have the ability to degrade a wide range of compounds. Initial studies were carried out with 11 bacterial isolates to assess the utilisation of CYN in Biolog MT2 plate assay. Results showed that out of 11 bacterial isolates examined, six of them gave a

high absorption variance. The reaction occurred in the Biolog plates could be the utilisation of CYN as the only carbon source present. The three different concentrations of CYN toxin evaluated in Biolog assay did not show that enhances the metabolism of CYN. It was interesting to see the slow growing *Brevibacterium*. sp (F3) showed better metabolism rate in the Biolog assay. The other five isolates such as *Rhodococcus* sp. (C6), *Arthrobacter.*sp (F7), *P. toxinivorans, Sphingomonas.* sp and *Arthrobacter.*spp (R1) also showed pronounced utilisation of CYN. It was not confirmed in the Biolog MT2 plates that the degradation of CYN had occurred. It was just a screening tool to examine the utilisation of desired compound, here CYN. This method was found to be cost effective and rapid screening tool for the assessment of bacterial metabolism with highly diverse respiratory substrates (Manage *et al*., 2009; Lawton *et al.,* 2011). Therefore, further investigation is needed to confirm the degradation CYN with these bacterial isolates.

To confirm that the positive response in the Biolog actually resulted in reduction in CYN samples were tested by HPLC-PDA. This confirmed degradation had occurred under these conditions as no CYN could be detected.

From the Biolog assay studies, six isolates were chosen for the batch culture experiments to confirm the degradation of the CYN. The batch culture experiments did not produce any positive results. Seven days sampling was carried out in this experiment. It was suggested that short term incubation may not be sufficient for the degradation of CYN. Studies with all the six isolates showed a steady toxin level over the 7 days sampling. It could also be that the characteristics of the CYN molecule prevent any easy breakdown.

One of the experimental parameters to notify here was the temperature. It was found that temperature had a pivotal role to play in the degradation of CYN. Studies were carried out to determine the effect of temperature on degradation of microcystin between 22 °C and 25 °C (Park *et al*., 2001; Wang *et al*., 2007). In the case of CYN, the experiments conducted showed rapid degradation at 25 °C (Smith *et al*., 2008). In general microorganisms posses a temperature range over which they are able to grow, especially those found in water usually with temperature optima for growth at ~25 °C. The current study utilised the same temperature for the shake flasks and found the degradation in two water bodies (River Carron and Loch Forfar). Three water bodies tested did not achieve any degradation of CYN. This could be that CYN showed high persistence in many waters due to its chemical stability and slow degradation (Wormer *et al.,* 2008).

To elucidate the reaction which occurred in the Biolog MT2 Plate assay more investigation has to be carried out. It was noted that the bacterial numbers in the Biolog assay was considerably higher than the batch culture study. In order to evaluate the effect of bacterial cell numbers on the degradation of the CYN different bacterial densities were evaluated. Results showed that there was no direct influence of bacterial cell numbers on the degradation.

Isolated bacteria assessed to evaluate the degradation of CYN produced no positive response. The next approach involved natural waters to check for CYN degrading bacterial communities. In order to do this, an experiment was carried out by collecting two natural waters, River Carron and Cowie. Results demonstrated that there was no effective CYN degraders in the waters examined. Eventhough a longer incubation of four weeks did not produce any degradation. So in this case further investigation has to also be carried out.

Again different water sources were examined for CYN degraders. In order to do so an experiment was carried out with natural waters from five locations. The initial results with bacterial cell density examined showed little influence at high bacterial cell numbers. In order to occupy maximum number of bacteria natural waters were concentrated and considered as treated water. The other set was performed without concentrating via less in bacterial cell numbers. But nutrients were supplied to all the samples. Three waters examined showed no degradation by keeping a steady toxin level over a period of 30 days. Interestingly, it was observed that two other waters, River Carron and Loch Forfar were found to contain CYN degraders. Both treated and non treated River Carron waters showed degradation of CYN after 15 days. In this aspect, one thing could be noticed that bacterial cell numbers really did not affect the degradation. On the other hand, Forfar treated water produced complete removal after 15 days but 30 days for the untreated one.

In order to investigate the bacterial strain that could be causing the degradation of CYN in previous studies, the experiment was carried out by collecting water from the same location. But this time large scale shake flask experiment was performed. The samples were kept for longer incubation over a period of 60 days because of the degradation tendency of the desired molecule was slow. Results showed no degradation of CYN in the waters examined. A steady toxin level appeared over the period of 60 days. The reason for no degradation could be the sampling time where the CYN degrading organisms not present in the waters.

The results of the extensive studies here confirm that CYN has a tendency to be relatively resistant to microbial degradation (Wormer *et al*., 2008). Many

previous studies have confirmed this with the main successful finding only observing biodegradation where microbial populations have previously been exposed to naturally occurring CYN (Smith *et al*., 2008). Clearly this is of a concern in environments or water treatment system where CYN occurrence may be sporadic with little opportunity for a reliable natural degrading community to develop or be maintained.

Conclusion

The main aim of this chapter was to investigate an alternative for the current methods to treat toxic CYN from waters. It was suggested that desired molecule shows high persistence in water bodies due to its chemical stability and slow degradation. To investigate a cost effective, environmental friendly alternative to degrade CYN, these studies were carried out. It was documented that degradation by-products are essentially non-toxic (Tsuji *et al.,* 2006). Aim of the study was to evaluate the efficiency of biodegradation of CYN. Initial screening assay revealed the utilisation of CYN in Biolog assay. Further studies were carried out to confirm the degradation of the CYN but did not produce any positive results. Studies were then carried out in natural waters to prove if there was effective CYN degrader present in the River Carron and Loch Forfar water. In order to investigate the particular strain that could degrade CYN, an experiment was carried out with water collected from the same location that produced no degradation. The reason could be climate changes or the different time periods of the sampling.

CHAPTER. 4

Titanium dioxide assisted photocatalysis of Cylindrospermopsin

TABLE OF CONTENTS

4. Introduction

Toxin producing cyanobacteria pose a serious health threat to both humans and animals. Production of secondary metabolites in the form of toxins is a major concern for researchers. Suitable methods for the degradation of contaminated drinking, ground, surface and waste waters containing toxic cyanobacterial secondary metabolites need to be investigated. Titanium associated photocatalysis has been successfully applied on organic pollutants and potent toxins (Fujishima *et al*., 2000). It has also been well studied on cyanotoxins including microcystins and nodularin, especially microcystin-LR (Lawton *et al.*, 1999; Cornish *et al.*, 2000; Liu *et al.*, 2002, Edwards *et al*., 2008; Torunska et al., 2008). However, high operating costs of UV radiation and the physico-chemical properties of the photocatalyst have to date hindered the wide application of advance oxidation processes with photocatalysts (Liu *et al.*, 2009). It was evident that little work has been carried out to develop water treatment methods for the efficient removal of CYN from drinking water. Conventional water treatment methods have either been untested or shown to be ineffective.

4.1.1. Semiconductor photocatalysis

The Photocatalysis may be described as a photo-induced reaction which is accelerated by the presence of a catalyst (Mills *et al*., 1997). The process contains two active phases solid and liquid. In which, the solid phase is a catalyst, usually a semiconductor. The molecular orbital of semiconductors possess two energetic band structures. The band with low energy called valence band (completely filled with electrons at 0 °K) and high-energy called

conductance band (empty at 0 °K) (Sobczyński and Dobosz, 2001). The energetic distance between the valence band to the conductance band is called band gap (Eg). It is the energy required to promote one electron from the valence band to the conductance band. Semiconductors may be photoexcited to form reducing sites (electron-donor) and oxidising sites (electron-acceptor), offering immense scope for redox reaction.

Photocatalytic reactions are activated by absorption of a photon with adequate energy (equal or higher than the band energy of the catalyst, Carp *et al*., 2004). The absorption leads to a charge partition due to an excited electron (e^-) from the valence band to the conduction band of the semiconductor catalyst, thus generating a positive hole (h^+) in the valence band (Gumy *et al.*, 2006; Herrmann *et al*., 2007). These electrons (e^-) – hole (h^+) pairs can either recombine in a few nanoseconds and the energy dispersed as heat or interacts separately with other molecules. In an aqueous suspension, this process reacts between positive holes (h^+) with adsorbed water and OH^- groups to give strong oxidising $OH^\bullet$ radicals. In addition, the photo-generated free electrons (e^-) react with adsorbed molecular O_2 to produce super oxide radical anion ($O_2^{\bullet -}$) that may cause the formation of $HO_2^\bullet$ radicals and H_2O_2 by contributing the production of $HO^\bullet$ radicals. The resulting $HO^\bullet$ radical, being a very strong oxidising agent, reacts with organic pollutants, a key reaction step leading to the mineralisation of these compounds (Vione *et al*., 2005; Akpan *et al.*, 2009) also leads to the formation of CO_2, H_2O, and inorganic ions (Robert and Malato 2002; Rajeshwar 1996; Ollis *et al*., 1991).

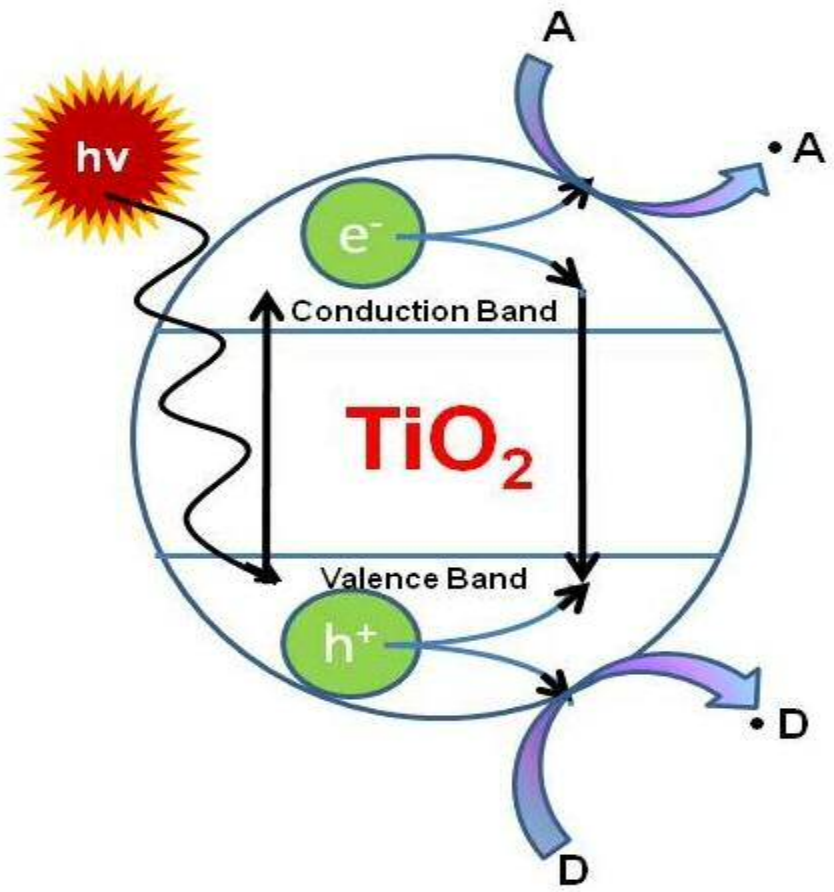
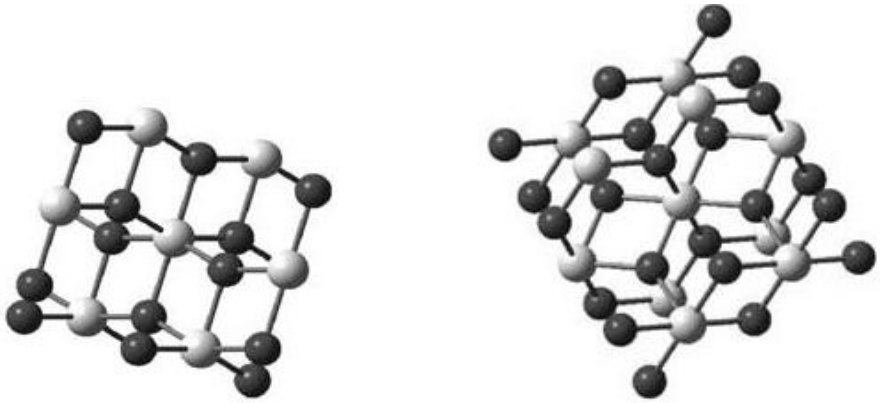


Fig.4.1. Hypothetical photocatalytic reactions occurring upon UV irradiation with TiO_2 alone. Note: A and ▪A represents acceptor molecules before and after reduction (for e.g., O_2 and ▪O_2). D and ▪D represents donor molecules before and after oxidation, respectively (for e.g., OH− and ▪OH). (Modified from Krishna *et al*., 2006)

Among all the semiconductors reported, the most widely used semiconductor catalyst in the photocatalytic process is Titanium dioxide. The advantages of this semiconductor catalyst includes, chemically and biologically inert, easy to produce and use, very cheap, photocatalytically stable, able to efficiently catalyse the reaction and there is no risk in environment or human (Carp *et al*., 2004).

Titanium dioxide (TiO_2) catalysts are very familiar and well-researched catalysts because of stability of its chemical structure, biocompatibility, optical,

physical and electrical properties. TiO_2 exists in four mineral forms such as, anatase (Fig. A), rutile (Fig. B), brookite and TiO_2 (B). The band gap energy of rutile and anatase are 3.00 eV and 3.23 eV respectively. Out of these three types only anatase and rutile display photocatalytic activity (Hanaor and Sorrell, 2011). A study reported that initial rate of degradation was affected mainly by the content of anatase in TiO_2. Pure anatase has exhibited the best catalytic efficiency, while pure rutile has exhibited the poorest catalytic efficiency (Tanaka *et al*., 1991).



(A) **(B)**

Fig.4.2. Cluster models of anatase (A) and rutile (B).
(Ref: Karvinen *et al*, 2003)

In the case of semiconductor photocatalysis, there are three types of catalyst application that have been demonstrated to date. These are powder form, pellets, or some form attached to the surface. i.e., as film sol gel or nanotubes (Sonawane *et al*., 2002). Aforementioned method has specific application with advantages and disadvantages. Powder forms generally perform well in the photo-decomposition of organic compounds. The main challenges faced when using the powder form was the removal from the suspension after

the photocatalytic treatment (Meyer *et al.*, 2003). As this problem exists, research conducted on cyanotoxins with pellet forms resolves the issues but shows less efficiency in degradation (Liu *et al.,* 2009). The other form of the catalyst usage was by attaching TiO_2 particles on the surfaces i.e., sol gel or nanotubes. In this type of process, issues such as, high production cost, relatively low yield of finished product, and the need of specialist equipment for the cost intensive manufacturing process are a concern (Meyer *et al*., 2003).

Catalysts are often given commercial names related to some of their properties for example the most widely used form of TiO_2 is Degussa P25 which is in powder form, with a composition of 75% anatase and a 25% rutile with a surface area (BET) 50 $m^2\ g^{-1}$. Degussa P25 is composed of 100% TiO_2 (Table 4.1). As compared to other TiO_2 types, Degussa P25 shows the degradation of pollutants much more rapidly under investigation procedures (Muneer and Bahnemann, 2001; Haque and Muneer, 2003; Bahnemann *et al*., 2007). Rapid degradation rate is thought to be because of their large surface area and complete distribution in the suspension. The second type of catalyst, mainly used in this research, was Hombikat K01/C which was pellet form (Fig.4.3), with a composition of 80% anatase and 20% rutile; it contains 100% TiO_2 with a surface area (BET) 95 $m^2\ g^{-1}$ (McCullagh *et al*, 2010).

A new approach to the application of TiO_2 is the annealing of the catalyst to hollow buoyant glass spheres, or magnetic pellets. The products called Photospheres™ have become commercially available with their size ranges from micro to millimetre. Photospheres™ possess a surface area (BET) ~ 47 $m^2\ g^{-1}$ and have 17% of TiO_2. These are hollow buoyant silica beads coated with TiO_2 (Fig.4.3) and were developed by Nanoparticulate Surface Adhesion Ltd (NSA

Ltd). These products are found to be easy to remove due to the buoyancy, while still offering high surface area. In addition, Photospheres™ are capable of repeated usage.

Table.4.1. Titanium dioxide catalysts particle comparison

TiO_2 Catalyst forms	Anatase (%)	Rutile (%)	BET surface area $m^2\ g^{-1}$	Particle size	TiO_2 (%)	References
Degussa P25	75	25	50	20-30nm	100	Frontistis *et al*., 2012
Hombikat K01/C	80	20	95	~ 15 nm	100	McCullagh *et al*, 2010
Photospheres™	>99		~ 47	10-85 µm	17	

Degussa P25 Hombikat K01/C Photospheres™

Fig.4.3. Different types of titanium dioxide photocatalysts.

There have been very few studies reported on the successful degradation of cylindrospermopsin by titanium dioxide photocatalysis. A study revealed the degradation of cylindrospermopsin, using titanium dioxide and UV radiation. In

their study two different powder form catalyst were evaluated. Degussa P25 found to be more efficient than the Hombikat UV-100. It was found that an influence of solution pH with high pH resulting in the best degradation rate. No adsorption of CYN to titanium dioxide particles found, which would adversely affect the degradation rate (Senogles *et al*., 2001).

This treatment has been effectively used on a wide range of organic pollutants including maleic hydrazide, propham, tebuthiuron, propachlor, chlortoluron, thiram, phenoxyacetic acid, 2,4,5-trichlorophenoxy acetic acid, 4-chlorophenoxy acetic acid, uracil, 5-bromouracil (Bahnemann *et al*., 2007). It was also successfully applied in the removal of other cyanotoxins nodularin and microcystins (Liu *et al*., 2005, 2009).

Aim of this section of work was to evaluate photocatalysis for the destruction of CYN in water, different catalysis formats will be explained with novel reactor design.

4.2. Materials and Method

4.2.1. Chemicals and reagents.

Proxy pollutant methylene blue received from PHIOBIO, England. Degussa P25 was obtained from Evonik, Degussa (UK), and used as received. Hombikat K01/C was acquired from Duisburg, Germany. Microspheres (40 micron Photospheres™) were provided by Microsphere Technology Ltd., Adare, Co. Limerick, Ireland. Cylindrospermopsin obtained from laboratory stocks, which were purified from laboratory cultures. HPLC solvent methanol and acetonitrile were obtained from Rathburn, Walkerburn, UK. Milli-Q Water was obtained from a Milli-Q system (purified to 18.2 MΩ, Millipore, Watford, UK).

4.2.2 Photocatalysis of proxy pollutant methylene blue using titanium dioxide.

Methylene blue is a model organic pollutant widely used to evaluate photocatalysis activity. Prior to commencing photocatalysis, the optimum concentration of methylene (PHIOBIO, England) had to be determined. This was such that removal by photocatalysis could be followed using a spectrophotometer. The concentration of metheylene blue preferred for this experiment was 10 mg/L as this gave an absorbance of just over 2.0 at 660 nm. A linear calibration for methylene blue was prepared as described in section 2.2.6. The initial experimental set up consisted of a UV light source (UVALINE xenon lamp; 360 nm– 500 nm), a glass beaker, a magnetic stirrer (supplied by Speed Safe™), methylene blue (PHIOBIO, England) and TiO_2 Degussa P25. After a series of dilutions, the optimal concentration of methylene blue was chosen to

be equal to 10 mg/L, while the optimal catalyst concentration was 1 g/L. A schematic diagram that delivers the experimental set up can be seen in Fig.4.4

Fig.4.4. Standard experimental design using a Xenon lamp with unidirectional irradiation of the reaction vessel (50 ml glass beaker) containing aqueous suspension of TiO_2 in the presence of test sample.

The first parameter considered for this experiment was light intensity. In order to determine the effect of light intensity on photocatalysis, a control was performed with methylene blue exposed to light. Experiments were performed by varying the distance between the light source and the glass beaker. The experiments were repeated using different distances e.g. 15, 20, 30, and 40 cm with a light intensity equal to 1735, 1230, 690 and 415 µmol s^{-1} m^{-2} respectively. The light source was illuminated on the sample containing the beaker and the sample was collected for measuring the optical density. To determine the photodegradation of methylene blue, TiO_2 catalyst Degussa P25 was added to

the beaker containing methylene blue, samples were collected every 5 min up to a total of 60 min. Samples (3 ml) were taken every 5 min and each sample was centrifuged at 15,000 x g for 5 min to remove the titanium dioxide. Prior to spectrophotometric analysis, the samples were covered with aluminium foil to avoid further illumination before the optical density was measured. Absorbance was measured by Novaspec® spectrophotometer at λ 660 nm in single use disposable cuvetts.

4.2.3. Standard Calibration Curve for Proxy pollutant Methylene blue.

A standard calibration curve was obtained for the proxy pollutant methylene blue (MB) which was used for the initial photocatalysis experiment. In order to perform the experiment a range of methylene blue concentrations (10.00, 8.00, 6.00, 4.00, 2.00, and 1.00 in mg/L) in Milli-Q water were used. Samples were always made by dilution of the stock solution in Milli-Q water. Sample concentrations were taken in the disposable cuvets and analysed spectrophotometrically (Pharmacia biotech Nova Spec II). The correlation coefficient was determined from the plotted graphs of concentration against absorbance at 660 nm.

4.2.4. Toxicity assay

In order to determine if the toxic effects associated with cylindrospermopsin were removed by photocatalysis, a simple brine shrimp (*Artemia salina*) bioassay was established (Campbell *et al*., 1994) and carried out.

Table.4.2 Brine shrimp medium (Harwig and Scott, 1971) was prepared as a concentrated stock solution containing the following chemicals.

COMPONENTS	g/1.25 L
Sodium chloride	300.00
Calcium chloride dehydrate	3.00
Magnesium chloride hexahydrate	15.00
Magnesium sulphate heptahydrate	5.00
Potassium chloride	8.00
Glycine	60.00
Disodium glycerophosphate	30.00

Each chemical was dissolved separately to prevent precipitation and the stock solutions were stored at 4 °C for future use. Brine shrimp stock solution (20 ml) was diluted with 140 ml distilled water and 100 mg of brine shrimp cysts (Sciento, Manchester, UK; stored at –20 °C until required) were added and incubated at 25 °C for 24 h.

Newly hatched larvae were separated from unhatched eggs and egg cases by placing the flasks in front of a light source that attracts the brine shrimp. Using a Pasteur pipette, hatched larvae were transferred to a glass universal bottle containing fresh medium. The medium was then mixed to give an even suspension of larvae. Aliquots (100 µl) containing around 15-25 individuals were placed in each well of 96 well Microtitre™ plate (Dynaguard, Dynex Technologies, UK). A dilution series of 0.1 to 20 µg/ml of toxin were prepared to determine the LC_{50}. The samples obtained from the different photocatalytic exposure time (100 µl) were added to each well containing brine shrimp. All solutions were tested in triplicate. Brine shrimp was incubated in the presence of test solution for 24 h at 25 °C after which, the number of dead larvae in each well were counted. Methanol (100 µl) was then added to each well to kill remaining larvae and the total number of organisms in the wells determined allowing the percentage mortality and subsequently LC_{50} values to be calculated.

4.2.5. Photocatalytic degradation of CYN using titanium dioxide Degussa P25.

Preliminary experiments with methylene blue confirmed the experimental optima, which were then used for the evaluation of CYN degradation during photocatalysis. Experimental set up was used as described in section 4.2.2. Initial concentration of CYN used for the experiment was 5 µg/ml with a catalyst (Degussa P25) concentration 1 mg/ml. The experiments were carried out with different light intensities (1735, 1230, 690 and 415 µmol s^{-1} m^{-2}). A glass beaker (50 ml) with a sample volume 10 ml was used for the experiment. CYN was then added to the beaker with a T_0 sample removed prior to illumination. Samples

were collected every 1 min up to a total 10 min in triplicate. From the reaction beaker, 200 µl samples were pipetted, and transferred to micro centrifuge tubes and covered with aluminium foil to avoid further light illumination. Samples were then centrifuged at 15,000 x g for 5 min to remove the titanium dioxide. HPLC analysis (section 2.2.6) was carried out by taking 100 µl from the supernatant.

4.2.6. Photocatalytic degradation of CYN by using Hombikat K01/C pelleted titanium dioxide catalyst.

Investigation of an alternative catalyst option to resolve the catalyst separation problem that Degussa P25 raised in the degradation of CYN. The pelleted form of TiO_2 Hombikat K01/C was evaluated in a similar manner to the previous experiment (Section 4.2.2) with four different light intensities. The same concentration of the catalyst as mentioned in the section 4.2.2 (1 g/L) resulted in a steady degradation that was sustained for hours. In order to improve the degradation rate, the catalyst concentration was increased 10 fold compared to that of the Degussa P25 concentration. All samples were processed as before and analysed by HPLC.

4.2.7. Degradation of CYN using Degussa P25 and toxicity confirmed by brine shrimp assay.

In order to confirm the toxicity removal, brine shrimp bioassay was carried out. In this experiment, concentration of titanium dioxide used was 1 mg/ml with a total reaction volume of 10 ml for each experiment. The optimal concentration of CYN used by Metcalf for the brine shrimp toxicity assay was 20 µg/ml (Metcalf *et al*, 2002). In this experiment, 500 µl of sample were removed

every 2 min and the samples were transferred to microcentrifuge tubes for centrifugation. The samples were fully covered by aluminium foil to avoid further light illumination. From every 500 µl, 80 µl were taken for HPLC analysis and 3 x 100 µl were taken to corresponding wells in microtitre plate for brine shrimp toxicity testing as described in the section 4.2.4.

4.2.8. Photocatalytic degradation of CYN using titanium dioxide Photospheres™ in a sparged system.

In order to find the efficiency of Photospheres^{TM} in the degradation of CYN, a pilot reactor model was designed. For the better mixing of the photosphere in the sample solution sparging was considered over stirring. To attain this, a hypodermic needle was inserted through the septum of the vial into the solution. Hypodermic needle was connected via silicone tubing to a rotameter (Influx Measurements, Alresford, UK) and then connected to an air pump (JUN – AIR, Norresundby, Denmark) (Fig.4.5)

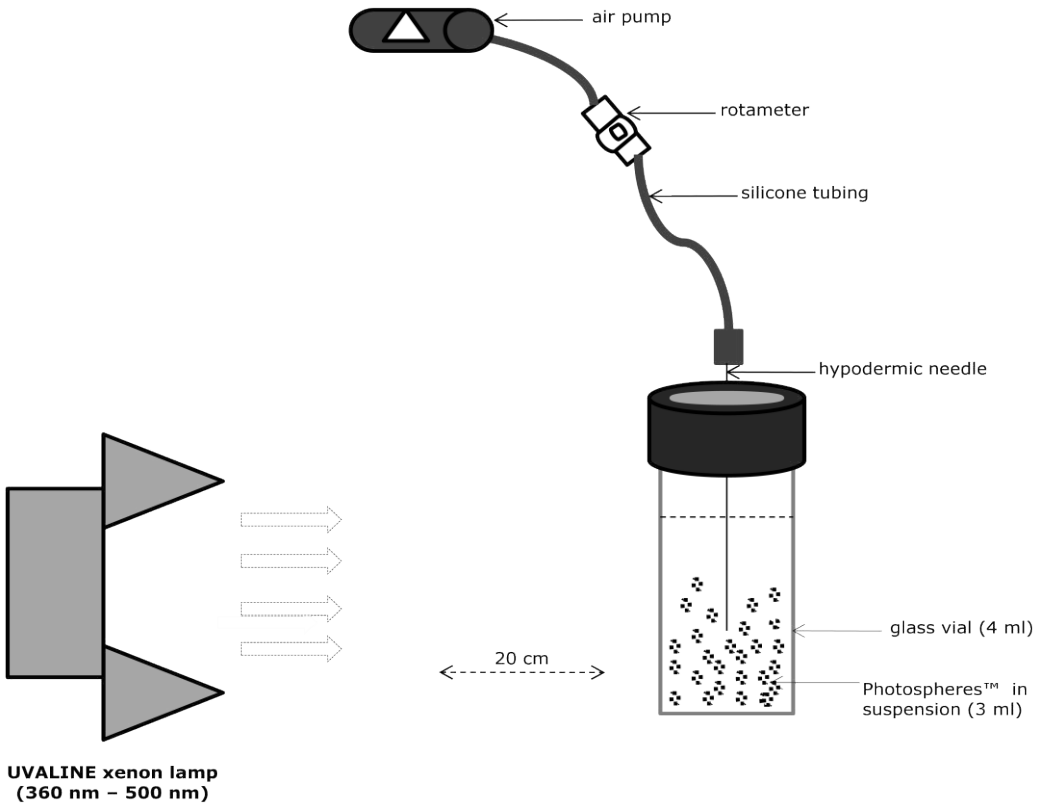


Figure.4.5: Experimental design of reactor model for the photodegradation of CYN by using Photospheres^{TM} in sparged system.

An aqueous suspension of CYN with a concentration of 20 µg/ml was used. Sample volume used for the experiment was 3 ml, and was placed in a 13 mm; 4 ml screw top vials (Kinesis, Beds, UK) with a plastic lid with self healing rubber septum. The distance between the glass vial and the light source (UV light, Xenon lamp 330-450 nm) was kept at 20 cm. Aliquots of 120 µl samples were taken at the beginning (T_0), and then the Photospheres™ were added (1% w/v). After 2 minutes of dark absorption samples was taken (T_{01}). The light was switched on and the samples were taken at intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 minutes respectively, all tests performed were in duplicate (n=2). All

the samples were placed in Spin-X filter tube (Corning). Samples were then centrifuged at 3000 rpm for 10 min at room temperature to remove the Photospheres™. The samples were freeze dried for 24 hrs and dissolved in methanol for HILIC analysis.

4.2.9. Photocatalytic degradation of CYN using titanium dioxide Photospheres™ by LED reactor.

The photodegradation of CYN by using Photospheres™ with pilot reactor design has revealed its efficacy similar to Degussa P25. Due to the buoyant characteristics of the Photospheres™, it was easily removable from the treated water. Investigation was then carried out to build a new reactor for the large scale experiment. While the light UV source originally used gave good removal, the energy demands of this source was high (i.e. 450 W). Recently low energy UV-LEDs have become available hence these were evaluated as an alternative. The problem that rose while using UV light in the environment led to investigate a more cost effective and efficient light source for the reactor design. To evaluate a lower energy light source, LED was considered. The first design of the photocatalytic reactor was built by cutting a commercially available polycarbonate sheeting (PLEXIGLAS ALTOP® SP, Evonik, Essen, Germany), with a guaranteed > 97 % UV light transmittance. The polycarbonate sheet was double walled and less compartmentalised. The sheet was cut size 300 mm (Length) x 190 mm (width) with 3 compartments. The reactor was sealed at both ends with a polycarbonate capping end–piece (Wickes, UK)) by cutting them to a length of 300 mm and sealed by using silicone sealant (Wickes, UK). Reactor consists of two openings at both ends such as an inlet and outlet. To

facilitate the water flow 0.5 cm segments were removed from the inner vertical dividers on alternate ends of the sheet. Water flows inside the reactor as shown in Fig 4.7 (4).

The reactor design consists of 5 L Pyrex glass beaker for the sample solution. Silicone tubing was connected to the reactor chamber through a peristaltic pump. The sample solution was carrying through the silicone pump to the reactor. An outlet silicone pump was carrying the sample back to the beaker. An aerator was introduced to give sparging in the sample solution. Light source was designed by arranging 90 LEDs in 9 rows (10 LEDs per row) parallel on a PCB (Printed Circuit Board). The LEDs (AT Technologies, Bath, UK) have a diameter of 5 mm, a 15° aperture, λ 360 nm, and a power output of 750 μW at 20 mA DC/3.8 V. Each chain of 10 LEDs was connected in parallel by soldering. The sampling was taken by using a 50 ml syringe (BD Plastipak^{TM}, Luer, Spain).

In this experiment, the reactor was kept in horizontal position and the light was introduced over the reactor with a distance 0.5 cm. Concentration of the CYN in the sample was 10 μg/L. The samples were taken at (T_0) before the light was switched on. Photospheres^{TM}, equal to 0.5% of (w/v) were added to 1.5 L of distilled water. Samples were run (one cycle) through the reactor before taking first sample. The UV-LED lights were switched on and the sample was run through the reactor. Samples were taken every 20 min from the beaker where samples were being recycled. The experiment was carried out for duration of 3 h. Samples (50 ml) were collected from the beaker every 20 min and kept for SPE analysis. Samples were then filtered using GF/C filters before the SPE analysis was carried out as described in section 2.2.13.

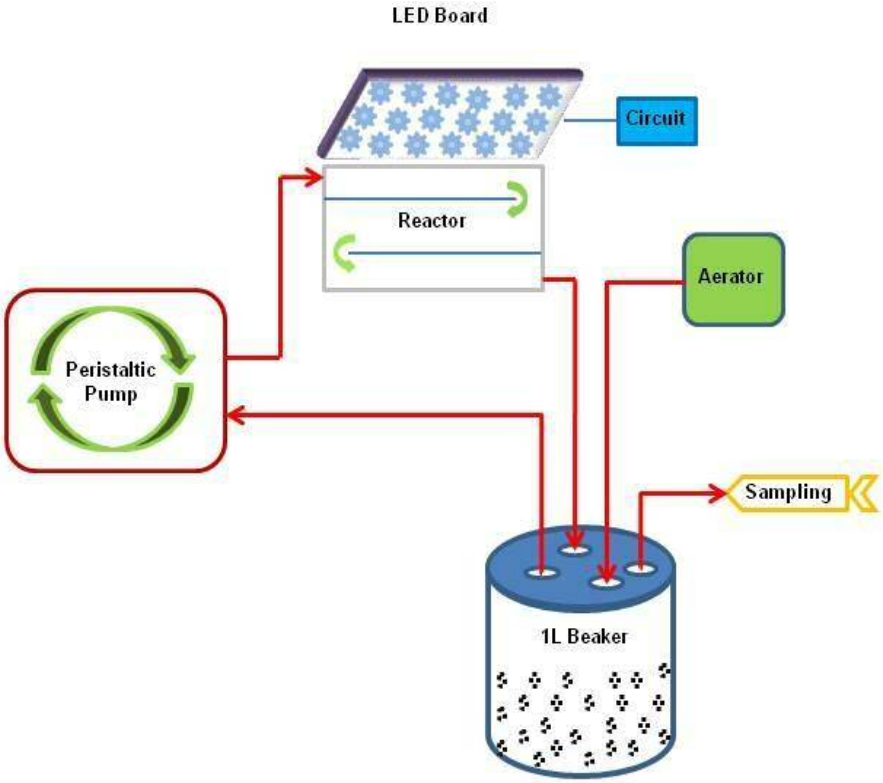


Fig.4.6. Schematic representation of novel LED photo reactor

Fig.4.7. Photographs represents the parts of the novel LED reactor **(1)**. UV LEDs arranged on PCB and connected with circuit board. **(2)**. Aerator. **(3)**. Peristaltic pump. **(4)**. Reaction container. **(5)**. 5 L glass beaker closed on top. Tubing in the beaker for sample intake and outtake, sampling (with syringe) and sparging (aerator).

4.2.10. Photodegradtion efficiency evaluated by placing LED photo reactor in vertical position.

Poor mixing of the catalyst was observed during horizontal operation of the reactor plate hence the reactor was adjusted to vertical position. The experiment was carried out as mentioned in section 4.2.9 except for changing the reactor position.

4.2.11. Photodegradation of CYN by Photospheres^{TM} using novel reactor with UV Xenon lamp as light source.

This study was conducted to compare the photodegradation efficiency of new LED reactor with UV Xenon lamp. All the parameters were kept constant and performed the same experiment as mentioned in section 4.2.10 except the light source used was different.

4.3. Results

The aim of the investigation was to evaluate the suitability of TiO_2 for better photodegradation of cylindrospermopsin and as a potential water treatment approach. The application of an alternate light source in the form of LED has also been investigated as part of this study.

4.3.1. Photocatalysis of proxy pollutant methylene blue by using titanium dioxide.

Initial experiments were performed to evaluate the photocatlytic efficiency of different light intensities using proxy pollutant methylene blue. The degradation of the dye was assessed by measuring the absorbance at 660 nm wavelength.

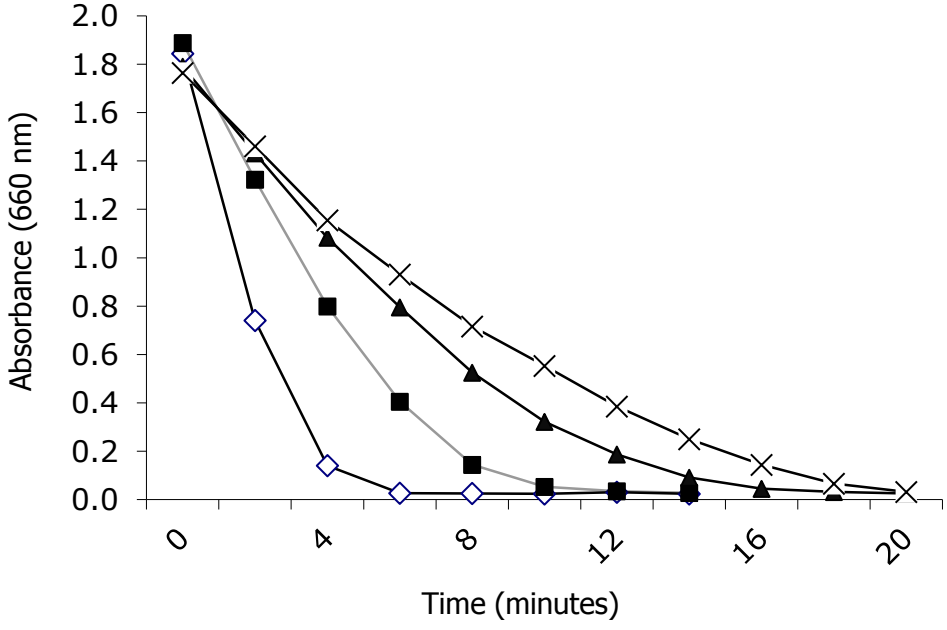


Fig.4.9. Photocatalytic degradation of methylene blue (10 mg/L) by using titanium dioxide catalyst P 25 (1 g/L) at range of different light intensities (◇ 1735 ■ 1230 ▲690 × 415 (µmol s^{-1} m^{-2}). Triplicates were used, i.e., n=3. Error bars equivalent to 1 standard deviation.

It was observed that the degradation was very rapid under high light intensity (1735 µmol s^{-1} m^{-2}) with a degradation of almost 60 % of the dye within 2 min and a complete degradation of the dye by 6 min. (Fig 4.9). The lowest light intensities used in these experiments were 690 $\mu mols^{-1}$ m^{-2} and 415 $\mu mols^{-1}$ m^{-2}. At these light intensities, CYN was degraded 100% after 16 min and 20 min respectively.

4.3.2. Standard calibration curve for methylene blue

Methylene blue was used as a proxy pollutant in the early development studies when evaluating TiO_2 photocatalysis. It was therefore important to establish a robust calibration for spectrophotometric analysis. This was achieved by taking concentration against absorbance response from spectrophotometer. Calibration curve obtained for the methylene blue demonstrated linearity and maintained a correlation coefficient (R^2) greater than 0.99 on their linear regression (Fig.4.8).

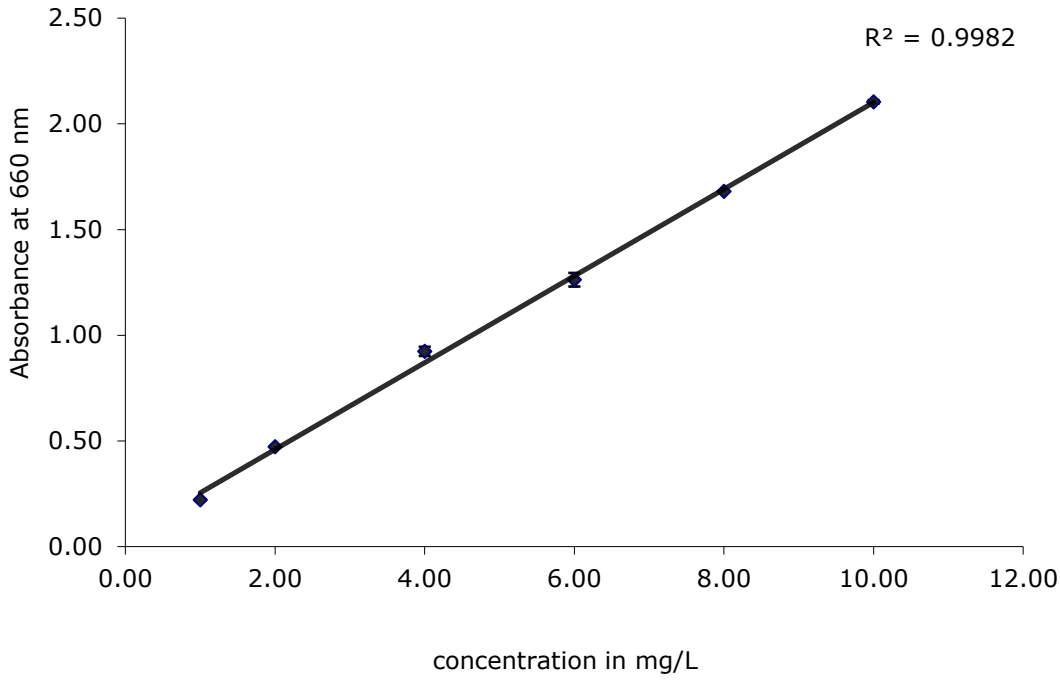


Fig.4.8. The calibration graphs for the proxy pollutant methylene blue (MB). Displayed with the graphs are the formula and the correlation coefficient (R^2 value) determined at 660 nm. Triplicates were used, i.e., n=3. Error Bars were equivalent to 1 standard deviation.

4.3.3. Degradation of cylindrospermopsin using Degussa P25 titanium dioxide catalyst.

The investigation of photodegradation of methylene blue with Degussa P25 demonstrated effective photocatalytic activity influenced by light intensity. This supported the potential application of TiO_2 photocatalysis for the removal of other pollutants specifically CYN which was of interest here. Results illustrate the influence of light intensity and the powder form catalyst on photodegradation of CYN. It was also indicated that in chromatogram analysis no significant degradation products were produced over the period of photocatalysis.

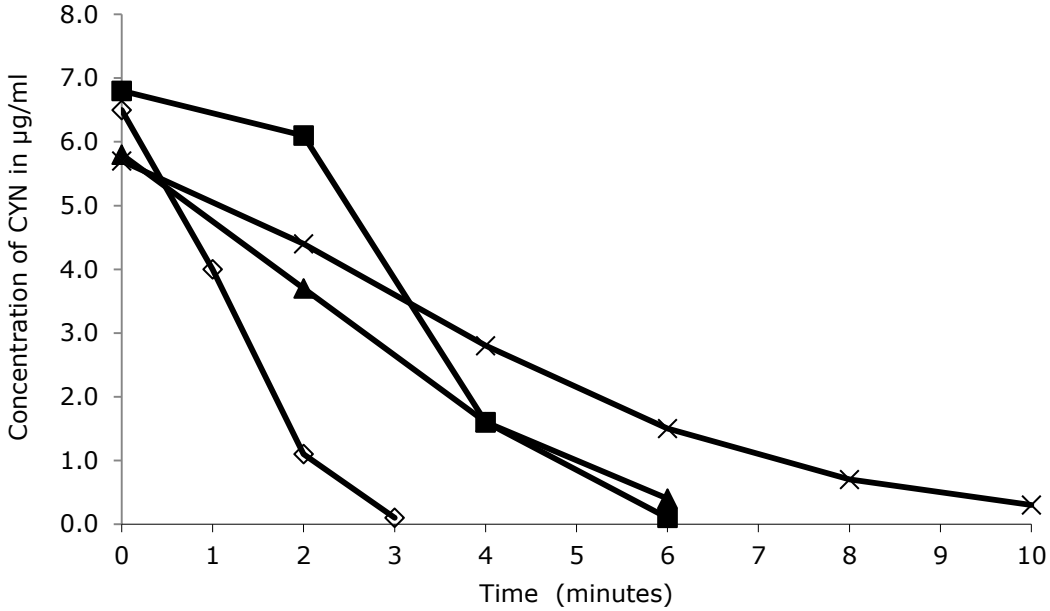


Fig.4.10. Photocatalytic degradation of cylindrospermopsin by using titanium dioxide catalyst P 25 Degussa at a range of light intensities (◇ 1735 ■ 1230 ▲ 690 × 415 µmol s^{-1} m^{-2}) Triplicates were used in the experiments, i.e., n=3. Bars equivalent to 1 standard deviation.

From Figure 4.10, it was observed that the highest light intensity resulted in a rapid degradation of CYN, and complete degradation (100%) occurred after 3 min. On the other hand, at lower light intensities, equal to 1230 µmol s^{-1} m^{-2} or 690 µmol s^{-1} m^{-2}, complete degradation of CYN occurred within 6 min of reaction time. At the lowest light intensity of 415 µmol s^{-1} m^{-2}, 60 % of the toxin was degraded after 6 min of reaction time, whereas, complete degradation of CYN was observed after 10 min. Collectively, these data showed that when TiO_2 was exposed to a high light intensity, the resulting jump of the electrons

generated a reduced environment that was optimum for the mineralisation of CYN.

Analysis of the CYN degradation was carried out by HPLC method. Chromatogram obtained by HPLC demonstrated the photo degradation of CYN. CYN peaks were identified on the basis of both their retention time and characteristic UV spectra at 262 nm. Fig.4.11 represents the chromatograms of the photodegradation of CYN obtained by HPLC which demonstrates the degradation of CYN by time.

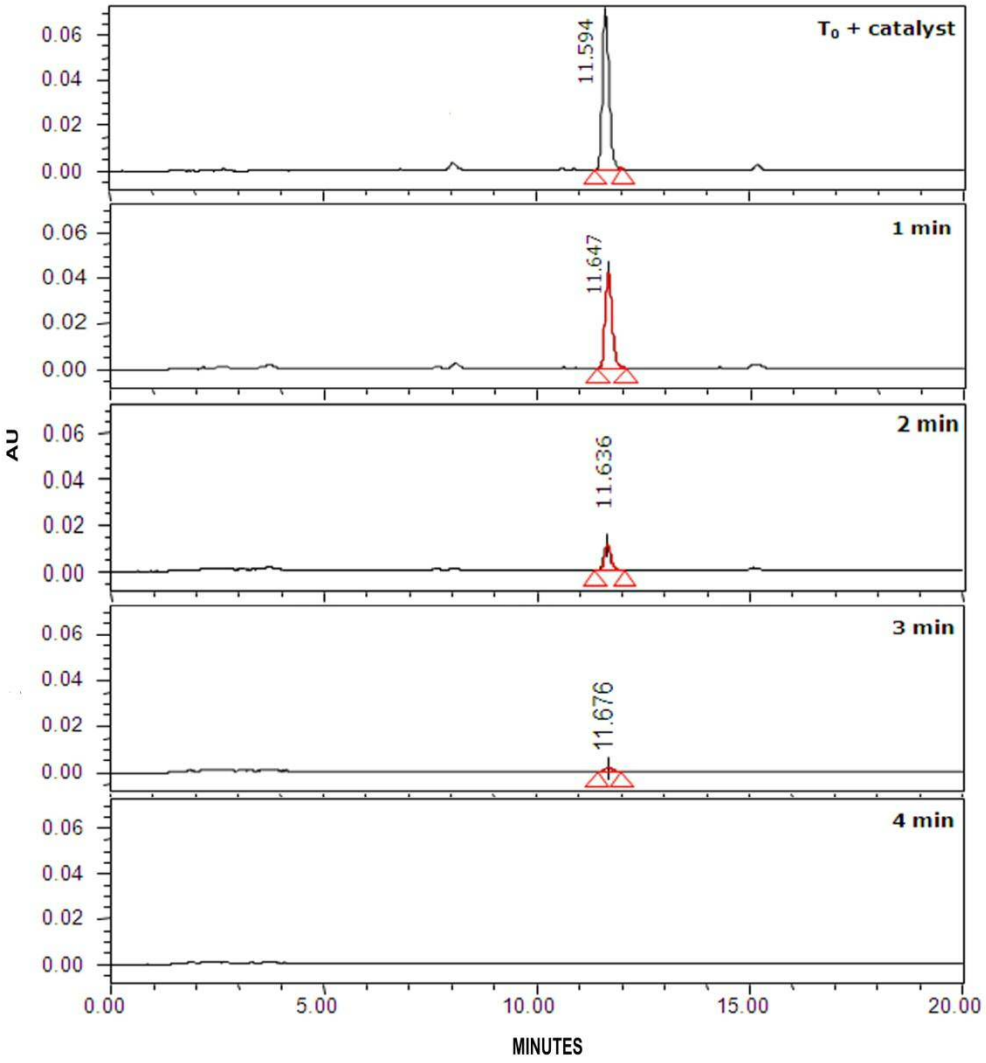


Fig.4.11. Chromatograms analysed by HPLC for the degradation of CYN with Degussa P25 catalyst at a light intensity 1735 μmol s^{-1} m^{-2}

4.3.4. Degradation of cylindrospermopsin by using Hombikat K01/C catalyst.

Results obtained from the previous experiment showed that Degussa P25 is efficient in the photodegradation of CYN. It was evident that TiO_2 photocatalysis catalyst has shown to be rapid in the degradation of CYN.

However, the difficulties encountered with catalyst removal after the treatment leads towards an investigation of an alternative option. A pellet form of TiO_2 was evaluated to resolve the post treatment removal difficulty. The same experimental protocol was performed as per the Degussa P25 but using Hombikat K01/C (Fig.4.12). This experiment resulted in a steady degradation rate that was sustained for hours.

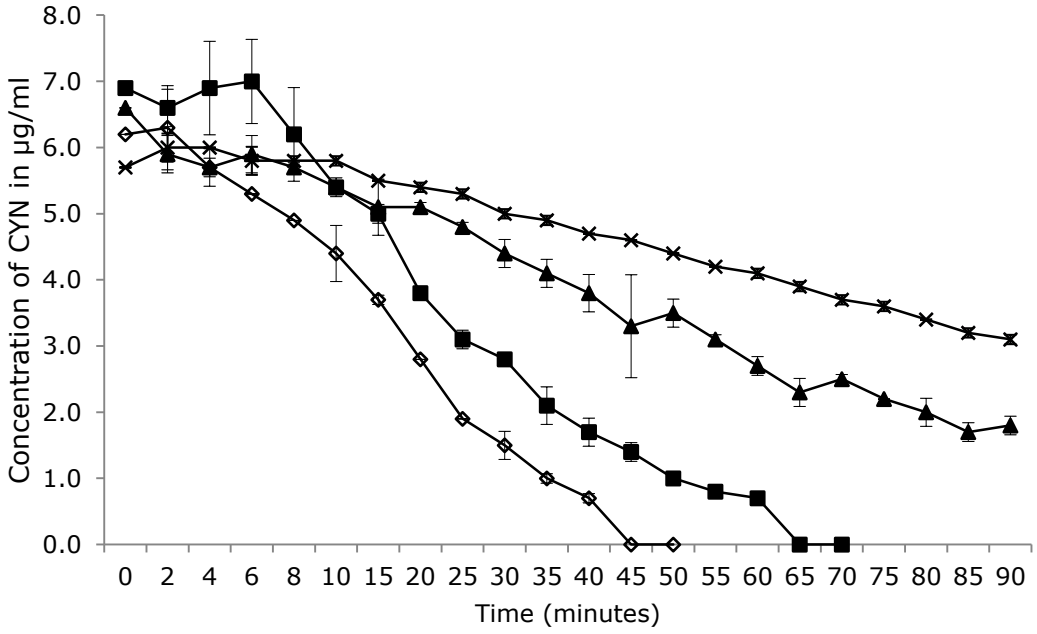


Fig.4.12. Photocatalytic degradation of cylindrospermopsin by using Hombikat K01/C catalyst load at a range of light intensities (◇ 1735 ■ 1230 ▲ 690 × 415 µmol s^{-1} m^{-2}) Triplicates were used in the experiments, i.e., n=3. Bars equivalent to 1 standard deviation.

While the Hombikat K01/C did degrade the CYN, the rate was extremely slow taking many hours (Data not shown). Hence the catalyst load was increased 10 fold (i.e. 10 g/L) and the degradation followed at different light intensities. Again degradation was slower than Degussa P25 although total

degradation was observed after 50 min with highest light intensity (1735 µmol s^{-1} m^{-2}).

4.3.5. Brine shrimp assay and LC_{50}

In order to confirm the elimination of toxicity following the degradation of CYN by photocatalysis, a brine shrimp assay was performed. This assay had previously been used in both the cases of microcystins and anatoxin-a (Kiviranta *et al*., 1991). Initially an LC_{50} assay using a range of CYN concentration was performed using the brine shrimp assay.

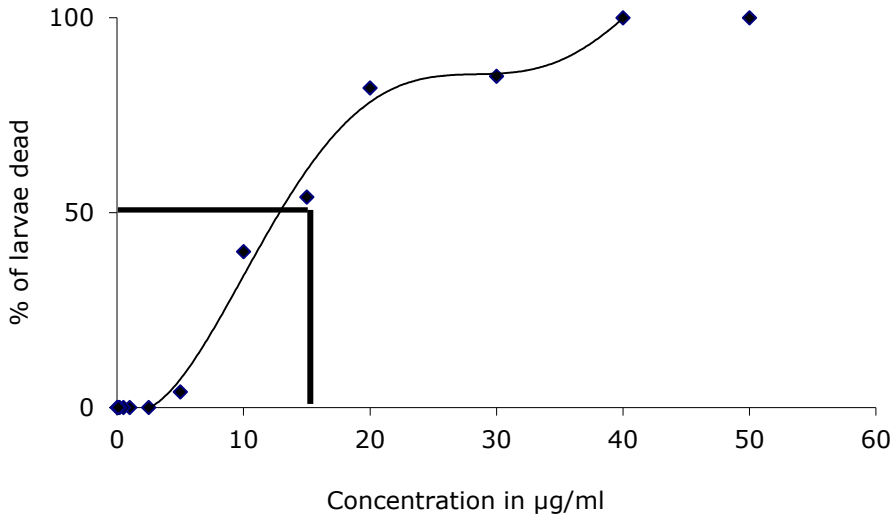


Fig.4.13. LC_{50} analysis results of cylindrospermopsin by brine shrimp assay.

LC_{50} was determined by taking the concentration of toxin that caused the death of 50% tested larvae. This assay produced an LC_{50} of 15 µg/ml (Figure.4.13) which is close to LC_{50} that Metcalf *et al.* reported in the bioassay.

Future experiments to evaluate the toxicity after the photodegradation process were carried out by using a toxin concentration equal to 20 µg/ml.

4.3.6. Photocatalytic degradation of CYN by using Degussa P25 determining toxicity using the brine shrimp assay.

To allow the degradation and subsequent loss of toxicity to be monitored by performing brine shrimp assay, a higher concentration of CYN had to be used. HPLC analysis of the reaction confirmed that even with the much higher CYN concentration, photocatalytic degradation using Degussa P25 degraded the toxin around 8 minutes. In order to confirm the toxicity had been removed from the degradation products, a brine shrimp assay was performed. HPLC can only analyse the presence or absence of the particular compound but did not reveal information on residual toxicity. Brine shrimp assay is one of the reliable methods to screen toxicity of cyanobacterial toxin and it was assesed in some of the marine cyanobacterial strains as well (Martins- Creuzburg *et al*., 2008).

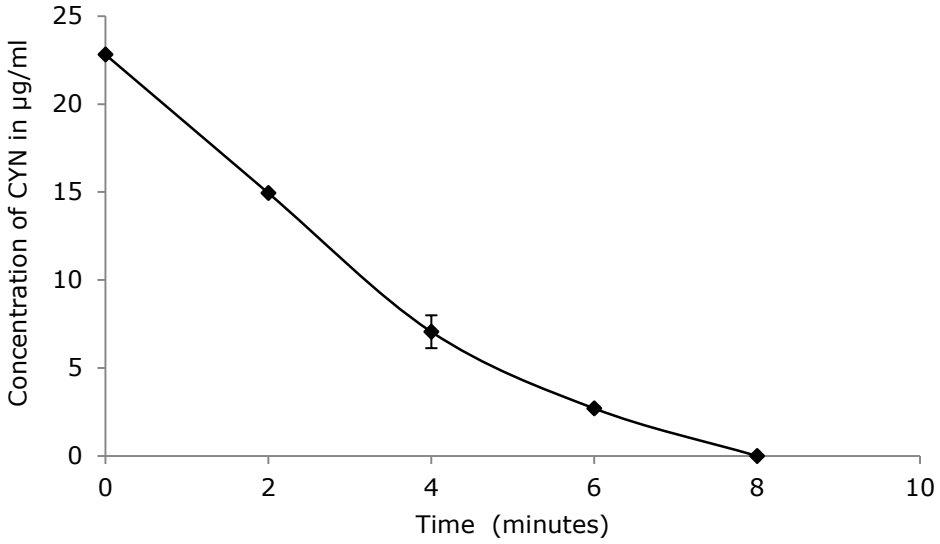


Fig.4.14. Photocatalytic degradation of cylindrospermopsin by using TiO_2 P25 Degussa catalyst with a light intensity 1230 µmol s^{-1} m^{-2}. Duplicates were used for the experiments, (n=2) ; error bars equivalent to 1 standard deviation.

The experiments performed were to support the CYN removal data obtained by HPLC. The results obtained by both HPLC and brine shrimp assay demonstrated a correlation in the toxicity removal. The degradation experiment of CYN toxin displayed the complete removal of toxin within 9 minutes both with brine shrimp assay (Table.4.3) and the HPLC data. These studies revealed that the brine shrimp assay can be used as an efficient method to confirm the toxicity of CYN. It has also clearly demonstrated that the photocatalysis treatment of CYN successfully eliminate the toxicity associated with this compound.

Table.4.3. Cylindrospermopsin toxicity removal by Degussa P25 catalyst and toxicity confirmed by performing brine shrimp assay.

Degradation Time (min)	Mortality-Brine shrimp assay	CYN concentration – HPLC (µg/ml)
0	84%	21.9
1	72%	17.9
2	60%	16.3
3	49%	12.4
4	45%	9.3
5	40%	6.7
6	30%	4.8
7	15%	2.8
8	10%	1.6
9	0%	0
10	0%	0

4.3.7. Photocatalytic degradation of CYN with Photospheres™ in a sparged system.

Previous studies on the photodegradation of CYN demonstrated the efficiency of powder and pellets form. Powder form was found to be difficult to remove from the treated water and pellet form showed less efficiency. It was important to investigate as an alternative to solve the problems raised while using these catalyst forms.

Evaluation of the performance of Photospheres™ demonstrated that at the same TiO_2 load (1% w/v) it performed with similar efficiency (Fig.4.15) to compared Degussa P25. However, due to the more demanding synthesis costs of

Photospheres™ it would be beneficial if a reduced amount of the Photospheres™ could be used. A range of dosing levels that were evaluated clearly demonstrated that degradation rate is dependent on load. However, the rates are still good particularly when compared to the alternative Hombikat K01/C.

Evaluation of the performance of Photospheres™ demonstrated that at the same TiO_2 load it performed with similar efficacy to that of Degussa P25, However due to the more demanding synthesis costs of Photospheres™ it would be beneficial if a reduced amount of the Photospheres™ could be used.

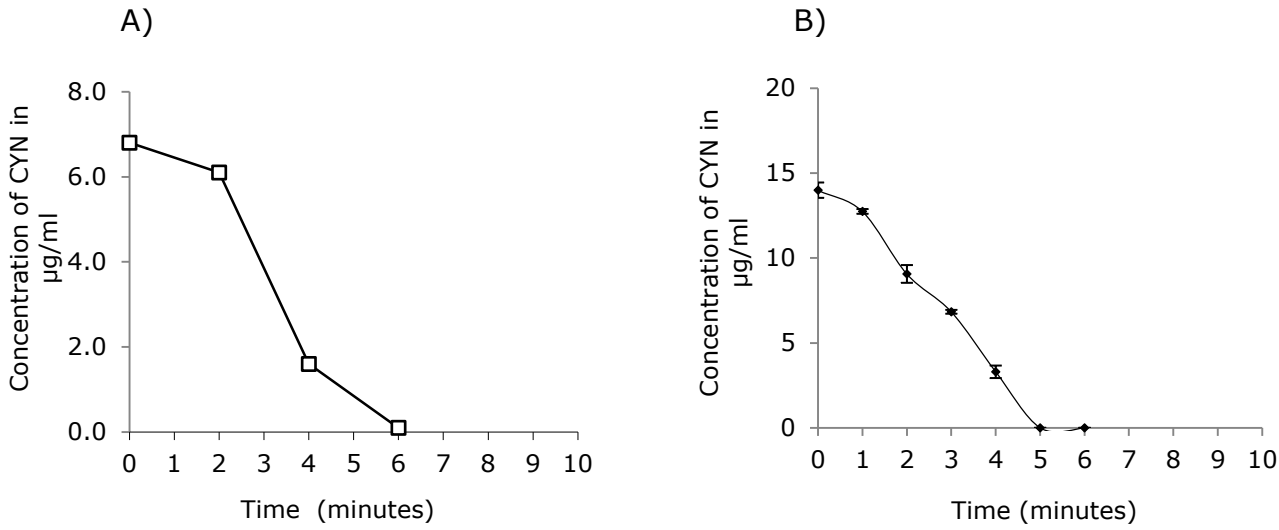


Fig.4.15. Comparison between two catalysts used for the photodegradation of CYN. With Degussa P25 (A) and Photospheres™ (B).

Results demonstrated that both Degussa P25 and Photospheres™ showed same efficacy in removing CYN.

A range of dosing levels that were evaluated clearly demonstrated that the degradation rate is dependant on load (Fig. 4.16) However; the rates are still good particularly when compared to the alternate Hombikat K01/C.

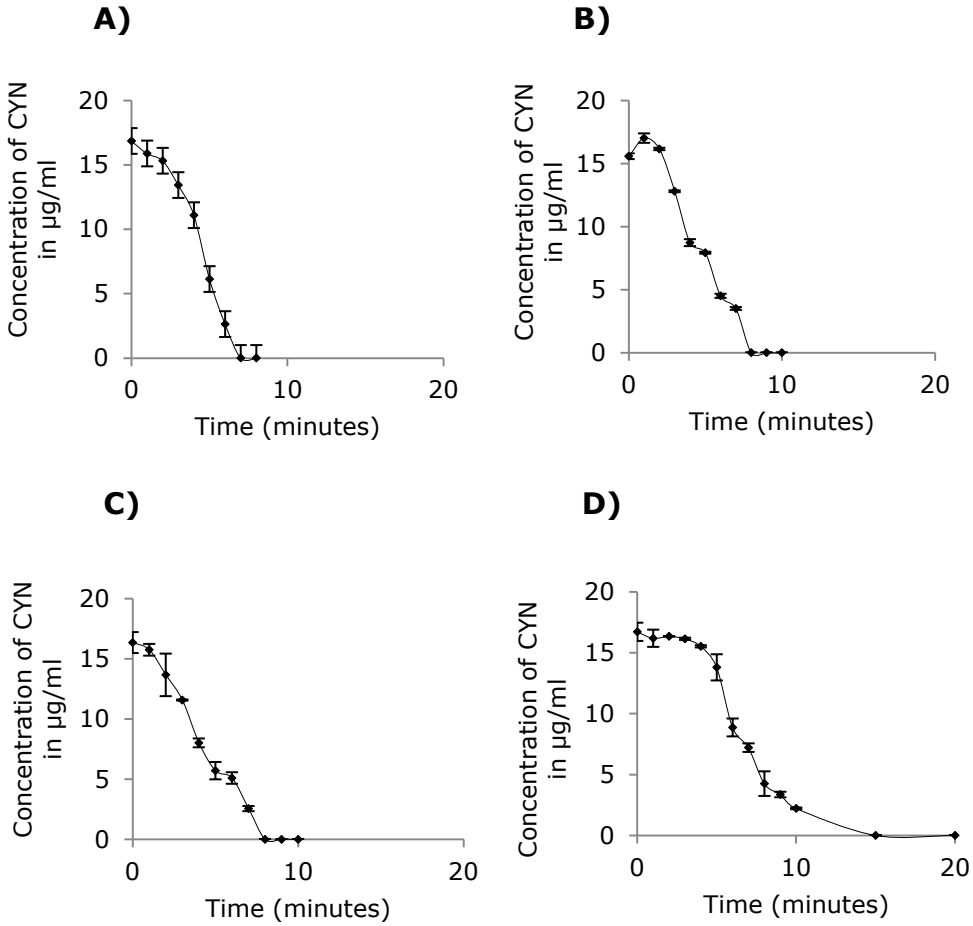


Fig.4.16. **A.** (0.8% w/v); **B.** (0.6% w/v); **C.** (0.4% w/v); **D.** (0.2% w/v) Photocatalytic degradation of CYN (20 µg/ml) by titanium dioxide variant photospheres™ at distance of 20 cm. Duplicates were used for the experiments, (n=2); Bars equivalent to 1 standard deviation.

Results showed that Photosphere™ has same efficacy as that of the Degussa P25 form tested. With different catalyst loading performed demonstrated rapid degradation of toxin and also showed the similarities in the loading.

Table.4.4. A comparison of the degradation rate of CYN with different Photosphere™ catalyst loading (w/v).

Photospheres™ catalyst loading (%)	50% Degradation achieved Time (min)	100% Degradation achieved Time (min)
1.0	5 min	3 min
0.8	7 min	5 min
0.6	8 min	5 min
0.4	8 min	4 min
0.2	15 min	6 min

The experiments indicated a relationship between the CYN degradation rate and the catalyst loading. As the catalyst load was reduced so the rate of degradation gradually dropped. The lowest loading of the catalyst was 0.2% (w/v) which resulted in a slower degradation rate taking 15 min to achieve 100% removal of the toxin. Catalyst loading of 0.4% w/v and 0.6% w/v showed the same degradation trend and both achieved complete CYN degradation by 8 min. The highest loading of the Photosphere™ in the experiment was 1.0% w/v which resulted in rapid CYN degradation when compared to the other loadings with 100% degradation in 5 min (Fig.4.15.B). The catalyst loading 0.4%, 0.6%, and 0.8% all resulted in very similar degradation rates. With three different loadings 100 % degradation had occurred in 4-5 min and 50% degradation occurred in 7-8 min. (Table 4.4). These finding suggests that significantly lower catalyst loads could be promising when considering the cost of the Photospheres™.

4.3.8. Photocatalytic degradation of CYN with titanium dioxide Photospheres™ by plate LED reactor.

Photodegradation of CYN using Photospheres™ was evaluated with a newly constructed LED photo reactor. A cost effective, light efficient method has been performed on large scale experiments.

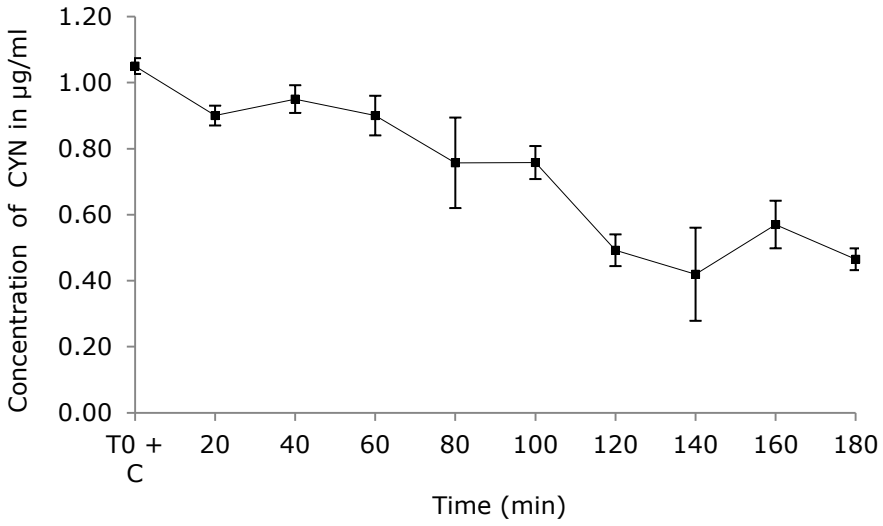


Fig.4.17. Photodegradation of CYN by using Photospheres™ with plate LED reactor (horizontal position). Duplicates were used for the experiments, (n=2); error bars equivalent to 1 standard deviation. All samples were concentrated by SPE prior to HPLC analysis.

This study demonstrated the degradation of the toxin achieved 50% by 2 h although the amounts detected fluctuated. Improper mixing of the sample solution and the catalyst has been noticed and this could be the reason for these variable findings.

4.3.9. Photodegradation efficiency evaluated by placing LED photo reactor in vertical position.

This study was carried out to achieve better mixing of the sample solution and catalyst inside the reactor. It was noted that in the vertical position the drop in toxin level was more consistent suggesting that more efficient mixing had taken place (Fig. 4.18)

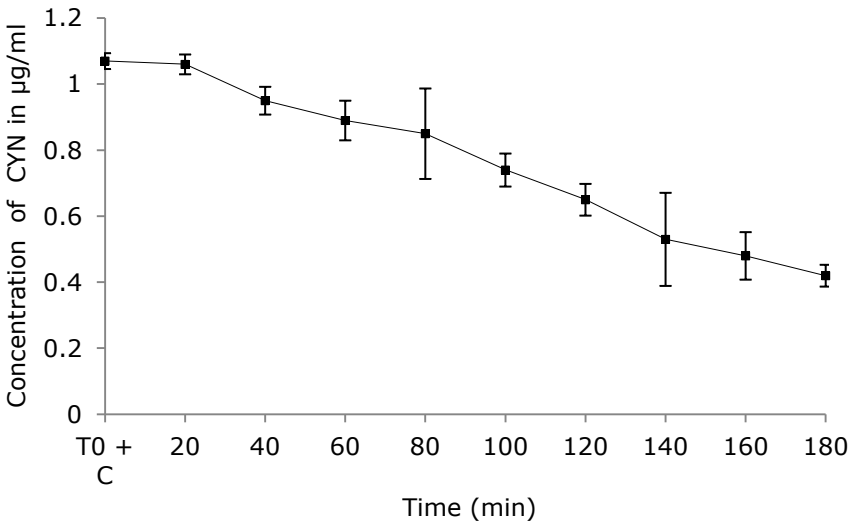


Fig.4.18. Photodegaradtion of CYN by using Photospheres^{TM} with newly constructed LED reactor (vertical position). Duplicates were used for the experiments, (n=2); error bars equivalent to 1 standard deviation. All samples were concentrated by SPE prior to HPLC analysis.

4.3.10. Photocatalytic destruction of CYN by Photospheres™ in the plate reactor with UV light source.

This experiment has been carried out in order to compare the efficiency of Photospheres™ with UV LED light source and conventional UV light source (UV Xenon lamp). As it was known to give better mixing the reactor was placed in a vertical position. Results showed that complete degradation of the CYN toxin occurred only after 40 min. It can be seen that a major percentage of the CYN degradation has occurred by 20 min or before and thereafter it gets slower.

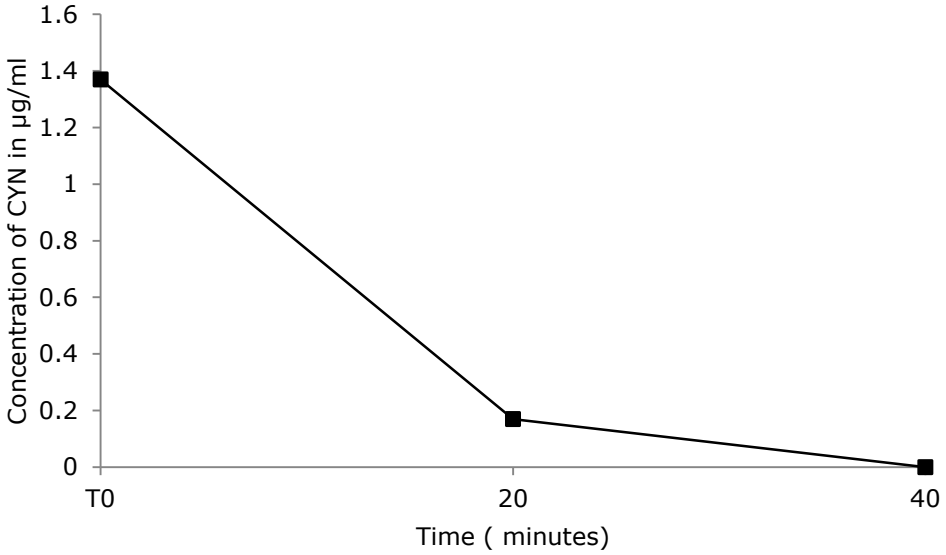


Fig.4.19. Photodegaradtion of CYN by using Photospheres™ with newly constructed reactor with UV xenon lamp as light source (reactor placed in vertical position). Duplicates were used for the experiments, (n=2); error bars equivalent to 1 standard deviation. All samples were concentrated by SPE prior to HPLC analysis.

4.4. Discussion

Compared to conventional water treatment methods semiconductor photocatalysis has now successfully proved its efficiency on removal of organic pollutants and potent toxins. A number of studies have shown its efficiency in treating cyanobacterial toxins including microcystin and nodularin (Liu *et al*., 2002, Liu *et al*., 2009). However, few studies have been reported on cylindrospermopsin degradation by semiconductor photocatalysis. Although CYN has been previously described to be degradable by UV irradiation (Senogles *et al*., 2000). A further study by these researchers reported that titanium dioxide, Degussa P25, was found to photocatalyse CYN more efficiently than Hombikat UV-100 (Senogles *et al*., 2001). However, photodegradation with powder form catalysts has its own drawbacks. Its nature as nanoparticulate powder form has showed difficulties in being removed from the water after the photocatalytic treatment (Liu *et al*., 2009). As an alternative to this, pellet form has been assessed but has been found to be less efficient in the degradation process. A novel buoyant product called Photospheres^{TM} has been tested as an alternative to Degussa P25. This product has a advantage over Degussa P25 as it was easily removable from the treated water.

To evaluate the photocatalytic efficiency of the titanium dioxide catalyst and allow the development of experimental design, a low cost proxy pollutant has been tested. Methylene blue has been widely used as a proxy pollutant the research due to its ready availability and ease of monitoring (i.e. direct spectrophotometry). Total degradation was observed at the highest light intensity in 6 min time total demonstrating, as has been shown before, that Degussa P25 is an effective photocatalyst. In this reaction, lattice distortion on

the anatase TiO_2 surface induced by chemical adsorption of methylene blue molecules is a crucial intermediate step during photocatalysis. The distorted lattice atoms absorb photons under illumination of light and tend to recover and break the molecule bond which causes the degradation of methylene blue (Zhang *et al.,* 2011).

To evaluate the efficiency of Degussa P25 in the photodegradation of CYN and also to confirm the findings of Senogles *et al.,* 2001, a range of experiments were carried out. Results here showed that Degussa P25 did indeed degrade CYN rapidly. At highest light intensity tested, a complete degradation was observed in 3 min. As compared to the previous studies our results showed a more rapid degradation. In their study, they used concentration of 0.1 µg/ml for both toxin and catalyst and found degradation in 5 min. However, the study omitted to mention the light intensity used. The current study using highest light intensity showed complete degradation of toxin with no detectable by-products in 3 min (Fig.4.9) which is promising.

The differences in the photocatalytic activity of TiO_2 are likely to be due to differences in the BET-surface, impurities, lattice mismatches or density of hydroxyl groups present on the catalyst's surface. The powder form Degussa P25 is known to be better photocatalyst. Its performance could be attributed to its nano-crystaline rutile form being dispersed within an anatase matrix. This helps in easy electron movement between the rutile and the anatase form making P25 more reactive (Hurum *et al*., 2003). The better efficiency of photocatalyst Degussa P25 may also due to 'quantum size effect' (Nozik *et al.,* 1993;). When the particles become too small, there is a 'blue shift' with an increase in the band gap energy, unfavourable to the near UV-photon

absorption, and an increase of the electron-hole recombination. In most cases it could be observed that the degradation of pollutants proceeds much more rapidly in the presence of Degussa P25 as compared with other TiO_2 catalysts (Muneer and Bahnemann, 2001; Haque & Muneer, 2003; Bahnemann *et al.,* 2007). The better photocatalytic activity of Degussa P25 has also been reported by Pizzaro *et al.,* in 2005.

In order to solve the problem suffered while using the powder form, another TiO_2 brand, pellet form Hombikat K01/C has been tested. Results showed less efficiency in the degradation process (Fig.4.11). it has been previously reported that adsorption in the surface area of the catalyst might play an important role in the photodegradation efficiency (Hoffmann *et al.*, 1995). It was also believed that this might be a reason for the slow degradation that was observed in our study. In the study conducted on microcystin degradation by granulated TiO_2 catalysts it suggests that not only is it the adsorption surface area but the surface area of the granulated particle exposed to the UV light that can determine the degradation efficiency (Liu *et al.,* 2009). As compared to the powder form, pellet form possesses less surface area (McCullagh *et al*., 2010). In the study conducted on MB, the concentration of MB in solution decreased more rapidly with a decrease in the particle size of anatase-type photocatalysts. Another study, especially so with the particle size of TiO_2 less than 30 nm showed significant increase of reaction rate (Xu *et al*., 1999). The powder form has a large surface area and is readily exposed to toxin for the reaction so that the toxin molecule can penetrate through the titanium dioxide molecule very easily. This particular affinity makes the powder form more efficient than the

pellet form. In this aspect it is evident that particle size could be the reason for the slower degradation rate when using Hombikat K01/C.

A biological sensitive assay was evaluated to confirm the toxicity removal of the photodegradation samples. It was evaluated by plotting concentration against the mortality. LC_{50} was assessed and found to be 15 µg/ml (Fig.4.6). This value was closely similar to that which Metcalf *et al* investigated. As it is a less sensitive detection compared to HPLC assay, further experiments conducted to check the toxicity removal by this assay used a concentration of 20 µg/ml. This assay was compared with HPLC analysis data against brine shrimp assay (Section 4.3.5, Fig.4.7) and showed a good correlation. It is reassuring in the development of this treatment method for the production of drinking water that the toxicity associated with CYN is eliminated.

In order to examine a catalyst with the same efficacy as that of Degussa P25 but with ease of separation the buoyant catalyst (Photospheres™) were tested. Photospheres™ compared very favourably to Degussa P25 further confirming that small particle size and good mixing are essential for rapid toxin destruction. The results with different catalyst loading showed some interesting aspects. The catalyst loading 0.4%, 0.6% and 0.8% and 1% all resulted in a similar degradation trend over time. This suggests that in an application less than 1% could still be used to achieve the same amount of toxin removal. This was promising since the cost of the catalyst can be a concern hence efficient dosing will help minimise this. There are studies which discussed the catalyst loading and photocatalytic efficiency (Andreozzi *et al.,* 2000; Pekakis *et al.,* 2006).

The use of UV light sources can add a high energy cost to the treatment of water. Using photocatalysis can be an economic issue hence reactor developments have been made available with low cost UV-LEDs as a potential alternative. In order to achieve this, light emitting diodes were assessed. These are low power devices that emit monochromatic levels of light. They have a long life span of at least 100,000 h compared to the gas discharge source (Natarajan *et al.,* 2011) which are typically one to two thousand hours. LEDs generally do not produce much heat emission so there is no heat energy loss. At the same time they convert all the electric energy into spectral output (Natarajan *et al.,* 2011).

A novel LED photo reactor constructed for the experiment to assess the efficiency of photospheres in the CYN removal. As compared to the results obtained by using Photospheres^{TM} in the presence of UV light source, this experiment could only achieve 50% of CYN removal by 180 min. It was believed that the light intensity used while using UV light was far higher than the current LED reactor. Improper mixing while keeping the reactor in horizontal position was also noticed. The results (Fig 4.16) also supported that not a steady reduction found. On the other hand while placing the reactor in vertical position, keeping all other parameters constant obtained a steady reduction in the graph plotted (Fig.4.17). In order to compare the LED light source with UV xenon lamp, another experiment was performed by keeping all the parameters constant but only changing the light source. Results showed that complete degradation was achieved in 40 min time (Fig.4.18).

The reason for the slow degradation occurred while using LED reactor was that the total light intensity was exposed on the reactor. By calculating the

total intensity of 1200 μW was exposed on the reactor which is less than UV xenon lamp (1735 μmol s^{-1} m^{-2}). Another point to take in to consideration was the catalyst's characteristics. Generally there is a difference between the Photosphere™ products and P 25, P 25 is > 98 % TiO2, whereas a substantial part of the mass of the Photospheres™ was accounted by the silica sphere. Thus 1 % (w/v) of P25 was not equal to 1 % (w/v) of Photospheres™ in terms of the actual amount of TiO_2. The actual amount of TiO_2 in the Photospheres™ was found to be 17 %. In addition, with comparison of both light sources, they were found to posses same light intensities performed similar. With all the advantages that LED posses over UV light source, better opportunity is provided for the LED assisted photo reactors.

Potential prospects of the reactor design would be convenient removal of CYN using Photospheres™ in a pilot system. In order to achieve this a pilot scale reactor can be designed as represented in the schematic diagram (Fig.4.19). Advantages of the design could be the Photosphere™ removal by placing a separator, also UV LEDs provided as the light source.

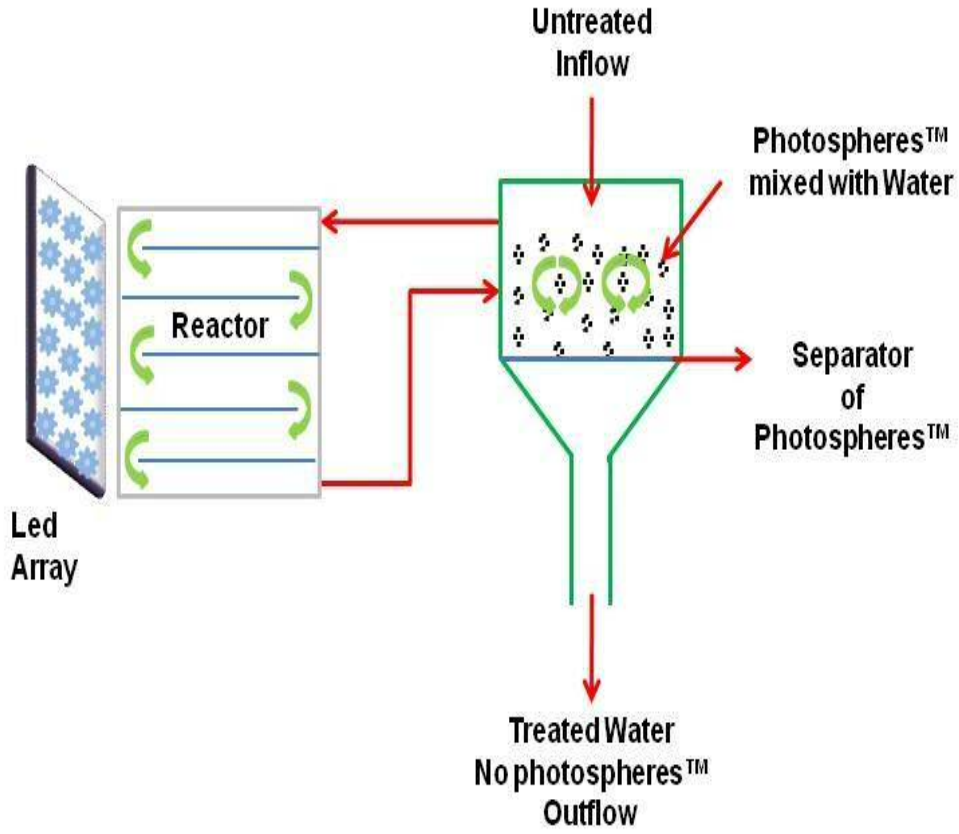


Fig.4.19. Schematic diagram of potential pilot system for water treatment

Conclusion

This chapter mainly discussed the application of semiconductor photocatalysis in the effective removal of CYN. Initial experiments successfully assessed rapid removal of CYN with Degussa P25. Removal of the catalyst from the treated water was found to be difficult thus leading to check the use of pellet form. The results demonstrated that pellet form was less efficient. Photospheres™ were found to have a similar efficacy as that of Degussa P25 and showed rapid degradation of toxin with a small pilot reactor tested. As far as the removal problem was concerned it was a better option. The brine shrimp was successfully used to confirm that TiO2 eliminated the toxicity associated with the presence of CYN. A novel photoreactor has demonstrated the effective use of LED assisted light sources in the photodegradation and was found to be a better alternative over one generally used UV xenon lamp.

CHAPTER 5

GENERAL DISCUSSION

General Discussion

The increase in the occurrence of cyanobacterial blooms, which contain potent toxins are of major concern to both humans and animals. Hepatotoxic alkaloid cylindrospermopsin (CYN) produced by cyanobacteria *cylindrospermopsis raciborskii* (Chiswell *et al*., 1999) has been found in almost all continents. Recent reports suggested that increasing water temperatures due to global warming in middle Europe could cause cost the proliferation of CYN producing species from warmer regions. This may lead to a high concentration of CYN in surface waters which, in turn, contaminates drinking water. Hence, an efficient elimination of CYN has to be investigated.

Conventional water treatments have not been well studied or have been found to have limited efficiency in removing dissolved cyanotoxins (Svreck *et al*., 2004). They have also been found to produce toxic substance which may be carcinogenic or mutagenic (Ishii *et al*., 2004). In this case, it would prove helpful to find a cost effective and sustainable approach. Significant interest has been shown towards the application of microbes for the elimination of cyanotoxins from the water supplies. But fewer studies have been reported in the case of CYN.

The present study aimed to investigate the exploitation of microbes for the removal of CYN from water supplies. Initial results demonstrated the utilisation of CYN in Biolog MT2 assay. Ten of the bacterial isolates demonstrated microcystin degrading qualities (Manage *et al*., 2009). In this study six isolates out of ten demonstrated the utilisation of CYN. A redox reaction occurred in the Biolog MT2 plates. Metabolism performed particularly well resulting in the formation of formazan, and producing a colour change in the tetrazolium dye.

This could have occured in the six isolates which showed the utilisation of CYN in Biolog MT2 plates.

In the present study, it was clear that Biolog MT2 plate assay could be used as an easy method for screening the isolates utilising CYN. But it was not confirmed that degradation of CYN occurred. Hence, further investigative study is needed. The result obtained from the Biolog assay has led the design of a batch culture experiment to confirm the degradation of CYN which resulted no degradation. It could be due to chemical stability and the slow degradation (Wormer *et al*., 2008). Another reason may be the redox chemicals and the high bacterial cell numbers utilised in the microtitre plates which showed that the utilisation of CYN was not present in the batch culture experiments. This study also relates to the previous researchers' findings that biodegradation may initiate quickly with a pre exposure of *C. raciborskii* blooms (Smith *et al*., 2008). In contrast, a 40 day period study performed by Wormer *et al* (2008) on CYN degradation did not discover any influence of pre-exposure of toxin. This suggests the stability of the CYN toxin that makes it difficult for the biodegradation to occur. Degradation studies with higher cell numbers of bacterial isolates were examined to check for any influence but no positive signs were displayed. It was suggested that bacterial cell numbers may not be the reason for lack of degradation in batch culture experiments.

The present study to investigate the degradation of CYN was carried out in natural waters. Two natural waters were utilised and the experiment was performed over a period of four weeks demonstrating no degradation of CYN. The lack of CYN degradation may be due to no CYN degraders within the endemic microbial population in the waters. As per the previous studies

reported, pre-exposure to *C*. *raciborskii* blooms has some influence in the CYN degradation process. In the current study, none of the selected waters were pre-exposed to C. *raciborskii* bloom. Studies were carried out by collecting water from five different locations to identify any CYN degraders present. Results demonstrated that two water sources such as River Carron and Loch Forfar waters performed showing CYN degradation. The results obtained conflicted with studies performed by Smith *et al*., 2008. The present study indicates that bacteria or bacterial population could use CYN as a carbon source. Thus, the endemic microbial population present in the two water bodies may consist of CYN degraders. It was not confirmed that a particular bacterium or a bacterial consortia were capable of degrading the CYN. In order to examine further the experiment was carried out in the same waters but in a large scale batch culture experiment. Interestingly, results showed no degradation of CYN over a period of the 60 days of conducting the experiment. It was strange that the same water had been used for the previous experiment yet was found to eliminate CYN with treated water. One reason for this may be suggested that due to different sampling times which may cause the absence of CYN degraders within the waters.

The present biodegradation studies demonstrate that Biolog MT2 assay can be used as an easy and rapid screening method to detect the CYN metabolism. In order to confirm whether CYN degraded in biolog plate's a batch culture experiment was carried out. Two water sources showed the elimination of CYN but could not be traced to particular bacteria or consortia. Further investigation carried out to identify the degraders showed no degradation and an inability to detect any.

The difficulties suffered while analysing the environmental samples by HPLC, means the present study has had to include the development of purification, concentration and analysis. The HILIC method was developed to achieve an alternative option for better analysis of environmental samples. This is the method generally used for the analysis of highly polar analytes. The zwitterionic character of CYN makes this component highly water soluble. The charge present in the molecule itself gives a dipole effect, which suits the polar solvent. The developed method showed improved retention behaviour of the component. However, the chromatogram obtained with environmental samples showed poor results. So the present study chose to stay C_{18} with HPLC analysis.

In order to avoid or reduce matrix interference that was observed in the analysis of environmental samples by HPLC the decision was taken to use pre concentration step. SPE method was developed to pre concentrate the environmental samples prior to HPLC analysis. The developed SPE method was used for the pre concentration step and found to be efficient in removing other cross contaminants. ENV+ column is composed of resin based non-polar sorbent and has the ability to retain water soluble CYN. 100% methanol was found to be the best performing elution solvent, however, good recovery was observed in the increased concentration of MeOH used as elution solvent. In order to elute the low concentration of toxin and elute in small volume of sample, 100% of methanol was found to be the best solvent. Metcalf *et al.*, 2002 used 100% methanol supplemented with 0.1% (v/v) TFA found good recoveries using Hypercarb cartridge. By using 100 mg cartridge it was found that the analyte was eluted in a smaller elution volume 1.5 ml. Previous studies performed by Norris *et al*., 2001 and Metcalf *et al*., 2002 obtained a total recovery in about 8

ml. Norris *et al* used 300 mg but Metcalf *et al* did not mention the size. Another study by Wormer *et al*., 2009 reported a total recovery in 4 ml by using 500 mg cartridge size. All used graphitised carbon with C_{18} while the current study used ENV+ cartridges.

The present work also investigated the effect of photocatalytic treatment of CYN removal from environmental waters. Basically the work has utilised three types of titanium dioxide photocatalysts. In the presence of conventional UV radiation sources, titanium dioxide powder form Degussa P25 observed to be efficient in the elimination of CYN. The majority of the studies reported that anatase was the most effective photocatalyst and rutile less efficient. However, studies demonstrated that mixtures of anatase and rutile were more efficient than 100% anatase (Miyagi *et al.,* 2004). Degussa P25 is composed of 80% anatase and 20% rutile. Increased activity of this form documented interaction between two forms, reducing bulk recombination. The mineralisation of the toxin can be ascribed to the formation of reactive oxygen species (ROS) such as O_2^- and $OH^\bullet$. There were studies proposing that $OH^\bullet$ are responsible for the killing of Bacteria (Salih *et al*., 2002; Cho *et al.*, 2004, 2005). The rapid efficiency of the powder form can also be attributed to their highest surface area.

Pellet form Hombikat K01/C showed slow degradation of CYN in the presence of conventional UV light source. It was previously suggested that adsorption in the surface area of the catalyst might play an important role in photocatalysis efficiency (Hoffmann *et al*., 1995). In the pellet form catalyst adsorption is not equal to its surface area when exposed to UV light. While contributing to the total surface area of the pellet form, there is an inner matrix which may be shielded from the UV light and hence not activated. It was not

possible to justify the efficiency of the pellet form by only taking adsorption surface. Both adsorption surfaces with light exposed surface area could explain the slow degradation of pellet form. Also toxin may find it hard to penetrate through the pellet and reach the TiO_2 surface. However, this form of the catalyst solved the problem of removal from the treated water.

Another form of the catalyst used for the study was Photosphere™. Interestingly, it was found that degradation occurred with the same efficacy as that of Degussa P25. Catalyst loading was also performed and resulted in the lower loading still achieving better degradation of CYN. The actual amount of TiO2 in Photospheres™ was found to be 17%. It was interesting to see the degradation was the same as Degussa 25 where it composed of 100%. Small scale experiments carried out were found to be impractical with improper mixing and deficiency of oxygen leading to the design of a large scale UV LED reactor.

Studies with Novel LED reactor demonstrated the degradation of 50% CYN by using Photospheres™ over a time of 3 h. It was found to be slow. However, considering the energy output of the LEDs (67.5×10^{-3} W) this is several orders of magnitude lower than that of the conventional xenon lamp (450 W) used for the experiment. With using the conventional xenon lamp, degradation was achieved in 40 min. In comparison to, the previous Photospheres™ experiments that performed showing the same efficacy as Degussa P25, the present design has little in common. Photospheres™ (1% w/v) has been used in the previous experiment with a 3 ml of sample volume. For both UV LED and conventional UV light source the difference in performance is all too obvious. But it has been noticed, however, that more than half of the toxin was removed with 2000 times less energy expended. Further optimisation

has to be used perhaps by increasing the treatment time needs to be longer, also longer residence time in the reactor. Periodic illumination could be another option to achieve better performance with UV LEDs (Tokode *et al*., 2012). It can be highly recommended that the use of LEDs over conventional UV light sources as it consume less energy so the reduced energetic cost.

The current research has clearly demonstrated the limitations and inconsistency of biodegradation in the reduction of CYN in water, however, TiO_2 photocatalysis proved very successful and is worthy of further investigation to develop pilot scale treatment system. The findings of this research clearly indicate the benefits of TiO_2 Photocatalysis for the removal of CYN from water. This supports the previous findings that this method is useful for the removal of other cyanotoxins (Microcystin and Nodularin) also taste and odour compounds (Geosmin). Works now needs to be carried out to construct and evaluate an operation scale reactor to evaluate the suitability of this novel treatment method in the routine removal of these toxic hazards. It is envisaged that significant developments in the application of this technology will occur in the near future.

CHAPTER 6

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