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TITLE: The destruction of the cyanobacterial toxin microcystin-LR by semiconductor photocatalysis.

YEAR: 2000

THE DESTRUCTION OF THE CYANOBACTERIAL TOXIN MICROCYSTIN-LR BY SEMICONDUCTOR PHOTOCATALYSIS

By

Benjamin J P A Cornish

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

The Robert Gordon University, February 2000

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Dedication

For my Dad, always missed

Acknowledgements

I would like to thank my supervisors Dr. Linda A Lawton and Dr. Peter K. J. Robertson for their guidance and support during this project. My thanks also to Dr Marcel Jaspers and Dr Christine Edwards for their assistance in carrying out the project. I thank the many people in the department who have helped and advised me over the years. A special thanks to the departments technicians and to my fellow lab partners over the years; Sadie, Jacqui and Gail, I thank you.

Finally a very special thank you to my Mum, Dad, Sister, Nana and John for all their love, support and encouragement over these last few years.

Publications

The following publications have been produced during the duration of this project.

Robertson, P.K.J., Lawton, L.A., Cornish, B.J.P.A. and Jaspers, M., (1998) "Processes Influencing the Destruction of Microcystin-LR by TiO_2 Photocatalysis." *J. Photochem. Photobiol. A: Chem.* **116**, 215-219.

Lawton, L.A., Robertson, P.K.J., Cornish, B.J.P.A. and Jaspers, M., (1999) "Detoxification of Microcystins (cyanobacterial hepatotoxins) using TiO_2 Photocatalytic Oxidation." *Environ. Sci. Technol.* **33**, 771-775.

Robertson, P.K.J., Lawton, L.A., Munch, B. and Cornish B.J.P.A., (1999) "The Destruction of Cyanobaterial Toxins by Titanium Dioxide Photocatalysis." *J. Adv. Oxid. Technol.* **4**, 20-26.

Robertson, P. K. J., Lawton, L. A. and Cornish, B. J. P. A., (1999). "The involvment of phycocyanin pigment in the photodecomposition of the cyanobacterial toxin, microcystin-LR." *J. Porphyrins Phthalocyanines* **3**, 544-551.

Cornish, B. J. P. A, Lawton, L. A. and Roberson, P. K. J., (2000). "Hydrogen peroxide enhanced photocatalytic oxidation of microcystin-LR using titanuium dioxide." *Appl. Cataly. B: Environ.* **25**, 59-67.

Abstract

In fresh waters where cyanobacteria (blue-green algae) flourish, dense growths known as blooms occur. Such blooms present a threat to human and animal health as many of these cyanobacteria produce toxins. One such group of toxins are the microcystins which are hepatotoxic resulting in haemoraging and tumour promotion in the liver. There have been several reports of human poisonings resulting from the presence of cyanotoxins in potable waters, some of which have resulted in fatalities. The most frequently cited cyanotoxin in these poisonings has been microcystin-LR, which has prompted the World Health Organisation (WHO) to set a guideline for the recommended safe level of this toxin in drinking water of 1 $\mu g\ l^{-1}$.

Removal of microcystin-LR from potable waters has proven to be inefficient using conventional water treatment techniques such as coagualtion, filtration and chemical oxidation using chlorine. While activated carbon adsorption and membrane filtration have been shown to physically remove microcystin-LR from water the toxin is not destroyed. Recently the use of photocatalysis was shown to rapidly degrade microcystin-LR even at high concentrations. The process involves the illumination of a titanium dioxide catalyst with ultraviolet (UV) light to produce highly oxidising hydroxyl radicals in solution. While several researchers have demonstrated the process's effectiveness in degrading the toxin none have determined the fate of the compound, or if the toxicity related to microcystin-LR has been removed. This study was carried

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out to determine if photocatalytic oxidation of microcystin-LR was suitable as a treatment method for potable water supplies. Analysis of treated toxin samples by high performance liquid chromatography (HPLC) with photo-diode array detection (PDA) and mass spectroscopy established that the toxin was not completely degraded during photocatalysis. A simple toxicity assessment however indicated that by-products were non-toxic. Using the data from this work a proposed pathway for toxin destruction was produced giving the speculative identity of some of the by-products.

The use of hydrogen peroxide to enhance UV mediated destruction of microcystin-LR has been previously reported. There have also been reports of the enhancement of photocatalytic reaction in the presence of this oxidant. The work carried out in this study demonstrated that the destruction of microcystin-LR by photocatalysis was both more rapid and more efficient when hydrogen peroxide was present in the system.

The use of a fixed film flow reactor was also investigated for microcystin-LR destruction. While degradation of the toxin occurred it was demonstrated that batch reactors were more efficient as a treatment method. The effectiveness of the photocatalytic process on microcystin-RR, -LW and -LF was also investigated. While destruction of all the variants occurred during photocatalytic treatment each microcystin demonstrated different rates and efficiencies of photooxidation.

It was concluded from this study that photocatalysis is a promising treatment method for the removal of microcystin-LR and other variants from potable waters. Further research however is required to assess if the tumour promoting effects of microcystin-LR are rendered inactive and to determine the behaviour of the toxins degradation in natural water supplies. The study also allowed for speculation as to how the degradation of the toxin occurred during the photocatalytic process.

CHAPTER 1

Introduction

1.1 Cyanobacteria

Cyanobacteria are commonly referred to as 'blue-green algae' due to their similar morphology to algae and their ability to photosynthesise. They are a group of prokaryotic microalgae that are known to exist in a wide range of environments and were first recognised by botanists over 200 years ago (Skulberg *et al.*, 1993). Fossil records have indicated their existence 3.3 to 3.5 billion years ago and it has been suggested that they contributed to the oxygenation of Earth's primitive atmosphere through oxygenic photosynthesis. Cyanobacteria contain chlorophyll *a* and metabolise energy through oxygenic photosynthesis associated with photosystems I and II (Castenholz and Waterbury, 1989). It is widely believed that some of these early cyanobacteria were incorporated into other microbes leading to the evolution of chloroplasts which are the photosynthetic organelles in plants.

Cyanobacteria share a number of features with both green algae and bacteria which has led to confusion regarding their taxonomy (Mur *et al.*, 1999). While cyanobacteria may share similar morphologies to algae and are photosynthetic they do not possess nuclei and their cell walls are composed of peptidoglycan and lipopolysaccaride layers as opposed to cellulose (Carmichael and Falconer, 1993). As a result there has been some confusion as to how cyanobacteria should officially be classified either according to the International Code of Botanical Nomenclature (ICBN) (Greuter *et al.*, 1994) or by the International Code of Nomenclature of Bacteria (ICNB) (Sneath, 1992). Currently morphology is exclusively used to identify and classify cyanobacteria

although developments in molecular biology are also being utilised by taxonimists.

Found in fresh, salt and brackish waters cyanobacterial growth is influenced by various environmental factors including temperature, light intensity and nutrient status. In waters which are rich in nitrogen and phosphorus and when optimum light and temperature conditions prevail, such as in summer, cyanobacteria flourish forming dense surface growths known as blooms. Due to eutrophication caused by nutrients from agricultural effluents and human wastes the occurrence of such blooms has increased (Bell and Codd, 1994). Certain species of cyanobacteria are known to produce toxins and where such species form blooms there is a considerable associated health risk to both animals and humans.

1.2 Cyanobacterial toxins

Cyanobacterial toxins have been identified as the cause of many wild and domestic animal deaths in different areas of the world following ingestion of waters where toxic cyanobacterial blooms accumulate (Carmichael, 1992). There are three types of cyanobacterial toxins that have been identified: hepatotoxins, neurotoxins and lipopolysaccaride endotoxins. Hepatotoxins and neurotoxins are intercellular toxins which are produced by specific strains of cyanobacteria. When large cyanobacterial blooms breakdown and the cells are lysed there may be a high concentration of these toxins released into the

aquatic environment. Lipopolysaccaride endotoxins are present on the outer membrane of the cell wall and are common to all cyanobacteria.

1.2.1 The cyclic peptide hepatotoxins: microcystins and nodularins

The cyclic peptide hepatotoxins known as microcystins, as they were first isolated from *Microcystis* spp. are the most commonly occurring of the cyanobacterial toxins. Produced by various genera of cyanobacteria including *Microcystis*, *Anabaena*, *Oscillitoria* (*Planktothrix*), *Anabaenopsis*, and *Nostoc* they are found in both fresh and brackish waters globally (Carmichael, 1994). These cyclic heptapeptides share a common structure containing three D-amino acids (alanine, erythro-β-methylaspartic acid and glutamic acid), two variable L-amino acids, and two unusual amino acids; N-methyldehydroalanine and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Botes *et al.*, 1982). Different microcystins are named according to the two variable amino acids located at positions X and Z (Figure 1.1, Carmichael, 1997) and may also be distinguished by other structural features such as methylation and variations in the Adda structure (Carmichael *et al.*, 1988a and 1988b). The most commonly occurring microcystin contains the amino acids leucine (L) and arginine (R) in positions X and Y and is therefore called microcystin-LR. Over 60 microcystin variants have been characterised to date from both naturally occurring blooms and from isolated cyanobacterial strains (Sivonen and Jones, 1999).

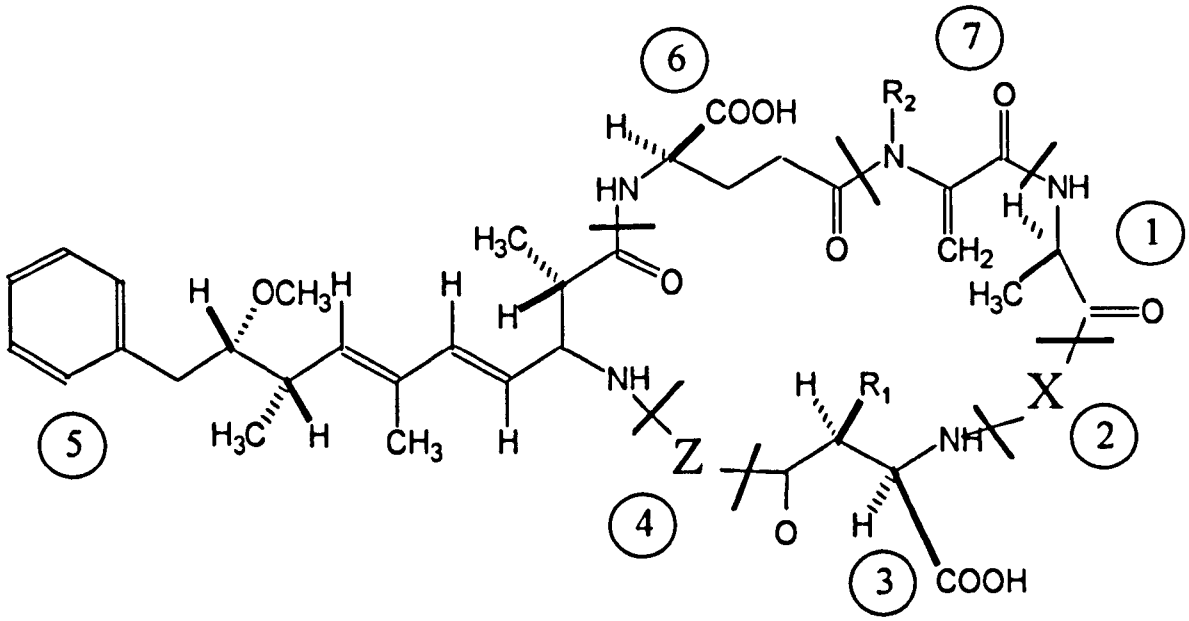


Figure 1.1: General structure of the cyclic heptapeptide hepatotoxins, microcystins, showing the most frequently found variations: cyclo-(D-Ala^1 -X^2 -D-MeAsp^3 -Z^4 -Adda^5 -D-Glu^6 -Mdha^7).

X and Z = the 2 variable amino acids.

R_1 and R_2 = H (demethylmicrocystins) or CH_3.

D-MeAsp = D-erythro-β-methylaspartic acid.

Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

Mdha = N-methyldehydroalanine.

The cyanotoxin nodularin is produced by the cyanobacterium *Nodularia spumigena* which is found predominantly in sea and brackish waters (Rinehart *et al.*, 1988) and several variants have also been identified. Nodularins are cyclic pentapeptides similar in structure to microcystins (Figure 1.2) and share the same mode of toxicity (Namikoshi *et al.*, 1993). The first recorded account of intoxication linked to cyanobacteria was in 1878 by George Francis (Francis, 1878) who described fatalities of horses, sheep, pigs and dogs that had ingested water from Lake Alexandria, South Australia containing a bloom (later identified as *N. spumigena*). By exposing a calf to bloom material samples from this lake he was able to confirm that ingestion of cyanobacteria was the cause of these animals deaths.

Acute exposure to these hepatotoxins results in liver damage in mammals leading to death (Beasley *et al.*, 1989, Runnegar *et al.*, 1988). Due to the water soluble nature of most of these toxins they are unable to enter cells through hydrophobic cell membranes. However following ingestion they may be transported to the liver by bile-acid carriers in hepatocytes and in the cells lining the small intestine (Runnegar *et al.*, 1991, Falconer *et al.*, 1992).

Toxicity is characterised by the disruption of cytoskeletal components in liver cells leading to the shrinking and separation of hepatocytes allowing blood to accumulate in the liver (Carmichael, 1994). Symptoms of intoxication following ingestion of high quantities of these compounds include weakness, diarrhoea and vomiting (Carmichael, 1992). Intoxication can culminate in intrahepatic haemorrhage and hypovolaemic shock leading to death within several hours or

several days (Theiss *et al.*, 1988). Apart from incidents involving the death of dogs and sheep there are also reports of fatalities of birds (Carmichael and Falconer, 1993) and fish (Kotak *et al.*, 1996) resulting from these toxins. Andersen *et al.* (1993) has also demonstrated hepatocyte damage in Atlantic salmon following microcystin intoxication. However in quails the spleen has been found to be the target organ for microcystins (Takahashi and Kaya, 1993) instead of the liver. The toxic effects of both microcystins and nodularins are associated with the inhibition of protein phosphatases 1 (PP1) and 2A (PP2A) (Eriksson *et al.*, 1990; Yoshizawa *et al.*, 1990). These enzymes are present in all eukaryotic cells and control the activities of proteins by catalysing the dephosphorylation of serine and threonine residues (Cohen, 1989). Inhibition of PP1 and PP2A by these toxins results in excessive phosphorylation of cytoskeletal proteins in the liver by protein kinases leading to hepatocyte deformation (Eriksson *et al.*, 1990). Both microcystins and nodularins have been shown to inhibit PP1 and PP2A with the same potency (Yoshizawa *et al.*, 1990; Honkanen *et al.*, 1994). Structural studies have shown that inhibition is mediated through interactions between the Adda-glutamine portion of the toxins with a hydrophobic pore in the enzyme molecule (Goldberg *et al.*, 1995; Rudolph-Böhner *et al.*, 1994). The binding of microcystins to PP1 has been shown to be particularly stable due to covalent bonding between the methyldehydroalanine residue of the toxin and the sulphur atom of Cysteine-273 in the protein phosphatase molecule (Goldberg *et al.*, 1995; MacKintosh *et al.*, 1995).

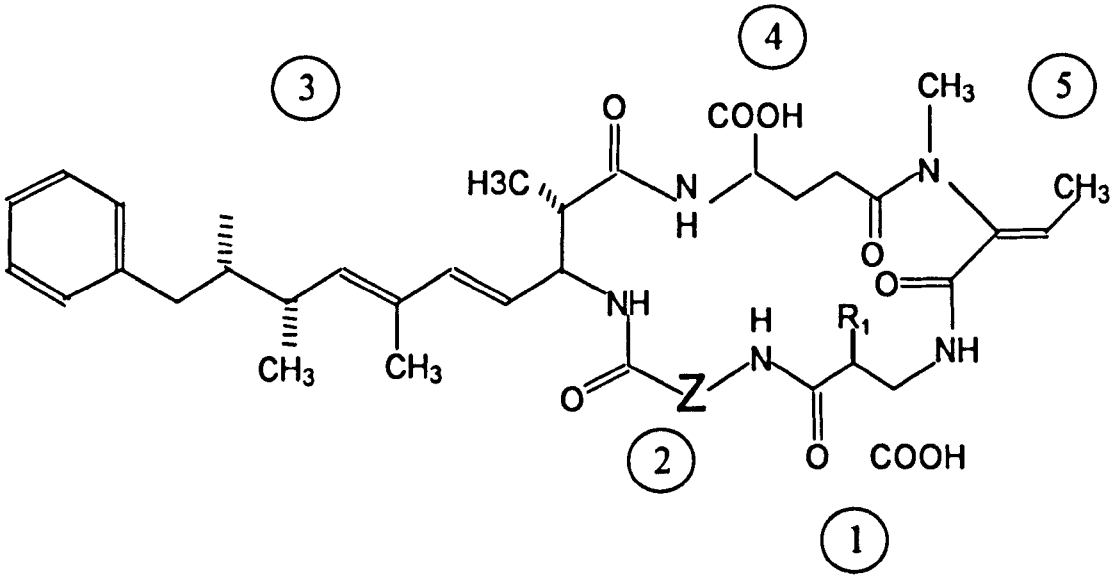


Figure 1.2: Structures of nodularins: cyclo-(D-MeAsp^1 -Z^2 –Adda^3 -D-Glu^4 – Mdhb^5).

Z = L-arginine

R_1 and R_2 = H or CH_3.

D-MeAsp = D-erythro-β-methylaspartic acid.

Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

Mdhb = N-methyldehydrobutyrin.

Long term low level exposure to cyanobacterial hepatotoxins also presents a health risk to humans and animals. These cyanotoxins have been shown to behave in a similar manner to the diarrhetic shellfish toxin, okadaic acid which is a potent tumour promoter (MacKintosh and MacKintosh, 1994). The tumour promoting activity of microcystin-LR has been demonstrated in rats (Nishiwaki-Matsushima *et al.*, 1992) which had been pre-treated with a tumour initiator while Ito *et al.* (1997) observed nodular formation in mouse livers even though no tumour initiator had been used. This tumour promotion in mouse livers was achieved by intraperitoneal injection of subleathal doses of the toxin over a 28 week period, no nodule formation was observed when the mice were administered the toxin orally.

1.2.2 The cytotoxic alkaloid cylindrospermopsin

Cylindrospermopsin is a hepatotoxin with a different structure and mode of toxicity to microcystins and nodularins. The toxin is a cyclic guanidine alkaloid (Figure 1.3) originally associated with the cyanobacterium *Cylindrospermopsis raciborskii* found in tropical and subtropical waters. Toxicity studies have shown that mice injected with cylindrospermopsin survive longer than mice administered microcystin or nodularin (Bell and Codd, 1996). Unlike microcystins and nodularin this hepatotoxin does not inhibit PP1 and PP2A and appears to have toxic effects on a number of other vital organs as well as on the liver (Ohtani *et al.*, 1992). Terao *et al.* (1994) have shown that cylindrospermopsin intoxication using pure toxin causes lipid

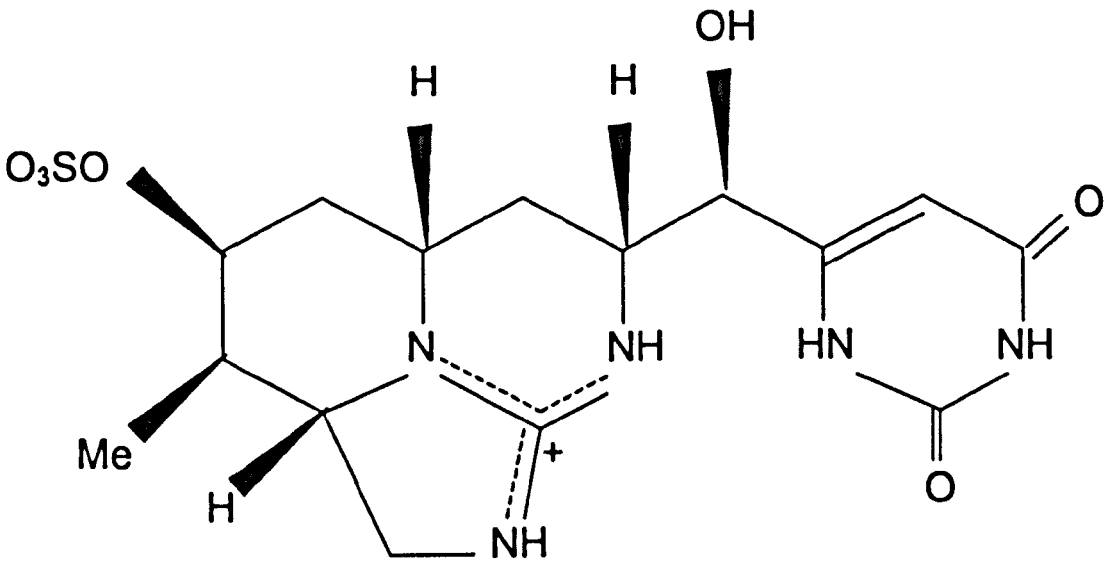


Figure 1.3: Structure of cylindrospermopsin.

accumulation in mouse liver cells. Hawkins *et al.* (1985) found more widespread damage including kidney, heart, lung and spleen necrosis when mice were exposed to crude material from lysed cells of *C. raciborskii.*

1.2.3 The neurotoxic alkaloids: anatoxin and saxitoxin

Cyanobacterial alkaloid neurotoxins are produced by certain species of *Anabaena*, *Oscillitoria* (*Planktothrix*), *Aphanizomenon* (Carmichael, 1997) and *Cylindrospermposis* (Lagos *et al.*, 1997). There are three classes of neurotoxins; saxitoxins, anatoxin-a and anatoxin-a(s).

Saxitoxins are also known as paralytic shellfish toxins (PST) and were originally isolated from shellfish where it has accumulated from feeding on dinoflagellates in the marine environment (Hashimoto and Noguchi, 1989). They are a group of carbamate alkaloids with a general structure (Figure 1.4) of which over 20 variations exist and are divided into three distinct groups. Non-sulphanated saxitoxins (STX) are the most highly toxic and include saxitoxin and neo-saxitoxin. Singly sulphanated (gonyautoxins – GTX) which are of intermediate toxicity and doubly sulphanated (C-toxins) which are the least toxic of the saxitoxin family. The toxin acts by blocking sodium channels and inhibiting the action of neurones causing muscle cells to receive no stimulation, effectively paralysing them (Kao, 1993). Saxitoxins were implicated in the death of over 1000 cattle and sheep drinking from the Darling

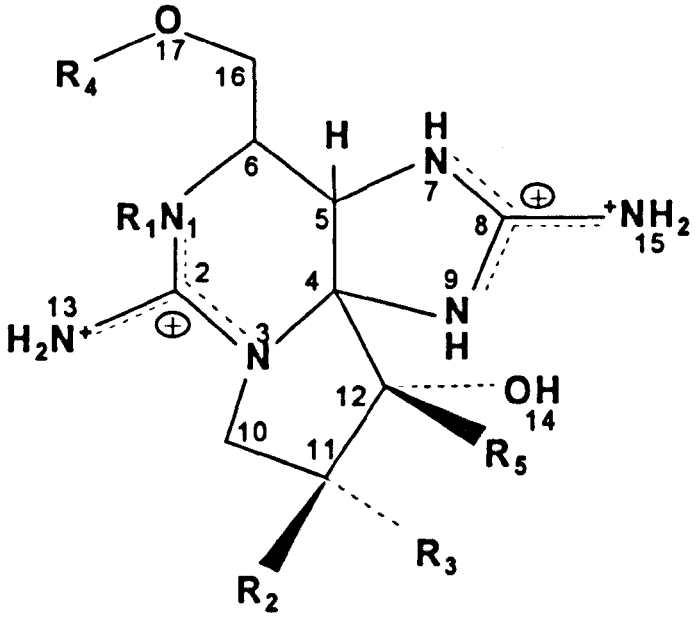


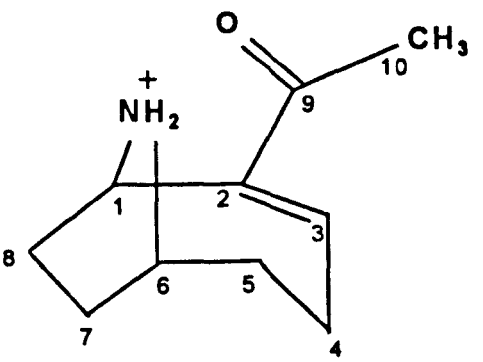
Figure 1.4: General structure of saxitoxins. $R_{1\text{-}5}$ represents variable groups distinguishing between non-sulphanated saxitoxins (STX), singly sulphanated (gonyautoxins-GTX) and doubly sulphinated (C-toxins).

River in Austraila in 1991 where a cyanobacterial bloom was present (Humpage *et al.*, 1994).

Anatoxin-a is a low molecular weight secondary amine (Figure 1.5 a) and was the first cyanotoxin to be chemically characterised (Devlin *et al.*, 1977). It is produced by cyanobacteria of the genus *Anabaena*, *Aphanizomenon* and *Oscillatoria* and has resulted in the poisonings of animals exposed to waters contaminated with these toxins (Carmichael and Gorham, 1978; Edwards *et al.*, 1992). The toxin activates the flow of ions which induces muscle contractions by mimicking the neurotransmitter acetylcholine. Normally muscles return to a resting state following degradation of acetylcholine by an enzyme (acetylcholinesterase) however, anatoxin-a is not degraded by this mechanism. The neurotoxin therefore continues to act on the muscle causing overstimulation and eventually the muscle cells stop functioning resulting in paralysis, respiratory arrest and death (Carmichael, 1997).

Anatoxin-a(s) is produced by *Anabaena flos-aquae* (strain NRC 525-17), (Matsunaga *et al.*,1989) and by certain strains of *Anabaena lemmermannii* (Henriksen *et al.*, 1997). The neurotoxin is a phosphate ester (Figure 1.5 b) and has a similar mode of toxicity to organophosphate insecticides such as malathion (Carmichael, 1994). Unlike anatoxin-a which mimics acetylcholine causing muscles to overstimulate, anatoxin-a(s) permits acetylcholine to bind to receptors as normal. However the anatoxin-a(s) inhibits the enzyme acetylcholinesterase which breaks down the neurotransmitter thereby

(a)



(b)

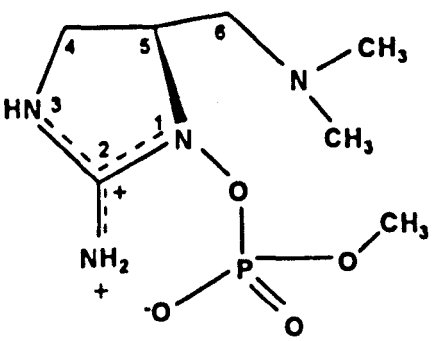


Figure 1.5: Structure of (a) anatoxin-a and (b) anatoxin-a(s)

preventing the muscles from relaxing, thus leading to overstimulation. Studies have shown anatoxin-a(s) to be about 10 times more toxic than anatoxin-a (Carmichael, 1992) with symptoms of intoxication being similar in both cases. Mahmood and Carmichael (1986) indicated that when anatoxin-a(s) was present in animal poisoning excessive salivation occurred, this symptom is not present during intoxication with anatoxin-a. Like anatoxin-a this cyanotoxin has also been implicated in the deaths of dogs (Mahmood *et al.*, 1988) and also in birds (Onodera *et al.*, 1997) where they have been exposed to cyanobacteria producing these toxins.

1.2.4 The lipopolysaccaride endotoxins

As with all other Gram-negative bacteria cyanobacteria possess cell walls which are composed of lipopolysaccarides. Considerably less toxic than either the hepatotoxins or neurotoxins these compounds are pyrogenic (fever causing) (Weckesser and Drews, 1979). Studies by Raziuddin *et al.* (1983) have indicated that these compounds are ten times less potent than lipopolysaccarides of other bacteria such as *Salmonella*. These cyanotoxins may cause skin irritations, allergic reactions and have also been known to cause gastrointestinal disorders when ingested (Keleti *et al.*, 1979).

1.3 The occurrence of cyanobacteria in water bodies

Surveys carried out by Rapala and co-workers (1994) determined that over 50% of cyanobacterial blooms occurring globally were toxic. A monitoring programme carried out by the National Rivers Authority in the UK showed that 68% of blooms investigated contained cyanotoxin producing species (Pearson *et al.*, 1990). It is therefore necessary to control the occurrence of cyanobacterial blooms and in cases where they have already formed take action in order to prevent poisoning of animals or humans.

1.3.1 Control and treatment of blooms

Studies have shown that growth of cyanobacteria is influenced by a number of environmental factors including temperature, light intensity, nutrient availability and pH. It has also been shown that toxin production is optimised in conditions that favour cyanobacterial growth. Watanabe and Oishi (1985) demonstrated that microcystin and anatoxin-a production in cyanobacterial cells was favoured at temperatures of 18-25 °C while the influence of nutrient availability has been shown by many researchers (Kotak *et al.*, 1995; Sivonen, 1990). Limiting those conditions which promote the growth of cyanobacteria prevents bloom formation in the natural environment. As eutrophication is a primary cause of such blooms, minimising input of nitrogen and phosphorus into water bodies form agriculture and industry would be an ideal preventative

method. However such an undertaking would be ineffective in waters that are naturally high in nutrients and other approaches are required. Visser *et al.* (1996) implemented the use of artificial aeration which mixes and destratifies water bodies and inhibits growth by limiting light availability.

In the case of blooms that have already formed the use of algicides has been employed to break-up cyanobacterial masses. The most commonly used chemical algicide is copper sulphate although Newman and Barrett (1993) advocated the use of barley straw which is thought to release algicidal phenolic compounds when it decomposes. However studies have shown this technique to be of variable success, and when used in a field trial by Cheng *et al.* (1995) a cyanobacterial growth was unaffected by barley straw for a six month period. Algicidal treatment of blooms has however been condemned by some researchers as it leads to high levels of toxins in the water body being treated (Watanabe *et al.*, 1992, Kenefick *et al.*, 1993). Cyanobacterial toxins are predominantly intracellular with only a small percentage being extracellular (Sivonen, 1990). Following algicide treatment the cells lyse and high concentrations of toxins are released into the water body. These releases of toxins into fresh water reservoirs have been implicated in several outbreaks of human illness.

1.3.2 Persistence and degradation of toxins in water

It has been shown by many researchers that intracellular microcystins are relatively stable in laboratory cultures and this would therefore appear to be the case in the natural environment (Sivonen, 1990). Jones *et al.* (1995) found that dried cyanobacterial crusts present on lake shores have high microcystin levels even after several months. Jones (1990) also demonstrated that once released from cyanobacterial cells' degradation of microcystin-LR occurred in surface waters, however this was found to take from three days to three weeks. Tsuji *et al.* (1994) indicated that microcystins in water bodies were more rapidly decomposed in the presence of photosynthetic pigments and sunlight suggesting that photodegradation influences persistence. Cousins *et al.* (1996) showed that low concentrations of microcystin-LR (10 $\mu g\ l^{-1}$) were degraded by mixed bacterial populations present in reservoir waters in less than a week. Currently the majority of persistence studies of toxins in fresh waters have been concerned with microcystins, fewer studies have been carried out on cylindrospermopsin or the neurotoxins. Rapala *et al.* (1994) demonstrated the ability of bacterial populations to bring about the degradation of both microcystins and anatoxin-a. However this study was performed on toxins purified from laboratory cultures and has yet to be demonstrated in natural fresh waters. Jones and Negri (1997) indicated that saxitoxins released from cells persisted for over 90 days in river water making these toxins the most persistent known to date.

1.4 Human health aspects

There have been a number of incidents where animal deaths have been linked to ingestion of waters containing toxic cyanobacteria. There have also been reports from around the world of human poisonings and fatalities linked to cyanobacterial toxins via exposure from both potable water supplies and other alternative routes.

1.4.1 Exposure via drinking water

In 1931 there were reports of gastro-enteritis in residents from towns situated along the Ohio River although no pathogenic organisms were detected in the water (Tisdale, 1931). The water supplies for these towns had been contaminated by a cyanobacterial bloom that had entered from a side branch of the river and this was believed to be the cause of the illness. In 1979 a more serious incident involving cyanobacterial toxins occurred in a town served by water from a dam on Palm Island situated off the north-eastern coast of Australia. Following exposure the community experienced a severe outbreak of hepato-enteritis that resulted in the hospitalisation of 10 adults and 140 children (Blyth, 1980). The poisonings were attributed to a cyanobacterial bloom which had been present in the water supply and had been treated with copper sulphate. The bloom material was identified as *C. raciborskii* which produces the cyanotoxin cylindrospermopsin. A more lethal epidemic of gastro-enteritis occurred following the flooding of a newly constructed dam in

Brazil in which 88 deaths were reported (Teixera *et al.*, 1993). Repeated low level exposure of microcystins has also been implicated in the high occurrence of liver cancer in south east China (Yu, 1995). Liver cancer mortalities were found to be higher (100 per 100,000 population) in areas where water was acquired from eutrophic ponds and water bodies found to be affected by an abundance of cyanobacterial blooms. This was in comparison to a lower level of 20 liver cancer mortalities per 100,000 population in areas where water was extracted from wells. To date, there have been no reports of human intoxication due to the presence of neurotoxins in contaminated fresh waters.

1.4.2 Alternative routes of exposure

Aside from exposure to cyanobacterial toxins through drinking water there have been a number of reports of human poisonings from alternative routes of exposure. Skin contact with bloom material by swimmers has resulted in illness, irritation and contact dermatitis (Codd *et al.*, 1989). Allergic reactions such as asthma and hay fever have also been associated with contact with cyanobacterial blooms (Falconer, 1994). Accidental ingestion of toxic cyanobacteria in recreational waters has also resulted in human illnesses. In 1989 a bloom of *M. aeruginosa* in Rudyard Lake, Staffordshire, UK was believed to be responsible for the poisoning of army recruits who used the lake for swimming and canoeing (Turner *et al.*, 1990). Diarrhoea, vomiting, mouth blisters and throat irritation were among symptoms experienced by individuals

following exposure while two recruits required hospitalisation after developing pneumonia like symptoms due to inhalation of water containing microcystis.

One of the most recent human poisoning incidents involving cyanotoxins occurred in a haemodialysis clinic in Caruaru, Brazil. Following treatment 86% of the patients experienced visual disturbances, nausea and vomiting prior to developing cholestatic liver disease. Examination of liver tissues of those patients affected indicated similar hepatocyte abnormalities to those seen in laboratory animals exposed to microcystins. Following analysis of the activated carbon filter of the clinic's water treatment system the presence of several microcystins including microcystin-LR was reported. More than forty of those patients affected in the incident have since died due to microcystin poisoning (Dunn, 1996, Jochimsen *et al.*, 1998).

Humans are most commonly exposed to the neurotoxin saxitoxin through consumption of shellfish, such as clams and mussels which accumulate the toxin by ingesting dinoflagellates. Negri and Jones (1995) showed that certain species of freshwater mussels accumulate saxitoxins from the cyanobacterium *Anabaena circinali.* Ingestion of large amounts of saxitoxins by humans leads to acute illness known as paralytic shellfish poisoning (PSP). Symptoms range from numbness or a tingling sensation in the mouth and lips, paralysis and death resulting from respiratory failure. Of over 1000 reports of PSP cases in North and Central America in the last decade approximately 10% have resulted in death (Kuiper-Goodman *et al.*, 1999). Such accumulation in shellfish is not however restricted to saxitoxins. Microcystins have been shown

to accumulate in zooplankton (Watanabe *et al.*, 1992), crayfish (Lirås *et al.*, 1998), freshwater mussels (Eriksson *et al.*, 1989, Vasconcelos, 1995) and fish (Carbis *et al.*, 1997).

A more recent concern to human health has been the possibility of cyanotoxins accumulating in food crops irrigated by water containing these toxins. While no poisonings have been reported by toxin consumption via this route current research has indicated that plants are capable of accumulating toxins.

1.5 Detection and analysis of cyanobacterial toxins in water

Increased incidents of cyanobacteria in potable water supplies has led to the need for analytical methods capable of detecting, identifying and quantifying cyanotoxins which may be present. While research is still ongoing in the field of cyanotoxin detection there are currently a number of techniques available.

1.5.1 Biological assays and detection methods

The longest established biological detection method for cyanobacterial toxins in blooms has been the mouse bioassay. Laboratory bred mice (male Swiss albino) are administered bloom lysate by intraperitoneal injection observed for symptoms of intoxication then killed using approved methods (Falconer, 1993). The quantification of toxicity is determined as the concentration of material that

is lethal to 50% (LD_{50}) of the animals exposed to the material. Samples are not taken to be toxic at an LD_{50} value greater than 1000 mg cell dry weight per kg mouse body weight (Lawton *et al.*, 1994). The assay not only allows quantification of overall toxicity of a sample but observation of symptoms may indicate types of toxin present. Death of animals within short periods of exposure (often less than 15 minutes) implies the presence of neurotoxins. The use of the mouse bioassay has provided data on the potencies of various cyanotoxins both within toxin groups (such as hepatotoxins and neurotoxins) and overall. Research had shown that toxicity of microcystins is dependent on the cyclic nature of the compound (Rinehart *et al.*, 1994) a conserved D-glutamic acid (Stotts *et al.*, 1993) and Adda moiety (Harada *et al.*, 1990). The main problem associated with the mouse bioassay is its non-specific nature. Quick death induced by the presence of neurotoxins in the sample will mask the presence of slower acting hepatotoxins resulting in the underreporting of the sample's toxin profile. Public opposition to the use of animals in laboratory testing has led to the decreasing use of this biological assay and is not permitted by law in certain countries (Falconer, 1993). Legislative restrictions in the use of the mouse bioassay has led to the requirement for developing new bioassay detection methods (Heinze, 1996).

A suitable replacement for the mouse bioassay has been the use of *in vitro* mammalian cells. Microcystin is well documented as causing acute liver damage which has prompted the use of freshly isolated rat hepatocytes (liver cells) for the bioassay (Aune and Berg, 1986). Good correlation was reported between results from the mouse bioassay and toxicity measured by leakage of

the enzyme lactate dehydrogenase from the hepatocytes. Two other such bioassays using hamster fibroblast cells (Codd *et al.*, 1989) and blood cells (Carmichael and Bent, 1981) were found to indicate microcystins but were not deemed suitable mouse bioassay replacements. An *in vitro* cell bioassay has also been developed for detecting saxitoxins. The use of the neuroreceptor binding assay (Davio and Fontelo, 1984: Doucette *et al.,* 1994) uses radiolabelled saxitoxin and has been shown to correlate well with the mouse bioassay (Cembella *et al.*, 1995).

Microcystins and nodularins have also been detected using the protein phosphatase inhibition assay which is based on the biochemical activity of the toxins. Two types of the assay exist, the first is based on the quantification of ^{32}P-phosphate released from a radiolabelled substrate by the activity of the protein phosphatase enzyme (PP1 and PP2A), an activity which is inhibited by the cyanotoxins (MacKintosh *et al.* 1990; Holmes, 1991). Lambert *et al.* (1994) successfully quantified microcystins in drinking water using this method however there is a need for radioactivity that requires specialised laboratory equipment and regulations which has deterred its use. The colorimetric protein phosphatase inhibition assay is also based on the release of phosphate from phosphorylated protein substrates. However this method is based on quantification of the chromogenic substrate *p*–nitrophenol phosphate (An and Carmichael, 1994; Ash *et al.*, 1995) as opposed to a radiolabeled substrate and therefore does not require the need for radioactivity. Although the colorimetric method is less sensitive than the radioactive assay it has been

shown to correlate well with high performance liquid chromatography (HPLC) detection of microcystin (Ward *et al.*, 1997).

An immunological detection assay has been developed which is shown to be ideal for rapid microcystin detection due to its sensitivity, specificity and ease of use. The Enzyme-Linked Immunosorbent Assay (ELISA) employs the use of either polyclonal or monoclonal antibodies raised against microcystins. An ELISA technique has been demonstrated to be suitable for use in water quality testing (Chu *et al.*, 1990).

The introduction of the invertebrate bioassays has provided an alternative biological detection assay which can be carried out in basic laboratories, requires no specialised handling or equipment and is deemed more ethical than using mammals. The use of the brine shrimp (*Artemia salina*) bioassay for the detection of hepatotoxins has proved favourable as it is inexpensive, simple to perform and compares well with the mouse bioassay and HPLC analysis (Lawton *et al.*, 1994). Brine shrimp larva are exposed to a dilution series of test samples then incubated for 18 hours before toxicity is determined and expressed as the concentration of sample required to cause 50% mortality (LC_{50}). A similar invertebrate bioassay using *Daphnia* has been investigated for hepatotoxin detection. The method is again inexpensive and compares well with the mouse bioassay however difficulty has been experienced due to varying sensitivities between species and culturing conditions (Baird *et al.*, 1989).

Bioassays utilising insects have also been developed for cyanotoxin detection. Microcystin-LR has insecticidal properties with a potency comparable to insecticides malathion and rotenone (Delaney and Wilkins, 1995). Turell and Middlebrook (1998) demonstrated that adult female mosquitoes (*Culex pipiens*) were sensitive to very low levels of the toxin following intrathoracic microinjection. An alternative assay was developed using fruit flies (*Drosophila melangaster*) as the mosquitoes were difficult to handle for toxin administration (Swoboda *et al.*, 1994). The fruit fly assay was a more practical assay as the toxin was administered orally rather than through injection and could therefore be carried out in laboratories without specialist equipment. The assay gave data that correlated well with the mouse bioassay for hepatotoxicity detection in bloom material. The method however was not able to provide detailed information such as dose response, but has been recommended for toxicity indication. Ross *et al.* (1985) developed a detection assay for saxitoxins using adult houseflies (*Musca domestica*). While the assay has proved comparable to the mouse bioassay it involves microinjection administration of toxin extracts which requires expertise. The use of locusts has also been investigated for the detection of saxitoxins (McElhiney *et al.*, 1998). These insects have been shown to be easier to handle with toxin administration by injection. The locust assay however has been shown not to be sensitive to either anatoxin-a or microcystin-LR.

Bacterial based bioassays have also been investigated in cyanotoxin detection. The basis of the assay is that toxins will inhibit bioluminescence or pigment production by certain types of bacteria. The Microtox

bioluminescence assay is a commercially available system that utilises the luminescence of *Photobacterium phosphoreum*. Test solutions are incubated with the bacteria and toxicity is expressed as EC_{50} (effective concentration resulting in 50% reduction in emitted light). Dierstein *et al.* (1989) demonstrated that the production of the pigment prodigiosin by the bacterium *Serratia marcescens* was inhibited by microcystins and saxitoxins. The assay provided a dose dependent response with extracts from *M. aeruginosa* and *Aphanizomenon flos-aquae*, however, when purified microcystin was used the results did not correlate. This suggested that other compounds were present in the extracts that resulted in pigment inhibition giving false positives.

1.5.2 Analytical techniques

A number of analytical techniques have been developed for the detection of cyanotoxins that are both quantitative and qualitative. The most commonly employed analytical system is HPLC. When used in conjunction with photodiode array detection (PDA) reverse phase HPLC has proven to be a very successful analytical method for microcystins. Typically in such a system microcystins are separated on a C18 silica column then detected by PDA at 238 nm followed by identification by their characteristic adsorption profiles between 200 and 300 nm (Lawton *et al.*, 1994). The reverse phase HPLC-PDA system has also been successfully used for the detection of cylindrospermopsin at a wavelength of 262 nm (Hawkins *et al.*, 1997). Anatoxin-a has also been detected in samples using the system and the toxin

is identified by its characteristic UV absorbance at 227 nm (Harada *et al.*, 1989; Edwards *et al.*, 1992). The use of HPLC-PDA has also been investigated to monitor levels of cylindrospermopsin (Harada *et al.*, 1994; Hawkins *et al.*, 1997).

HPLC-PDA relies on both retention times and UV adsorption profiles to identify microcystin variants leading to a limited ability to distinguish between individual microcystins as most of these compounds have similar spectra. Separation and identification of microcystins may be carried out simultaneously using liquid chromatography/mass spectroscopy (LC-MS) (Kondo *et al.*, 1992; Edwards *et al.*, 1993). The use of MS allows distinction between microcystin variants due to the different molecular weights of individual microcystins.

The detection of saxitoxins can be achieved using reverse phase HPLC in conjunction with fluorescence detection. Fluorescent saxitoxin analogues are formed following the oxidation of the toxin which is carried out either before the sample is injected onto the separation column (prechromatographic) or after separation on the column (postchromatographic). An HPLC method using prechromatographic oxidation of shellfish samples with either peroxide or periodate has been developed by Lawrence *et al.* (1995) which has been used in the analysis of shellfish extracts for a range of saxitoxin variants. Following sample oxidation the toxins are separated by reverse phase HPLC and detected using a fluorescence detector set at 330 nm (excitation) and 400 nm (emission).

The detection of saxitoxins has been investigated using capillary electrophoresis (CE) and involves the separation of toxins according to molecular size and charge (Bell and Codd, 1996). Furthermore, this method has been used in combination with MS for the detection of saxitoxins in dinoflagellate and shellfish extracts (Thibault *et al.*, 1991).

1.6 Removal of cyanotoxins from drinking water

With the increasing concern over human poisonings resulting from consumption of cyanotoxins in drinking water, studies have been carried out to determine if water treatment processes are removing these compounds. The most frequently cited cyanotoxin in human and animal poisonings has been microcystin-LR (Carmichael, 1997) therefore the majority of investigations have focused on its removal. A guideline for the recommended safe level of this toxin in drinking water of 1 $\mu g\ l^{-1}$ has recently been adopted by the World Health Organisation, (WHO, 1998). It is therefore important to ascertain if water treatment processes currently being used, as well as newer technologies, are capable of reducing toxin levels below the guideline value.

1.6.1 Removal by physical treatment methods

Conventional water treatment methods such as coagulation, flocculation and

sedimentation processes, which rely on physically removing pollutants, have been shown to effectively remove intact cells of *M. aeruginosa* from potable waters. Chow and co-workers (1999) found that cells treated with aluminium sulphate for coagulation followed by flocculation were not affected by the process and could be successfully removed without cell damage and hence toxin release. However it is not always the case that cyanotoxins are contained within cells, algicide treatment and natural degradation of bloom material may result in the contamination of water bodies with substantial quantities of soluble toxins.

The processes of coagulation and flocculation have however been shown to be ineffective in treating dissolved toxins (Hoffman, 1976; Drikas, 1994; Rositano and Nicholson, 1994). Sand filtration was demonstrated to remove over 80% of microcystins, 30-65% removal of toxins from *Oscillatoria* and nearly 70% of anatoxin-a (Keijola *et al.*, 1988). It was believed however that this removal of toxin was not as a result of physical removal by adsorption to the sand but instead due to degradation by bacterial populations present in the system.

An effective method for the physical removal of dissolved cyanotoxins in water is the use of activated carbon. Powdered activated carbon (PAC) has been demonstrated by researchers in laboratory studies to remove over 95% of microcystin-LR and nodularin (Donati *et al.*, 1994; Keijola *et al.*, 1988;). Lambert *et al.* (1996) demonstrated that an average of 82% of microcystins were removed from raw waters in a full scale water treatment plant. Studies

using PAC have shown that high doses of material are required with sufficient contact time to make the process effective. It was also found that in waters with high organic content the effectiveness of the process was reduced due to competition for adsorption. Granular activated carbon (GAC) has also been studied for cyanotoxin removal with similar results for those obtained using PAC (Lambert *et al.*, 1996). However GAC was demonstrated to have a limited life span due to competitive adsorption of organic matter in raw waters with only between 40 – 60% microcystin removal. One problem encountered with the use of activated carbon is that the toxins are not destroyed and when the activated carbon is spent it must be treated as toxic (Lawton and Robertson, 1999).

The removal of microcystins from drinking water using activated carbon on a domestic scale has also been reported (Lawton *et al.*, 1998). Filter jug systems that utilise granulated activated carbon and ion exchange resin were tested for their ability to remove microcystin-LR from tap water. The removal efficiency was found to be variable and performance declined with the increasing volume of water passed through the cartridge. It was however unclear whether the activated carbon or the exchange resin was the primary removal agent.

Reverse osmosis (RO) semi-permeable membranes are used in water treatment to desalinate brackish and estuarine waters to provide potable water. Similar to filtration, it separates suspended or dissolved components from the water being treated and is capable of removing organic compounds

with molecular weights above 100. The molecular weight of microcystins is around 1000 and therefore it can be assumed that this process will efficiently remove these compounds (Lawton and Robertson, 1999). Neumann and Weckesser (1998) showed that microcystin-LR and -RR gave removal efficiencies of greater than 95% while other researchers demonstrated the removal of nodularin (Vuori *et al.*, 1997). However, as with activated carbon adsorption the cyanotoxins have not been degraded and the process of RO results in the formation of wastewater containing high concentrations of toxin that must then be treated further.

1.6.2 Removal by chemical treatment methods

The effectiveness of water treatment chemicals to degrade cyanotoxins has also been investigated. Nicholson and co-workers (1994) investigated the use of the water disinfecting chemicals chlorine and chloramines for the removal of cyanotoxins. While chlorine appeared to remove microcystin-LR and nodularin in waters with pH less than 8, chloramines had little effect on toxin concentrations. Previous work on the effect of chlorine on microcystins had shown the chemical to be ineffective (Hoffman, 1976; Keijola *et al.*, 1988, Himberg et al., 1989). Nicholson however, had found that chlorine destruction was pH dependent and that previous studies were carried out at pH values shown to inhibit destruction. The levels of chlorine used by Nicholson *et al.* for toxin destruction was also relatively high compared to that generally used in water treatment (chlorine residual of at least 0.5 mg ml^{-1} for 30 minutes contact

time). The use of such high chlorine levels in water containing high organic contents may lead to the formation of organic halogens (i.e. trihalomethane) which are carcinogenic (Bellar *et al.*, 1974). While toxin destruction was observed there has still to be research undertaken as to how the toxins are degraded and if any harmful by-products are formed. The pH dependent destruction of the toxin may also be difficult to implement on a larger water treatment scale.

Other chemical oxidising agents investigated for cyanotoxin removal are potassium permanganate and hydrogen peroxide (H_2O_2). Potassium permanganate was shown to remove 95% of microcystin-LR (200 $\mu g\ l^{-1}$) within 30 minutes (Rositano *et al.*, 1998). When compared with chlorine it was found that this treatment method was more effective. Little work has been carried out on the effectiveness of H_2O_2 on the oxidative destruction of cyanotoxins. Researchers have shown that H_2O_2 was ineffective for removing microcystin-LR from water. Rositano and co-workers (1998) demonstrated that there was no reduction in initial toxin concentration (1 mg l^{-1}) after 10 minutes of treatment using a 2 mg l^{-1} H_2O_2 solution. Drikas (1994) however achieved 17% toxin removal after 1 hour of treatment using a 20 mg l^{-1} peroxide solution.

The oxidising agent ozone has also been investigated for the destruction of microcystins and was shown to be 100% efficient at removing the toxin (Keijola *et al.*, 1988). Rositano *et al.* (1998) demonstrated that ozone was a more effective oxidising agent for removing microcystin-LR from water compared to chlorine, H_2O_2 and potassium permanganate. As with chlorine pH dependence

was observed for the ozonation process with acid conditions being more favourable. They believed that this was due to the oxidising potential of ozone being greater in acidic conditions (2.07 volts vs NHE) than in alkaline solutions (1.24 volts vs NHE).

It was also determined that when used in combination with H_2O_2 the effectiveness of ozone treatment was greatly enhanced (Rositano *et al.*, 1998). There are no reports of the combination of any other chemical treatment method with H_2O_2 for the destruction of cyanotoxins.

The use of ultraviolet (UV) light has also been found to degrade microcystin-LR with total destruction being observed within 10 minutes, however, the dose required was substantially higher than that typically used in water treatment (Tsuji *et al.*, 1995). Other researchers have shown that UV illumination of microcystin-LR resulted in the formation of isomers of the toxin with only 50% reduction in concentrations over about 75 minutes (Kaya and Sano, 1998). The use of UV illumination in the presence of H_2O_2 was demonstrated to be an effective method of toxin removal (Rositano and Nicholson, 1994). It was believed by these researchers that this increased rate of destruction was due to the photodissociation of the peroxide to form highly oxidising hydroxyl radicals.

Robertson *et al.* (1997) have demonstrated that UV light in the presence of titanium dioxide (TiO_2) catalyst effectively removed microcystin-LR at high concentrations (200 $\mu g\ ml^{-1}$) within 30 minutes. This photocatalytic method of

microcystin-LR degradation was also demonstrated to degrade microcystin-YR and -YA (Shepard *et al.*, 1998). These preliminary findings demonstrated that this method of toxin removal has great potential for water treatment and therefore warrants further investigation.

1.7 Photocatalysis

Photocatalysis is a process which involves the promotion of reactions by a light-activated catalyst that is not itself consumed during these reactions. The process is dependent on the formation of oxidising species formed by the catalyst on its illumination which initiate the destruction of compounds present in solution. The use of semiconductor photocatalysis has been demonstrated in many applications; most notable is the photodegradation of hazardous waste products.

1.7.1 Mechanism of photocatalytic oxidation

In a semiconductor overlapping atomic orbitals form molecular orbitals that are closely packed giving rise to continuous bands. The valence band (VB) is composed of occupied orbitals while the conductance band (CB) is formed by unoccupied orbitals. Separating these bands is an energy gap (E_g) which is unoccupied by orbitals and is defined by the energy difference between the bottom edge of the conductance band and the top edge of the valence band.

When illuminated with light (hν) of greater energy than this band gap an electron (e^-) is promoted from the valence band to the conductance band leaving a positive hole (h^+) in the valence band (Figure 1.6). There are three possible reactions that may then occur in a semiconductor once photoexcitation has occurred.

$$e^- + h^+ \rightarrow \text{heat} \qquad \text{Equation 1.1}$$

$$O + h^+ \rightarrow O^{\bullet +} \qquad \text{Equation 1.2}$$

$$e^- + R \rightarrow R^{\bullet -} \qquad \text{Equation 1.3}$$

Recombination is a process in which the electron and the hole combine to produce heat and the molecule returns to an unexcited state (Equation 1.1). However where recombination does not occur the hole in the valence band may accept electrons in oxidation reactions (Equation 1.2) while the electron in the conductance band may be donated giving rise to reduction reactions (Equation 1.3). The oxidation and reduction potentials of the valence and conductance bands for different semiconductors are shown in Table 1.1. The redox potentials for these materials ranges from between 4.1 and –2.3 volts vs Normal Hydrogen Electrode (NHE). Therefore these semiconductors are capable of oxidising or reducing a wide range of substances.

Table 1.1: Oxidising and Reducing powers of the valence and conductance bands of some common semiconductor materials (Robertson, 1996).

Semiconductor	Valance Band (V vs NHE)	Conductance Band (V vs NHE)
TiO_2	+3.1	-0.1
SnO_2	+4.1	+0.3
ZnS	+1.4	-2.3
WO_3	+3.0	+0.2
CdSe	+1.6	-0.4
GaAs	+1.0	-0.4
GaP	+1.3	-1.0

The maximum wavelength of light energy required to promote electron excitation is determined by equation 1.4:

$$\lambda = \frac{hc}{E_g} \qquad \text{Equation 1.4}$$

where λ is wavelength, h is Planck's constant, c is the speed of light and E_g is the band gap energy between the valence and conductance bands of the catalyst.

One problem faced by some researchers in the choice of semiconductor is the process of photocorrosion. This is a process in which the photogenerated holes in the valence band break the bonds between the surface atoms of the material and leads to the semiconductor dissolving. Due to this process the number of materials suitable for photocatalysis is restricted.

TiO_2 is one of the most commonly used semiconductors for photocatalytic reactions as it is not prone to photocorrosion. The material has a band gap of 3.2 eV and therefore photoexcitation will occur with adsorption of light at near ultraviolet wavelength (~380 nm). It has been shown that the use of TiO_2 as a photocatalyst for the destruction of pollutants in water is an effective process. In the presence of the semiconductor catalyst and illumination at the appropriate wavelength pollutants present will be transformed either by oxidation or reduction reactions. TiO_2 has been shown to degrade a wide range of both organic (Ku *et al.*, 1996; Chen and Ray, 1998; Schmelling and

Gray, 1995), and inorganic compounds (Mills *et al.,* 1996) as well as bacteria (Butterfield *et al.*, 1997).

The exact mechanism of the destruction or transformation of pollutants by photocatalysis is still an area of debate. It is believed that hydroxyl radicals ($OH^{\bullet}$) are generated at the valence band via oxidation which subsequently oxidise the pollutant (Turchi and Ollis, 1990). The electron promoted to the conductance band is donated to oxygen giving rise to a superoxide radical anion ($O_2^{\bullet-}$). This process of water purification is illustrated in Figure 1.7.

It is however believed by some researches that the pollutant undergoes oxidation by direct transfer of an electron to the valance band of the catalyst (Prairie *et al.*, 1993). However, despite which of the mechanisms is occurring, the oxidation potential of both hydroxyl radicals (2.8 V) or the valence band hole (3.1 V) is far greater than that of most oxidising agents used in conventional water treatment such as ozone (2.07 V), chlorine (1.36 V) and hydrogen peroxide (1.78 V).

1.7.2 Uses of TiO_2 for water treatment

In 1976 Carey and co-workers demonstrated the degradation of biphenyl and chlorobiphenyls using TiO_2 photocatalysis. This successful destruction of organic pollutants led to an increase in research for the use of TiO_2 to remove

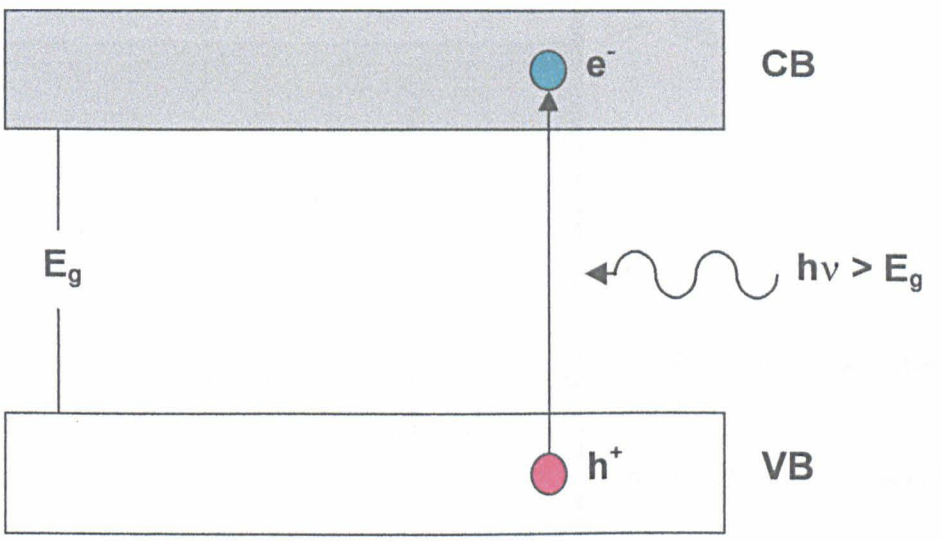


Figure 1.6: The promotion of an electron (e^-) from the valence band (VB) to the conductance band (CB) of a semiconductor by light energy.

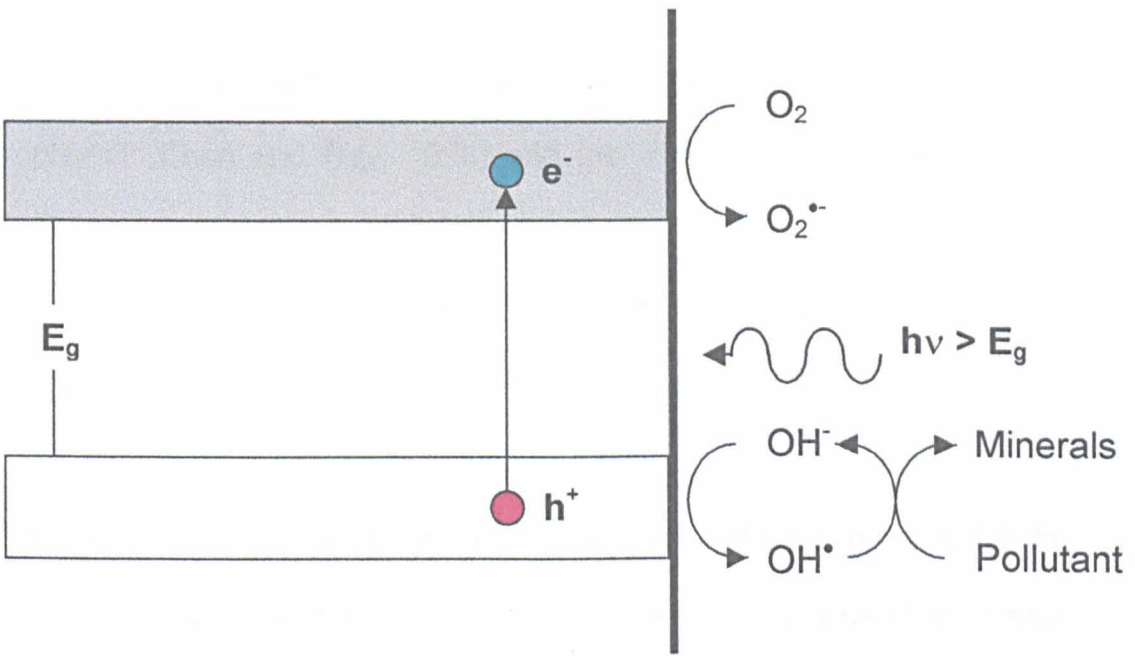


Figure 1.7: The process of semiconductor photocatalysis for water purification.

other pollutants from both waste and potable waters. Fox and Chen (1981) reported the oxidation of aromatic hydrocarbons when they were adsorbed onto the surface of the catalyst then illuminated with UV light. Other examples of pollutants successfully treated with photocatalysis include the fungicide metalaxyl (Topalov *et al.*, 1999), the insecticide monocrotophos (Hua *et al.*, 1995), organics pollutants such as 2-chlorophenol (Ku *et al.*, 1996) and 4-nitrophenol (Chen and Ray, 1998) and the explosive 2,4,6-trinitrotoluene (TNT) (Schmelling and Gray, 1995). TiO_2 was also shown to be effective in destroying biological organisms such as *Escherichia coli* and *Clostridium perfingens* (Butterfield *et al.*, 1997).

TiO_2 photocatalysis was successfully used in combination with ozonation for the removal of acetic acid and phenol in waste waters (Tanaka *et al.*, 1996). Tanaka and co-workers indicated that the photocatalyst enhanced the rate at which these waste materials were degraded when treated with ozone. Similarly some researchers found that the addition of the chemical oxidant hydrogen peroxide (H_2O_2) to the photocatalytic system enhances photocatalysed destruction of pollutants (Grätzel *et al.*, 1990; Tanaka *et al.*, 1990; Jakob *et al.*, 1993). Crittenden *et al.* (1997) utilised activated carbon impregnated with TiO_2 to produce a self-regenerating adsorbant to purify groundwater. While little regeneration occurred when the system was in operation the catalyst was capable of degrading waste material once it was flushed from the adsorbent.

Photocatalysis has also been shown to be effective in the treatment of pollutants in potable waters. Eggins *et al.* (1997) demonstrated the decomposition of humic substances in potable waters using TiO_2 as a replacement for coagulation and filtration. The process successfully degraded the organics present in the water, however only 50% removal was achieved after 1 hour illumination and the presence of break-down by-products suggest that this technique requires further development. Mills and co-workers (1996) also demonstrated the removal of bromate from drinking water using platinised TiO_2.

As previously reported, UV illumination with TiO_2 catalyst efficiently removes microcystin-LR even at concentrations of 200 $\mu g\ ml^{-1}$ (Robertson *et al.*, 1997). These preliminary studies indicated that toxin concentrations were reduced rapidly with levels almost undetectable after 30 minutes. However there were no indications as to whether breakdown products were formed or if the toxicity of treated samples had been removed.

The aim of this thesis is to determine the effectiveness of the photocatalytic process for the oxidation of microcystin-LR and to determine how destruction occurs. Treated samples were analysed by HPLC and mass spectroscopy to determine the presence of photocatalytic by-products and a simple invertebrate bioassay was used to determine if the process removed the toxicity associated with microcystin-LR. Using the data provided for by-products and information obtained from optimisation experiments a pathway and mechanism for microcystin-LR destruction was then proposed.

As the degradation of microcystin-LR by UV light has been shown to be enhanced by H_2O_2, studies were carried out to determine the oxidant's effects on the photocatalytic process. The use of a small scale fixed layer flow reactor was also investigated for the removal of microcystin-LR in both domestic and raw waters to determine the system's effectiveness in natural waters. Furthermore the process of photocatalytic degradation was also studied for the treatment of the microcystin variants -RR, -LW and -LF. Finally, the effect of initial solution pH was determined for each of these variants to optimise degradation.

CHAPTER 2

Purification and Analysis of Microcystins

2.1 Introduction

2.1.1 Purification of microcystin toxins

Purified microcystin standards are becoming increasingly important as the need for research into toxicological effects, detection and destruction methods grows. Chemical synthesis of cyanobacterial toxins has been investigated with some success (Namikoshi *et al.*, 1989; Zetterström *et al.*, 1995) however these techniques are yet to be fully developed. Humphrey *et al.* (1996) recently synthesised the hepatotoxin microcystin-LA which was identical to the authentic compound as analysed by NMR and HPLC. However the process is very time consuming requiring the synthesis of 42 compounds before the toxin is produced.

The separation and concentration of toxins extracted from cyanobacterial cells must therefore be performed to acquire purified microcystins. A number of procedures have been developed for extracting and purifying microcystins however many involve numerous complicated time consuming stages (Harada *et al.*, 1991; Namikoshi *et al.*, 1992; Martin *et al.*, 1990). Several different liquid phases have been used to extract toxins from cyanobacterial cells including 5% acetic acid, methanol acidified with triflouroacetic acid (TFA), butanol-methanol-water and methanol (Harada *et al.*, 1996, Krishnamurthy *et al.* 1986). Lawton *et al.* (1994b) advocated the use of methanol over acetic acid and butanol-methanol-water for extracting microcystins due to its efficiency at extracting microcystins of varied polarity. It has recently been

suggested that methanol gives poor recovery of more polar microcystins such as microcystin-RR and that the addition of a small percentage of water (approximately 20%) increases extraction of these toxins (Fastner *et al.*, 1998). The purification of sub-gram quantities of microcystins was achieved by Edwards *et al.* (1996a, 1996b) following the concentration and preliminary purification of methanol extracts using reversed-phase flash chromatography. The procedure facilitates the concentration of toxins extracted from cyanobacterial cells onto a pre-packed C18 flash chromatography solid-phase extraction cartridge. Microcystins are then eluted from the cartridge using a step gradient from 0% to 100% (v/v) aqueous methanol giving fractions containing microcystins of similar polarity. Recently Lawton *et al.* (1999) utilised flash chromatography to purify microcystins -LW and -LF which had previously coeluted in the same fraction and required specialised equipment to further separate them.

Solid Phase Extraction (SPE) is a process that concentrates and purifies analytes by removing them from solution by sorption onto a solid-phase cartridge using either normal phase, reverse phase or ion exchange mechanisms. The analyte may then be selectively removed from this cartridge by an appropriate solvent. Traditionally this process was carried out by liquid-liquid extraction which involved large quantities of organic solvent and led to problems with formation of emulsions when used with aqueous samples. During the mid 1970's the concept of SPE, shown in Figure 2.1, was introduced as an alternative to liquid-liquid extractions. The SPE cartridge is first conditioned with a solvent that wets the packing material removing any

trapped air and solvates the functional groups of the sorbent (Figure 2.1a). The sample containing the analyte is then applied to the cartridge and the analyte is concentrated onto the sorbent (Figure 2.1b), however, other component's in the sample may also be retained in the cartridge. These interferences may be removed from the cartridge by washing the sorbent with an appropriate solvent to elute these impurities (Figure 2.1c). The final stage is to elute the analyte from the cartridge with an appropriate solvent that will disrupt the analyte-sorbent interaction. The analyte can then be analysed and the toxins quantified, e.g. by HPLC. Microcystins of varied hydrophobicity have been successfully concentrated using C18 reverse phase SPE (Lawton *et al.,* 1994b). However the results gave poor recoveries for the more hydrophobic toxin microcystin-LW especially in raw water samples. The procedure for C18 SPE involves acidifying samples with TFA prior to sample concentration and the low recoveries suggest that microcystin-LW is unstable in such acidic conditions.

OASIS™ HLB SPE cartridges (Waters, Waters Corporation, Millford, USA) were introduced to replace the existing C18 reverse phase cartridges and the new sorbent has been reported to give distinct advantages over C18. The OASIS cartridge contains a hydrophilic/lipophilic balance (HLB) co-polymer which allows retention of both polar and non-polar compounds and the cartridge has no pH restrictions. Another advantage is that the OASIS cartridge performance is not affected if the packing dries out prior to sample application; one of the main drawbacks of the C18 SPE cartridge.

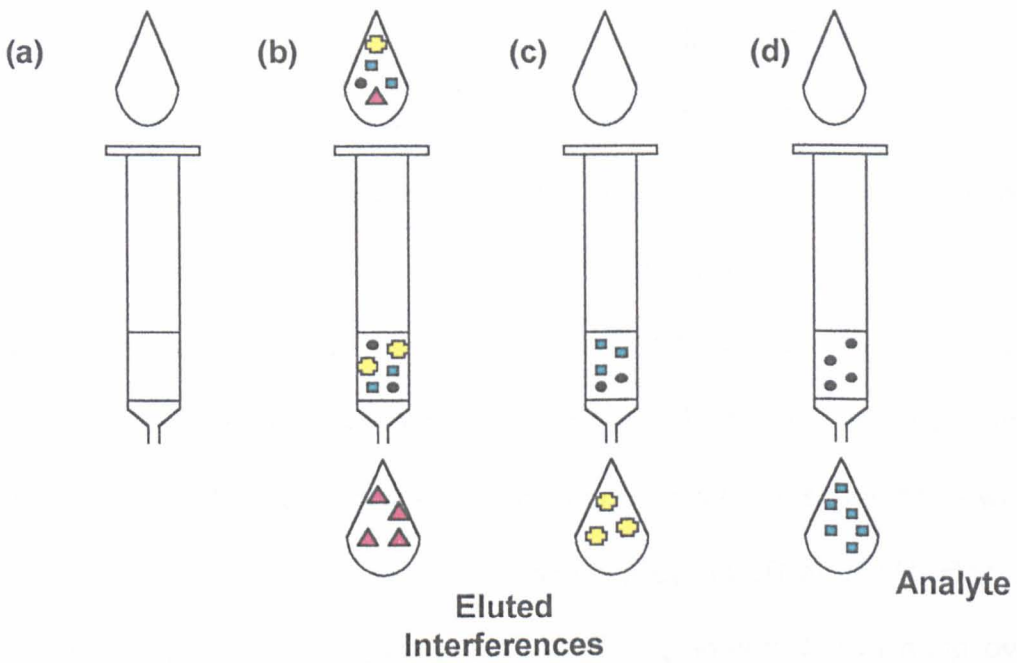


Figure 2.1: The process of reverse phase solid phase extraction, (a) cartridge is conditioned with appropriate solvent, (b) sample containing analyte and interferences is loaded onto cartridge, (c) cartridge is washed with solvent to remove interferences, (d) purified analyte is eluted from the cartridge in appropriate solvent.

2.1.2 HPLC-PDA detection of microcystin toxins

Reverse phase high performance liquid chromatography (HPLC) coupled with photo-diode array detection (PDA) has been used by many researchers for the analysis of microcystins. Guo *et al.* (1986a and 1986b) were some of the first researchers to utilise reverse phase HPLC for peptides and developed procedures for predicting retention times of amino acids using water-acetonitrile-0.1% TFA gradients. Different procedures have since been developed by researchers for microcystins using different column types and sizes, mobile phases and separation gradients (Siegelman *et al.,* 1984; Kungswan *et al.*, 1987). One of the problems arising with these methods were coelution of toxins of similar polarity such as microcystin-LR and -YR (Harada *et al.*, 1988). Also in some cases mobile phase gradients fail to elute over broad enough polarity ranges resulting in the under detection of more hydrophobic microcystins such as microcystin-LW and -LF (Meriluoto *et al.*, 1990).

Lawton *et al.* (1994b) used a gradient of 30%–70% aqueous acetonitrile over 30 minutes with 0.05% TFA added to both solvents as mobile phases for separation of microcystins on a C18 column. These mobile phases were similar to those used in the original peptide separation experiments by Guo *et al.* (1986b). The method provides chromatograms with well separated peaks over a 45 minute period for microcystins of varying polarity.

HPLC-PDA relies on both retention times and the UV spectra to identify microcystin variants. This leads to a limited ability to differentiate between these variants as most of these compounds have similar UV spectra.

Analytical HPLC-PDA allows for both quantitative and tentative identification of microcystins. By altering the gradient of the mobile phase it is possible to develop short timesaving gradients for just one or two microcystins. These rapid methods are particularly suitable for samples of relatively high purity as a wash cycle is not required.

This chapter presents the procedures that were used to provide and analyse microcystins of suitable purity for use in this study. It also presents studies which were carried out to compare traditionally used C18 SPE cartridges and the OASIS HLB SPE cartridge to determine if it was a suitable substitute for microcystin trace analysis.

2.2 Methods

2.2.1 Growth and harvesting of cyanobacterial material

Batch cultures of *Microcystis aeruginosa* PCC7820 (Pasteur Culture Collection, Paris, France) were grown in BG-11 medium (Table 2.1, Stanier *et al.*, 1971) plus nitrate (8.8 mM). All cultures were grown in 10 litre flasks for 5 weeks under continuous illumination (approximately 20 μmol m^{-2} s^{-1}) with cool white fluorescent tubes (36 W), and sparged with sterile air throughout. Cells were harvested after the five week growth period by tangential flow filtration (Pellicon-2; fitted with three 0.22 μm, type GVPP-V filters, Millipore) then centrifuged to give a wet pellet. Harvested cells were stored at -20°C until required.

2.2.2 Flash purification of microcystins

Microcystins were extracted from a wet pellet of cyanobacterial cells using methanol (Lawton *et al.* 1994b). The cells were extracted in 2 litres of methanol (Rathburn, Walkerburn, UK) for 30 minutes with regular agitation then centrifuged at 1500 x g for 30 minutes and the supernatant decanted. The residual pellet was then re-extracted a further two times. The pooled supernatants were diluted with Milli-RO water (Millipore, Watford, UK) to 20% methanol (v/v) then left to stand for 30 minutes allowing time for precipitation

Table 2.1: Composition of BG-11 growth medium plus nitrates (Stanier *et al.*, 1971).

$NaNO_3$	0.750 g l^{-1}
K_2HPO_4	0.040 g l^{-1}
$MgSO_4.7H_2O$	0.075 g $^{-1}$
Na_2CO_3	0.020 g l^{-1}
$CaCl_2.2H_2O$	0.036 g l^{-1}
EDTA	0.001 g l^{-1}
$FeSO_4.7H_2O$	0.006 g l^{-1}
Citric acid	0.006 g l^{-1}
Trace element solution †	1 ml l^{-1}

† Trace element solution

H_3BO_3	2.680 g l^{-1}
$MnCl_2.H_2O$	1.810 g l^{-1}
$ZnSO_4.7H_2O$	0.222 g l^{-1}
$Na_2MoO_4.2H_2O$	0.390 g l^{-1}
Cu $(NO_3)_2.6H_2O$	0.079 g l^{-1}
Co $(NO_3)_2.6H_2O$	0.049 g l^{-1}

to occur prior to removal by tangential flow filtration. A sample (1 ml) was removed from the diluted extract for HPLC analysis.

Purification of the microcystin extract was carried out using a Biotage Flash 40 system (Biotage, a Division of Dyax Corporation, Charlottesvile, VA, USA) shown in Figure 2.2. Nitrogen pressure (20-30 p.s.i.) was applied to the solvent reservoir to achieve a flow rate of around 40 ml min^{-1}. A KP-C18-HS flash cartridge (15 x 4 cm I.D., 35-70 μm particle size, 60 Å pore size) was conditioned with 1 litre of methanol followed by 1 litre of Milli-Q water prior to applying the extract. Microcystins were eluted from the cartridge using a step gradient of 0 to 100% methanol in 10% increments (1 litre per step). Each fraction was collected separately then analysed by HPLC. Following use the flash C18 cartridge was cleaned with 1 litre dichloromethane (DCM- Rathburn, Walkerburn, UK) then 1 litre methanol and stored in methanol for future use.

2.2.3 Purification of closely eluting microcystins

HPLC analysis of reversed-phase flash fractions obtained from the extraction described in 2.2.2 indicated that microcystin-LW and -LF were eluted in the 60% methanol fraction. This fraction was diluted with 1 litre of water, loaded onto a conditioned KP-C18-HS™ flash cartridge then eluted in 500 ml of 100% methanol. Using a method adapted from Lawton *et al.* (1999) the sample was then dried by rotary evaporation and resuspended in 20 ml of methanol. A KP-Sil™ silica cartridge (15 x 4 cm I.D., 32-63 μm, 60 Å pore size; Biotage) was

prepared by flushing with 1 litre methanol, 1 litre DCM and 1 litre of the initial mobile phase (95% A:5% B, where A is DCM and B is methanol:acetic acid (10:2 v/v)). Nitrogen pressure (15 p.s.i.) was applied to achieve a flow rate of 50 ml min^{-1}. The sample was applied to the cartridge in 100 ml of DCM (120 ml total volume) and the microcystins were then eluted using the gradient shown in Table 2.2.

Aliquots (250 μl) were removed from each fraction, dried, resuspended in 250 μl of methanol then analysed by HPLC. The collected fractions were left uncovered in a fume hood overnight to allow the DCM to evaporate. Fractions found to contain a single microcystin with purity greater than 80% were pooled, diluted to 20% methanol (v/v) and passed through a KP-C18-HS™ flash cartridge (7.5 x 4 cm I.D., 35-70 μm particle size, 60Å pore size; Biotage, USA). Toxins were eluted from the cartridge by step gradient from 0 to 100% methanol in 10% increments (500 ml per step) and analysed by HPLC. Fractions containing a single purified microcystin were rotary evaporated to dryness then resuspended in methanol.

Table 2.2. Gradient used for the separation of microcystin-LW and -LF using a pre-packed flash KP-Sil™ silica cartridge (15 x 4 cm I.D.), as described in Section 2.2.3. Mobile phase was composed of DCM (A), and methanol:acetic acid (10:2 v/v) (B).

Mobile phase composition	Elution volume (ml)	Number of fractions (50 ml) collected
95% A: 5% B	360	7
92% A: 8% B	1200	24
90% A:10% B	2000	40

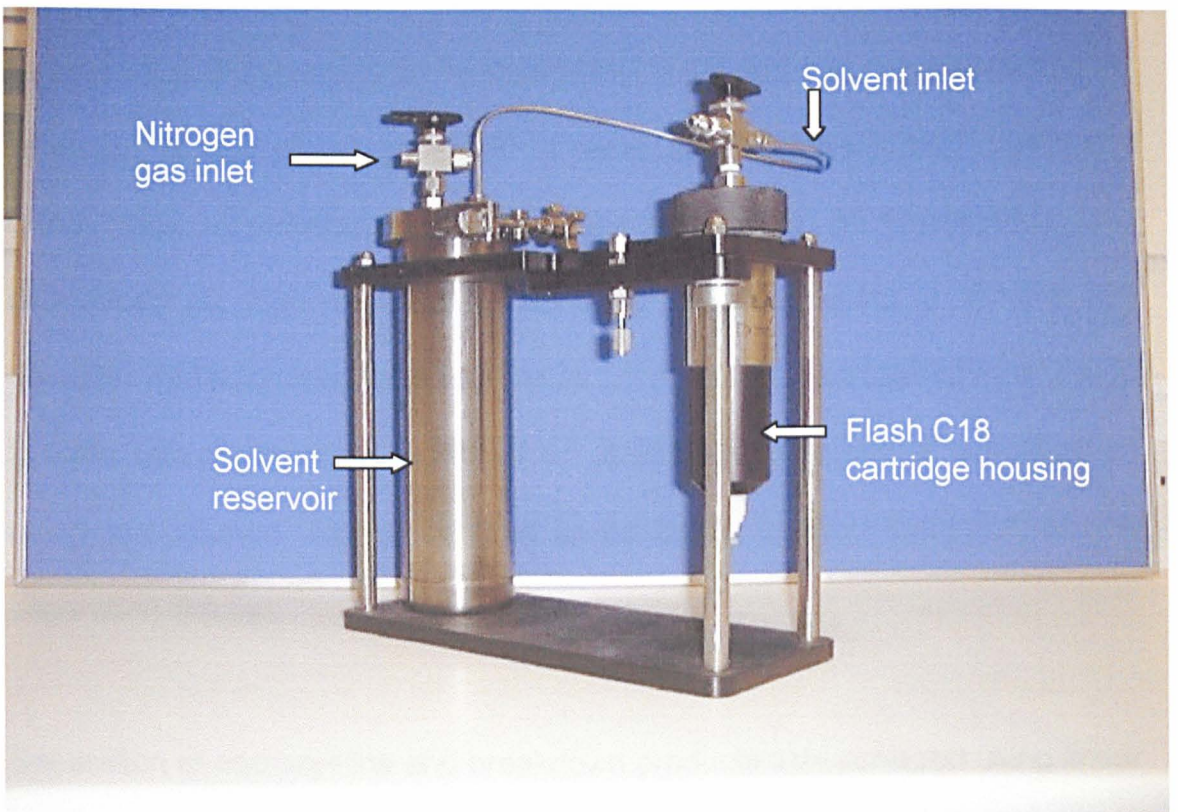


Figure 2.2: Biotage Flash 40 system comprised of a stainless steel solvent reservoir under pressure which drives solvent/sample through a cartridge (packed with stationary phase) housed in an axial compression module.

2.2.4 HPLC analysis of microcystins

Microcystins throughout the study were analysed by HPLC as described by Lawton *et al.* (1994b) with the following modifications. Samples were separated on a Symmetry C18 column (250 x 4.6 mm I.D.; 5 μm particle size, Waters). Mobile phase consisted of water and acetonitrile both containing 0.05% TFA. Quantification, identification and purity of microcystins was determined by high-resolution diode-array detection using a Waters 996 detector. Detector resolution was set at 1.2 nm and data acquired from 200 to 300 nm with chromatograms monitored at 238 nm. The limit of quantification using this method was determined to be 0.005 μg microcystin-LR on the separation column.

Separation of microcystins and breakdown products was achieved using linear gradients starting at 30% (v/v) aqueous acetonitrile increasing to 35% over 10 minutes followed by an increase to 70% over the next 30 minutes at a flow rate of 1 ml min^{-1}.

For the rapid detection of microcystin-LR a shortened linear gradient starting at 30% (v/v) aqueous acetonitrile increasing to 60% over 10 minutes was used also at a flow rate of 1 ml min^{-1}. A linear gradient starting at 30% (v/v) aqueous acetonitrile increasing to 35% over 10 minutes was used to obtain rapid detection of the microcystin-RR. A linear gradient starting at 30% (v/v) aqueous acetonitrile increasing to 70% over 7 minutes was used to obtain rapid detection of the microcystin-LW and -LF.

2.2.5 Trace analysis of microcystins using SPE

Water samples including tap water (from Aberdeen and Dinnet, Aberdeenshire) and raw water obtained from Loch Loirston (Aberdeen) and Loch Kinord (Aberdeenshire) were filtered using GF/C filter disks (110 mm, Whatman International Ltd, Maidstone, UK) to remove particulates. The water samples were then treated with sodium thiosulphate (100 µl per 500 ml sample) to remove any residual chlorine then spiked with a mixture of microcystin-RR, -LR and -LW to give a concentration of 0.5 µg l^{-1} and 5 µg l^{-1} of each of the toxins.

Triplicate samples (500 ml) for each water type and concentration were then applied to C18 and OASIS extraction cartridges as decribed in Table 2.3. The procedure was carried out using a VacMaster Vacuum Manifold System (Figure 2.3). The recovery of the three microcystins by each cartridge was then determined by HPLC and compared.

Normal phase clean-up of samples was carried out using silica SPE cartridges (IsoluteTM, International Sorbent Technology, UK). Tap water from Aberdeen and Stonehaven (Aberdeenshire) and raw water from Loch Kinord and Loch Rescobie were spiked with toxin as before and applied to OASIS cartridges. Following the methods of Tsuji *et al.* 1994, samples eluted from the OASIS cartridges with methanol were applied directly to silica cartridges. HPLC analysis of the sample that passed through the cartridge indicated that

breakthrough of the microcystins was occurring, i.e. they were not retained by the silica.

Following elution from the OASIS reverse phase cartridge the sample was dried then resuspended in 200 µl of methanol as before. Silica cartridges were conditioned with 5 ml of methanol followed by 5 ml of DCM. The 200 µl sample was added to 10 ml of DCM (a less polar solvent than methanol) then applied to the cartridge followed by a further 10 ml of DCM. The microcystins were then eluted from the silica cartridge firstly with 5 ml of methanol then with 5 ml of 0.1% TFA in methanol. These fractions were dried under nitrogen and resuspended in 200 µl 80% aqueous methanol then combined into one sample prior to analysis by HPLC. The use of 80% aqueous methanol was found in this study to give better recovery of microcystin-RR due to this variant's hydrophilic nature.

Table 2.3: Experimental procedure for the trace analysis of microcystins using solid phase extraction cartridges.

Cartridge type	C18	OASIS
Sample pre-treatment	Add 5 ml 10% aqueous TFA. Filter through GF/C filter disk then add 5 ml methanol.	None
Cartridge pre-treatment	10 ml Methanol followed by 10 ml distilled water.	1 ml Methanol followed by 1 ml distilled water.
Pressure	20 psi	10 psi
Cartridge wash	10 ml 10% methanol followed by 10 ml 20% methanol followed by a further 10 ml 30% methanol.	1 ml 10% methanol followed by 1 ml 20% methanol followed by a further 1 ml 30% methanol.
Drying time prior to elution	30 minutes	None
Elution	3 ml 0.1% TFA in Methanol	1 ml methanol
Following elution all samples were dried under nitrogen then resuspended in 200 μl of 80% (v/v) aqueous methanol.		

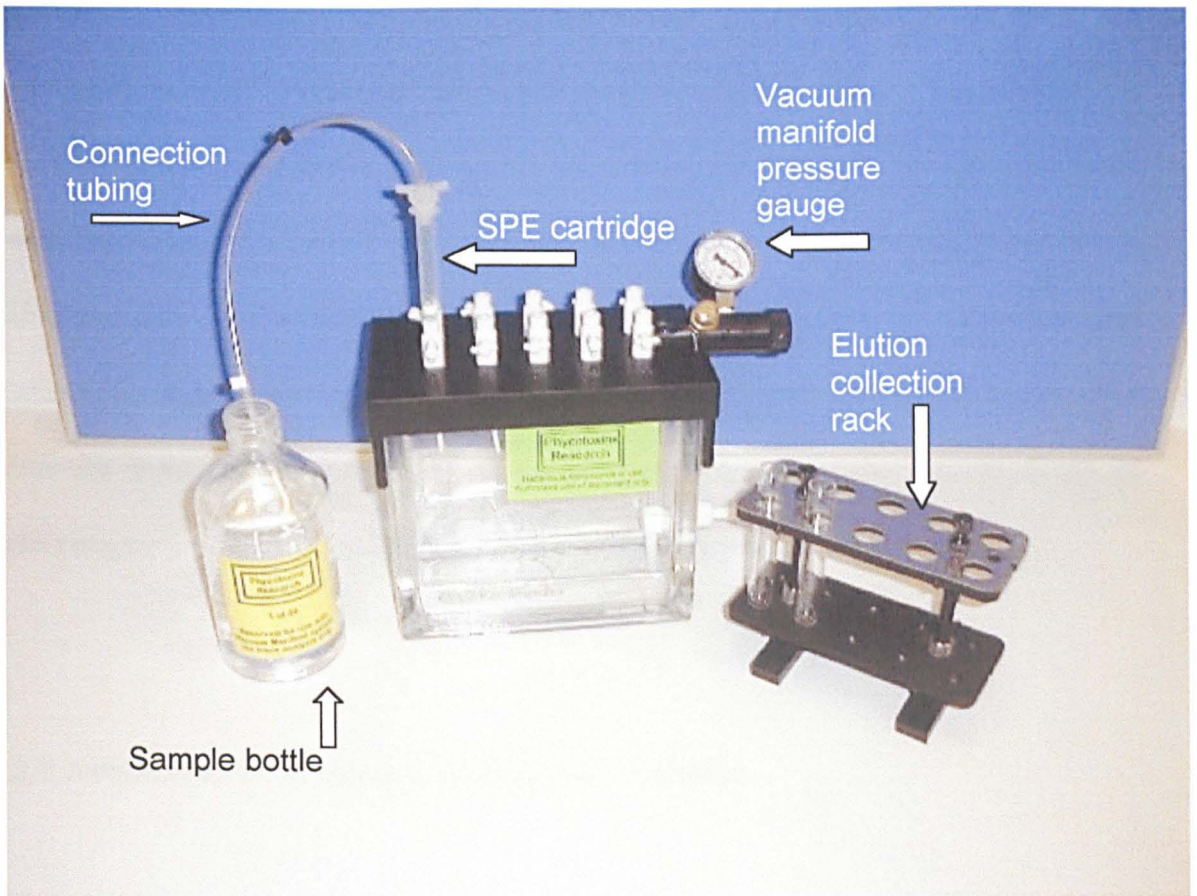


Figure 2.3: The Vacmaster^{TM} Vacuum Manifold system

2.3 Results

2.3.2 Flash purification of microcystins

Following extraction of the *M. aeruginosa* PCC7820 cells three main microcystins are present in the crude extract, microcystin-LR, -LW and -LF (Figure 2.4). HPLC-PDA analysis of the fractions eluted from the C18 flash cartridge indicated that microcystin-LR was present in 40% aqueous methanol (Figure 2.5). The two hydrophobic microcystins, -LW and -LF were coeluted with 60% aqueous methanol (Figure 2.6). Analysis of the waste eluent that passes through the cartridge indicated that there was no breakthrough of microcystins following sample loading.

2.3.3 Purification of closely eluting microcystins

Separation of microcystin-LW and -LF occurred in fractions 30 to 65 during the final step of the gradient using the 90% A:10% B mobile phase (Figure 2.7). A significant number of fractions containing microcystin-LF were of purity greater than 80% however there were fewer pure microcystin-LW fractions obtained (Figure 2.8). Following further purification using C18 flash purity of the individual microcystins was increased to over 95% (Figures 2.9 and 2.10).

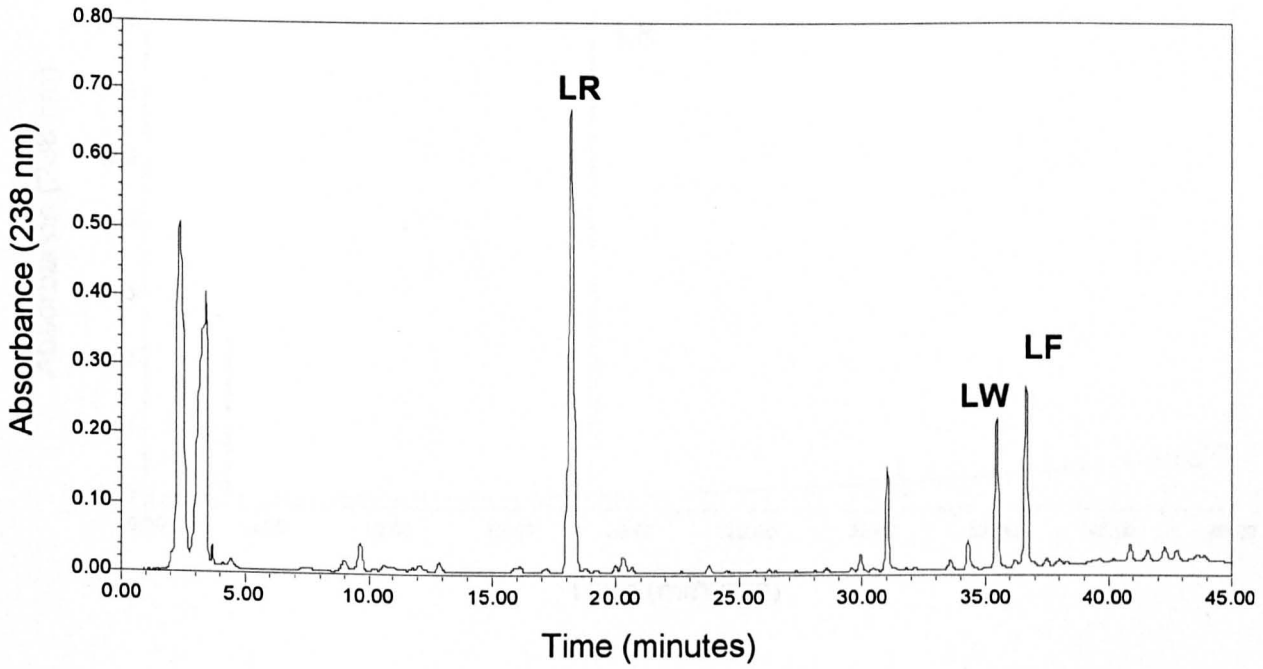


Figure 2.4: Reverse phase HPLC chromatogram of methanol extract of *M. aeruginosa* PCC7820 prior to application to C18 flash cartridge. The chromatogram shows three of the microcystins used in this study: microcystin-LR, microcystin-LW and microcystin-LF.

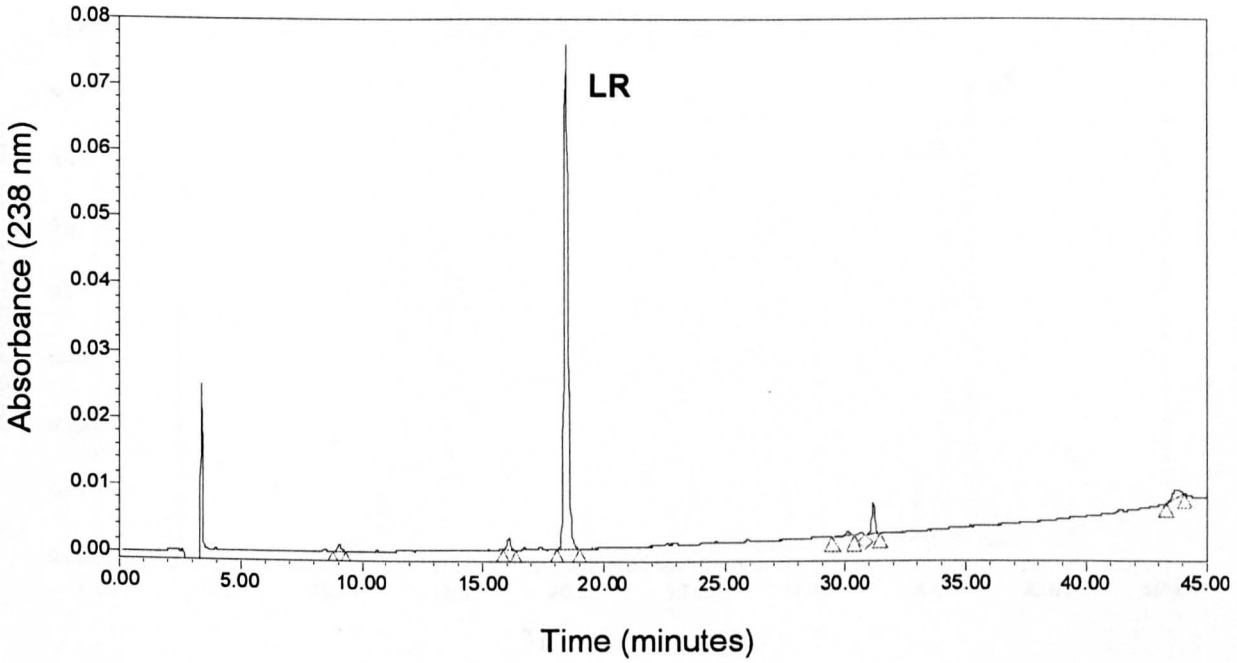


Figure 2.5: Reverse phase HPLC chromatogram of microcystin-LR eluted from the C18 flash cartridge with 40% aqueous methanol.

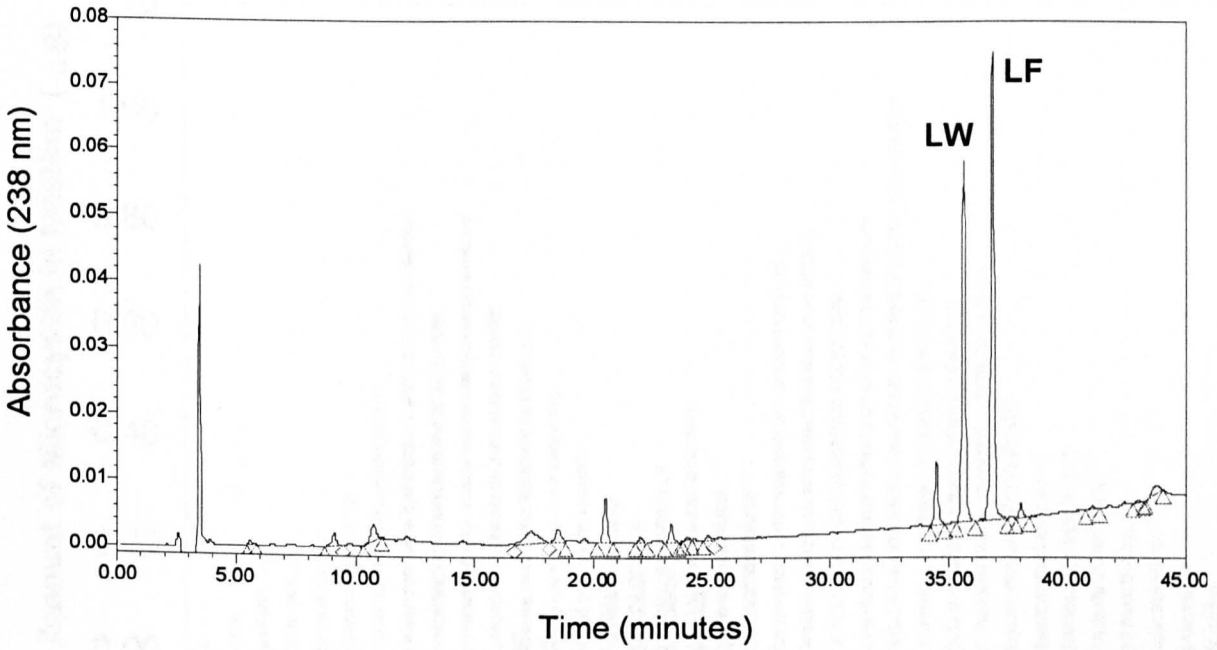


Figure 2.6: Reverse phase HPLC chromatogram of microcystin-LW and -LF co-eluted from the C18 cartridge with 60% aqueous methanol.

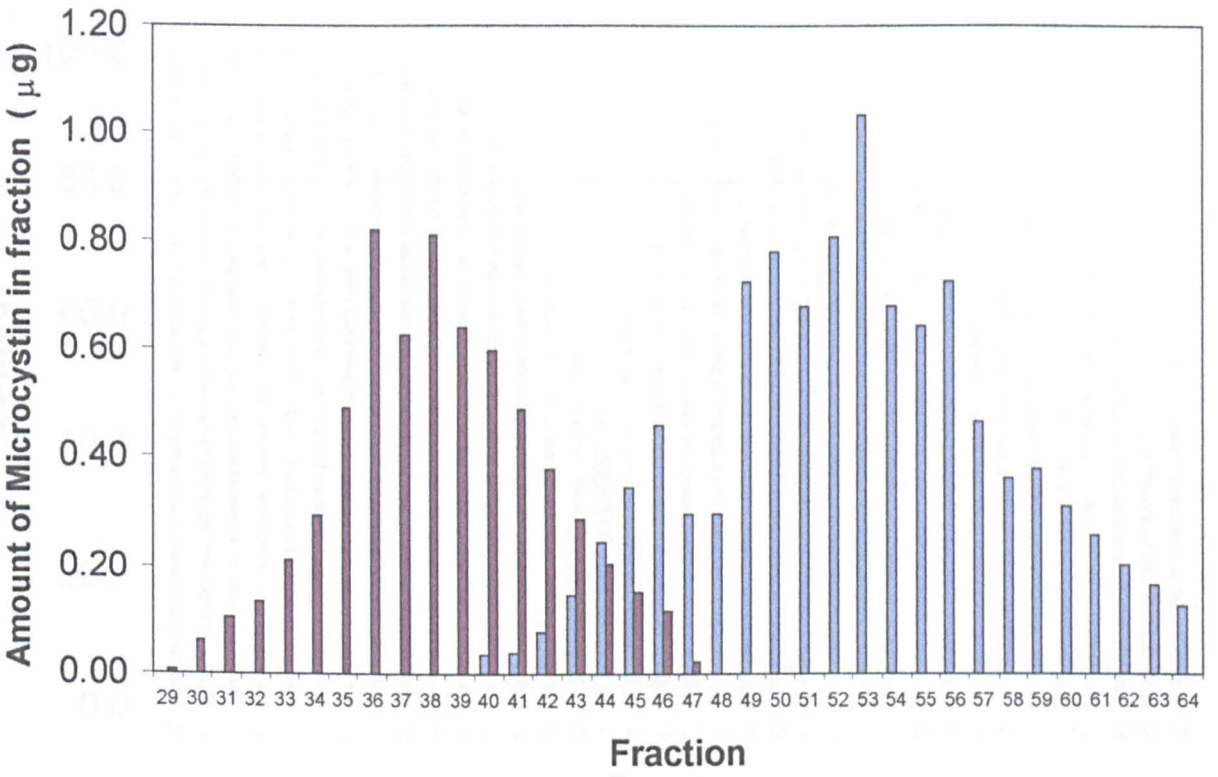


Figure 2.7: HPLC analysis of fractions collected from normal phase flash separation showing amounts of microcystin-LF (♦) and microcystin-LW (♦) in each fraction.

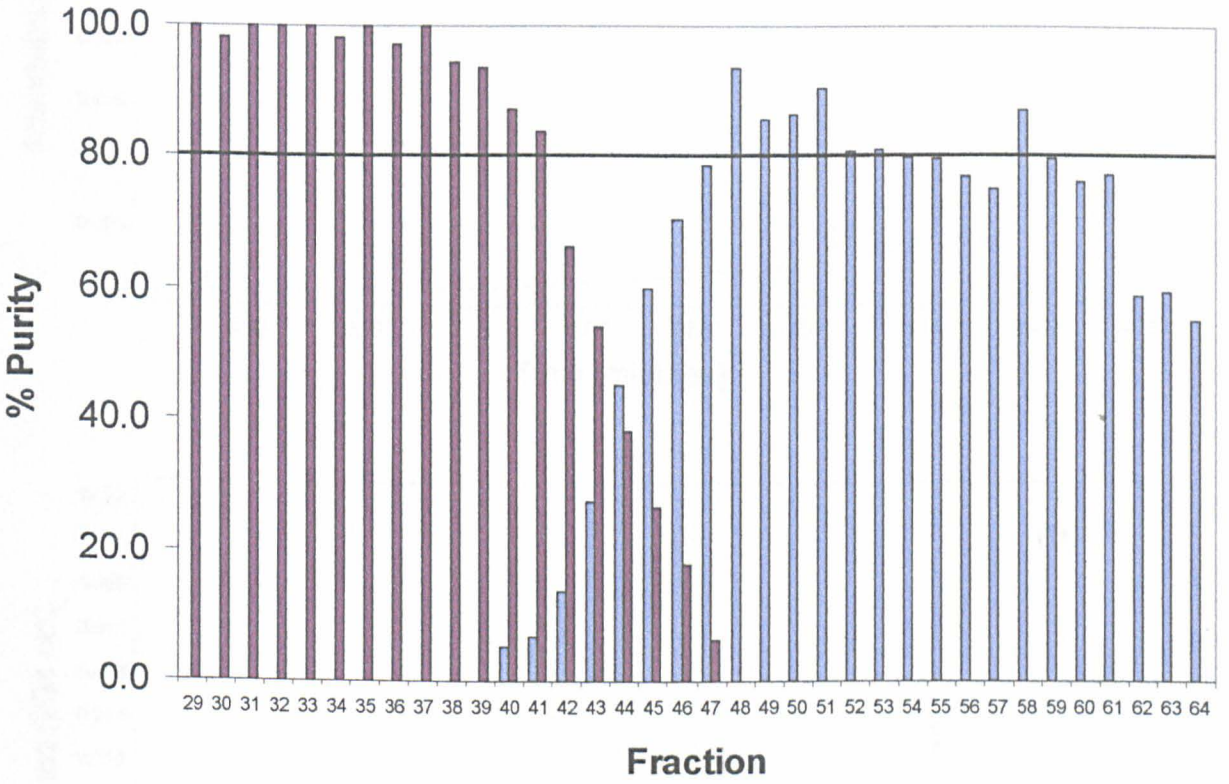


Figure 2.8: HPLC analysis of fractions collected from normal phase flash separation showing purity of microcystin-LF (♦) and microcystin-LW (♦) in each fraction. The line indicates samples above 80% purity.

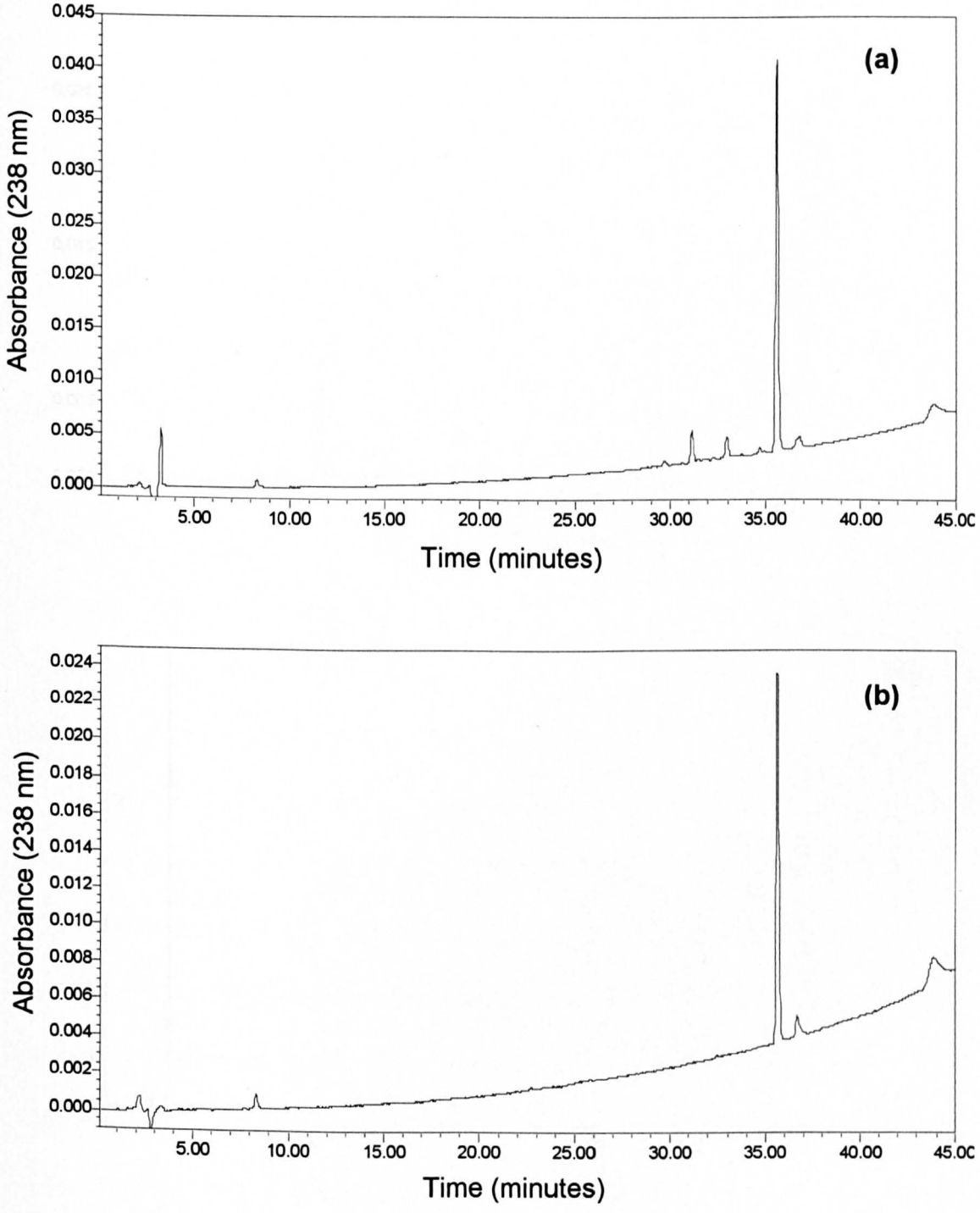


Figure 2.9: Reverse phase HPLC chromatogram of microcystin-LW eluted from (a) the normal phase separation silica cartridge and (b) reverse phase C18 cartridge.

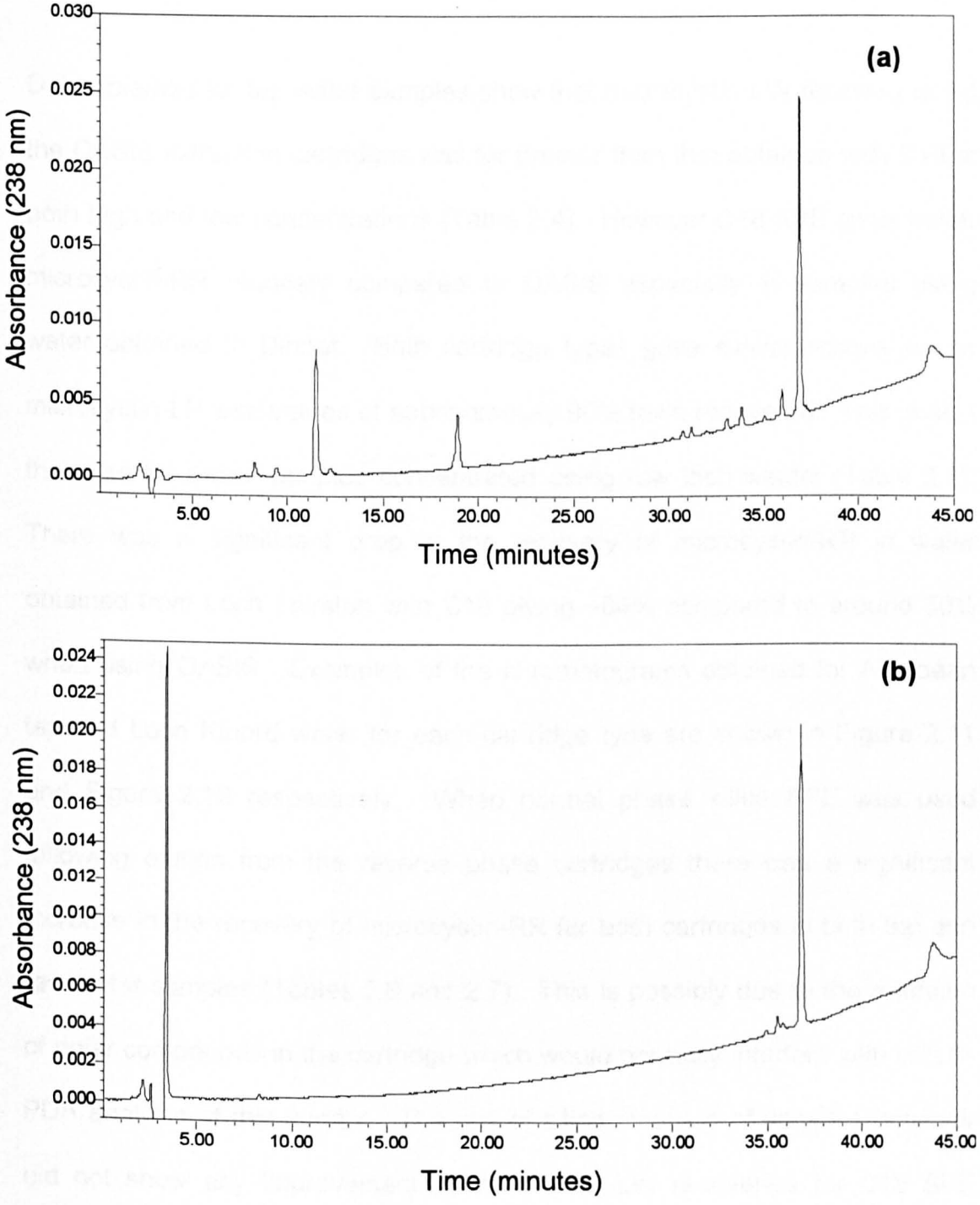


Figure 2.10: Reverse phase HPLC chromatogram of microcystin-LF eluted from (a) the normal phase separation silica cartridge and (b) reverse phase C18 cartridge.

2.3.5 Trace analysis of microcystins using SPE

Data obtained for tap water samples show that microcystin-LW recovery using the OASIS extraction cartridges was far greater than that obtained with C18 at both high and low concentrations (Table 2.4). However C18 SPE gives better microcystin-RR recovery compared to OASIS especially in samples using water obtained in Dinnet. Both cartridge types gave similar recoveries for microcystin-LR with values of approximately 90% toxin recovered. This is also the case for those samples concentrated using raw loch waters (Table 2.5). There was a significant drop in the recovery of microcystin-RR in water obtained from Loch Loirston with C18 giving ~84% compared to around 30% when using OASIS. Examples of the chromatograms obtained for Aberdeen tap and Loch Kinord water for each cartridge type are shown in Figure 2.11 and Figure 2.12 respectively. When normal phase silica SPE was used following elution from the reverse phase cartridges there was a significant increase in the recovery of microcystin-RR for both cartridges in both tap and raw water samples (Tables 2.6 and 2.7). This is possibly due to the retention of polar compounds in the cartridge which would normally interfere with HPLC-PDA analysis of this variant. The use of silica clean-up of samples however did not show any improvement in microcystin-LW recoveries for C18 SPE while recoveries of microcystin-LR were again comparable between cartridge types. Examples of the chromatograms obtained for Aberdeen tap and Loch Kinord water for each cartridge type following silica clean-up are shown in Figure 2.13 and Figure 2.14 respectively.

Table 2.4: Recovery of microcystins in tap water obtained from (a) Aberdeen city and (b) Dinnet, Aberdeenshire using C18 and OASIS SPE cartridges as determined by HPLC analysis.

(a)

Microcystin variant	Initial conc. $\mu g\ L^{-1}$	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	67.3	5.7	76.8	19.7
	5.0	68.0	1.6	75.1	7.1
LR	0.5	82.8	3.7	103.5	16.4
	5.0	79.5	1.9	93.4	0.9
LW	0.5	58.4	2.3	97.0	16.3
	5.0	47.2	14.5	91.2	0.8

(b)

Microcystin variant	Initial conc. $\mu g\ L^{-1}$	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	78.8	3.0	48.0	17.4
	5.0	72.9	7.4	56.0	4.6
LR	0.5	94.1	7.4	91.0	2.4
	5.0	82.5	7.7	94.8	8.7
LW	0.5	76.7	13.0	97.8	1.9
	5.0	66.7	9.2	99.9	7.4

Table 2.5: Recovery of microcystins in raw waters obtained from (a) Loch Loirston and (b) Loch Kinord using C18 and OASIS SPE cartridges as determined by HPLC analysis.

(a)

Microcystin variant	Initial conc. μg L^{-1}	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	84.1	8.7	32.2	7.9
	5.0	83.1	0.8	29.4	5.0
LR	0.5	82.8	9.0	83.8	8.5
	5.0	81.6	0.3	85.7	2.6
LW	0.5	48.6	2.3	94.5	12.0
	5.0	66.5	2.4	88.1	4.7

(b)

Microcystin variant	Initial conc. μg L^{-1}	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	82.0	15.7	67.0	1.9
	5.0	83.2	1.3	68.4	12.5
LR	0.5	95.5	9.5	92.0	2.7
	5.0	89.5	0.2	100.1	7.0
LW	0.5	73.9	7.8	84.6	3.7
	5.0	70.5	5.5	96.0	5.5

Table 2.6: Recovery of microcystins in tap waters obtained from (a) Aberdeen city and (b) Stonehaven using C18 and OASIS SPE cartridges followed by silica clean-up as determined by HPLC analysis.

(a)

Microcystin variant	Initial conc. $\mu g\ L^{-1}$	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	123.7	6.1	113.7	4.0
	5.0	113.6	6.5	121.8	4.1
LR	0.5	92.2	4.9	88.1	2.0
	5.0	87.3	6.5	91.9	1.2
LW	0.5	ND	ND	ND	ND
	5.0	59.7	5.1	86.9	1.0

ND indicates no data available

(b)

Microcystin variant	Initial conc. $\mu g\ L^{-1}$	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	114.3	6.5	107.2	3.6
	5.0	123.7	8.3	112.1	0.7
LR	0.5	91.7	9.7	81.2	0.6
	5.0	92.2	8.2	86.7	0.8
LW	0.5	62.3	1.4	81.6	0.1
	5.0	63.2	9.8	83.2	1.0

Table 2.7: Recovery of microcystins in raw waters obtained from (a) Loch Kinord and (b) Loch Rescobie using C18 and OASIS SPE cartridges followed by silica clean-up as determined by HPLC analysis.

(a)

Microcystin variant	Initial conc. $\mu g\ L^{-1}$	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	141.3	3.4	92.9	0.5
	5.0	120.6	7.9	106.6	0.4
LR	0.5	106.0	1.1	78.0	3.3
	5.0	95.2	6.4	85.9	0.7
LW	0.5	63.3	3.9	74.1	0.5
	5.0	67.7	2.5	82.4	0.1

(b)

Microcystin variant	Initial conc. $\mu g\ L^{-1}$	**C18** % recovery	CV %	**OASIS** % recovery	CV %
RR	0.5	140.0	3.1	92.1	1.2
	5.0	122.8	0.1	106.0	0.1
LR	0.5	86.0	4.9	73.2	0.1
	5.0	93.6	3.1	84.5	0.7
LW	0.5	60.7	3.7	77.0	3.7
	5.0	68.3	2.3	83.2	1.3

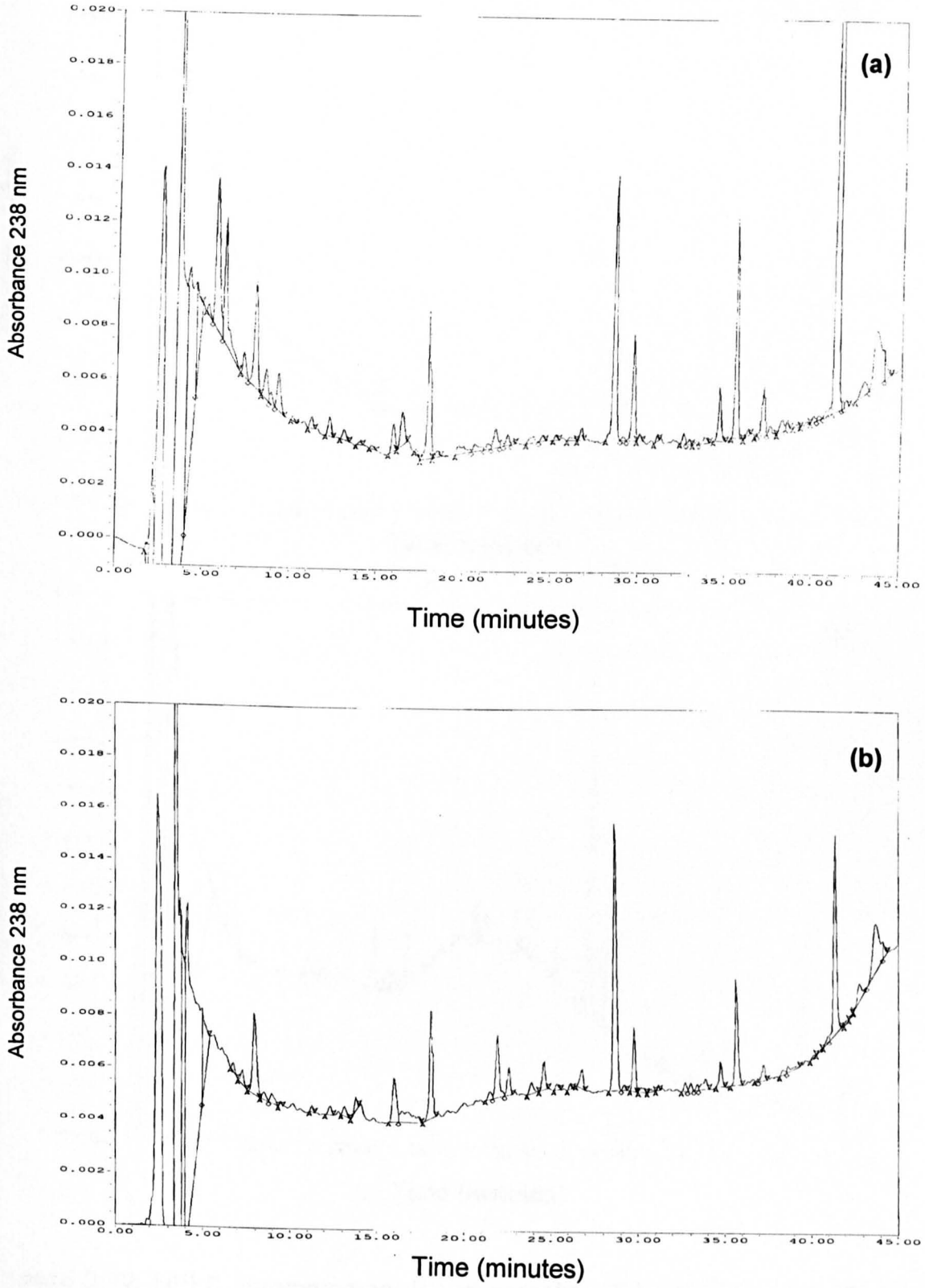


Figure 2.11: HPLC chromatogram for microcystins (0.5 μg l^{-1}) in Aberdeen tap water concentrated using (a) OASIS HLB and (b) C18 SPE cartridges.

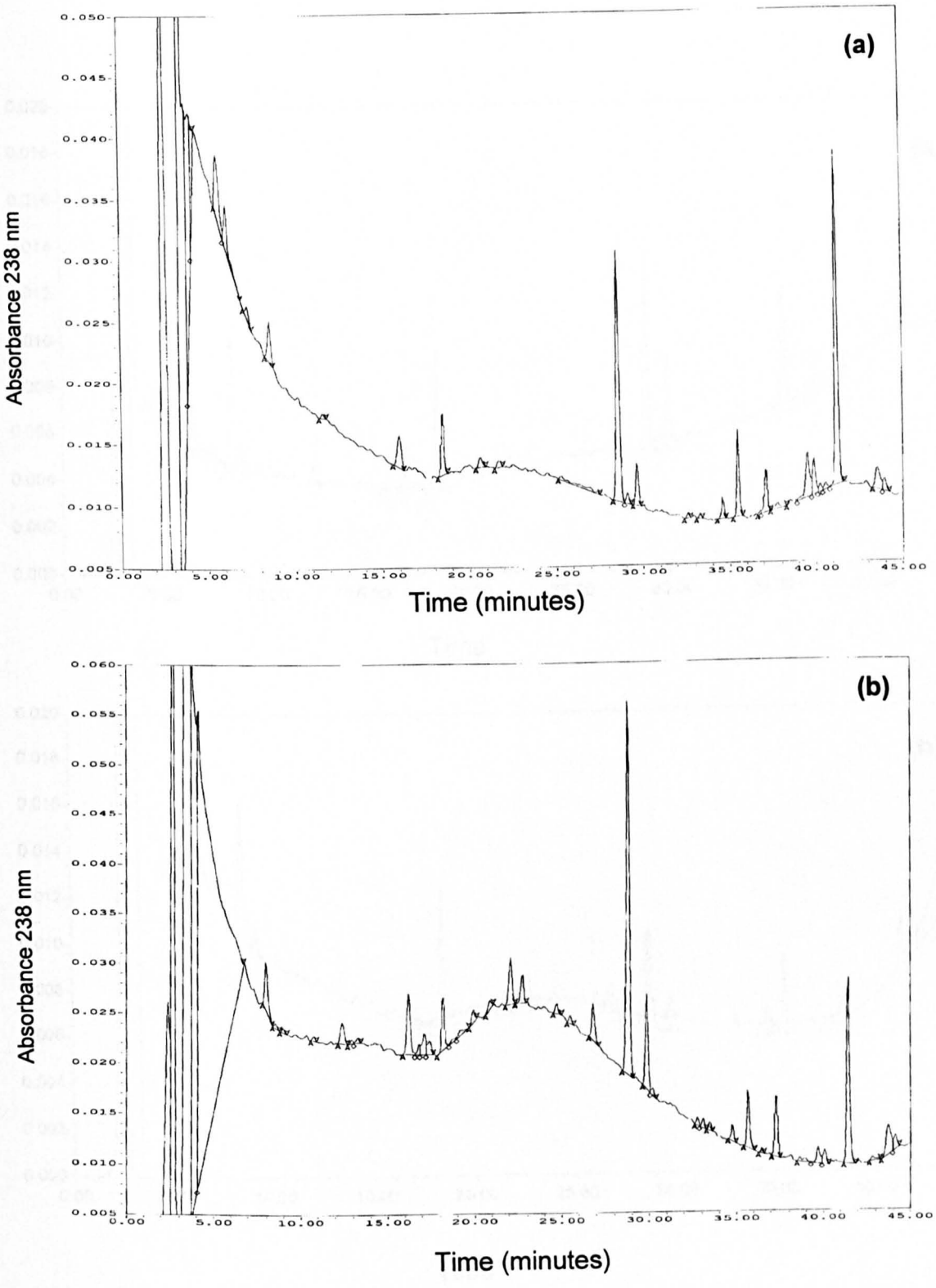


Figure 2.12: HPLC chromatogram for microcystins (0.5 μg l^{-1}) in Loch Kinord raw water concentrated on (a) OASIS HLB and (b) C18 SPE cartridges.

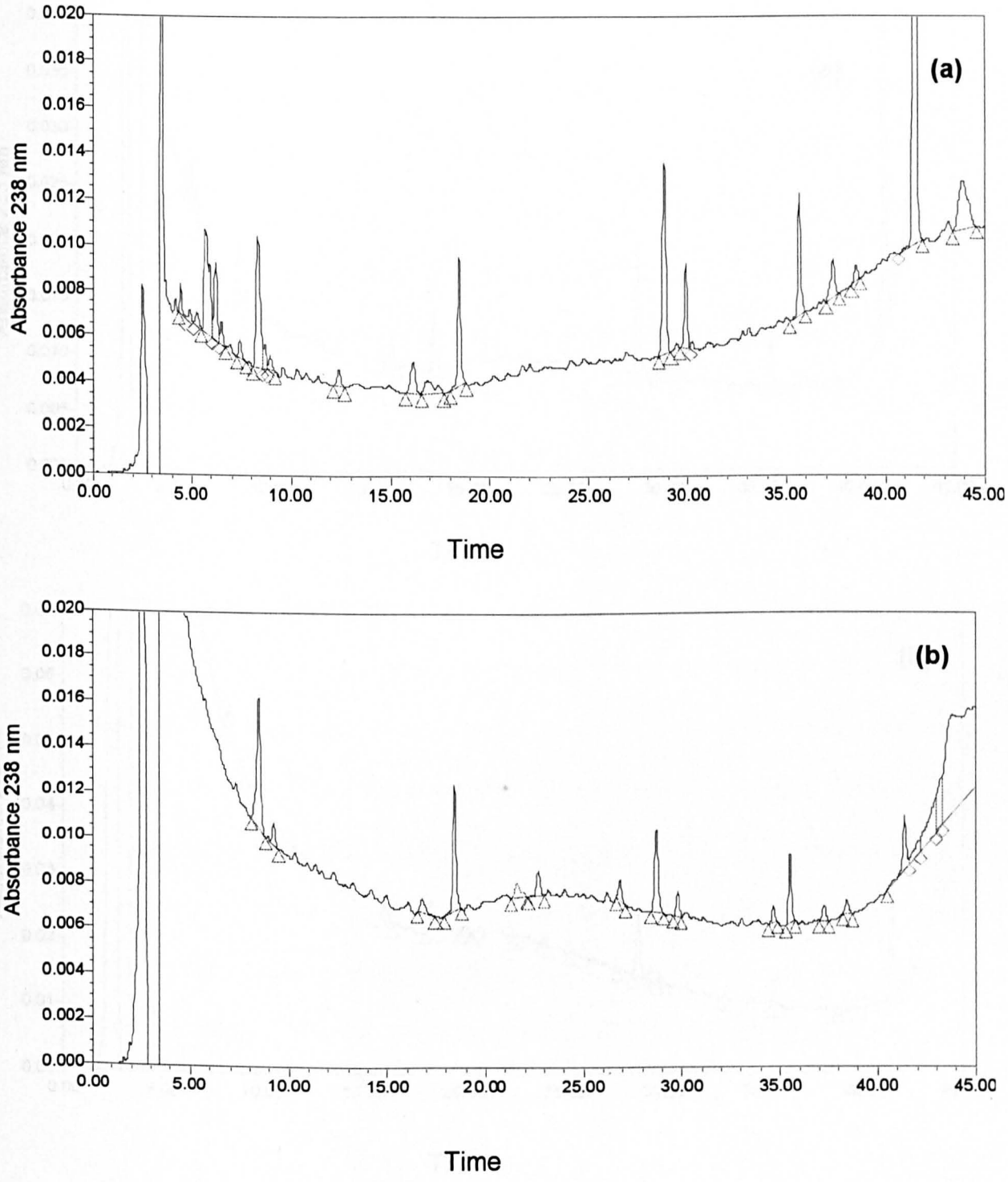


Figure 2.13: HPLC chromatogram for microcystins (0.5 μg l^{-1}) in Aberdeen tap water concentrated on (a) OASIS HLB and (b) C18 SPE cartridges followed by silica clean-up.

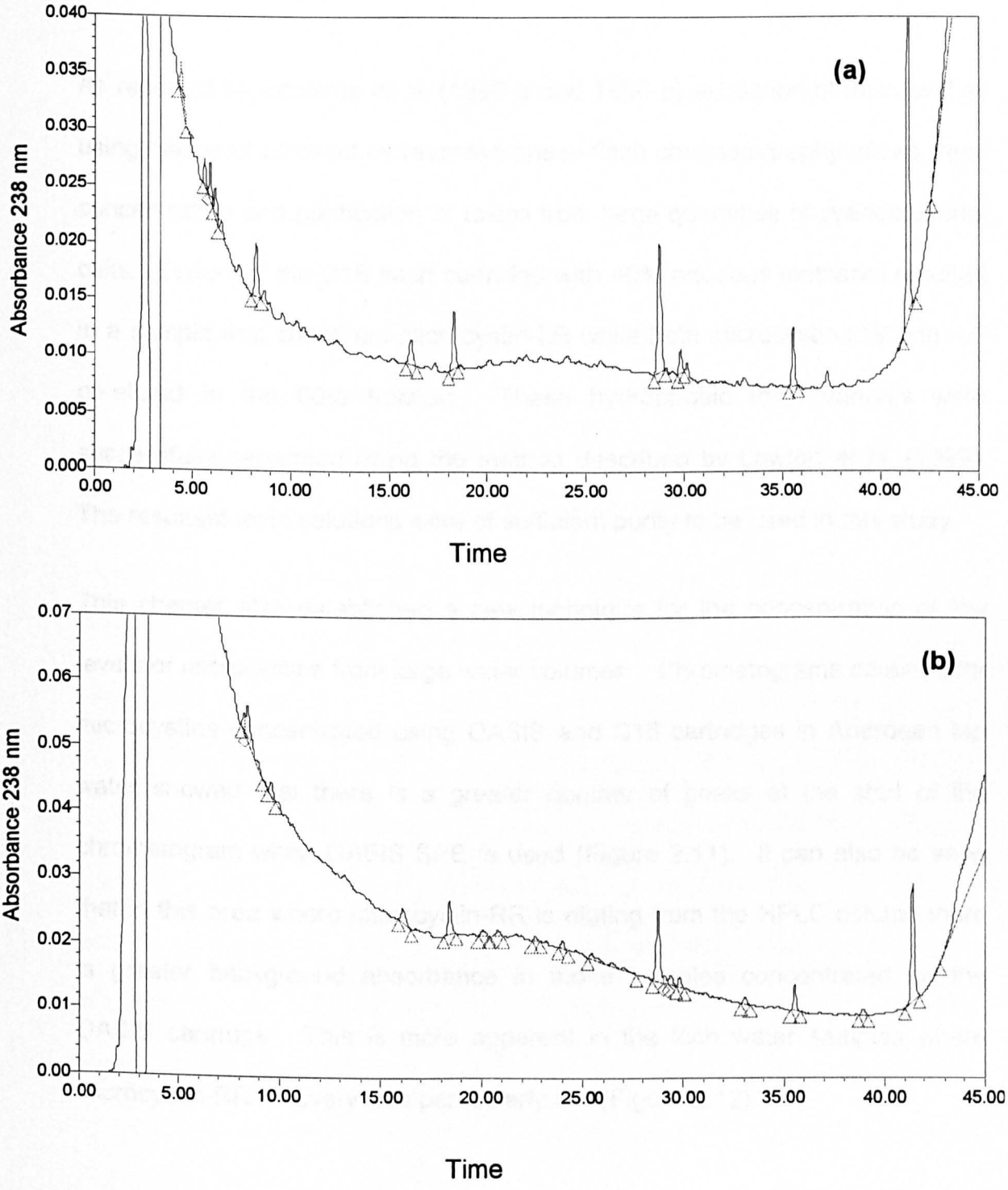


Figure 2.14: HPLC chromatogram for microcystins (0.5 μg l^{-1}) in Loch Kinord raw water concentrated on (a) OASIS HLB and (b) C18 SPE cartridges followed by silica clean-up.

2.4 Discussion

As reported by Edwards *et al.* (1996 a and 1996 b) extraction of microcystins using methanol followed by reversed-phase flash chromatography allows rapid concentration and purification of toxins from large quantities of cyanobacterial cells. Elution of the C18 flash cartridge with 40% aqueous methanol resulted in a sample that contained microcystin-LR while both microcystin-LW and -LF co-eluted in the 60% fraction. These hydrophobic toxin variants were successfully separated using the method described by Lawton *et al.* (1999). The resultant toxin solutions were of sufficient purity to be used in this study.

This chapter also established a new technique for the concentration of low levels of microcystins from large water volumes. Chromatograms obtained for microcystins concentrated using OASIS and C18 cartridges in Aberdeen tap water showed that there is a greater number of peaks at the start of the chromatogram when OASIS SPE is used (Figure 2.11). It can also be seen that in this area where microcystin-RR is eluting from the HPLC column there is greater background absorbance in those samples concentrated on the OASIS cartridge. This is more apparent in the loch water samples where microcystin-RR recovery was particularly low (Figure 2.12).

The apparent low levels of microcystin-RR recovered using OASIS cartridges were therefore believed to be as a result of coelution of polar compounds that were eluting at the same time as the toxin during HPLC analysis. Once the samples had been passed through the normal phase silica cartridge there was

an increase in microcystin-RR recovery for both cartridge types. Chromatograms obtained for OASIS samples gave less background absorption than those obtained prior to clean-up and again compared to C18 SPE (Figure 2.13). This was more apparent in those samples concentrated from raw loch water (Figure 2.14).

The study has shown that the OASIS HLB cartridge is a suitable alternative to the C18 SPE cartridge for the trace analysis of microcystins. However it was noted that recovery of microcystin-RR was greatly increased following sample clean-up through normal phase silica SPE. Overall OASIS gives good recoveries of microcystin toxins over a broad range of polarities. Whereas C18 SPE may not require normal phase clean-up for the more polar toxins the new OASIS cartridge is both easier to use and provides HPLC chromatograms which have smoother baselines. When the OASIS cartridges were first introduced, only small volumes of packing material were available, recently however cartridges with packing sizes equivalent to the C18 sorbent have been manufactured. It is possible that with increased sorbent volume the recovery of microcystin-RR may be improved using OASIS cartridges. It was therefore decided to use the OASIS SPE cartridges for trace analysis in this study due to their ease of use and efficient recovery of microcystins of varied polarities.

CHAPTER 3

TiO_2 Photocatalysis of Microcystin-LR

3.1 Introduction

Increased toxic cyanobacterial blooms brought about by continuing environmental eutrophication is causing concern due to the potential contamination of potable water supplies. Many of the cyanobacterial blooms that have been observed contain microcystins – the hepatotoxic cyanotoxins. It has been found that most conventional water treatments are not effective in removing microcystins and consequently alternative novel methods need to be investigated. Photocatalysis using TiO_2 has been investigated for the treatment of many contaminants in drinking water including chemicals such as bromate (Mills *et al.*, 1996) and organics e.g. humic substances (Eggins *et al.*, 1997). UV illumination of TiO_2 in solution produces oxidising hydroxyl radicals ($OH^{\bullet}$) which are believed to degrade compounds. Hydroxyl radicals are more oxidising than any other reagents currently being used in potable water treatment (Robertson, 1996). Some researchers (Okamoto *et al.*, 1985; Anpo *et al.*, 1991) have also proposed that superoxide radicals produced by the catalyst can play a role in pollutant destruction.

It has been previously shown that photocatalysis using TiO_2 effectively removes microcystin-LR from aqueous solution (Robertson *et al.*, 1997). This initial study showed that even high concentrations (200 $\mu g\ ml^{-1}$) of the toxin were rendered undetectable by HPLC analysis within a 40 minutes period. Robertson *et al.* (1997) also investigated the kinetics of the reaction. It was found that the rate that the toxin was destroyed was influenced by the initial concentration with the higher concentrations giving faster rates. Destruction

rates were so rapid that at lower toxin concentrations (< 50 μg ml^{-1}) microcystin-LR was reduced to below the limit of quantification after 2 minutes. At higher concentrations of toxin (200 μg ml^{-1}) the levels of microcystin-LR were reduced by almost three orders of magnitude to < 0.2 μg ml^{-1} within 30 minutes treatment time. This study suggested that TiO_2 photocatalysis might be an ideal method of eliminating microcystins from drinking water. However the fate of the toxin molecule or whether the toxicity related to microcystin-LR had been removed has not been elucidated.

The effectiveness of this process on microcystin-LR was also shown by Feitz *et al.* (1999) while Shephard *et al.* (1998) demonstrated successful degradation of microcystin-LR, -YR and -YA by TiO_2 photocatalysis. These studies however were carried out using crude extracts of the toxin which contained cyanobacterial cellular material. While this was done to simulate natural water content and reduce the effort required to purify significant quantities of toxin it was felt the use of purified toxin in this study was necessary to clearly elucidate the destruction process.

Research is necessary to determine whether photocatalytic degradation of the toxin results in full mineralisation or if breakdown by-products are formed as a result of the process. It must also be established that the toxicity of microcystin-LR has been removed hence rendering the water safe for human consumption before TiO_2 photocatalysis can be considered as a viable treatment method.

The following chapter presents studies that were carried out to determine the degree to which microcystin-LR was degraded and consequently investigates the presence of reaction products formed during photocatalysis. The toxicity of the microcystin-LR solution following photodegradation was determined using a simple invertebrate bioassay. Lawton *et al.* (1994a) assessed the brine shrimp assay using 21 hepatotoxic cyanobacterial bloom samples (including species containing microcystins) and found the results to be comparable to the previously used mouse bioassay.

Factors influencing photocatalysis such as solution pH and oxygen availability were also investigated here. Furthermore, preliminary determination of the mechanism by which degradation of microcystin-LR is thought to occur was also carried out. The concentration of microcystin-LR used in this study was higher than might be expected to occur in the natural environment enabling direct quantification by HPLC analysis without multistep processing (e.g. Lawton *et al.*, 1994b) which is required to quantify the lower levels found in the environment. Turchi and Ollis (1989) showed that increasing the concentration of TiO_2 catalyst increased the rate of the photocatalytic process. However their findings indicated that at concentrations above 1% (w/v) the rate begins to drop due to the scattering of the UV light by the catalyst in solution therefore this concentration was adopted. The TiO_2 used in this study is P25 (Degussa) which is the most commonly used form of the catalyst as its structure (70% anatase: 30% rutile) is thought to inhibit recombination of photogenerated holes used to generate the oxidising $OH^{\bullet}$ radicals.

3.2 Methods

3.2.1 Photocatalysis of microcystin-LR

Aqueous solutions of microcystin-LR (10 ml; ~200 μg ml^{-1}) were prepared in glass universal bottles using Milli-Q high purity water to which TiO_2 catalyst (1% w/v) was added and stirred using a magnetic stirrer for 5 minutes to equilibrate.

Solutions where then illuminated, in the presence of air and with constant stirring, using a xenon UV lamp (280 W UVASpot 400 lamp, Uvalight Technology Ltd; spectral output 330 - 450 nm) situated 30 cm from the reaction vessel. Samples (0.5 ml) were taken prior to catalyst addition and at timed intervals of 0, 2, 4, 6, 8, 10, 20 and 30 minutes of illumination then centrifuged (12,800 x g) for 10 minutes to remove catalyst prior to HPLC analysis (see section 2.2.4).

Microcystin-LR solutions were illuminated in the absence of TiO_2 over the same time period. Solutions were also prepared with and without catalyst and placed in a darkened area over the 30 minute experiment period.

3.2.2 Mineralisation of microcystin-LR

The extent to which microcystin-LR is mineralised during photocatalytic destruction was monitored using a method based on those described by Matthews *et al.* (1990) and Byrne (1997). The method involves CO_2 generated by minerilisation reacting with a $Ba(OH)_2$ solution in the conductivity cell resulting in the formation of insoluble $BaCO_3$ (equation 3.1).

$$Ba(OH)_2 + CO_2 \rightarrow BaCO_3 + H_2O \qquad \text{Equation 3.1}$$

As a consequence the conductivity of the $Ba(OH)_2$ solution is reduced, the drop in conductivity is then used to determine the amount of CO_2 produced.

The conductivity cell was calibrated by passing predetermined quantities of CO_2 through the solution and measuring the conductivity change (Oyster Conductivity meter). This was achieved by injection of Na_2CO_3 solutions of known concentration (0 – 3000 ppm) into 2 M H_2SO_4 in a sealed flask. 1 ppm Na_2CO_3 added is equivalent to 1 ppm CO_2 produced. The CO_2 generated in the flask was carried over to the conductivity cell by continual purging with CO_2 free nitrogen. The resulting change in conductivity was subsequently measured. The calibration curve (Appendix 1) was prepared by plotting change in conductivity against the quantity of carbon added to the H_2SO_4.

A 50 ml solution of 100 $\mu g\ ml^{-1}$ microcystin-LR was placed in a 100 ml 3 port round bottomed flask to which TiO_2 (1% w/v) was added. A carrier gas was

passed through the flask forcing the generated CO_2 along the system to the conductivity cell (Figure 3.1). The carrier gas used was pure oxygen and was first passed through a bottle containing $Ba(OH)_2$ to remove CO_2 that may be present. Samples were taken from the flask via the sample port prior to TiO_2 addition, 5 minutes after catalyst addition, then at 10 minute intervals for 1 hour of UV illumination. These samples were analysed by HPLC to confirm toxin destruction. At each sampling point the conductivity was recorded allowing the amount of CO_2 produced (i.e. mineralisation) to be determined.

3.2.3 HPLC-PDA analysis of photocatalytic breakdown products

Using Millennium[32] Chromatography Manager software (Waters, Watford, UK) chromatograms were monitored at 238 nm allowing the detection of microcystin-LR and other compounds that absorb at this wavelength. PDA absorbance spectral information between 200 and 300 nm were also obtained for each peak on the chromatogram. The similarity of any peak's absorbance spectrum to that of authentic microcystins was evaluated by use of a spectral library. The spectral library contained absorbance spectra of microcystin-LR, -LF, -LW, and -RR. Closeness of fit between the two spectra is indicated by a match angle, where the closer this value is to zero the nearer the spectral match. All peaks detected at 238 nm were assessed for their spectral closeness to the parent microcystin giving an indication of the degree of degradation.

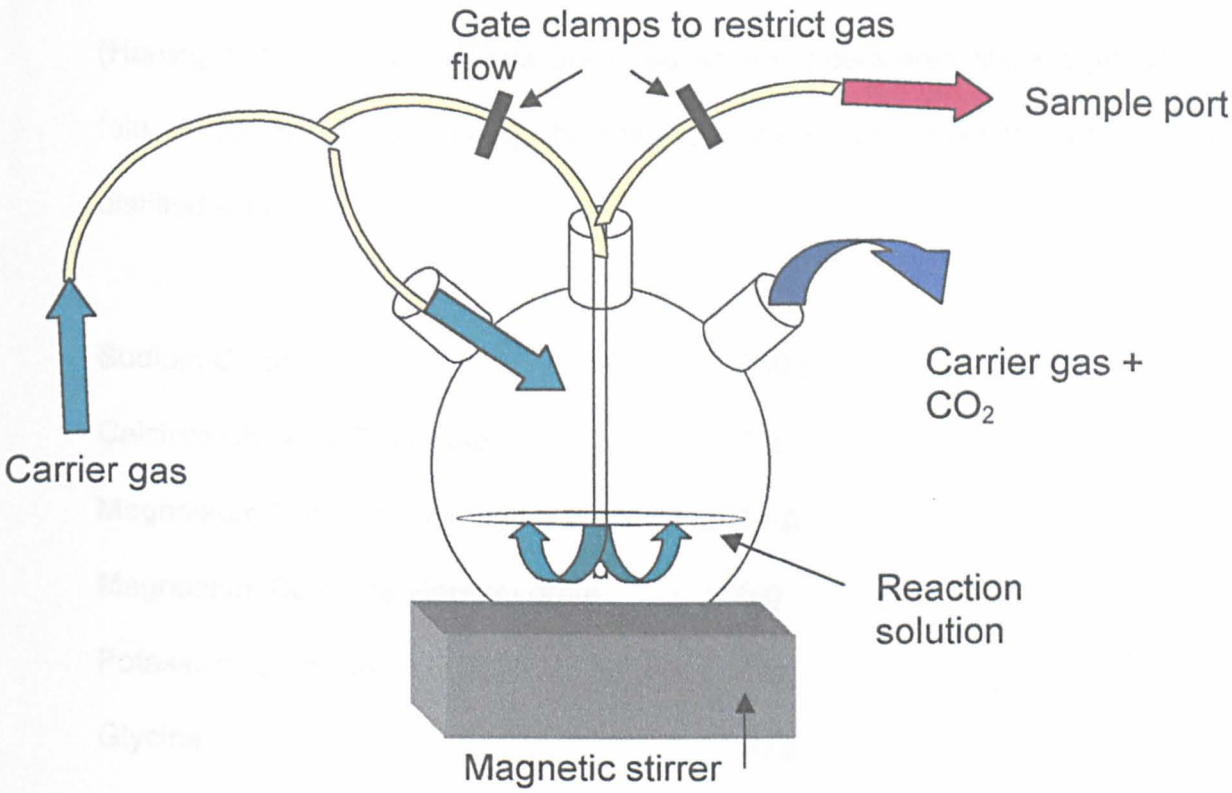


Figure 3.1: Apparatus used to monitor mineralisation of microcystin-LR. The carrier gas flows through the round bottomed flask where it collects the CO_2 produced by mineralisation and passes it through to the conductivity cell.

3.2.4 Toxicity assessment of by-products

To determine if the toxic effects associated with microcystin-LR were removed by photocatalytic oxidation a simple brine shrimp (*Artemia salina*) bioassay developed by Campbell *et al.* (1994) was carried out. Brine shrimp medium (Harwig and Scott, 1971) was prepared as a concentrated stock solution (8-fold concentration) containing the following chemicals added to 1.25 litres of distilled water:

Sodium Chloride	300 g
Calcium Chloride Dihydrate	3 g
Magnesium Chloride Hexahydrate	15 g
Magnesium Sulphate Heptahydrate	5 g
Potassium Chloride	8 g
Glycine	60 g
DiSoduim Glycerophosphate	30 g

Each chemical was dissolved separately to prevent precipitation and 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 brine shrimp cysts (Sciento, Manchester, UK; stored at -20°C until required) were added and incubated at 25°C for 48 hours.

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 30 individuals were placed in each well of a 96-well Microtitre™ plate (Dynaguard, Dynex Technologies, UK).

Microcystin-LR solutions before and after photocatalysis were filtered (0.22 μm) to remove any remaining particles of catalyst. Test solutions were adjusted to the same salt concentration as the media by addition of the appropriate amount of undiluted stock brine shrimp media. A dilution series from 0 to 100 mg ml^{-1} in 10 mg ml^{-1} increments was prepared for each of the photocatalytic exposure times (0, 2, 4, 6, 8, 10, 20 and 30 min.) and for the starting solution of microcystin-LR before the addition of catalyst. Four replicates were prepared for each of the dilution series and exposure times with 100 μl of test solution added to each well containing brine shrimps.

Brine shrimps were incubated in the presence of test solutions for 18 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 the LC_{50} values to be calculated.

3.2.5 Mass spectral-analysis of by-products

To investigate by-products of the photocatalytic destruction microcystin-LR the reaction mixture was analysed by electrospray MS. A Finegan Masslab Navigator with electrospray ionisation was utilised. The instrument uses a quadrupole mass filter enabling measurements up to 1600 m/z. Analysis was carried out in collaboration with Dr Marcel Jaspers, Aberdeen University.

3.2.6 Influence of pH on the photocatalysis of microcystin-LR

The effect of initial solution pH on the destruction of microcystin-LR was investigated as in the method 3.2.1 with the following modifications. Either nitric acid or sodium hydroxide was added to attain the required pH levels of 1, 5, 7, 10 and 12. A pH meter (Oyster) was used to determine when the required pH had been reached. For control purposes microcystin-LR solutions at each pH were illuminated in the absence of catalyst.

3.2.7 Photooxidation under a nitrogen atmosphere

The influence of oxygen on the photooxidation process was carried out to determine the importance of the presence of oxygen during photocatalysis of microcystin-LR. Photocatalysis was carried out as in section 3.2.1 with the

following modifications. The experiment was performed in a glove bag through which a constant flow of nitrogen was maintained to prevent oxygen being introduced to the system. Reaction solutions were sparged with nitrogen for 10 minutes prior to addition of catalyst to remove dissolved oxygen. Sparging continued throughout the duration of the experiment. TiO_2 was roasted in a furnace (Carbolite, Keison Products, Chelmsford, UK) at 650°C to eliminate oxygen from the catalyst's surface then allowed to cool in the nitrogen atmosphere within the glove bag prior to addition to the microcystin-LR solution. Samples were taken prior to catalyst addition then at timed intervals of 0, 10, 20, 30, 40, 50 and 60 minutes of UV illumination. For control purposes the experiment was repeated as above but with air being passed into the glove bag instead of nitrogen.

3.2.8 Photooxidation of microcystin-LR in D_2O (heavy water)

The photocatalytic destruction of microcystin-LR was carried out in heavy water (deuterated water, Fluorochem Ltd, Derbyshire, UK) as opposed to normal water (see section 3.2.1) to investigate its effect on the rate of the photocatalytic process. The objective of the kinetic isotope studies was to help elucidate the mechanism of the photocatalytic reaction. Samples were taken prior to catalyst addition then from 0 to 100 minutes in 10 minute intervals with a final sample taken after 200 minutes illumination. The experiment was carried out in water over the same time period but only up to the 100 minute illumination period.

3.3 Results

3.3.1 Photocatalysis of microcystin-LR

Results confirm that microcystin-LR was rapidly degraded on exposure to TiO_2 and UV light (Figure 3.2). Destruction was not observed when microcystin was illuminated in the absence of the catalyst or when TiO_2 was present with no illumination. There was an initial decline in toxin concentration of over 50% prior to UV illumination after the addition of the catalyst (Figure 3.2). This was previously observed by Robertson *et al.* (1997) and referred to as the dark reaction that is thought to correspond to the adsorption of microcystin-LR onto the surface of the TiO_2 catalyst.

3.3.2 Mineralisation of microcystin-LR

The calibration graph for the system (Appendix 1) shows the conductivity drop in solution corresponding to the amount of CO_2 produced. Using this simple conductivity method it was found that only 6.4% (377 ppm CO_2) of the microcystin-LR has been mineralised. This suggests that the photocatalytic process has not completely broken the toxin down and that reaction products that do not under-go complete degradation are present.

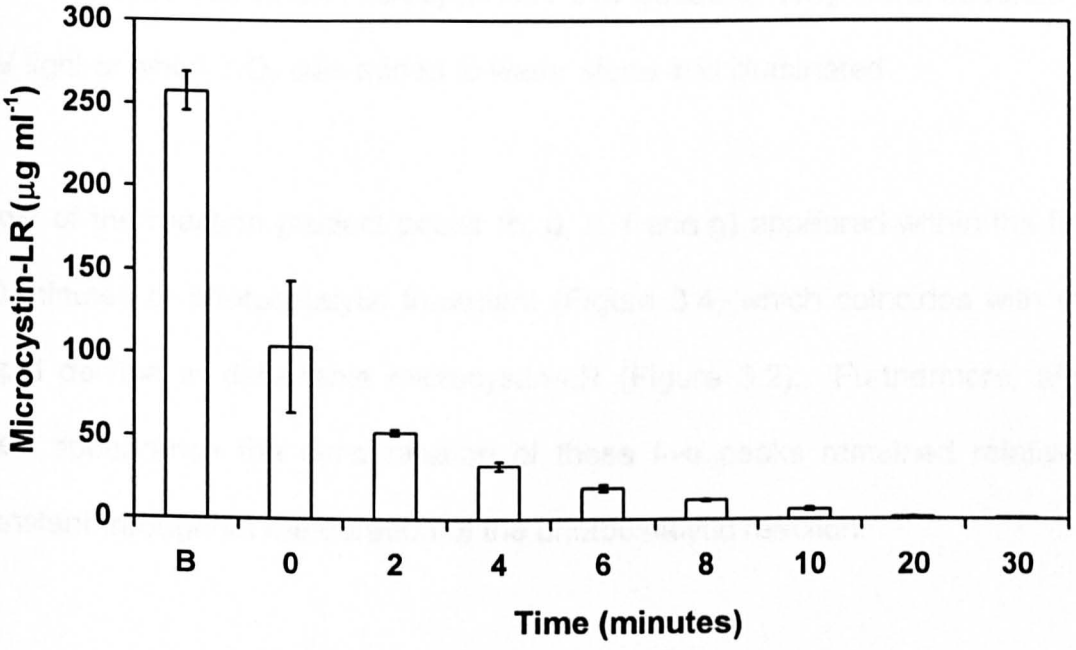


Figure 3.2: Destruction of microcystin-LR by TiO_2 photooxidation monitored by HPLC. B indicates the concentration of microcystin-LR prior to the dark adsorption resulting from the addition of the catalyst. Bars are equivalent to one standard deviation (n=2).

3.3.3 HPLC-PDA analysis of photocatalytic breakdown products

HPLC analysis of the reaction mixture following photocatalytic treatment revealed a number of UV absorbing products. The chromatogram at 238 nm after 10 minutes treatment (Figure 3.3c) shows seven distinct peaks that were not present in the microcystin-LR starting solution (Figure 3.3a). These peaks were not observed when microcystin-LR was added to TiO_2 in the absence of UV light or when TiO_2 was added to water alone and illuminated.

Most of the reaction product peaks (b, d, e, f and g) appeared within the first 10 minutes of photocatalytic treatment (Figure 3.4) which coincides with the rapid decline in detectable microcystin-LR (Figure 3.2). Furthermore, after their appearance the concentration of these five peaks remained relatively constant throughout the duration of the photocatalytic reaction.

Peak (a) was seen to increase gradually during the first 30 minutes of illumination after which it shows a slight decline. However the data was highly variable, particularly in the later part of the reaction period.

Peak (c) behaved quite differently to the other reaction products in that, although it appeared rapidly as did the other peaks, it gradually degraded over the duration of the reaction (Figure 3.4).

The absorbance spectrum between 200-300 nm (see insets in Figure 3.4) for each of these peaks was compared to that of microcystin-LR. Four of the

peaks (a,b,d and f) gave no match as determined by a match angle which were found to be >10.000, however all retained λ_{max} which were less than 10 nm different to microcystin-LR (Figure 3.4). Of the remaining three peaks, two were found to have spectra relatively similar to those in the library, with match angles of 3.196 for peak (c) and 4.021 for peak (g). Peak (e) gave a match of 9.996 which was just below the cut-off, and when studied more closely its spectrum is very similar to the closely eluting peak (d). Peak (c) not only gave the closest spectral match (3.196) to that of an authentic microcystin but was previously shown to degrade over the time of illumination. It can therefore be speculated that this product is only a slight modification of the microcystin-LR and that it is itself sensitive to photocatalytic degradation.

3.3.4 Toxicity assessment of by-products

Toxicity data determined using the brine shrimp assay corresponded well with the HPLC findings (Table 3.1). Reduction in toxicity mirrored the reduction in the concentration of microcystin-LR, which was seen to occur over the first 10 minutes of the photocatalytic treatment. As with the HPLC analysis, toxicity dropped on addition of the TiO_2 catalyst, which was explained by the dark reaction. On UV illumination toxicity dropped further with all samples tested after 8 minutes of very low toxicity and therefore an LC_{50} value could not be determined.

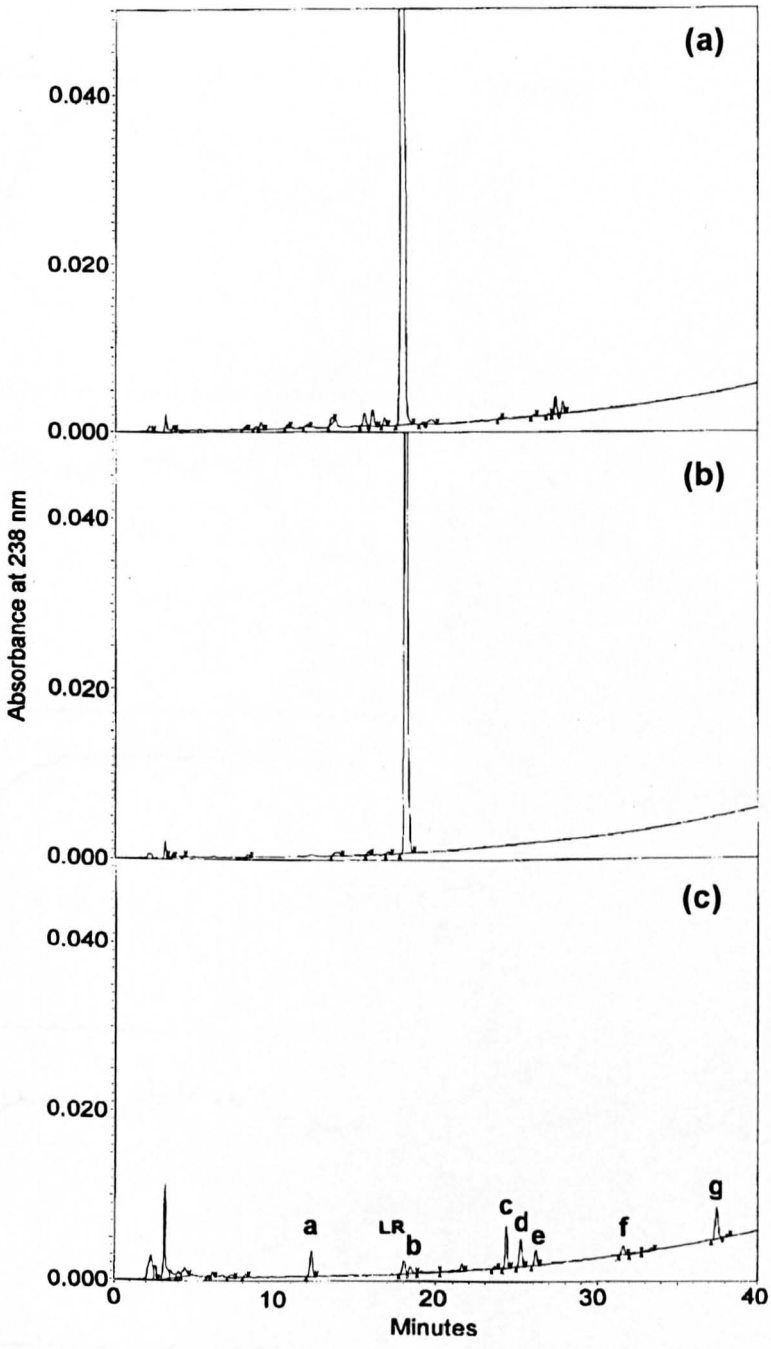


Figure 3.3: HPLC chromatogram at 238 nm of (a) microcystin-LR prior to addition of TiO_2, (b) after addition of TiO_2, not illuminated, and (c) after 10 min. photocatalytic oxidation with TiO_2.

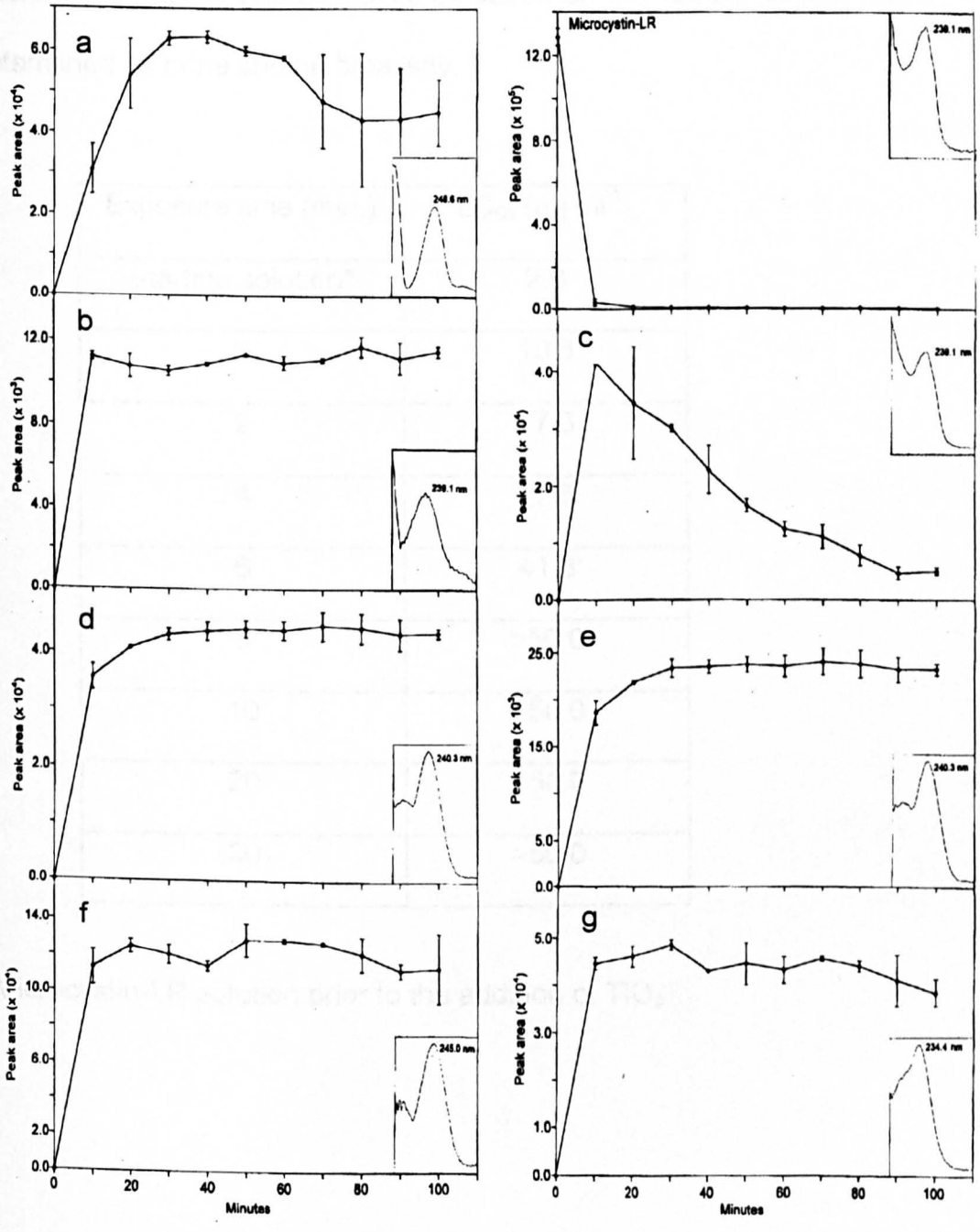


Figure 3.4: Presence of microcystin-LR and seven reaction products detected by HPLC during photocatalysis, inserts show absorbance spectra (200-300 nm) of each peak and λmax.

Table 3.1: Effect of photocatalytic oxidation on the toxicity of microcystin-LR, determined by brine shrimp bioassay.

Exposure time (min.)	LC_{50} ($\mu g\ ml^{-1}$)
Starting solution*	2.0
0	10.8
2	17.3
4	27.5
6	41.3
8	>50.0
10	>50.0
20	>50.0
30	>50.0

* Microcystin-LR solution prior to the addition of TiO_2

3.3.5 Mass spectral-analysis of by-products

Mass spectral data indicated several fragments of interest that are shown in Figure 3.5. The peak in the starting material at 995 m/z (Figure 3.5a) indicates the presence of the parent compound, microcystin-LR.

After 10 minutes of photocatalytic treatment (Figure 3.5b) a peak at 1029.7 m/z was observed which was still present after 30 minutes (data not shown) treatment before disappearing. Also at 10 minutes a peak at 835.5 m/z becomes prominent along with a smaller peak at 817.5 m/z. Following a further 10 minutes of photocatalysis at 20 minutes treatment time (Figure 3.5c) the peak at 835.5 m/z disappears and the peak at 817.5 m/z becomes dominant. This peak remains present in the sample right up to 100 minutes of photocatalyst (Figure 3.5e) indicating that this is a very stable by-product.

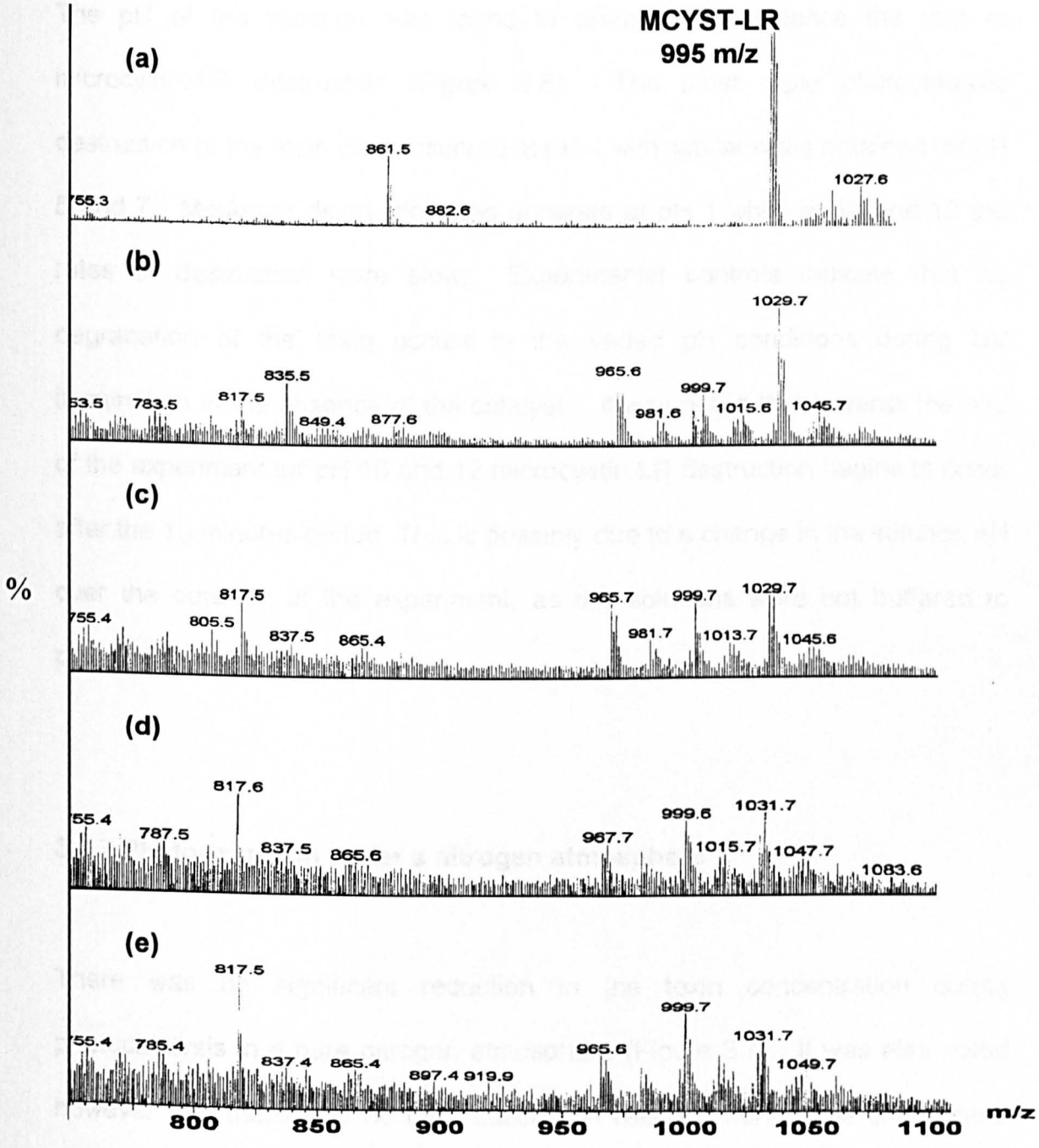


Figure 3.5: Mass Spectral data of (a) microcystin-LR prior to photocatalysis; (b) after 10 minutes photocatalytic oxidation with TiO_2; (c), (d) and (e); after 20, 60 and 100 minutes respectively of photocatalytic oxidation.

3.3.6 Influence of pH on photocatalysis of microcystin-LR

The pH of the reaction was found to dramatically influence the rate of microcystin-LR destruction (Figure 3.6). The most rapid photocatalytic destruction of the toxin was observed at pH 4 with similar rates obtained for pH 5 and 7. Moderate destruction was obtained at pH 1 while at 10 and 12 the rates of destruction were slow. Experimental controls indicate that no degradation of the toxin occurs in the varied pH conditions during UV illumination in the absence of the catalyst. It was noted that towards the end of the experiment for pH 10 and 12 microcystin-LR destruction begins to occur after the 10 minutes period. This is possibly due to a change in the solution pH over the duration of the experiment, as the solutions were not buffered to prevent pH drifts.

3.3.7 Photooxidation under a nitrogen atmosphere

There was no significant reduction in the toxin concentration during photocatalysis in a pure nitrogen atmosphere (Figure 3.7). It was also noted however that there was no dark adsorption reaction during this experiment. This was similarly observed in the oxygen atmosphere control, yet despite the loss of the dark adsorption reaction the toxin was still destroyed as expected (data not shown).

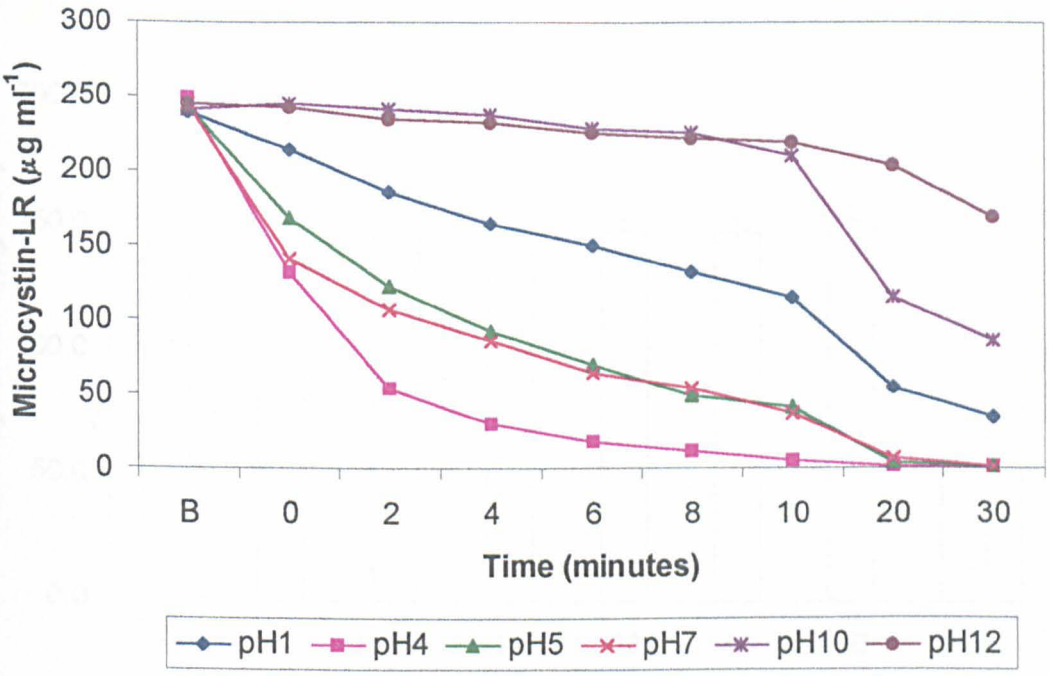


Figure 3.6: Destruction of microcystin-LR at different pH, monitored by HPLC. B indicates the concentration of microcystin-LR prior to the dark adsorption resulting from the addition of the catalyst.

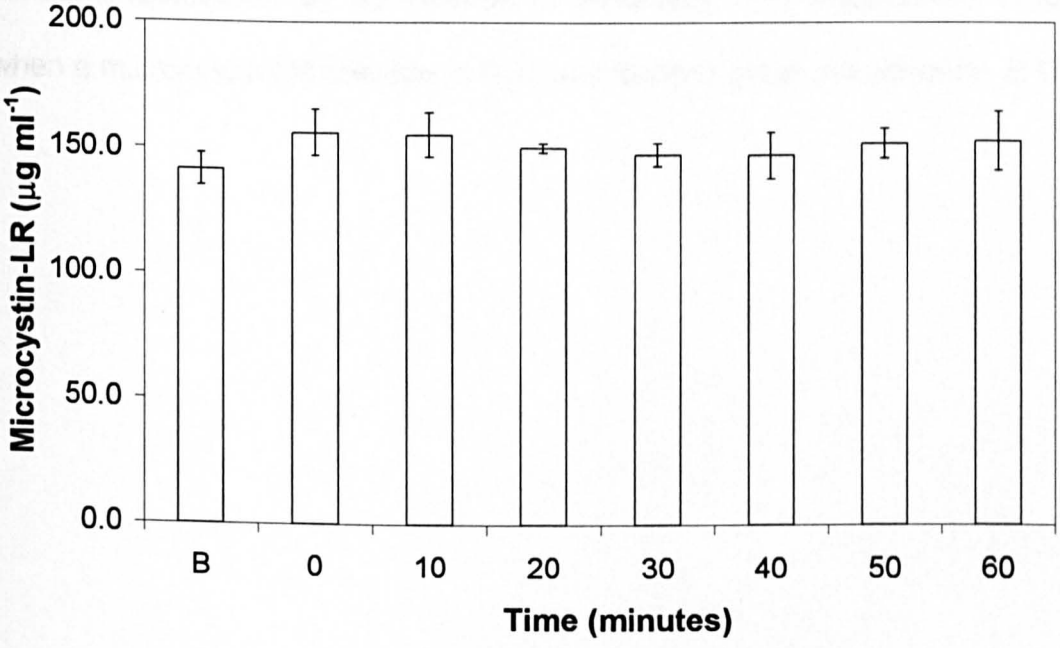


Figure 3.7: Destruction of microcystin-LR by TiO_2 photooxidation in a nitrogen atmosphere monitored by HPLC. B indicates the concentration of microcystin-LR prior to the dark adsorption resulting from the addition of the catalyst. Bars are equivalent to one standard deviation (n=2).

3.3.8 Photooxidation of microcystin-LR in D_2O (heavy water)

It was found that the rate of destruction of microcystin was greatly reduced when photocatalysis was carried out using D_2O as a solvent (Figure 3.8). The results show that the toxin was still present even after 200 minutes of photocatalytic treatment in D_2O. In normal water toxin concentrations are almost undetectable by 30 minutes of treatment. No degradation occurred when a microcystin-LR solution in D_2O was illuminated in the absence of TiO_2.

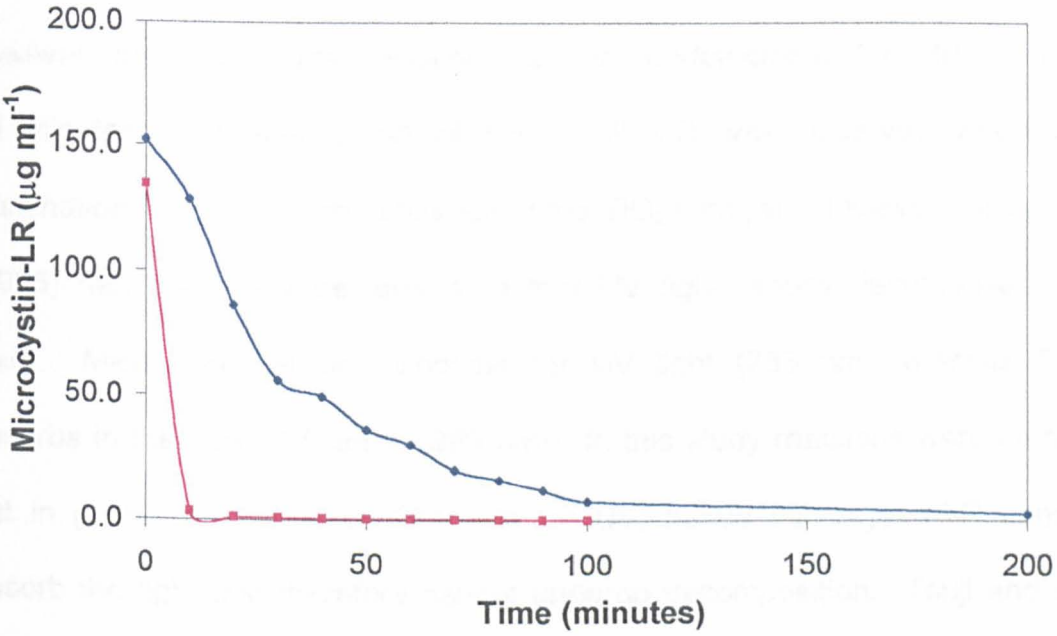


Figure 3.8: Destruction of microcystin-LR by TiO_2 photooxidation in H_20 [■] and D_2O [◆] solvents monitored by HPLC.

3.4 Discussion

The results confirm that TiO_2 photocatalysis rapidly breaks down microcystin-LR. Concentrations of toxin used in this study were much higher (almost 1000 times) than used by Shephard *et al.* (1998) and Feitz *et al.* (1999) however the toxin was rendered almost undetectable by HPLC after 30 minutes. No destruction of microcystin-LR was observed when UV illumination was used in the absence of the TiO_2 catalyst. However Tsuji *et al.* (1995) had previously demonstrated that UV light readily decomposed the toxin. Microcystin-LR only absorbs far UV light (238 nm) whereas TiO_2 absorbs in the near UV range (380 nm). In this study reactions were carried out in glass vessels which filter out far UV, hence microcystin-LR cannot absorb the light and therefore cannot undergo decomposition. Tsuji and co-workers used quartz vessels which permit the far UV wavelengths to pass into solution to be absorbed by the toxin to initiate decomposition. TiO_2 utilises near UV wavelength sources which are less energy intensive and hence more economic then if far UV was required for direct photolysis of the microcystin-LR.

Most compounds subjected to photocatalytic destruction have been found to be completely mineralised (Turchi and Ollis, 1989; Schmelling and Gray, 1995) although the findings here indicate that this is not the case for microcystin-LR. Only 6.4% mineralisation of the toxin was determined and PDA results also indicated the presence of several breakdown by-products. Yet the results have shown that TiO_2 photocatalysis does eliminate the toxicity associated

with microcystin-LR suggesting that these by-products are of limited threat to human health.

A pathway for the formation of some of the breakdown products of microcystin-LR has been postulated based on the mass spectral data obtained in section 3.3.5. (Figure 3.9). The initial reaction is believed to be the dihydroxylation of either of the double bonds of microcystin-LR (Figure 3.9, structure 1), this is indicated by the increase from 995.7 m/z $[M + H]^+$ to 1029.7 m/z $[(M + 2OH) + H]^+$ (Figure 3.9, structures 2 and 3). This suggests that in the case of microcystin-LR photocatalytic destruction occurs via $OH^\bullet$ rather that direct band oxidation. The peak at 835.5 m/z $[M + H]^+$ is possibly due to cleavage occurring at the Adda CH=CMe bond (Figure 3.9, structure 4). The smaller peak at 817.5 m/z $[M + Na]^+$ which appears alongside this peak is consistent with cleavage occurring at the Adda CH=CH bond (Figure 3.9, structure 5).

The reason that the 835.5 m/z peak disappears altogether can be attributed to the fact that a further cleavage step may occur at the Adda CH=CH bond via structure 6 giving rise to structure 5. This is the final structure in the pathway as this peak is present up to 100 minutes of photocatalytic treatment.

The mass spectral fragmentation data is supported by the UV spectra acquired during the HPLC run (Figure 3.4 insets). The calculated value of λ for microcystin Adda is 240 nm (including solvent correction), which is

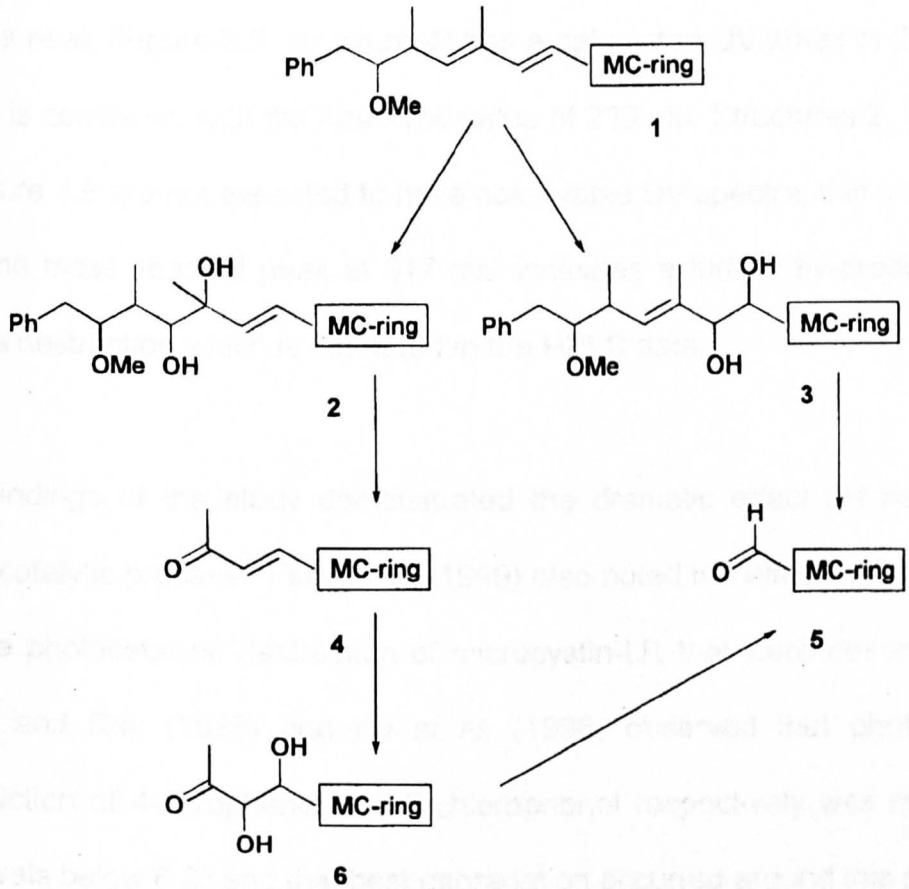


Figure 3.9: Scheme of proposed microcystin-LR breakdown products following TiO_2 photocatalysis based on mass spectral data.

identical to that of the observed value. The rapid increase of peak (c) in Figure 3.4 followed by its rapid decrease is similar to the behavior of the 835 m/z peak in the mass spectral data (Figure 3.5). The suggested structure for this peak (Figure 3.9, structure 4) has a calculated UV λmax of 235±5 nm, which is consistent with the observed value of 239 nm. Structures 2, 3, 5 and 6 in Figure 3.9 are not expected to have observable UV spectra, this would imply that the mass spectral peak at 817 m/z indicates a further by-product of the toxin's destruction which is not noted in the HPLC data.

The findings of the study demonstrated the dramatic effect pH has on the photocatalytic process. Feitz *et al.* (1999) also noted the effects of solution pH on the photocatalytic destruction of microcystin-LR that were observed here. Chen and Ray (1998) and Ku *et al.* (1996) observed that photocatalytic destruction of 4-nitrophenol and 2-chlorophenol respectively was reduced at pH levels below 6.25 and that best degradation occurred around this point.

Microcystin-LR has been shown to be stable at both acid and alkali conditions (Harada *et al.* 1996) hence solution pH has no direct effect on the destruction of the toxin. This stability was confirmed here as no destruction of the toxin was observed when solutions at different pH were illuminated in the absence of the catalyst. Consequently the findings in this study and in those by other researchers must be due to the effect of pH on the surface charge of the catalyst itself and the charge of the pollutant.

The zero point of charge (pH_{zpc}) for the TiO_2 catalyst is pH 6.25 (Hoffman *et al.*, 1995). Below this pH the surface charge of the TiO_2 is positively charged and will attract anions, above this pH and the catalyst acts in the opposite manner. Microcystin-LR has an isoelectric point of pH 2, below this pH the toxin is positively charged and above negatively. Therefore there is a pH range in which the adsorption of microcystin onto the catalyst's surface is highly favoured (Figure 3.10), outwith this area adsorption is less likely to occur as both compounds have similar charge and will repel one another.

The experimental results agree with this as pH 4 and 5 fall within the area of best adsorption and pH 10 and 12 well outwith this region. The moderate destruction rate observed at pH 1 may be due to the fact this pH is at the borderline of the ideal adsorption range. Despite the fact that pH 7 is outwith the area of ideal adsorption the rate of destruction was comparable to pH 4 and 5. This is probably due to a pH drift into the ideal adsorption range at the start of the reaction as the test solutions were not buffered. These results suggest that surface adsorption plays an important role in the photocatalytic degradation of the toxin, at the pH range of maximum adsorption the highest reaction rates were achieved.

This was not the case however in section 3.3.7 in which the complete loss of dark adsorption, following the roasting of the catalyst at high temperatures, did not effect the degradation of the toxin. The surface of P25 TiO_2 is hydrated and it is possible that adsorbed water on the catalyst surface may have a role

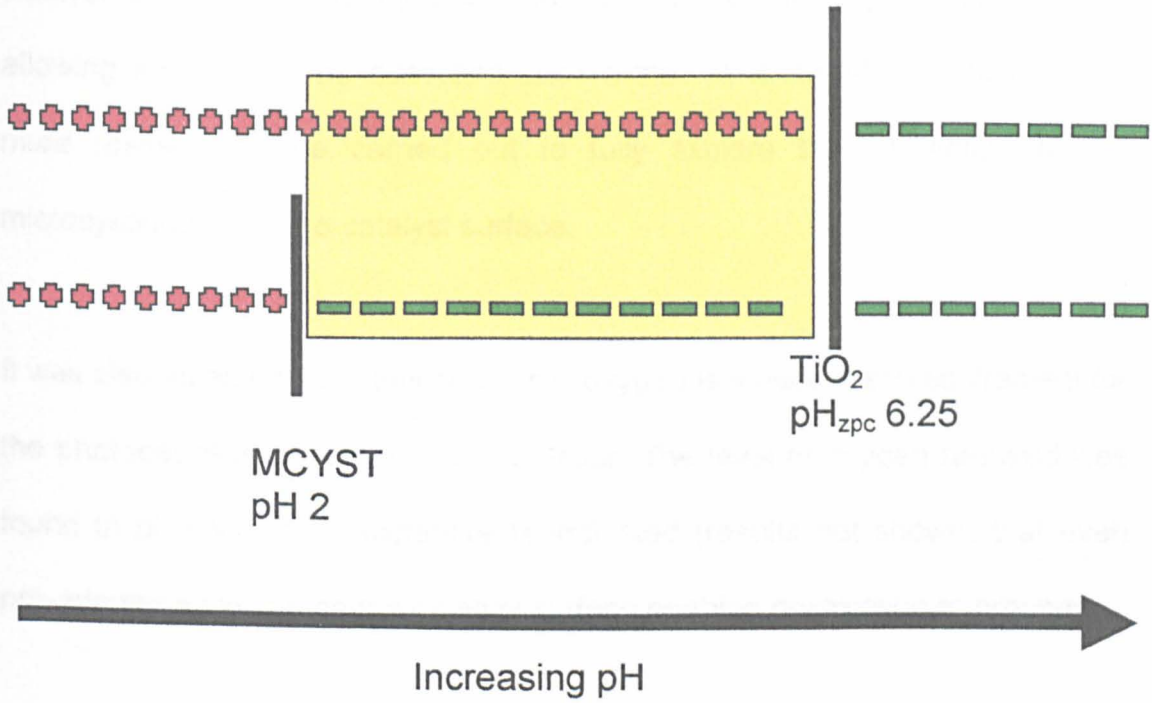


Figure 3.10: Effect of solution pH on the charge of microcystin-LR and TiO_2 [+] indicates a positive charge [-] negative charge. The yellow area indicates where attraction of microcysin-LR (with positive charge) to the TiO_2 surface (with negative charge) occurs. Outwith this area both compounds have the same charge and no adsorption occurs.

in the dark adsorption reaction as shown in Figure 3.11. When the TiO_2 was roasted this surface water would have been evaporated off and was therefore not available to react with the microcystin-LR. However on illumination of the catalyst in the presence of oxygen degradation of the toxin was rapid despite this loss of dark adsorption. This is possibly due to the rehydration of the catalyst surface caused by the activation of the TiO_2 by UV light hence allowing the toxin to be adsorbed once more. It is therefore necessary for more research to be carried out to fully explore the interaction of the microcystin-LR with the catalyst surface.

It was also established in this study that oxygen is a necessary requirement for the photodegradation of the toxin to occur. The level of oxygen required was found to be very small, experiments indicated (results not shown) that even pre-adsorbed oxygen on the catalyst surface enabled destruction to proceed.

It can be concluded from the results that neither the toxin nor any of the intermediate or final by-products of degradation are capable of acting as an electron acceptor in place of oxygen. Therefore electrons formed in the catalyst will recombine with electron holes preventing the formation of the $OH^{\bullet}$ radicals required for photodegradation to occur (see section 1.6.2). It is also the belief by some researchers that the products of oxygen conductance band reduction (e.g. $O_2^{\bullet-}$, $HO_2^{\bullet}$ and H_2O_2) play a role in the photooxidation process (Okamoto *et al.*, 1985; Anpo *et al.* 1991). The loss of degradation may be a result of the loss of these agents in solution. It is possible that photodegradation occurs via hydroxyl radicals generated as a result of the

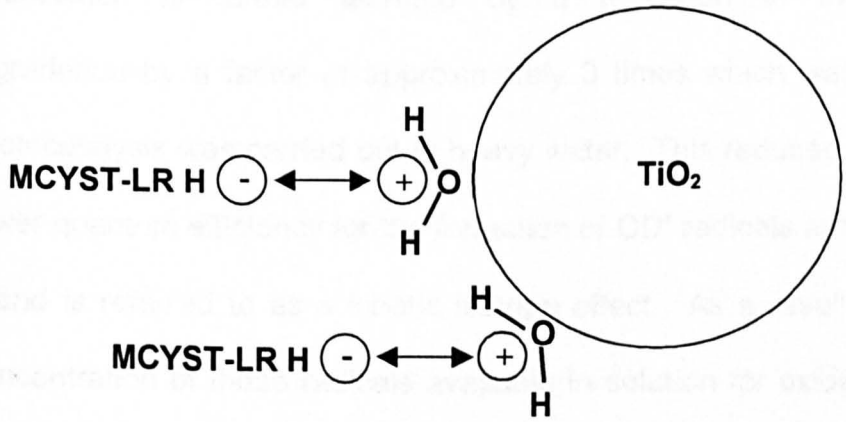


Figure 3.11: Diagram demonstrating the possible role of water on the surface of TiO_2 in the adsorption of microcystin-LR (MCYST-LR).

superoxide radical anion ($O_2^{\bullet-}$) being hydrated by the solvent. Therefore the rate determining step may in fact be the generation of this superoxide radical at the catalyst surface, not the formation of the $OH^{\bullet}$ radical. However since hydroxylated by-products were detected by mass spectroscopy it would appear that in the case of microcystin-LR the photooxidation process is initiated by hydroxyl radical attack on the microcystin.

This speculation is further affirmed by a reduction in the rate of photodegradation by a factor of approximately 3 times which was observed when photocatalysis was carried out in heavy water. This reduced rate is due to the lower quantum efficiency for the formation of $OD^{\bullet}$ radicals at the catalyst surface and is referred to as a kinetic isotope effect. As a result there is a lower concentration of these radicals available in solution for oxidation of the microcystin than there would be of $OH^{\bullet}$. This effect has also been noted by other researchers including Cunningham and Srijaranai (1988) who obtained an identical kinetic isotope effect value of 3 for the destruction of isopropanol using TiO_2. This would not only imply that the hydroxyl radicals are the main agent in the toxin's destruction but also indicates that the formation of the $OH^{\bullet}$ radicals is the rate determining step in the photocatalytic process.

In contrast however some researchers (Draper and Fox, 1990; Mao *et al.*,1991) have proposed that the formation of hydroxylated products does not necessarily mean that hydroxyl radicals are involved. They believe that the substrates may undergo direct hole oxidation at the catalyst's surface and are

then hydrated by the solvent. Likewise the kinetic isotope effect may be due to the reduced interaction of the deuterium with the superoxide anion compared to water.

Overall the experimental evidence seems to indicate that hydroxyl radicals are the main oxidant in the photocatalytic destruction of microcystin-LR. This is supported by the formation of hydroxylated products and the observed kinetic isotope effect. However the rate determining step in the destruction of the microcystin-LR has yet to be confirmed.

In conclusion, TiO_2 photocatalysis has proven to be a reliable and efficient treatment method in the destruction of microcystin-LR. Despite the fact that the process results in by-products both UV and MS detectable, the toxicity of the microcystin-LR solution has been shown to have been removed. A tentative breakdown pathway has been derived using data gathered throughout the study and consequently the mechanism by which photodestruction occurs has been theorised. Further studies must now be carried out to improve the process and determine the effectiveness of this treatment method in natural water samples.

CHAPTER 4

H_2O_2 Enhanced Photocatalysis of Microcystin-LR

4.1 Introduction

The use of hydrogen peroxide (H_2O_2) in combination with UV light has been investigated by many researchers studying the degradation of organic and chemical pollutants (Bose *et al.*, 1998; Beltrán *et al.*, 1997). Equation 4.1 shows how UV light enhances the oxidative power of H_2O_2 by dissociating the molecule resulting in highly oxidative hydroxyl radical generation (Baxendale and Wilson, 1957).

$$H_2O_2 + \text{UV light} \rightarrow 2OH^{\bullet} \qquad \text{Equation 4.1}$$

Furthermore some researchers have found that the addition of H_2O_2 to the photocatalytic system enhances photocatalysed destruction of pollutants (Grätzel *et al.*, 1990; Tanaka *et al.*, 1990; Jakob *et al.*, 1993). Most of the researchers who investigated the $UV/H_2O_2/TiO_2$ system compared the rate enhancing effects of H_2O_2 only with UV/TiO_2 data (Martin *et al.*, 1995; Grätzel *et al.*, 1990), no comparison was made with UV/H_2O_2 data.

Recently however, Wang and Hong (1999) studied the effects of the UV/TiO_2, UV/H_2O_2 and $UV/TiO_2/H_2O_2$ systems on the degradation of 2-chlorobiphenyl. They found that although H_2O_2 enhanced the rate of photocatalytic destruction when used in combination with UV/TiO_2 the destruction with the UV/H_2O_2 system was the most efficient. They demonstrated that TiO_2 photocatalysis was not being enhanced by the H_2O_2 but in this case the catalyst was inhibiting the oxidising power of the H_2O_2. These researchers speculated that this was

due to interaction of the peroxide with the $OH^{\bullet}$ radicals formed by the TiO_2 thereby reducing the concentration of these oxidising agents in solution.

Not much work has been carried out on the effectiveness of H_2O_2 in destroying microcystin-LR with or without UV illumination. Rositano and Nicholson (1994) found some degradation of the toxin occurred in the presence of H_2O_2 however a UV/H_2O_2 system achieved almost 50% removal.

This chapter investigates the use of TiO_2 photocatalysis combined with H_2O_2 as a means of enhancing the photodegradation of microcystin-LR. The study compares the efficiency of the $UV/TiO_2/H_2O_2$ system with both UV/TiO_2 and UV/H_2O_2 systems.

4.2 Methods

4.2.1 Photocatalysis of microcystin-LR in the presence of H_2O_2

Photocatalysis was carried out as in section 3.2.1 with the following modifications. Hydrogen peroxide concentrations of 0.01, 0.1 and 0.6% (v/v) in aqueous microcystin-LR (200 μg ml^{-1}) solutions were obtained by dilution of a 6% hydrogen peroxide solution (Fisher Scientific UK Ltd., Leicestershire, UK). All solutions were prepared using Milli-Q water (Millipore, Watford, UK).

4.2.2 Determination of optimum hydrogen peroxide level

Photocatalysis was carried out as previously shown on 100 μg ml^{-1} microcystin-LR solutions. Hydrogen peroxide concentrations of 0.001, 0.005, 0.01, 0.05, 0.1 and 0.5% (v/v) in aqueous microcystin-LR solutions were obtained by dilution of a 6% hydrogen peroxide solution (Fisher Scientific UK Ltd., Leicestershire, UK). All solutions were prepared using Milli-Q water (Millipore, Watford, UK).

4.2.3 Mineralisation of microcystin-LR in the presence of H_2O_2

Mineralisation of microcystin-LR by photocatalysis in the presence of 0.1% (v/v) H_2O_2 was carried out as in section 3.2.2.

4.2.4 HPLC-PDA analysis of photocatalytic solutions

Microcystin-LR was detected and quantified by HPLC with Photo Diode Array (PDA) detection. Chromatograms at 238 nm and absorbance spectra were obtained as in section 3.2.3.

4.2.5 Toxicity assessment

The toxicity of photocatalysed solutions of microcystin-LR using TiO_2 in the presence of 0.01% (v/v) H_2O_2 was determined using the brine shrimp bioassay. The method is the same as described in section 3.2.4.

4.2.6 Quantification of hydrogen peroxide levels

The levels of H_2O_2 in solution over the duration of UV illumination were monitored by a self-indicating potassium permanganate titration. A 100 ml

sample of 0.1% (v/v) H_2O_2 containing TiO_2 (1% w/v) was exposed to UV light (280 W UVASpot 400 lamp, Uvalight Technology Ltd; spectral output 330 - 450 nm) and 10 ml samples were taken at timed intervals of 0, 2, 4, 6, 8, 10, 20 and 30 minutes. Samples were transferred to 250 ml conical flasks to which 100 ml water and 10 ml H_2SO_4 (2M) was then added. Solutions were titrated using standard potassium permanganate solution (0.02 M) to the first permanent pink colour. The percentage of H_2O_2 in each sample was then calculated and consequently the drop in oxidant levels determined. This procedure was repeated with a H_2O_2 solution containing catalyst in the absence of illumination and then for solutions of H_2O_2 without TiO_2.

4.2.7 Effect of hydrogen peroxide on dark adsorption

To determine the effect of H_2O_2 in solution on the adsorption of microcystin-LR onto the TiO_2 surface the following experiments were carried out.

(1) A 10 ml microcystin-LR solution (200 μg ml^{-1}) was prepared to which 1% (w/v) TiO_2 was added and allowed to equilibrate for 5 minutes. A 0.5 ml sample was removed then centrifuged to remove the catalyst, the toxin level was determined by HPLC analysis as before.

(2) A 10 ml microcystin-LR solution (200 μg ml^{-1}) containing 0.6% (v/v) H_2O_2 was prepared and allowed to equilibrate for 5 minutes after which 1% (w/v)

TiO_2 was added. A sample was processed as before to determine microcystin-LR concentration.

(3) A 10 ml solution of 1% (w/v) TiO_2 and 0.6% (v/v) H_2O_2 was prepared and left for 5 minutes to equilibrate. After this time microcystin-LR was added to the solution to give the required starting concentration (200 $\mu g\ ml^{-1}$). Following a further 5 minutes a sample was removed and the toxin level determined.

(4) A 10 ml microcystin-LR solution (200 $\mu g\ ml^{-1}$) containing 1% (w/v) TiO_2 was prepared and allowed to equilibrate for 5 minutes after which H_2O_2 was added to give a concentration of 0.6% (v/v). Following a further 5 minutes a sample was removed and the toxin level determined.

4.3 Results

4.3.1 Photocatalysis of microcystin-LR in the presence of H_2O_2

The $UV/TiO_2/H_2O_2$ system using 0.6% (v/v) H_2O_2 was found to degrade microcystin-LR much more rapidly than either UV/TiO_2 or UV/H_2O_2 with no toxin detected after 20 minutes of treatment (Figure 4.1). Both the UV/TiO_2 and UV/H_2O_2 systems are capable of rapid toxin destruction, however the former was the more efficient of the two (Figure 4.1a). It was also noted that there is a higher degree of variability with the UV/H_2O_2 system (Figure 4.1b). No destruction was observed when microcystin-LR was illuminated in the absence of TiO_2 or when in the presence of H_2O_2 without illumination.

Despite rapid toxin destruction comparable to that found with photocatalysis without H_2O_2 it was noted that there was a marked decline in the dark adsorption. On the addition of the TiO_2 catalyst in the absence of H_2O_2 the initial decline in toxin concentration due to dark adsorption is over 60% but in the presence of H_2O_2 this is reduced to as little as 15%. However, the results indicate that a decline in the dark adsorption had no influence on the microcystin-LR destruction which was similar to the effect noted when the catalyst was roasted and the dark adsorption lost (see section 3.3.5).

Photocatalysis was carried out using a lower H_2O_2 level to determine if inhibition of dark adsorption was concentration dependent. Lower H_2O_2 doses of 0.1% (v/v) and 0.01% (v/v) destroyed microcystin-LR more rapidly than 0.6%

(v/v) when used in combination with TiO_2 (Figure 4.2). There was also an increase in the dark adsorption reaction when the lower concentration of H_2O_2 used. The results indicate that a H_2O_2 concentration of 0.1% (v/v) is the most efficient when used in conjunction with TiO_2 photocatalysis.

4.3.2 Determination of optimum hydrogen peroxide level

Optimum enhancement of the photocatalytic reaction was found to occur between peroxide levels of 0.005 and 0.1% (v/v) (Figure 4.3). At concentrations above 0.5% H_2O_2 there is a drop in reaction rate below that achieved with photocatalysis in the absence of the oxidant suggesting that an inhibition effect is occurring.

4.3.3 Mineralisation of microcystin-LR in the presence of H_2O_2

The degree to which microcystin-LR has been mineralised by photocatalytic oxidation enhanced with H_2O_2 was determined to be approximately 18%. This is almost three times the degree of mineralisation observed for microcystin-LR in the absence of H_2O_2.

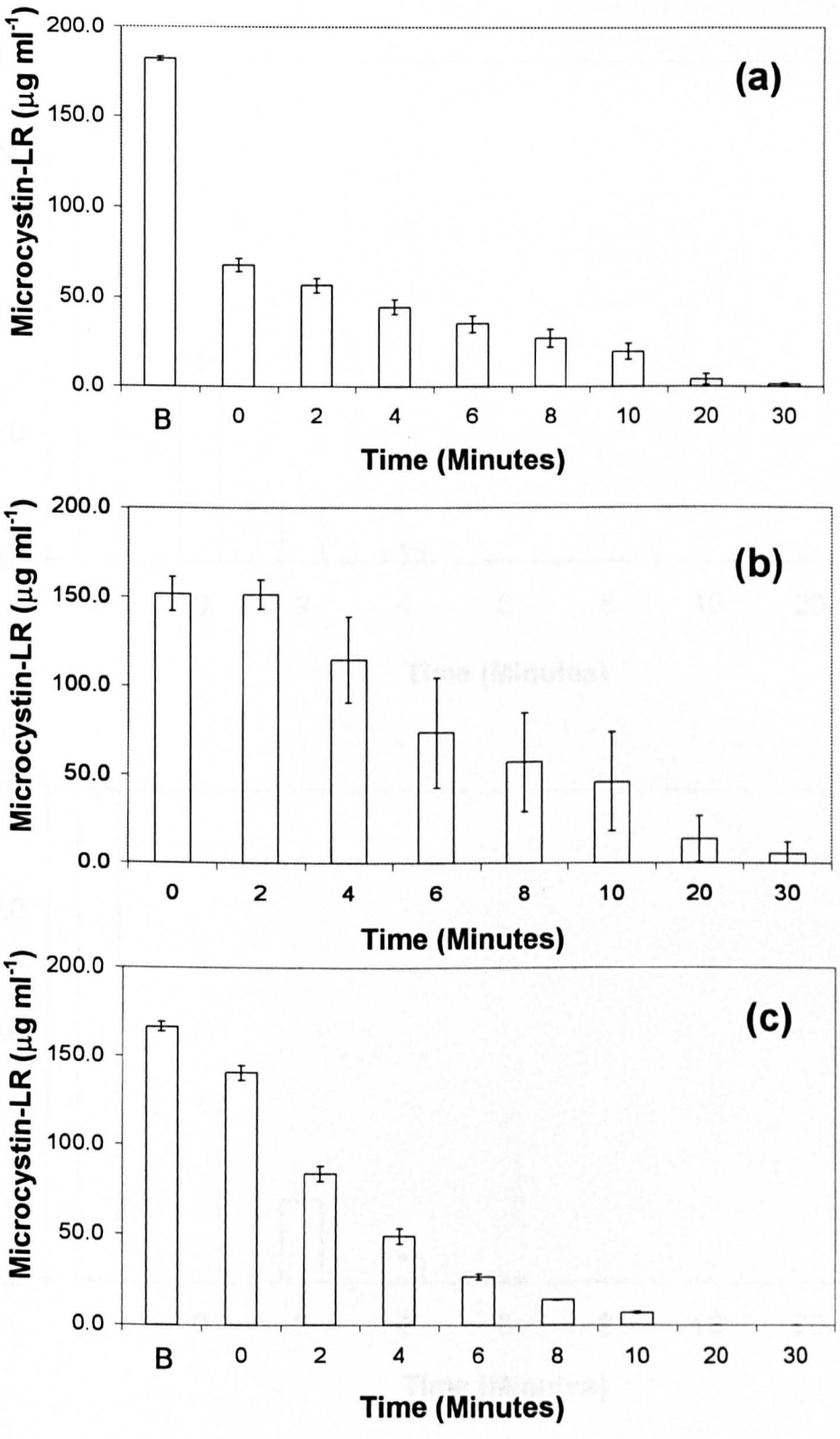


Figure 4.1: Destruction of microcystin-LR by (a) TiO_2 photooxidation, (b) 0.6% H_2O_2 with UV illumination and (c) TiO_2 photooxidation with 0.6% H_2O_2. Microcystin-LR concentration monitored by HPLC, bars are equivalent to 1 SD (n=2). B indicates MCYST-LR concentration prior to catalyst addition.

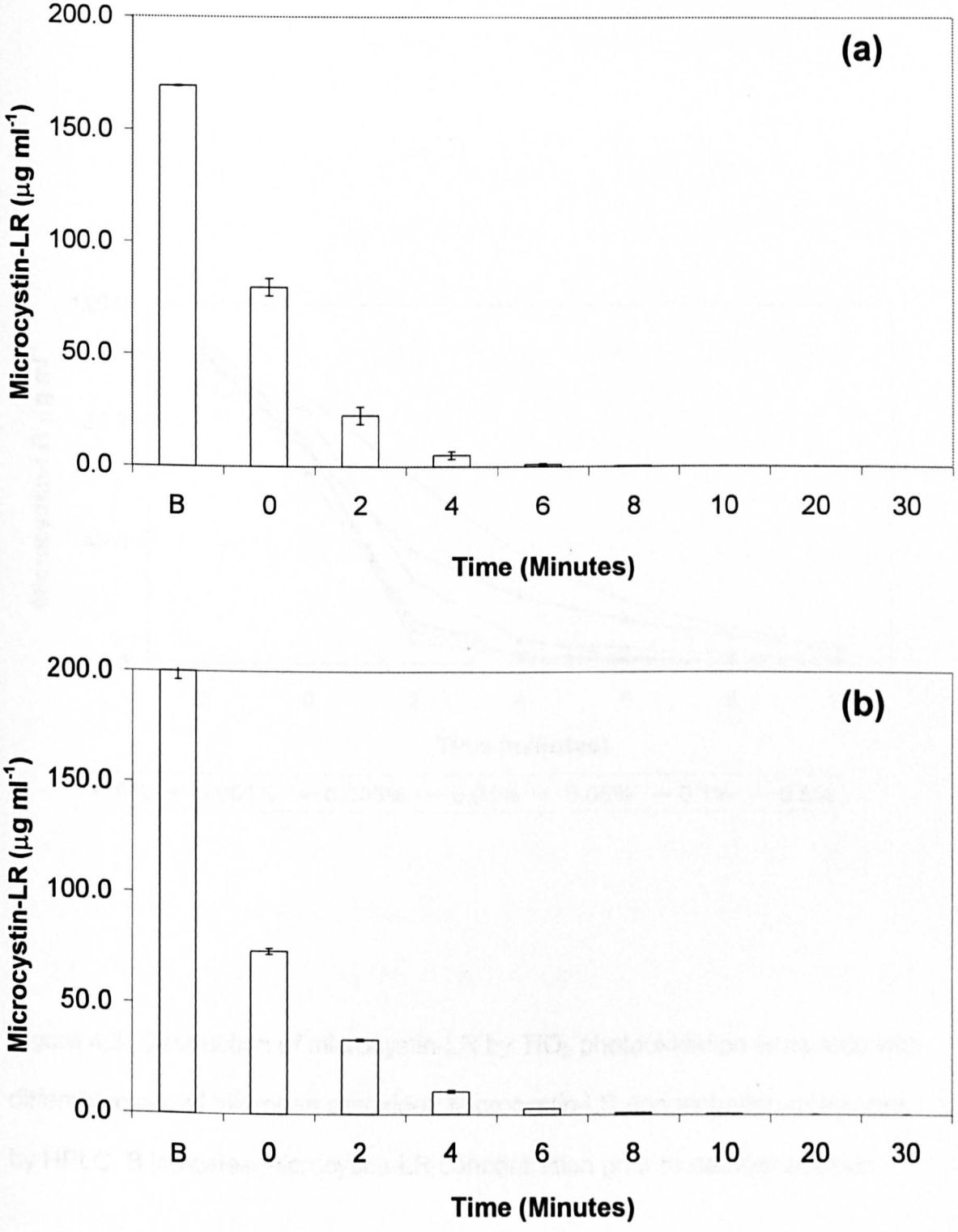


Figure 4.2: Destruction of microcystin-LR by TiO_2 photooxidation with (a) 0.1% H_2O_2 and (b) 0.01% H_2O_2. Microcystin-LR concentration monitored by HPLC, bars are equivalent to 1 SD (n=2). B indicates MCYST-LR concentration prior to catalyst addition.

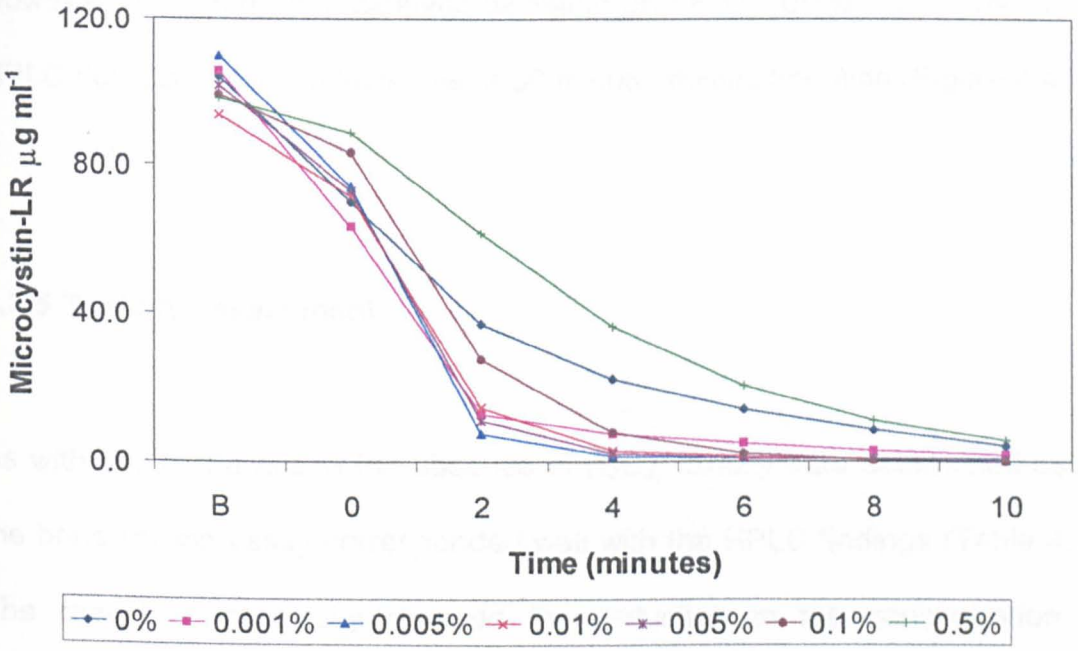


Figure 4.3: Destruction of microcystin-LR by TiO_2 photooxidation enhanced with different levels of hydrogen peroxide. Microcystin-LR concentration monitored by HPLC, B indicates microcystin-LR concentration prior to catalyst addition.

4.3.4 HPLC-PDA analysis of photocatalytic breakdown products

The photocatalytic oxidation of microcystin-LR with UV/TiO_2 results in the formation of several HPLC detectable breakdown products (see section 3.3.1). However combined photocatalytic oxidation and H_2O_2 (0.1% v/v) results in no HPLC detectable by-products over a 30 minute illumination time (Figure 4.4).

4.3.5 Toxicity assessment

As with photocatalysis in the absence of H_2O_2, toxicity data determined using the brine shrimp assay corresponded well with the HPLC findings (Table 4.1). The reduction in toxicity mirrored the reduction in the concentration of microcystin-LR, which occurred over the first 4 minutes of the enhanced treatment. As with the HPLC analysis, toxicity dropped on addition of the TiO_2 catalyst, which is due to the dark adsorption reaction. Toxicity dropped further with all samples tested after 8 minutes giving toxicity levels too low for an LC_{50} value to be determined. Controls established that the presence of the oxidant in the test solutions had no effect on the assay with no mortality detected even in the starting concentration.

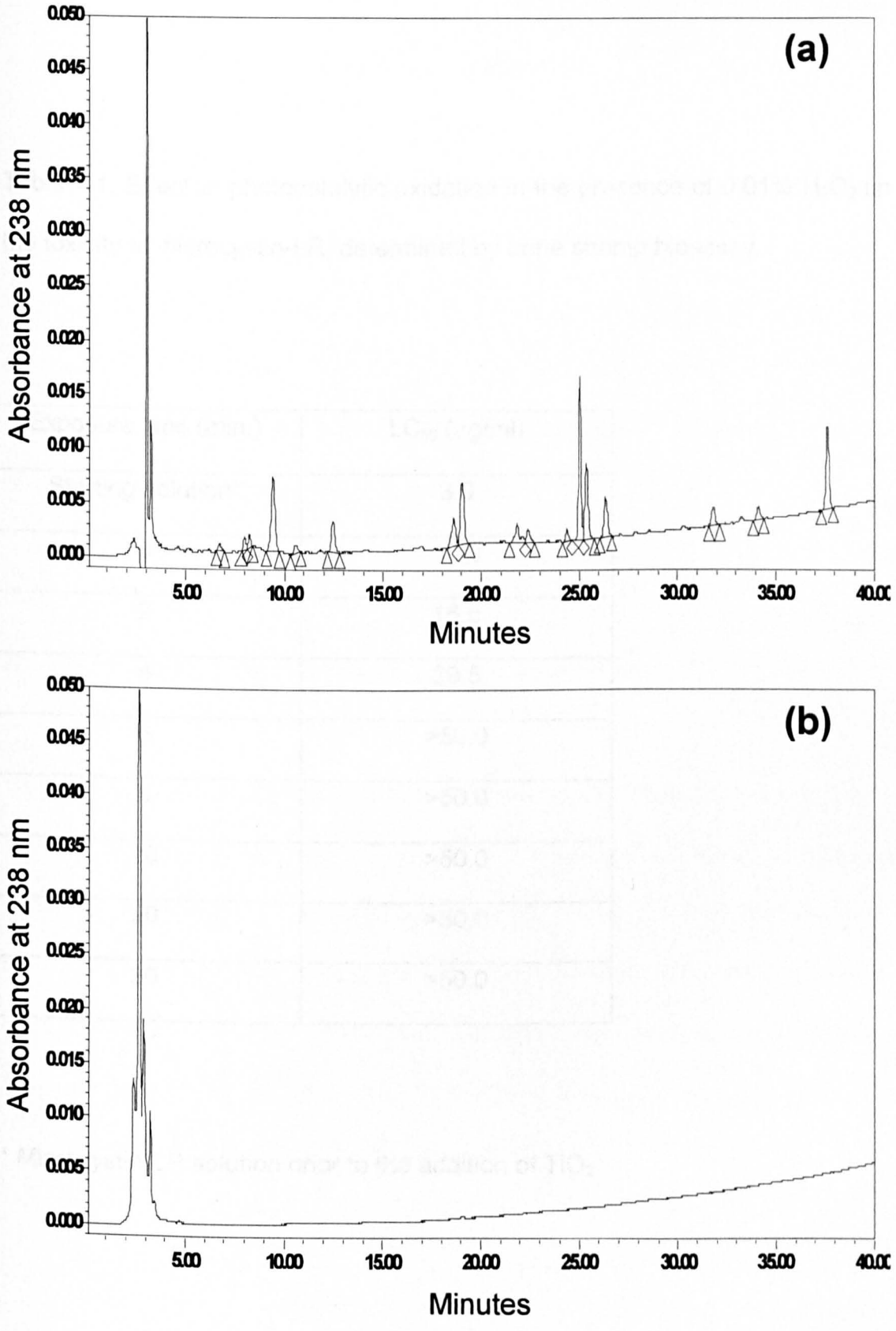


Figure 4.4: HPLC chromatogram after 30 minutes photocatalytic oxidation with TiO_2 (a) in the absence of H_2O_2 and (b) in the presence of 0.1% (v/v) H_2O_2

Table 4.1: Effect of photocatalytic oxidation in the presence of 0.01% H_2O_2 on the toxicity of microcystin-LR, determined by brine shrimp bioassay.

Exposure time (min.)	LC_{50} (μg/ml)
Starting solution*	3.0
0	10.0
2	16.5
4	29.5
6	>50.0
8	>50.0
10	>50.0
20	>50.0
30	>50.0

* Microcystin-LR solution prior to the addition of TiO_2

4.3.6 Quantification of hydrogen peroxide levels

On UV illumination of a solution of H_2O_2 (0.1% v/v) the oxidant levels drop by approximately 8% over the 30 minute experiment time (Figure 4.5). However upon UV illumination of 0.1% H_2O_2 in the presence of TiO_2 (1% w/v) the levels drop much more steeply with a 75% decrease in 30 minutes. No H_2O_2 degradation was observed in the presence of TiO_2 with no illumination or in the absence of both the catalyst and illumination.

4.3.7 Effect of hydrogen peroxide on dark adsorption

Regardless of the order in which the catalyst and the H_2O_2 are introduced to the microcystin-LR solution the inhibition of the dark adsorption reaction observed in section 4.3.1 remained (Figure 4.6). Dark adsorption of the toxin in a TiO_2/water solution is approximately 60% however this has dropped to only 10% when 0.6% H_2O_2 is in solution. Even when the H_2O_2 was introduced after the catalyst to the toxin solution the dark adsorption was low indicating that the adsorbed toxin must have been displaced from the TiO_2 surface.

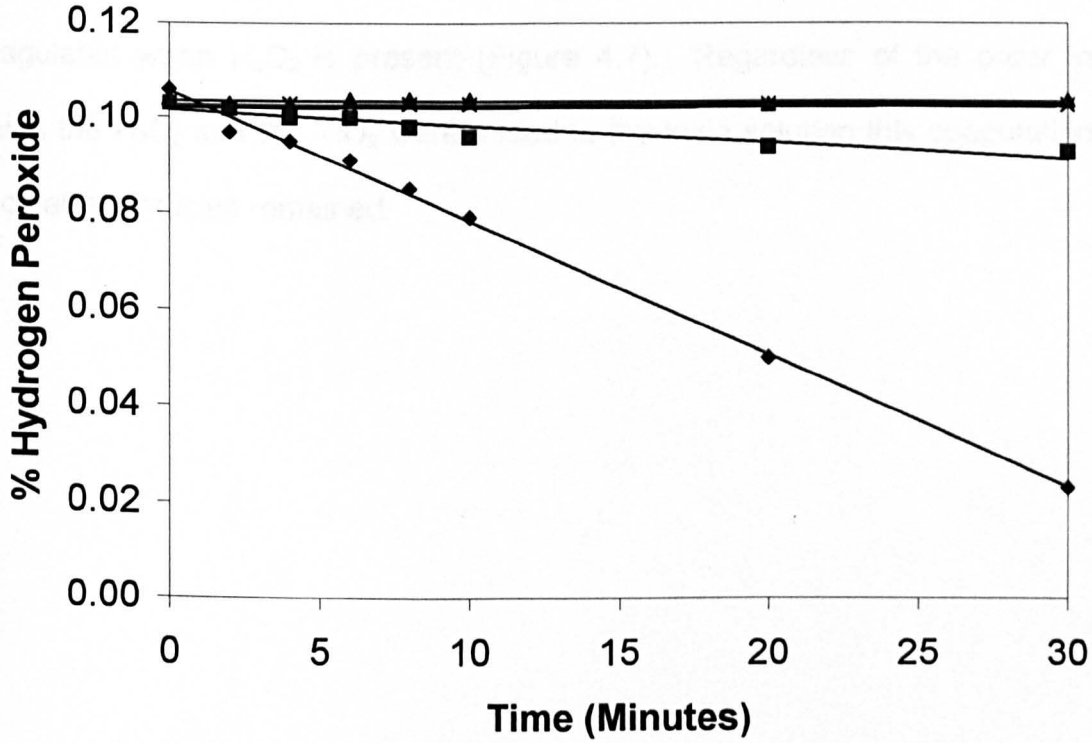
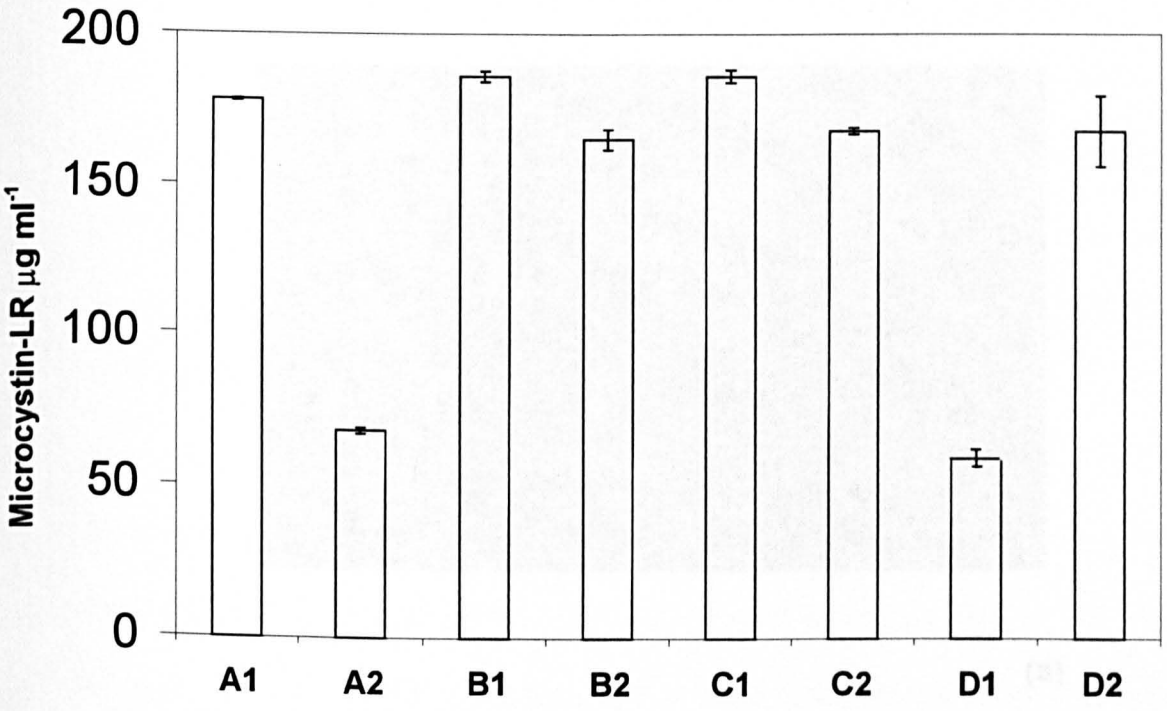


Figure 4.5: Hydrogen peroxide monitored by potassium permanganate titration over 30 minutes [♦] TiO_2 + UV, [■] no TiO_2 + UV, [▲] TiO_2 in dark, [x] no TiO_2 in dark.

It was also observed that when H_2O_2 was present at high concentrations in a TiO_2 suspension the suspension went from white to pale yellow and the catalyst fell out of solution easily. On microscopic inspection of 1% (w/v) TiO_2 suspended in both water and 0.6% (v/v) H_2O_2 it was observed that the catalyst coagulates when H_2O_2 is present (Figure 4.7). Regardless of the order in which the H_2O_2 and the TiO_2 were added to the toxin solution this coagulation of catalyst particles remained.



A1 = Toxin concentration prior to the addition of TiO_2.

A2 = Toxin concentration 5 minutes after addition of TiO_2.

B1 = Toxin concentration in a H_2O_2 solution prior to the addition of TiO_2.

B2 = Toxin concentration in a H_2O_2 solution 5 minutes after addition of TiO_2.

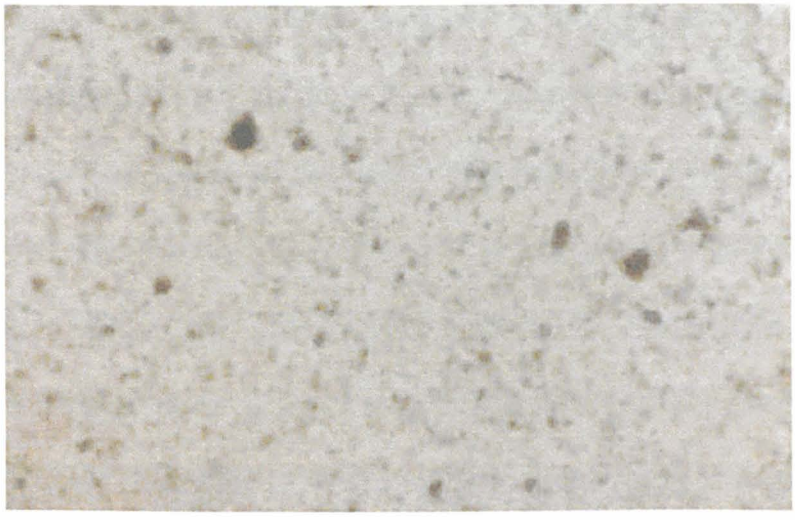
C1 = Toxin concentration prior to the addition of a TiO_2/H_2O_2 slurry.

C2 = Toxin concentration 5 minutes after the addition of a TiO_2/H_2O_2 slurry

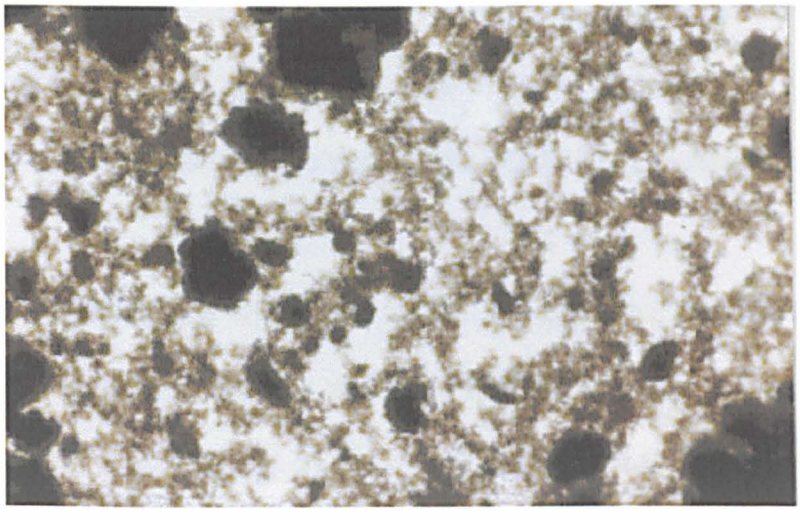
D1 = Toxin concentration in a TiO_2 solution prior to the addition of H_2O_2.

D2 = Toxin concentration in a TiO_2 solution 5 minutes after the addition of H_2O_2.

Figure 4.6: Dark adsorption of microcystin-LR to TiO_2 in the presence of 0.6% (v/v) H_2O_2. Microcystin-LR concentration monitored by HPLC, bars are equivalent to 1 SD (n=2).



(a)



(b)

Figure 4.7: Photomicrographs of (a) TiO_2 in water and (b) TiO_2 in 0.6% H_2O_2, (magnification x 1000).

4.4 Discussion

The results clearly show that photocatalysis of microcystin-LR is enhanced when H_2O_2 is present in the system. Toxin destruction was observed with the UV/H_2O_2 system (as found by Rositano and Nicholson, 1994) however the standard errors were found to be higher than those found for both UV/TiO_2 and UV/TiO_2/H_2O_2. This would indicate that reliability for this system may not be consistent enough to be considered as a treatment method in itself. The toxin treated by UV/TiO_2/H_2O_2 has been shown to produce no HPLC detectable by-products and has mineralised microcystin-LR to a greater degree than when UV/TiO_2 is used. However as only 18% mineralisation has occurred it must be assumed that breakdown by-products not detectable by PDA are present in the reaction solution. Toxicity studies however confirm that the test solutions are rendered non-toxic at a faster rate than when the UV/TiO_2 system is used.

As hydroxyl radicals are believed to be the primary oxidising agent in photocatalytic oxidation it can be assumed that the H_2O_2 is acting as an extra source of these oxidising agents in the UV/TiO_2/H_2O_2 system. H_2O_2 is dissociated by UV illumination to produce two highly reactive hydroxyl radicals (Equation 4.1). However it was shown in section 4.3.4 that on UV illumination of a 0.1% (v/v) solution of H_2O_2 the levels drop by only 8% due to photolytic dissociation. This is in comparison to a drop of over 75% over the same illumination period of a 0.1% H_2O_2 solution in the presence of TiO_2. This suggests that there is another process in which H_2O_2 is being reduced to $OH^{\bullet}$, one that involves the photocatalyst.

During photocatalysis oxygen is reduced at the conductance band of the TiO_2 molecule forming the superoxide anion. However H_2O_2 acts as an alternative electron acceptor to oxygen and H_2O_2 reduction is thermodynamically a more favourable reaction (Jaeger and Bard, 1979) due to its higher reduction potential (E^0 = -0.13 for O_2 and 0.72 for H_2O_2). As a consequence of this increased reduction rate there is an increase in the rate of formation of $OH^\bullet$ radicals at the valence band. Combined with the additional formation of these radicals at the conductance band due to H_2O_2 reduction (Equation 4.2) there is an overall increase in the rate of photocatalytic oxidation (Wang and Hong, 1999) of the microcystin-LR.

$$e^-_{CB} + H_2O_2 \rightarrow OH^- + OH^\bullet \qquad \text{Equation 4.2}$$

Crittenden *et al.* (1999) indicated that H_2O_2 can also accept electrons from the superoxide anion (O_2^-) produced at the conductance band when oxygen acts as the electron acceptor, this again leads to the formation of $OH^\bullet$ radicals (Equation 4.3).

$$O_2^- + H_2O_2 \rightarrow OH^- + OH^\bullet + O_2 \qquad \text{Equation 4.3}$$

Regardless of which reaction is most prevalent both produce additional hydroxyl radicals that are available to oxidise the microcystin-LR hence increasing the efficiency of the photocatalytic process.

However the concentration of H_2O_2 was found to influence the degree of photocatalytic enhancement with concentrations of 0.005 - 0.1% (v/v) H_2O_2 giving the most efficient destruction compared to >0.5% of the oxidant. The UV absorbance spectra (200 – 400 nm) of 0.1 and 0.6% (v/v) H_2O_2 solutions were obtained and are shown in Figure 4.8. The spectra show that at the higher concentration of H_2O_2 there is greater absorbance in the area where TiO_2 is activated by the UV light (~380 nm). This may indicate that UV light at that wavelength normally used to activate the catalyst is being absorbed by the H_2O_2 in solution preventing formation of the photoexcited electron holes needed for photocatalysis to occur. However the absorbance is very low and its effects are more likely to be insignificant.

Alternatively Wang and Hong (1999) have proposed that at high levels of oxidant, H_2O_2 adsorbed onto the TiO_2, can scavenge $OH^{\bullet}$ radicals produced at the catalyst surface (Equation 4.4 and 4.5). H_2O_2 can also react with photogenerated holes (h_{CB}^{+}) at the catalyst surface (Equation 4.6) these holes are responsible for the generation of $OH^{\bullet}$ radicals by illuminated TiO_2.

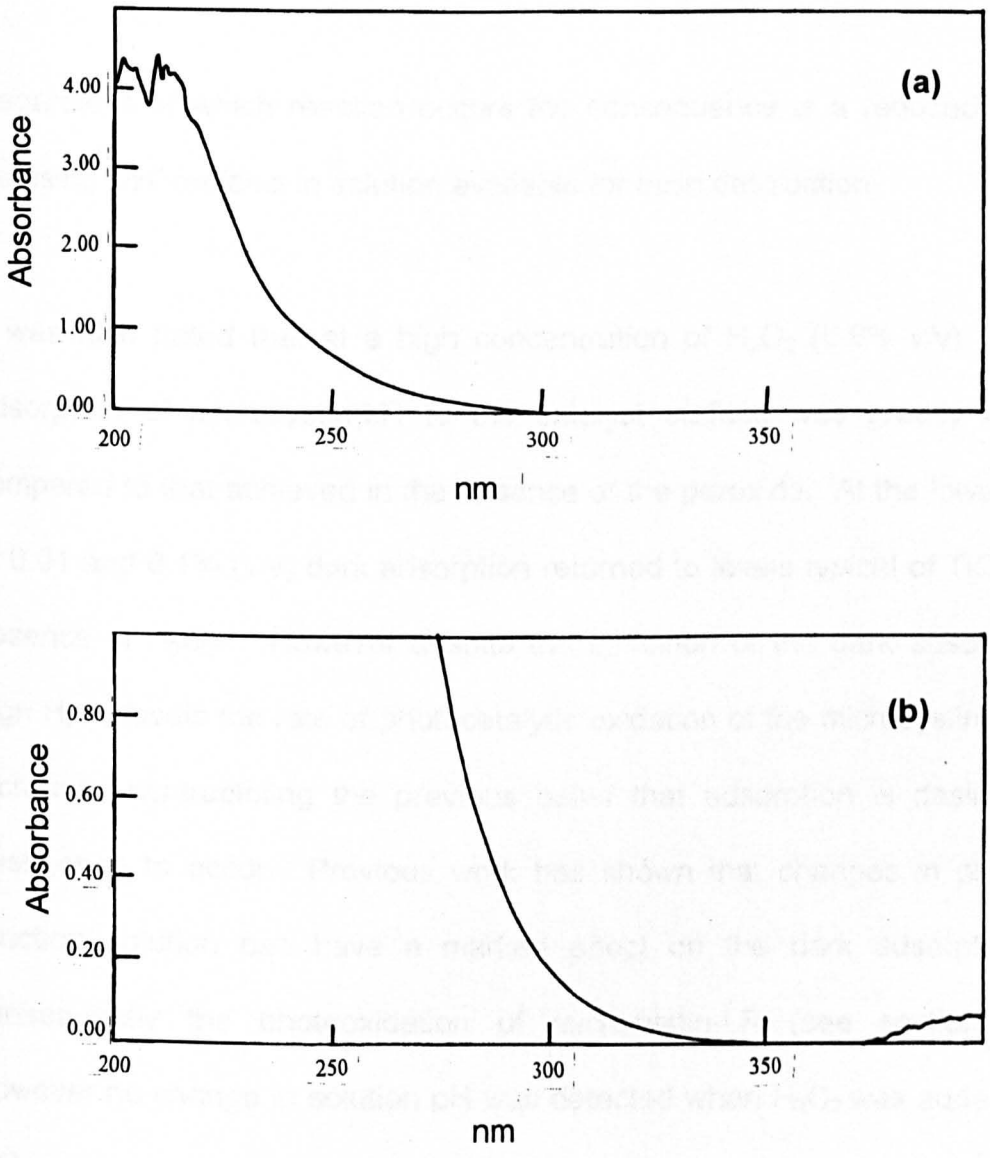


Figure 4.8: Absorbance spectra of (a) 0.1% hydrogen peroxide and (b) 0.6% hydrogen peroxide.

$$H_2O_2 + OH^{\bullet} \rightarrow H_2O + HO_2^{\bullet} \qquad \text{Equation 4.4}$$

$$HO_2^{\bullet} + OH^{\bullet} \rightarrow H_2O + O_2 \qquad \text{Equation 4.5}$$

$$H_2O_2 + h_{CB}^{+} \rightarrow HO_2^{\bullet} + H^{+} \qquad \text{Equation 4.6}$$

Regardless of which reaction occurs the consequence is a reduced level of oxidising $OH^{\bullet}$ radicals in solution available for toxin destruction.

It was also noted that at a high concentration of H_2O_2 (0.6% v/v) the dark adsorption of microcystin-LR to the catalyst surface was greatly reduced compared to that achieved in the absence of the peroxide. At the lower levels of 0.01 and 0.1% (v/v) dark adsorption returned to levels typical of TiO_2 in the absence of H_2O_2. However despite the inhibition of the dark adsorption at high H_2O_2 levels the rate of photocatalytic oxidation of the microcystin-LR was increased contradicting the previous belief that adsorption is desirable for destruction to occur. Previous work has shown that changes in pH of the reaction solution can have a marked effect on the dark adsorption and consequently the photooxidation of microcystin-LR (see section 3.3.6). However no change in solution pH was detected when H_2O_2 was added to the TiO_2 solution.

Another possible explanation of the reduced dark adsorption is believed to be a result of coagulation of the TiO_2 particles observed in section 4.3.6. The study has shown that pre-adsorbed microcystin-LR on the catalyst surface is displaced when H_2O_2 is introduced to the system. This indicates that there is strong competition between the oxidant and the toxin for surface sites on the

TiO_2 surface and that H_2O_2 attaches preferentially to the catalyst. Consequently the surface charge of the TiO_2 will be altered enabling the particles to coagulate. This would lead to a decrease in the surface area available for the microcystin to adsorb to the catalyst and subsequently reduce the amount of dark adsorbed toxin. The colour change from white to yellow is believed to be a result of the formation of Ti(IV) peroxide species which is a standard analytical test for Ti(IV) ions (Vogel, 1989).

It is therefore important to find and maintain the right concentration of H_2O_2 to enhance the photocatalytic process with a given TiO_2 concentration otherwise maximum efficiency of this system will not be achieved. The $UV/TiO_2/H_2O_2$ system has however proved to be an improvement on the photocatalytic system giving greater mineralisation of the microcystin-LR. The process also effectively degrades the peroxide via UV initiated dissociation therefore no removal step would have to be introduced to a large scale treatment plant. However, as with the UV/TiO_2 system, further studies must be carried out to determine if treated water is safe for human consumption.

CHAPTER 5

Small Scale Photocatalysis Flow Reactor and Photocatalysis of Other Microcystin Variants

5.1 Introduction

The majority of research into photocatalytic reactions carried out in laboratories has been carried out in batch slurry reactors. These reactors involve introducing the TiO_2 into solutions containing the pollutant then exposing to UV radiation while constantly stirring the solution to give heterogeneous slurry. However this method may not be ideal in large scale water purification since it would involve separating the catalyst from the water either by filtration, sedimentation, centrifugation or coagulation. Some researchers have overcome this by utilising a fixed film flow reactor for laboratory investigations of photocatalytic degradation of pollutants. Such a system involves the use of a catalyst coated onto the surface of a suitable substrate. The solution containing the pollutant is then passed over the UV illuminated coated surface. One such reactor involves the coating of the interior of a glass spiral coil. A UV light tube passed through the middle of the coil provides the light source as illustrated in Figure 5.1 (Hua *et al.*, 1995; Matthews, 1993). Matthews indicated that when TiO_2 catalyst was allowed to dry on a glass surface the film was found to be difficult to remove by washing with water alone. He suggested that this strong adhesion is due to the electrostatic charge on the surface of the catalyst particle being attracted to the negatively charged surface of the glass.

A similar flow system to that reported by Matthews was constructed and evaluated as part of this study to determine its effectiveness in degrading

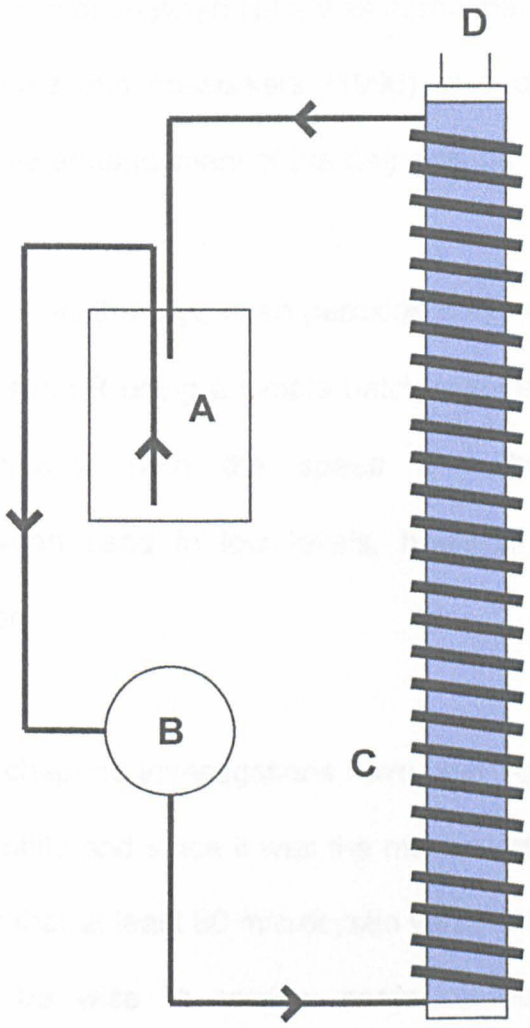


Figure 5.1: Schematic diagram of a simple photocatalytic fixed film flow reactor: (A) Sample reservoir; (B) pump; (C) glass spiral coated with catalyst and (D) UV tube light.

microcystin-LR. Fixed film reactors have also been used in combination with chemical treatments. The photodegradation of monocrotophos was shown to be enhanced in a flow system when H_2O_2 was introduced into the sample (Hua *et al.*, 1995). Tanaka and co-workers (1996) also demonstrated that the addition of ozone gave enhancement of the degradation of acetic acid.

It was shown (Chapter 4) that hydrogen peroxide enhanced the photocatalytic oxidation of microcystin-LR using a simple batch reactor design. The oxidant was found to enhance both the speed and the efficiency of the photodegradation when used in low levels, however at higher levels the reaction was inhibited.

In all the preceding chapters investigations have been focused on microcystin-LR due to its availability and since it was the most common occurring variant. However it is known that at least 60 microcystin variants exist (Sivonen, 1990). It would therefore be wise to confirm some of the findings with other congeners. Shephard and co-workers (1998) demonstrated the photocatalytic oxidation of microcystin-YR and -YA indicating that they achieved similar rates of destruction to that obtained for microcystin-LR. It is believed that since the microcystins share structural similarity then photocatalytic oxidation will be effective for all variants although it is not known if the presence of different amino acids will affect the reaction.

5.2 Methods

5.2.1 Construction of reactor coil

The inside surface of a glass coil (internal volume 250 cm^3, Scotia Glass Technology, Stirling, UK) was cleaned by filling with nitric acid (2M) and left for 2 hours. The coil was then rinsed with water and allowed to dry in an oven at 90°C.

A 10% w/v TiO_2/water slurry (200 ml) was prepared then sonicated for 10 minutes to provide a homogeneous solution. The coil was filled with the catalyst slurry, left for 2 minutes and then drained. The TiO_2 remaining on the inside surface of the glass was then dried by blowing hot air over the outside surface of the coil. This was carried out 3 times then the coated coil was allowed to dry in an oven at 90°C for 2 hours.

A UV strip light (15 WATT, Blak-Ray® BL-15 long wave ultraviolet lamp, UVP, CA, USA) was passed through the centre of the coated glass coil. To complete the reactor the coil/light apparatus was placed in a metal foil lined housing which when closed encases the reactor (Figure 5.2). The foil lining allows the UV light to be redirected back onto the coil maximising the amount of light available for the photocatalytic reaction.

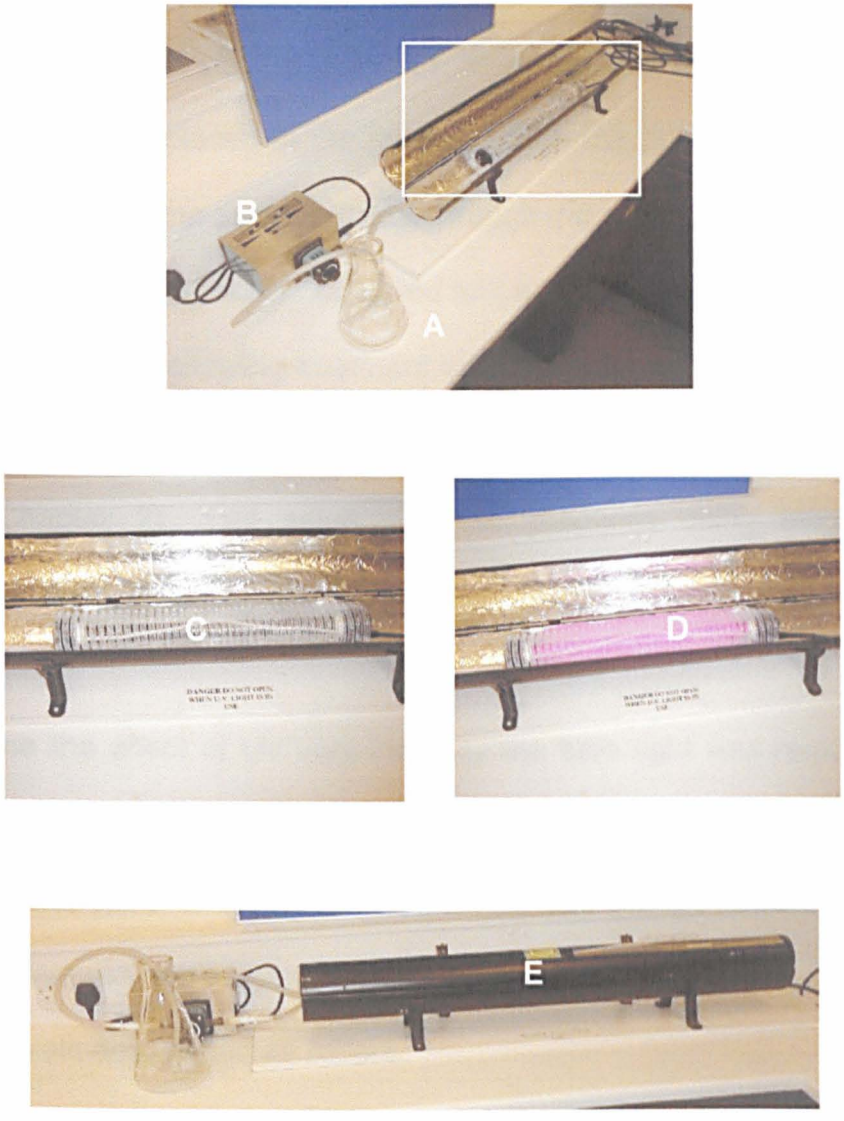


Figure 5.2: Completed fixed film flow reactor. (A) Sample reservoir; (B) pump; (C) glass spiral coated with catalyst; (D) UV tube light and (E) foil lined coil casing as closed during operation.

5.2.2 Photocatalytic destruction of microcystin-LR in the flow reactor

Milli-Q water samples (1 litre), spiked to a concentration of 10 μg ml^{-1} microcystin-LR, were placed in the reservoir then the pump was switched on and adjusted to allow the sample to flow through the coil at a constant flow rate of 1.5 L min^{-1}. The UV light was activated and samples were treated for 15, 30, 45 and 60 minutes. Following treatment each sample was concentrated using OASIS SPE (3cc, 60 mg, Waters, Watford, UK) cartridges prior to HPLC analysis (Chapter 2). All solutions were prepared using Milli-Q water (Millipore, Watford, UK).

To determine the effect of UV light intensity the tube light was removed from the core of the coil and the UV lamp (280 W UVASpot 400 lamp, Uvalight Technology Ltd; spectral output 330 - 450 nm) was placed in front of the coil assembly. Samples were illuminated for 15 minutes using both types of lamp and toxins levels determined as before.

5.2.3 H_2O_2 enhanced photocatalytic destruction of microcystin-LR in the flow reactor

This procedure was carried out as in section 5.2.2 with the UV tube light in place but also in the presence of 0.01% (v/v) hydrogen peroxide. The desired hydrogen peroxide concentration was obtained by dilution of a 6% hydrogen

peroxide solution (Fisher Scientific UK Ltd., Leicestershire, UK). All solutions were prepared using Milli-Q water (Millipore, Watford, UK).

5.2.4 Photocatalytic destruction of microcystin-LR in the flow reactor using raw and tap water

Raw (Loch Rescobie) and tap (Aberdeen) water samples (1 litre) were spiked to an initial concentration of 10 μg ml^{-1} microcystin-LR then treated in the coil as in section 5.2.2. The treatment was then repeated in the presence of 0.01% H_2O_2.

5.2.5 Photocatalytic oxidation of microcystin-RR, -LW and -LF

Photocatalytic oxidation of 200 μg ml^{-1} solutions of microcystin-RR (obtained from Dr Christine Edwards, Biotage, Hartford, UK) and microcystin-LW and -LF (purified as in Chapter 2) was carried out as in section 3.2.1. Samples were analysed by HPLC using the standard gradient to detect the presence of any breakdown products (see section 2.2.4).

5.2.6 Influence of pH on photocatalysis of microcystin-RR, -LW and -LF

The effect of initial solution pH on the destruction of microcystin-RR, -LW and -LF (100 μg ml^{-1}) was investigated as in the method described in 3.2.1 with the following modifications. Either nitric acid or sodium hydroxide was added to attain the required pH levels of 1, 5, 7, 10 and 12. A pH meter (Oyster) was used to determine when the required pH had been reached. For control purposes microcystin solutions at each pH were illuminated in the absence of catalyst.

5.3 Results

5.3.2 Photocatalytic destruction of microcystin-LR in the flow reactor

Using the fixed film reactor microcystin-LR was found to be degraded (Figure 5.3) however at a rate much lower than that achieved with the batch reactor (Chapter 3). Approximately 64% of the toxin was still present after 30 minutes treatment whereas in the batch system all the microcystin-LR was destroyed by this time. Substitution of the UV tube light with the more powerful lamp increased the rate of the photocatalytic degradation of the toxin but only slightly (Figure 5.4). Following the 15 minutes treatment time the tube light achieved ~38% microcystin-LR degradation while the lamp gave ~60% removal of the toxin.

5.3.3 H_2O_2 enhanced photocatalytic destruction of microcystin-LR in the flow reactor

The presence of the H_2O_2 in the sample appeared to inhibit the photooxidation of the microcystin-LR in the coil reactor (Figure 5.5). Over the first 30 minutes the degree of degradation is comparable to that achieved in the absence of H_2O_2. However over the next 30 minutes only a further 8% of the microcystin-LR is destroyed whereas a further 30% was removed during this time period in the peroxides absence.

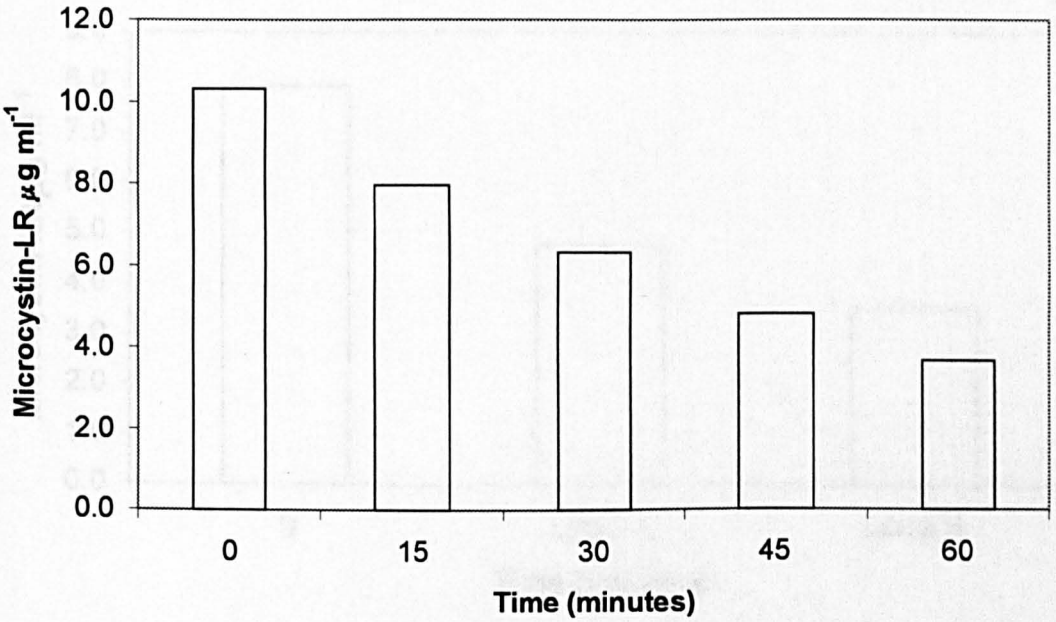


Figure 5.3: Destruction of microcystin-LR by TiO_2 photooxidation in a fixed film flow reactor monitored by HPLC.

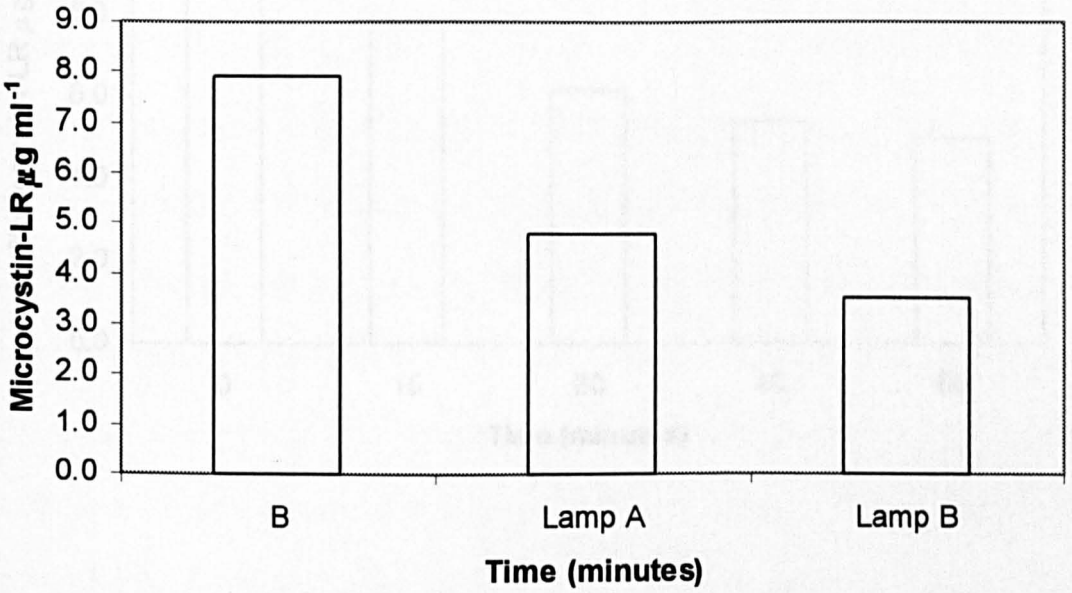


Figure 5.4: Destruction of microcystin-LR by TiO_2 photooxidation in a fixed film flow reactor after 15 minutes illumination using a UV tube light (Lamp A) and a UV lamp (Lamp B), levels monitored by HPLC. B indicates toxin concentration prior to UV illumination.

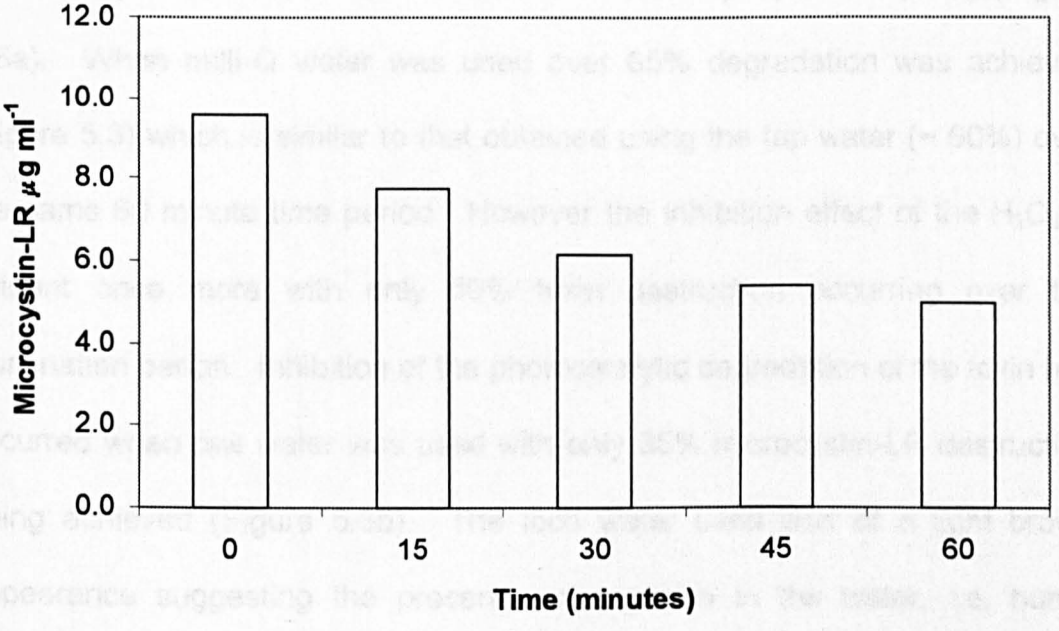
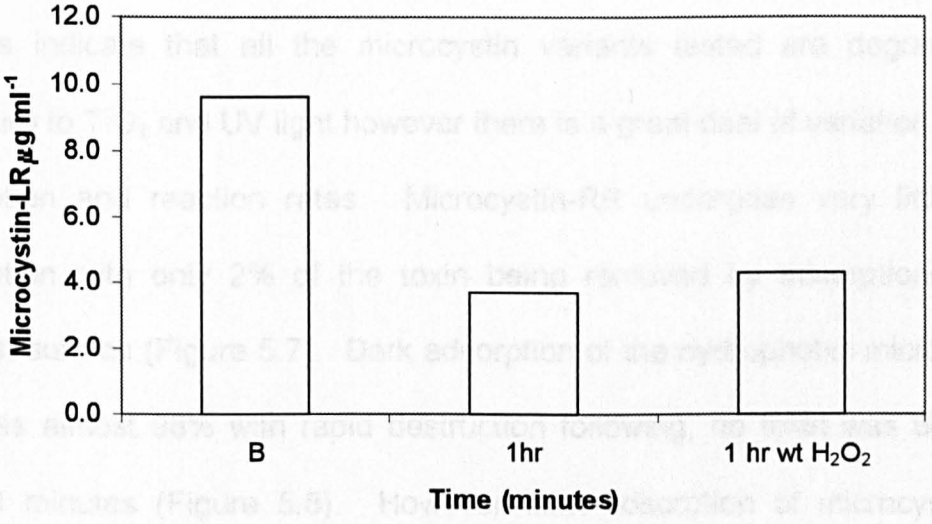


Figure 5.5: Destruction of microcystin-LR by TiO_2 photooxidation in the presence of H_2O_2 carried out in a fixed film flow reactor, levels monitored by HPLC.

5.3.4 Photocatalytic destruction of microcystin-LR in the flow reactor using raw and tap water

Following 60 minutes illumination there was no indication that the degree of photocatalytic destruction of the toxin was affected by tap water quality (Figure 5.6a). When milli-Q water was used over 65% degradation was achieved (Figure 5.3) which is similar to that obtained using the tap water (~ 60%) over the same 60 minute time period. However the inhibition effect of the H_2O_2 is evident once more with only 50% toxin destruction occurring over the illumination period. Inhibition of the photocatalytic degradation of the toxin has occurred when raw water was used with only 35% microcystin-LR destruction being achieved (Figure 5.6b). The loch water used was of a light brown appearance suggesting the presence of organics in the water, i.e. humic substances. It was observed that when H_2O_2 was present in the system the degree of colour removal was greater than in the absence of the oxidant. The presence of the H_2O_2 also increased the degree of toxin degredation to ~75% (Figure 5.6b) which was higher than that obtained using the milli-Q water (Figure 5.5).

(a)



(b)

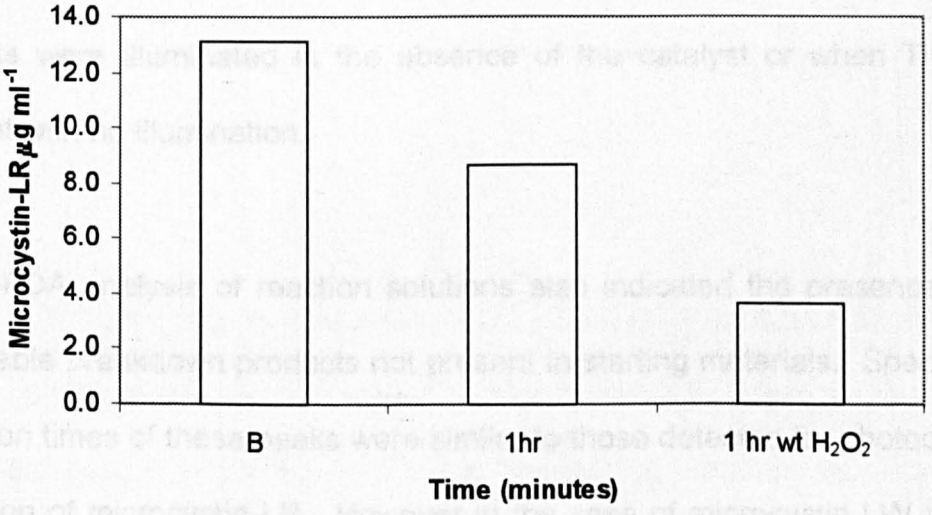


Figure 5.6: Destruction of microcystin-LR by TiO_2 photooxidation with and without H_2O_2 in a fixed film flow reactor using (a) tap water (Aberdeen) and (b) raw water (Loch Rescobie), monitored by HPLC. B indicates toxin concentration prior to UV illumination.

5.3.5 Photocatalytic oxidation of microcystin-RR, -LW and -LF

Results indicate that all the microcystin variants tested are degraded by exposure to TiO_2 and UV light however there is a great deal of variation of dark adsorption and reaction rates. Microcystin-RR undergoes very little dark adsorption with only 2% of the toxin being removed by adsorption to the catalyst surface (Figure 5.7). Dark adsorption of the hydrophobic microcystin-LW was almost 98% with rapid destruction following, no toxin was detected after 4 minutes (Figure 5.8). However dark adsorption of microcystin-LF, which is also thought to be relatively hydrophobic, was only 10% although on UV illumination rapid destruction occurs with no toxin detected after 30 minutes (Figure 5.9). Destruction was not observed when microcystin variants were illuminated in the absence of the catalyst or when TiO_2 was present with no illumination.

HPLC-PDA analysis of reaction solutions also indicated the presence of UV detectable breakdown products not present in starting materials. Spectra and retention times of these peaks were similar to those detected for photocatalytic oxidation of microcystin-LR. However in the case of microcystin-LW and -LF these products are undetected after 30 minutes of photocatalytic treatment. These peaks were not observed when microcystin variants were added to TiO_2 in the absence of UV light or when TiO_2 was added to water alone and illuminated.

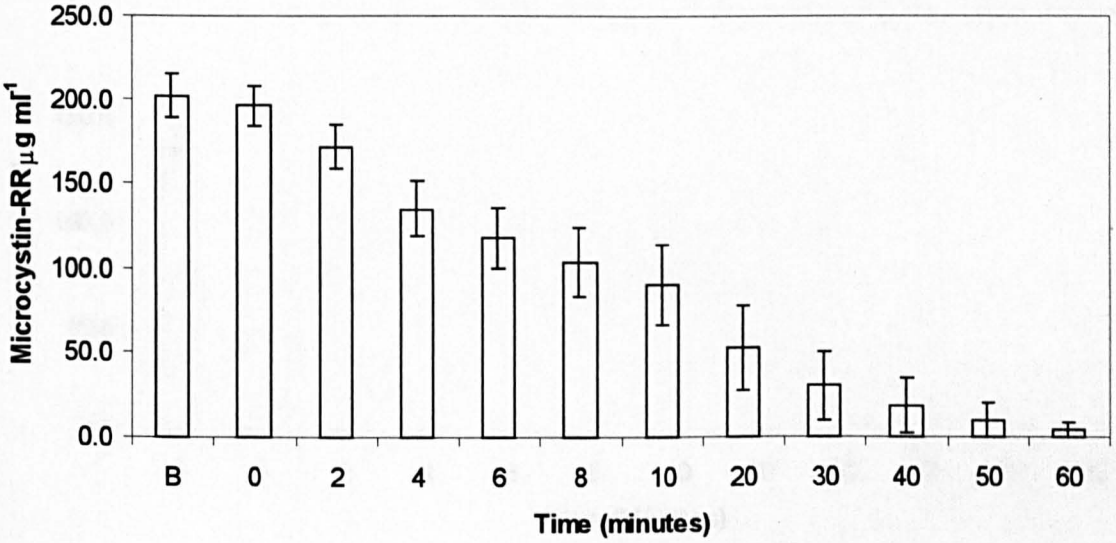


Figure 5.7: Destruction of microcystin-RR by TiO_2 photooxidation monitored by HPLC. B indicates the concentration of microcystin-RR prior to the dark adsorption resulting from the addition of the catalyst. Bars are equivalent to one standard deviation (n=2).

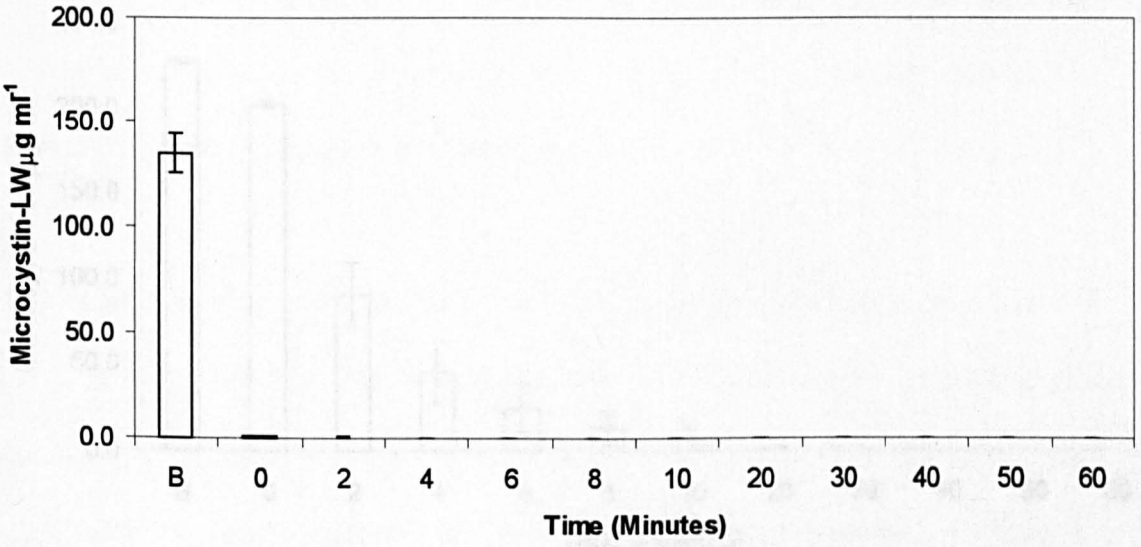


Figure 5.8: Destruction of microcystin-LW by TiO_2 photooxidation monitored by HPLC. B indicates the concentration of microcystin-LW prior to the dark adsorption resulting from the addition of the catalyst. Bars are equivalent to one standard deviation (n=2).

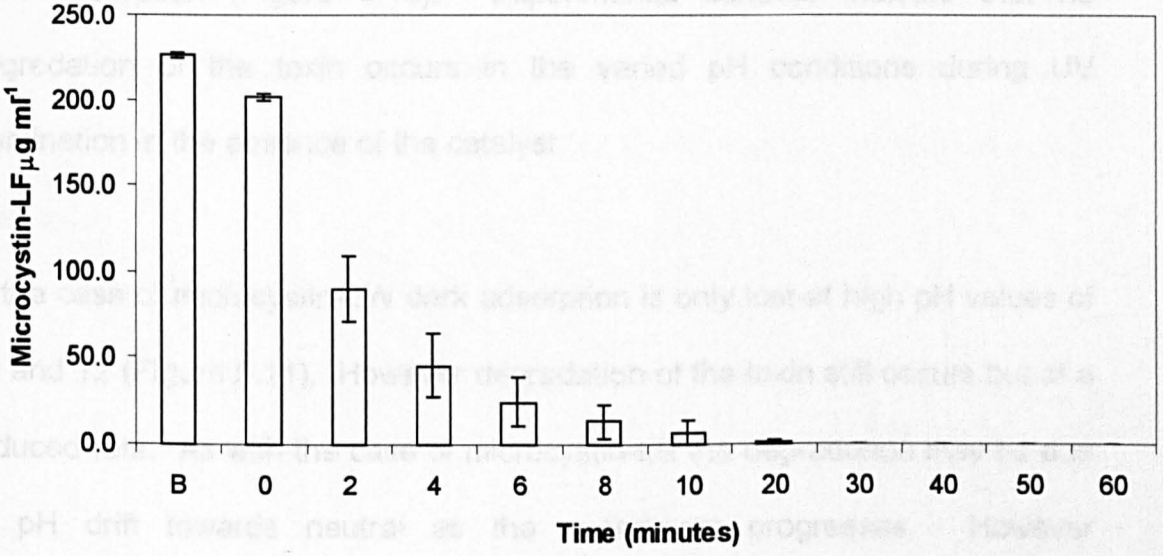


Figure 5.9: Destruction of microcystin-LF by TiO_2 photooxidation monitored by HPLC. B indicates the concentration of microcystin-LF prior to the dark adsorption resulting from the addition of the catalyst. Bars are equivalent to one standard deviation (n=2).

5.3.6 Influence of pH on photocatalysis of microcystin-RR, -LW and -LF

Unlike microcystin-LR the initial solution pH does not appear to influence rates of destruction of microcystin-RR to any great extent. Rates of destruction of microcystin-RR are similar for most pH values, there is also no variation in dark adsorption (Figure 5.10). Experimental controls indicate that no degradation of the toxin occurs in the varied pH conditions during UV illumination in the absence of the catalyst.

In the case of microcystin-LW dark adsorption is only lost at high pH values of 10 and 12 (Figure 5.11). However degradation of the toxin still occurs but at a reduced rate. As with the case of microcystin-LR the degradation may be due to pH drift towards neutral as the experiment progresses. However experimental controls indicated that at these pH values some degradation occurs during illumination in the absence of the catalysts. The degradation may therefore also be a result of the instability of these toxins at these pH levels. Controls also indicated that at pH 1 there is rapid degradation, almost 40% degradation on addition of the acid, of this toxin in the absence of the TiO_2 indicating that this microcystin variant is highly unstable in acidic conditions.

Microcystin-LF was found to be the most influenced by pH exhibiting similar behavior to microcystin-LR. Most rapid degradation occurs at initial pH values of 1 and 4 (Figure 5.12), this is also the area of greatest dark adsorption. As

pH increases there is a loss of the dark adsorption reaction however the rate of degradation is only slightly reduced.

It was also observed that at reaction pH 4 (solution concentration 100 μg ml^{-1}) there is a dark adsorption of approximately 95%. However at a solution concentration of 200 μg ml^{-1} dark adsorption was as low as 10% (section 3.3.5). This suggests that the dark adsorption reaction for this variant is concentration dependent, no other microcystin variant displayed such behaviour.

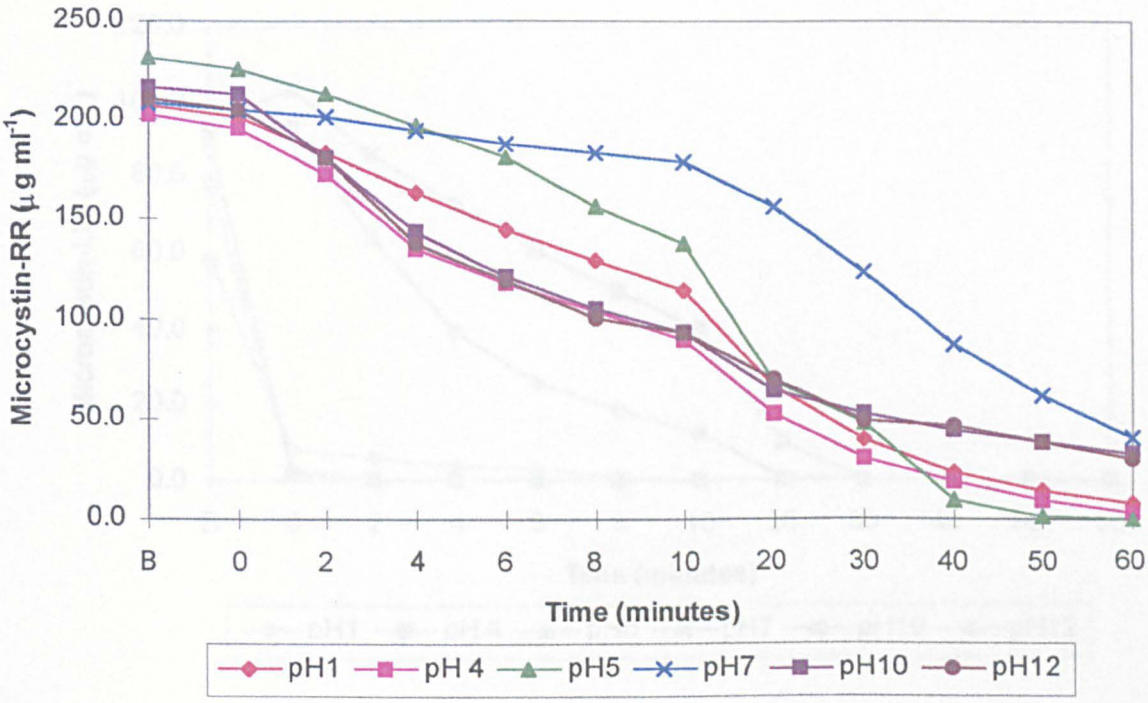


Figure 5.10: Destruction of microcystin-RR at different pH levels, monitored by HPLC. B indicates the concentration of microcystin-RR prior to the dark adsorption resulting from the addition of the catalyst.

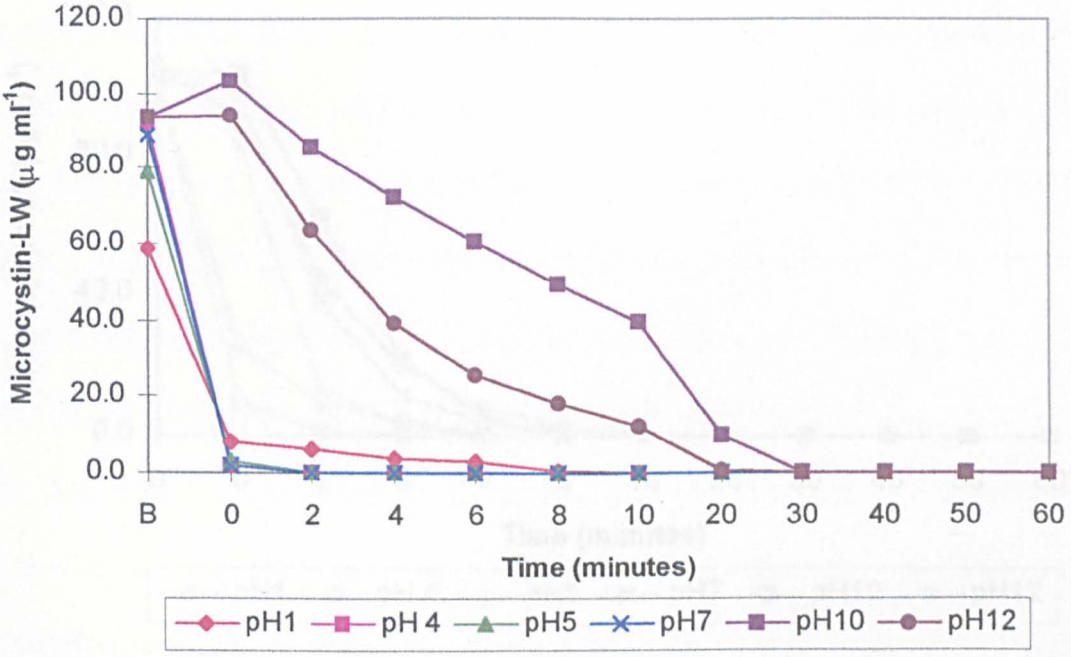


Figure 5.11: Destruction of microcystin-LW at different pH levels, monitored by HPLC. B indicates the concentration of microcystin-LW prior to the dark adsorption resulting from the addition of the catalyst.

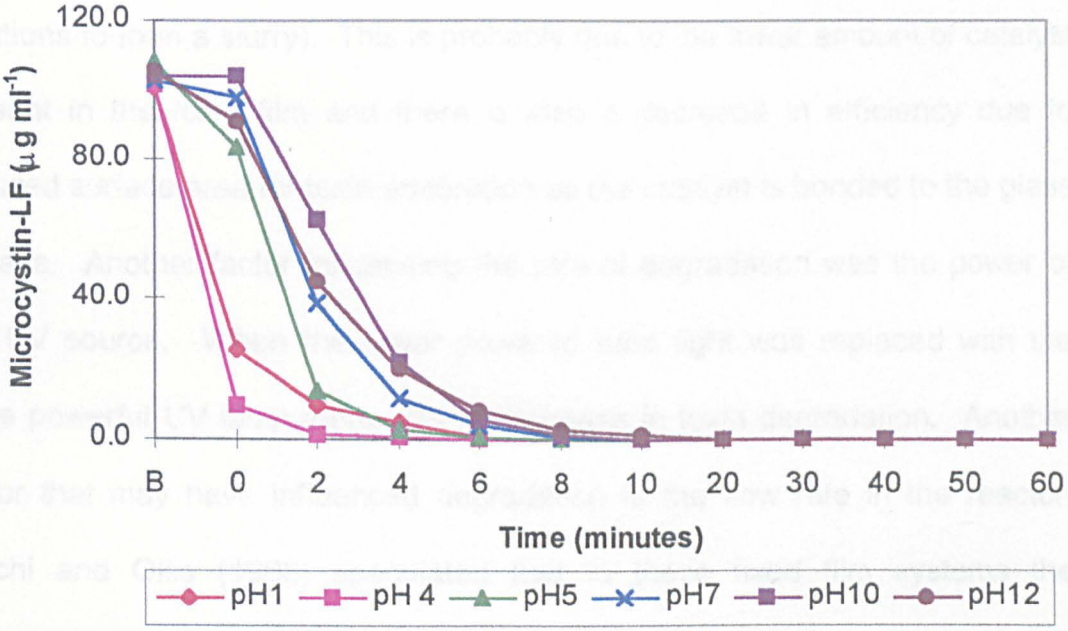


Figure 5.12: Destruction of microcystin-LF at different pH levels, monitored by HPLC. B indicates the concentration of microcystin-LF prior to the dark adsorption resulting from the addition of the catalyst.

5.4 Discussion

The results confirm that microcystin-LR is successfully degraded in the fixed film flow system. The rate of toxin destruction however is far lower than that demonstrated by batch reactors (i.e. when the TiO_2 catalyst is added directly to solutions to form a slurry). This is probably due to the lower amount of catalyst present in the fixed film and there is also a decrease in efficiency due to reduced surface area for toxin adsorption as the catalyst is bonded to the glass surface. Another factor influencing the rate of degradation was the power of the UV source. When the lower powered tube light was replaced with the more powerful UV lamp there was an increase in toxin degradation. Another factor that may have influenced degradation is the flow rate in the reactor. Turchi and Ollis (1988) speculated that in these fixed film systems the degradation reaction is limited to a thin water layer near the catalyst surface and therefore the rate would be controlled by the rate the pollutants passed over the coated surface.

It was also shown here that while H_2O_2 can be used to enhance the photodegradtion of microcystin-LR, if the concentration of the oxidant is too high the reaction was inhibited. Wang and Hong (1999) suggested that if present in high levels H_2O_2 adsorbed onto the TiO_2 surface can scavenge $OH^{\bullet}$ radicals produced at the catalyst surface. H_2O_2 can also react with photogenerated holes (h_{CB}^{+}) at the catalyst surface which are responsible for the generation of $OH^{\bullet}$ radicals by illuminated TiO_2. The results in this study have also indicated that there is strong competition between the oxidant and

the toxin for surface sites on the TiO_2 surface and that H_2O_2 attaches preferentially to the catalyst (Chapter 4). The concentration used in the flow reactor study was 0.01% (v/v), a level that did not inhibit degradation in the batch system but did so in the fixed film system. This suggests there is a relationship between the amount of catalyst present and the concentration of H_2O_2 required in the system, i.e. the less TiO_2 present the less H_2O_2 required otherwise inhibition of the photocatalytic reaction occurs. Therefore a lower concentration of the oxidant may have been required to observe enhancement of the fixed film photocatalytic system used here. It would be beneficial to determine exact catalyst load on the glass surface of any fixed film system to allow determination of the optimum H_2O_2 level required for enhancement of toxin degradation.

The work carried out with the fixed film coil reactor has also demonstrated that when other compounds such as humics are present in the photocatalytic system there is lower efficiency of toxin degradation. TiO_2 photocatalysis has been shown to degrade humic substances in potable waters (Eggins *et al.*, 1997). Following illumination in the flow system there was a noticeable decrease in the colour of the loch water indicating the breakdown of organic compounds present. Humic substances have also been shown to degrade microcystins by indirect photolysis (Welker and Steinberg, 1999). These researchers speculated that toxin destruction was initiated by singlet oxygen or peroxide formed on the illumination of humics. Robertson *et al.* (1999) demonstrated a similar method of indirect photolysis of microcystins using the pigment phycocyanin (present in cyanobacteria). It was also found that when

this pigment was present in a solution of TiO_2 and microcystin-LR the photocatalytic reaction was inhibited. This was thought to be due to the pigment competitively absorbing light preventing the activation of the TiO_2. Alternatively it was suggested that the pigment itself may be reacting with the photogenerated hole in the TiO_2 preventing the formation of the oxidising $OH^{\bullet}$ radicals. Once the pigment had been degraded by the UV light the photocatalytic degradation of the toxin occurred as normal. It is believed that a similar effect is occurring in the presence of the humic compounds in the loch water. It is also possible that these humic substances which made the water appear yellow were absorbing the light required to activate the TiO_2 catalyst or that they are interacting with the TiO_2 itself. When H_2O_2 was present the colour removal was much more evident suggesting that the presence of the oxidant was enhancing degradation of the humics present. The removal of the microcystin-LR was also improved in the raw water system when H_2O_2 was present. It is possible that levels of H_2O_2 were rapidly depleted, by degrading the humics in the raw water, to a level that was enhancing the photocatalytic reaction. This would explain why degradation was so efficient in this water quality compared to when H_2O_2 was used in pure and tap water.

The work also indicates that photocatalytic oxidation degrades other microcystin variants, namely microcystin-RR, -LW and -LF, which is important if this method is to be used in the treatment of waters contaminated with microcystins. While these toxins have been shown here to be degraded there is great variation of dark adsorption and degradation rates (Figure 5.13). The most readily adsorbed toxin was microcystin-LW which may be due to the

hydrophobic nature of this variant. Alternatively, microcystin-RR is relatively hydrophilic and the results have shown this toxin displayed very little dark adsorption (~ 2%). The polarity of microcystin-LR falls in-between these two variants and the dark adsorption of this toxin is approximately 60%. These results may suggest that the hydrophobicity of the microcystin variant plays a role in the rate of degradation of that specific toxin. Microcystin-LF is also relatively hydrophobic, however the toxin did not display high dark adsorption as would be expected if this was a factor, only 10% of the toxin was adsorbed to the catalyst surface. Yet at a lower concentration (100 $\mu g\ ml^{-1}$) the dark adsorption was a high as 95%, which would be expected if polarity played a role in surface adsorption. This was the only microcystin variant tested to display this concentration dependent dark adsorption. It may be that the catalyst surface was saturated at the high concentration of toxin and was unable to adsorb more toxin. This saturation point was not reached at the lower concentration hence there was a greater dark adsorption. On the whole the results indicated that for efficient degradation to occur there must be a high degree of dark adsorption. In particular for those toxins which undergo high levels of dark adsorption such as microcystin-LR and -LW there are higher rates of degradation (Figure 5.13). In the case of microcystin-RR little dark adsorption is followed by a slow degradation. Once more microcystin-LF behaves in a different manner to other variants, while it has only 10% dark adsorption it has a degradation rate comparable to microcystin-LR. The effect of pH on adsorption and degradation of each toxin varied. Initial solution pH had no effect on the dark adsorption or the degradation rates of microcystin-RR. This would suggest there is no surface charge influence like that

observed with microcystin-LR in the case of this variant. Dark adsorption of microcystin-LW was only inhibited at high pH values (10 and 12) and was followed by slow degradation. It was determined that this variant was unstable at these pH levels with degradation occurring even in the absence of the catalyst. The degradation at these levels may therefore have been due to this factor and not via photocatalysis. Microcystin-LF was found to be the most influenced by pH and exhibited similar behavior to microcystin-LR. Most rapid degradation occurred at initial pH values of 1 and 4, this also being the area of greatest dark adsorption. As pH increases there is a loss of the dark adsorption reaction however the rate of degradation is only slightly reduced. The interaction of microcystins with TiO_2 needs to be fully modelled in detail, currently it can only be speculated as to how the various microcystin variants interact with the catalyst surface and how it effects their degradation.

The results however have shown that photocatalytic degradation of microcystin variants does occur and that the extent of destruction has been shown to vary between the toxin variants although all were destroyed within one hour. The work implies that dark adsorption is an important reaction step although there are some contradictions. HPLC-PDA data suggests that breakdown products of the microcystin variants are similar to those obtained for microcystin-LR suggesting similar degradation pathways. However mass spectral analysis of reaction solutions would have to be applied to determine if these compounds were in fact the same. It would also be necessary to determine if the toxicity related with these microcystins had been removed. Further studies would also

have to be carried out to determine the effectiveness of this treatment method on these variants in natural water samples.

The work in this chapter has also shown that fixed film flow reactors are capable of degrading microcystin-LR, however, at rates much lower than that achieved using slurry batch reactors. It would be necessary to determine the optimum flow rate in such fixed film reactors to give the most efficient degradation as well as investigate the effect of catalyst loading. The investigation has also highlighted the fact that even though the presence of H_2O_2 in the system may enhance photooxidation there is a need to determine exact catalyst load to ensure the reaction is not inhibited by too high a dose of the oxidant. It has also been shown that the photodegradation process is affected by the presence of organics present in raw waters being treated. Where such compounds are present it may be necessary to remove them prior to photocatalytic treatment, alternatively the addition of H_2O_2 to the system would aid in their removal.

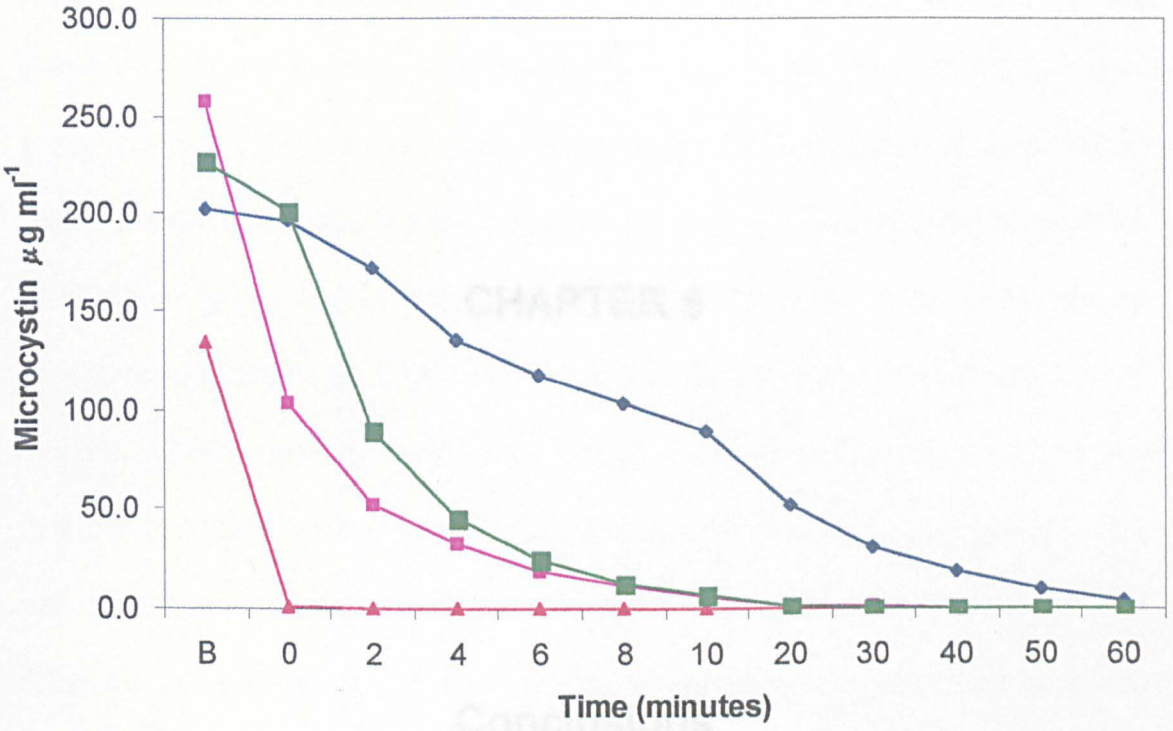


Figure 5.13: Photooxidation of microcytin-LR(■), -RR(♦), -LW(▲) and -LF(■), levels monitored by HPLC. B indicates the concentration of microcystin prior to the dark adsorption on addition of the catalyst.

CHAPTER 6

Conclusions

The use of UV light in the presence of a TiO_2 photocatalyst has previously been demonstrated to effectively degrade microcystin-LR at high levels (200 $\mu g\ ml^{-1}$) within 30 minutes (Robertson *et al.*, 1997). This study has confirmed that microcystin-LR is rapidly degraded upon UV illumination in the presence of TiO_2 even at high concentrations. It was determined that only 6.4% of the microcystin-LR was mineralised and HPLC-PDA analysis of reaction solutions indicated the presence of several UV absorbing breakdown products in the reaction solutions. However the use of the brine shrimp bioassay indicated that TiO_2 photocatalysis does eliminate the acute toxicity associated with microcystin-LR and suggests that these by-products, and any that may not be UV detectable, are not a direct threat to human health. The use of a protein phosphatase assay would have to be carried out to determine if the tumour promoting activity of the toxin over long term exposure was also removed by this process.

An advantage of the TiO_2 system is use of low energy UV sources compared to those required for direct UV treatment. Some researchers have also modified TiO_2 to allow the use of solar light instead of UV (Cheung *et al.*, 1998). If such a modified catalyst was used in a water treatment system the cost of operation would be greatly reduced. Another advantage is the fact that once the TiO_2 is removed no invasive chemicals have been added to the drinking water. TiO_2 itself is used as whitening agent in many foodstuffs and pharmaceuticals, therefore consumption of a small amount present in drinking water following treatment would pose no direct threat to human health.

One major disadvantage of a large scale photocatalytic process is the requirement for removing the catalysis from the water. Any large scale batch reactor system would therefore require a further step to remove the catalyst such as filtration, centrifugation or coagulation. One way to overcome this disadvantage is the use of a fixed film flow reactor design. Such a system involves the use of a catalyst coated to the surface of a material which the solution containing the pollutant is passed over whilst illuminating with UV light. Such systems have been demonstrated to be effective for the removal of several pollutants from water (Hua *et al.*, 1995; Matthews 1993). A simple fixed film coil reactor was constructed and tested for the destruction of microcystin-LR. The system, however, was found to degrade the toxin at a much lower rate than that determined using a batch reactor. It would therefore be more desirable to develop a simple separation process for a large scale batch reactor as such a system would be more efficient than a flow reactor. Using the fixed film flow reactor it was also found that the presence of organics in the water inhibited the degradation of the toxin. This is possibly due to the competition of these compounds for oxidation by the $OH^{\bullet}$ radicals formed by the catalyst or due to competition for adsorption to the catalyst surface.

Photocatalysis has also been reported to degrade microcystin-YR and -YA (Shepard *et al.*, 1998). This study found photooxidation to be effective in the degradation of microcystin-RR, -LW and -LF using a slurry batch reaction. While all the tested variants seemed to produce similar breakdown products to those formed by degradation of microcystin-LR each toxin demonstrated different dark adsorption behaviour and degradation rates. Further work must

be carried out to determine the exact nature of the breakdown products of these variants and to determine if their acute and chronic toxicity has been removed.

While TiO_2 photocatalysis has proved to effectively degrade a variety of microcystins including microcystin-LR, -LW, -LF and -RR the study was carried out mainly using pure water. It must therefore also be determined if the breakdown behaviour of the toxin is affected by the presence of organics in natural waters. While it was demonstrated that organics in raw water inhibited the degradation of microcystin-LR it must be established how these compounds affect the breakdown pathways. It must also be established whether any new breakdown products that are formed by the presence of organics are themselves toxic.

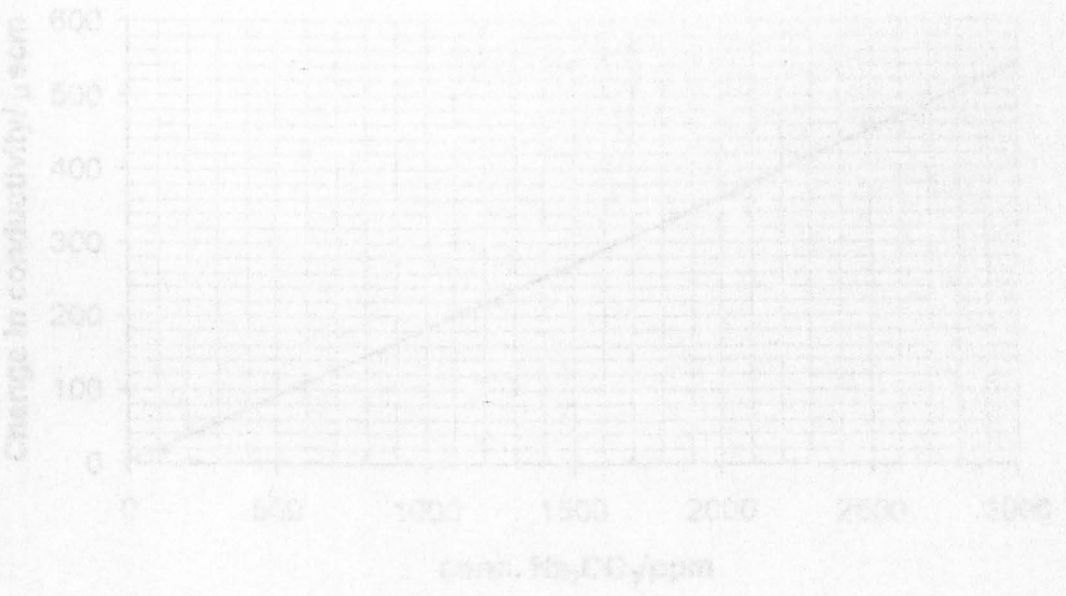
Another aspect of the study was to gain a greater understanding as to how the photocatalytic breakdown of microcystin-LR is initiated. The use of mass spectral data allowed a proposed pathway of toxin destruction to be speculated, some of which correlated well with the HPLC data. Furthermore, it was demonstrated that the presence of oxygen is essential for the process to occur and that when the reaction was carried out in heavy water (D_2O) there was a significant drop in reaction rates. Overall the results suggested that the oxidation of the toxin was being carried out by the $OH^{\bullet}$ radicals produced at the catalyst surface during UV illumination. The work carried out also established that the photocatalytic process was pH dependent. The optimum pH for toxin destruction falls within an area where attraction of the microcystin-

LR to the TiO_2 surface is occurring. At pH levels where both the toxin and the catalyst share a similar electrical charge, and hence repel each other, little or no degradation occurs. These findings suggest that adsorption of the toxin to the TiO_2 surface is highly favourable for photooxidation to occur. While further research would have to be carried out to determine the role of other species formed by the catalyst, the work here has given a greater understanding of the way in which the photocatalytic degradation of the toxin occurs in the system.

The use of UV illumination in the presence of H_2O_2 was demonstrated to enhance the rate of toxin removal compared to either UV or the oxidant used individually (Rositano and Nicholson, 1994). This was found to be the case in this study but levels of degradation with a UV/ H_2O_2 system were far lower than those achieved with photocatalysis. Some researchers demonstrated that the addition of H_2O_2 to the photocatalytic system enhances photocatalysed destruction of pollutants (Grätzel *et al.*, 1990; Tanaka *et al.*, 1990; Jakob *et al.*, 1993). It was suggested by these researchers that this increased rate of destruction was due to the photodissociation of the peroxide to form highly oxidising hydroxyl radicals. In combination with those hydroxyl radicals formed by the illumination of the TiO_2 there is a resultant increased amount of these oxidising species in solution. This study has shown that a UV/TiO_2/H_2O_2 system achieves greater rates of toxin degradation than either a UV/TiO_2 or UV/H_2O_2 system. It was also shown that the UV absorbing breakdown products detected up to 1 hour after UV/TiO_2 treatment were also degraded within 30 minutes when H_2O_2 was present in the system. Mineralisation of the toxin was found to have increased to 18% suggesting that there were still non-

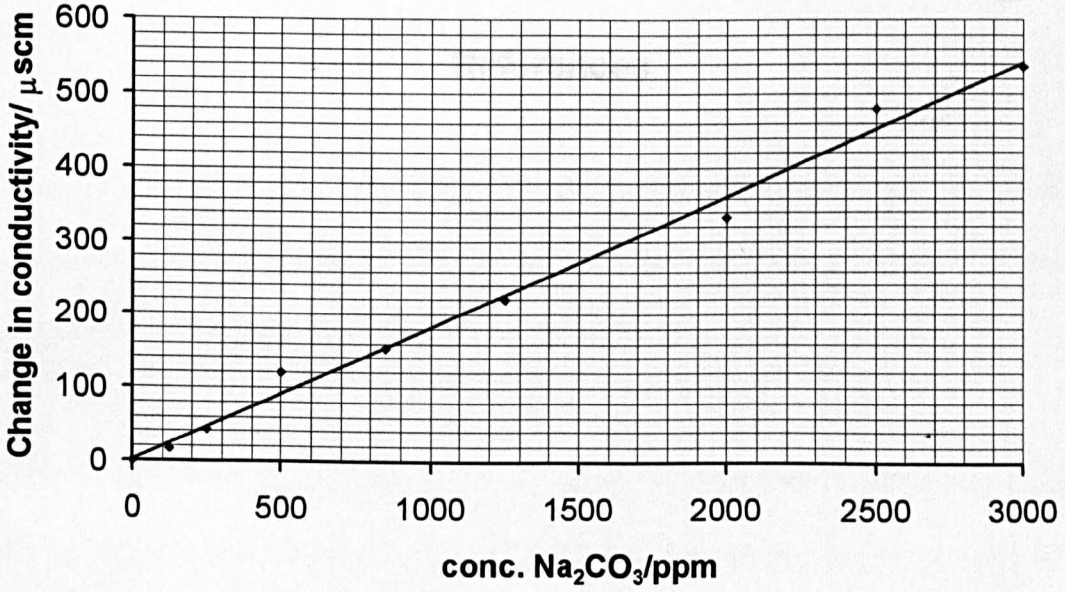
UV absorbing by-products present. Toxicity studies indicated that test solutions were rendered non-toxic after 6 minutes treatment. It was demonstrated that the enhancement of the reaction is dependent on concentration with most efficient enhancement shown to occur at oxidant concentrations between 0.01 – 0.1% (v/v). At higher levels (>0.5%) enhanced rates began to drop possibly due to competitive adsorption of the H_2O_2 and the microcystin-LR to the catalyst surface. This suggests that peroxide dosages are dependent on the catalyst load present in the reaction solution. In regards to large scale use of the UV/TiO_2/H_2O_2 system the H_2O_2 oxidant is effectively removed from the water by dissociation resulting from the UV illumination, as a result no chemical residuals are left in the treated water. However, this also means that oxidant levels in the batch system are constantly dropping making it necessary to investigate a system capable of monitoring H_2O_2 levels in solution and maintaining an optimum level in the water until all the toxin is removed. An important advantage of H_2O_2 being present in the batch system with regards to reactor design is the oxidant's ability to make the catalyst coagulate. The work here has shown that when H_2O_2 was present the catalysts drops out of solution due to the TiO_2 forming clumps. This would result in easier removal of the catalyst from solution by sedimentation or centrifugation. The fixed film flow reactor was also tested with H_2O_2 in the system, however, the levels were too high compared to the catalyst load and inhibition of the reaction occurred. While fixed film flow reactors have the advantage of no extra treatment steps to remove the catalyst batch reactors provide much more efficient toxin degradation.

In conclusion photocatalysis has shown to be a promising treatment method for the removal of microcystin-LR and other variants from potable waters. However further research is required to determine the behaviour of the toxins' degradation in natural water supplies. While the acute toxic effect of the microcystin-LR was removed it is necessary to investigate if the compounds tumour promoting effects are rendered inactive. Further research must also be applied to reactor design for large scale water treatment. Both batch and flow reactors have advantages over one another, however, most efficient degradation occurs in the small scale batch reactors. The study also allowed for speculation as to how the photooxidation of the toxin occurred.



Appendix 1

Calibration graph for the determination of the level of CO_2 produced during photocatalysis by the change in conductance of the conductivity cell (1 ppm Na_2CO_3 is equivalent to 1 ppm CO_2):



CHAPTER 7

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