CHAPTER 2

An overview of the impact of anthropogenic activity on water quality with special reference to human health, risks and detection of toxic compounds present in water bodies.

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2.1 Introduction

For most of history, human population and the level of technological development have been too low to drastically affect the global quality of air, soil, water and biological systems. However, with the world's population increasing to 6 billion, pressures on the environment, and especially the water resources is increasing. Approximately, 65% of the 1015 billion litres of freshwater withdrawn for all purposes in the United States in 1985 was obtained from surface water sources (Solley *et al*. 1988). While water quality is generally good in the United States, many municipal water supply systems are operating potentially harmful contaminant and pollution levels. In 1976, it was estimated that some 8 million US inhabitants were using potentially dangerous water from about 5 000 community supplies (U.S. Environmental Protection Agency, 1976), and with numerous reports of illnesses due to contamination of drinking water resources (see later Table 2.2), the importance of water quality is highlighted.

Anthropogenic disturbances change the physicochemical characteristics and resource availability of rivers from headwaters to mouth and influence community structure and function of stream biota. Assessment of stream system health cannot be fully achieved without a careful analysis of the benthic fauna and benthic processes (Reice & Wohlenberg 1993). Macroinvertebrates and phytoplankton are a critical component of every stream. They play an essential role in the food chain, productivity, nutrient cycling, and decomposition (Cummins 1988). Knowledge of the state of the macroinvertebrate community is, therefore, essential to the assessment of the health of the stream system.

2.2 Water quality

Water serves as the universal medium for all metabolic reactions, and life cannot exist without water. The quality of surface water is currently arousing considerable interest, due to the importance of this resource for human life and activities (U.S. Environmental Protection Agency 2004). Aquatic pollutants originate from point sources and/or from non-point sources. The former comprises effluents from sewers originating from human settlements or industries where one can easily identify the source of pollution. The non-point sources include surface runoff laden with agricultural chemicals such as pesticides and fertilizers. Domestic sewage disposal and urban runoff causes the most serious water pollution problems in the more densely populated areas throughout the world. Domestic sewage mainly increases biological oxygen demand and nutrient levels of the receiving waters (Mason 1996). Progressive deterioration of many surface water ecosystems has been reported all over the world. In the United States alone, one of every three lakes and nearly one-quarter of the nation's rivers contain enough pollution that people should limit or avoid eating fish caught there. Every state but Alaska and Wyoming issued fish advisories covering some and occasionally all of their lakes or rivers in 2003, according to a national database maintained by the U.S. Environmental Protection Agency (U.S. EPA 2004). A recent survey in 2002-2003, assessing the water quality of the Cache la Poudre River, Colorado, revealed the presence of pesticides; wastewater compounds and *Esterichia coli* bacteria; dissolved organic compounds; 89 volatile organic compounds and polycyclic aromatic hydrocarbons (PAHs) (e.g., coaltar residues, acetone, toluene and benzene) (Collins & Sprague 2005).

2.3 Urban runoff

Urbanization is inevitably associated with the greatly increase runoff problems due to the fact that roads, parking lots, rooftops and other such surfaces are impervious to water. Rainfall that lands on these areas has no chance to sink into the ground, as would be the case if the land were covered with vegetation. The percentage of land cover by impervious surface in business districts may approach 100% and in residential areas may be easily 50%. Thus, the amount of overland runoff from urban areas is much greater than in comparable rural areas. Furthermore, runoff from impervious surfaces is more rapid than from land covered with vegetation, since there is little to impede the flow of water over parking lots, streets, sidewalks or urban areas than from rural areas. Because the erosive power of water increase with the intensity of water flow and because there is more total runoff in urban areas, exposed land is eroded away more rapidly in urban than in rural areas. The crux of the problem is the fact that in many communities urban runoff, which may contain high concentrations of nutrients, oxygen-consuming wastes, pathogens and toxic substances such as pesticides, heavy metals, and oil is simply routed to the nearest convenient watercourse and discharged into a stormwater management pond or detention lake without treatment (Peterson *et al.* 1985). The limited available data on the presence of pollutants in urban runoff indicate that urban streams are the most vulnerable to contamination, with one or more wastewater chemicals found in 100% of the samples collected and tested from urban streams in Colorado (Sprague & Battaglin 2004).

2.4 Eutrophication

Nutrient pollution especially with phosphorus but also with nitrogen coming from urban runoff and sanitary sewer systems can lead to the eutrophication of the receiving water bodies (Stevens 2005). Eutrophication of water bodies has increased during the last century due to an increase in anthropogenic inputs. In Europe, Asia and America, more than 40% of lakes are now eutrophic and hence subject to algal proliferations (Bartram *et al.* 1999). The perturbation of the natural succession of phytoplankton in eutrophic and especially hypertrophic waters extends and intensifies the period of cyanobacterial dominance. Cyanobacterial water blooms can degrade water quality in many ways. A high cyanobacterial biomass can contribute to aesthetic problems such as scum formation that dramatically decreases the transparency of the water body and can also cause the production of undesirable tastes and odours in drinking water (WHO 1998). From an economic standpoint, the most important problem relates to taste and odours in drinking water, and the potential for this to happen in the US is high due to the fact that more water is utilized from surface water than ground water (Solley *et al.* 1988). During the decomposition of water blooms, deoxygenation of waters is observed which can affect the ability of aquatic animals to survive. One of the most serious effects of bloom formation can be the production of secondary metabolites by cyanobacteria, which can be toxic to humans and animals. In 1998, risks to humans, lead the World Health Organization (WHO) to propose a provisional guideline value of 1.0 µg/L for the levels of one of the most common cyanotoxins, the microcystin-LR in drinking water (WHO 1998).

Toxins of cyanobacteria are grouped in two main categories by Carmichael (1992) namely, biotoxins and cytotoxins based on the types of bioassays used to screen for

their activity. The presence of cytotoxins is detected by mammalian cell lines and biotoxins are assayed with small animals, e.g. mice or aquatic invertebrates. The primary types of cyanobacterial biotoxins include hepatotoxin (microcystins, nodularins, cylindrospermopsins), neurotoxin (anatoxins, saxitoxins) and dermatotoxins (lyngbyatoxin A, aplysiatoxins, lipopolysaccharides) (Codd & Poon 1988; Codd & Bell 1989), and in the toxicity standards, biotoxins are considered supertoxic (Table 2.1). Biotoxins of cyanobacteria are water-soluble and heat stable and they are released upon aging or lysis of the cells. The most frequently occurring hepatotoxins are microcystins. They are cyclic peptides of molecular weight from about 800 to 1 100. They contain seven amino acids. More than 60 different structural variations of microcystin have been characterized so far (Burns *et al*. 2004). The majority of microcystin variants differ by L-amino acids in positions 2 and 4. For microcystin-LR, the two variable amino acids are leucine and arginine that have the single letter abbreviations L and R, respectively (Codd & Poon 1988; Carmichael 1992).

Table 2.1 Comparison of toxicities of some biological toxins

Toxins	Sources	Lethal doses (LD_{50})	Reference
Saxitoxin	*Aphanizomenon flos-aquae*	10	(Oshima 1995).
Anatoxin-a(s)	*Anabaena flos-aquae*	20	(Falconer 1998).
Cobra toxin	*Naja naja*	20	(Bagchi 1996)
Nodularin	*Nodularia spumigena*	30	(Rinehart *et al*. 1994).
Microcystin LR	*Microcystis aeruginosa*	50	(Rinehart *et al*. 1994).
Anatoxin-a	*Anabaena flos-aquae*	200	(Carmichael 1992).
Brevetoxin	*Karenia brevis (dinoflagellate)*	500	(Morohashi *et al*. 1999).
Ciguatoxin	*Gambierdiscus toxicus (dinoflagellate)*	0.25	(Bagnis *et al*. 1980).
Cylindrospermopsins	*Cylindrospermopsins raciborskii*	2 100	(Ohtani *et al*. 1992).
Styrchnine	*Strychnos nuxvomica*	2 000	(Bagchi 1996).

Toxic cyanobacteria have caused mortalities amongst wild and domestic animals (Codd 1992). They also constitute a hazard to human health, for example, via ingestion and skin contact (Carmichael 1992). The first clearly documented human fatalities, which are ascribed to cyanobacterial toxins, occurred in 1996. More than 50 patients at a hemodialysis center in Caruaru died with hepatotoxic and neurotoxic symptoms (Pouria *et al*. 1998). Long-term chronic exposure of humans to cyanobacterial toxins may also occur, as some conventional water treatment processes are ineffective in the removal of toxins from drinking water (Himberg *et al*. 1989). The cyanobacterial hepatotoxins have also been shown to have tumor-promoting activity and can lead to primary liver cancer (Fujiki 1992). Cyanobacterial blooms are not always toxic. At the same sampling point, it is possible to find toxic and nontoxic strains of the same species of cyanobacteria.

2.5 Human health risks of long-term exposure to toxic compounds

2.5.1 Health risks of long-term exposure to low levels of microcystin

Little information is available on the effects of long-term exposure to low levels of microcystin toxins in humans (Table 2.2). We know that in experiments performed on a time-scale of minutes or hours, microcystin has obvious effects on the functions of plant and animal cells at concentrations as low as 3-10 nM that is equivalent to 3-10 µg for an adult female liver. In cells that take up microcystin freely, the maximum effects are visible at concentrations of around 1µM, the point at which all of the cellular PP1 and PP2A is saturated with toxin. This means that approximately 1 mg (equivalent to drinking to two liters of water per day at 32 µg/L microcystin over two weeks) would bind all of the PP1 and PP2A in an adult female human liver, provided

that the PP-microcystin complexes were stable (MacKintosh 1993). However, most of the available data about uptake and turnover of microcystins has been obtained from experiments carried out with rodents. In this regard, it should be noted that PP1 and PP2A from mice and humans amino acid sequences are 100 percent identical (Barker *et al*. 1993). In the case of mice low doses of microsystin cause progressive changes in liver tissue over time, including chronic inflammation, focal degeneration of hepatocytes and the accumulation of metabolites such as bilirubin in the blood, and tend to increase mortality (Hermansky *et al*. 1990). In South Africa liver damage and death of vervet monkeys has occurred following toxic *Microcystis* administration with signs of poisoning similar to those observed in live stock and mice (Tustin *et al*. 1973). These demonstrations of the susceptibility of primates to cyanobacterial poisoning are consistent with the results of an epidemiological study of a human population of the city of Armidale, New South Wales, Australia, which obtains its drinking water from the Malpas Dam reservoir. A clear pattern of admission of patients to the local hospital with liver complaints was identified which coincided with the seasonal production of a hepatotoxic *Microcystis aeruginosa* bloom in the reservoir. This correlation was confined to patients who had taken their drinking water from the Malpas Dam (Falconer *et al*. 1983).

Yu (1995) reported that the incidence of liver cancer is significantly higher for populations using cyanobacteria-infested surface water than those drinking groundwater in China. In Shanghai and its nearby regions where epidemiological studies showed that increased incidence of primary liver cancer is related to the consumption of microcystin contaminated water, the concentrations of microcystins in samples of pond-ditch water were within the range of 0.09-0.46 µg/L (Ueno *et al*.

1996). However, Zegura *et al*. (2002) showed that microcystin-LR induced oxidative DNA damage in HepG2 human cells at low concentrations (0.01 µg/mL) and this might be a mechanism by which chronic exposure to low concentrations of microcystins contribute to increase the risk for liver cancer development. A recent study in mice has shown that *Microcystis aeruginosa* extract provided in drinking water increased the area of aberrant crypt foci in the colon, suggestive that microcystins promote preneoplastic colonic lesions (Humpage *et al*. 2000a).

Table 2.2. Acute intoxications of humans from cyanobacteria.

Cases attributed to cyanotoxins in drinking water	
Year	**Report**
1931	United States; A massive *Microcystis* bloom in the Ohio and Potomac rivers caused illness in 5 000 to 8 000 persons whose drinking water was taken from these rivers. Low rainfall has caused the water of a side branch of the river to develop a cyanobacterial bloom, which was then washed by new rainfall into the main river. Drinking water treatment by precipitation, filtration, and chlorination was not sufficient to remove the toxins (Tisdale 1931; Veldee 1931).
1960-1965	Zimbabwe, Harare; Cases of acute gastroenteritis among European children admitted to the local hospital in Salisbury, Rhodesia (now Harare, Zimbabwe). In this instance, several supply reservoirs provided water to different regions of the city, but only the reservoir containing blooms of *Microcystis* supplied water to the affected population. (Zilberg 1966).
1968	United States; Numerous cases of gastrointestinal illness after exposure to mass developments of cyanobacteria was compiled by Schwimmer and Schwimmer (1968).
1975	United States; Hindman *et al*. (1975) reported the results of an investigation into 49 pyrogenic reactions in patients undergoing haemodialysis treatment in Washington, DC. They concluded that 'the cause of these reactions was traced to an increase in endotoxin contamination of the tap water used to prepare dialysate, possibly caused by an increase in the algae levels in the local water source.
1979	Australia; Combating a bloom of *Cylindrospermopsis raciborskii* in a drinking water reservoir on Palm Island with copper sulfate led to liberation of toxins from the cells into the water, thus causing serious illness with hospitalization of 141 persons supplied from this reservoir (Falconer 1993a, 1993b).
1981	Australia; In the city of Armidale, liver enzyme activities were elevated in the blood of the population that was supplied from surface water polluted by *Microcystis* spp. (Falconer *et al*. 1983).
1992	United States; Carmichael (1992) compiled case studies on nausea, vomiting, diarrhea, fever and eye, ear, and throat infections after exposure to mass developments of cyanobacteria.
1993	Australia; Ressom *et al.* (1994) estimated that more than 600,000 person-days are lost annually due to absence of their water source due in turn to toxic cyanobacterial blooms. China; The incidence of very high rates of liver cancer is related to water sources. The incidence is significantly higher for populations using cyanobacteria-infested surface waters than those drinking ground water. A cohort study showed that people who drank pond and ditch water had 121 deaths per 100 000 compared with 0 for those who drank well water (Yu 1994, 1995).
1994	Sweden Near Malmo; Illegal use of untreated river water in a sugar factory led to an accidental cross-connection with the drinking water supply for an uncertain number of hours. The river water was densely populated by *Planktothrix agardhii*, and samples taken a few days before and a few days after the incident showed these cyanobacteria contained microcystins. Of 304 inhabitants of

	the village, 121 became ill with vomiting, diarrhea, muscular cramps, and nausea (Cronberg *et al*. 1995).

Cases attributed to cyanotoxins in recreational water

Date	Description
1959	Saskatchewan, Canada; In spite of livestock deaths and warnings against recreational use, people did swim in a lake infested with cyanobacteria. Thirteen persons became ill (headaches, nausea, muscular pains, painful diarrhea). In the excreta of one patient – a medical doctor who had accidentally ingested 300 ml of water-numerous cells of *Microcystis* spp. And some trichomes of Anabaena circinalis could be clearly identified (Dillenberg & Dehnel 1960).
1989	England; In Staffordshire ten out of 20 soldiers became ill after swimming and canoe-training in water with a heavy bloom of *Microcystis* spp.; two of them develop severe pneumonia attributed to the inhalation of a *Microcystis* toxin and required hospitalization and intensive care. Sixteen develop sore throat, headache, abdominal pain, dry cough, diarrhoea, vomiting and blistered mouths (Turner *et al*. 1990). Swimming skills and the amount of water ingested appear to have been related to the degree of illness.
1995	Australia; Epidemiological evidence of adverse health effects after recreational water contact from a prospective study involving 852 participants who showed elevated incidence of diarrhea, vomiting, flu symptoms, skin rashes, mouth ulcers, fevers, and eye or ear irritations within 2 to 7 days after exposure. The sensitivity of individuals to allergic-type reactions at low cyanobacteria cell densities is greater than can be attributed to the toxin content of cyanobacteria (Pilotto *et al*. 1997).

Cases due to other exposure routes

Date	Description
1996	Caruaru in Brazil; One hundred and twenty six dialysis patients were exposed to microcystin through the water used for dialysis, and 60 of them eventually died, principally of liver failure, 6 had died by 2 weeks after exposure, 30 by 6 weeks, 44 by 10 weeks, and 55 by 27 weeks. At least 44 of these victims showed the typical common symptoms associated with microcystin, now referred to as 'Caruaru Syndrome' and the liver microcystin content corresponded to that of laboratory animals that received a lethal dose of microcystin (Jochimsen *et al*. 1998; Carmichael *et al*. 1996; Pouria *et al*. 1998).

2.5.2 Effect of long-term exposure to PAHs

Petroleum products like PAHs can adversely affect organisms by physical action, habitat modification and toxic action. The aromatic fraction of petroleum is considered to be responsible for most of the toxic effects, thus PAHs affect organisms through toxic action. The mechanism of toxicity is reported to be interference with cellular membrane function and enzyme systems associated with the membrane (Neff 1985). Although unmetabolized PAHs can have toxic effects, a major concern in animals is the ability of the reactive metabolites, such as epoxides and dihydrodiols of some PAHs to bind to cellular proteins and DNA. The resulting biochemical

disruptions and cell damage lead to mutations, developmental malformations, tumors, and cancer (Eisler 2000; Santodonato *et al*. 1981; Varanasi 1989). Four-, five-, and six-ring PAHs have greater carcinogenic potential than the two-, three-, and seven-ring PHAs (Eisler 2000; Neff 1985). The addition of alkyl groups to the base PAH structure often produces carcinogenicity or enhances existing carcinogenic activity. Some halogenated PAHs are mutagenic without metabolic activation (Fu 1999) and the toxicity and possibly the carcinogenicity of PAHs can be increased by exposure to solar ultraviolet radiation (Ren 1994; Arfsten *et al*. 1996). Cancerous and precancerous neoplasms have been induced in aquatic organisms in laboratory studies, and cancerous and noncancerous neoplasms have also been found in feral fish from polluted sites (Eisler 2000; Neff 1985; Baumann 1989; Chang *et al*. 1998).

2.6 Methods for detection of toxicity as a result of chemical pollutants and biological toxic compounds in raw water

2.6.1 Bioassays for detection of PAHs

In recent years the increasing desire to link exposure, to effect has drawn considerable attention to the 'biomarker approach'. Because chemical contaminants are known to evoke distinct measurable biological responses in exposed organisms, biomarker-based techniques are currently being investigated to assess toxicant-induced changes at the biological and ecological levels (Shugart 1996). Collectively the term biomarker refers to the use of physiological, biochemical, and histological changes as 'indicators' of exposure and effects of xenobiotics at the suborganism or organism level (Huggett *et al*. 1992).

However, indicators or biomarkers can be defined at any level of biological organization, including changes manifested as enzyme content or activity, DNA adducts, chromosomal aberration, histopathological alterations, immune-system effects, reproductive effects, physiological effects, and fertility at the molecular and individual level, as well as size distributions, diversity indices, and functional parameters at the population and ecosystem level. In the field of ecotoxicology, the use of biomarkers has emerged as a new and powerful tool for detecting both exposure and effects resulting from environmental contaminants (Huggett *et al*. 1992; Shugart *et al*. 1989, 1992a, 1992b; McCarthy & Shugart 1990; Peakall & Shugart 1992; Fossi & Leonzio 1993; Travis 1993). Unlike most chemical monitoring, biomarker endpoints have the potential to reflect and assess the bioavailability of complex mixtures present in the environment as well as render biological significance. Biomarkers provide rapid toxicity assessment and early indication of population and community stress and offer the potential to be used as markers of specific chemicals. In the European Inventory of Existing Commercial Substances (EINECS) about 100,000 chemicals are identified, of which approximate 10-20 chemicals are monitored in important European aquatic ecosystems. If a closer look is taken at the effects data for the chemicals in the EINECS, it appears that only for 20-30 chemicals adequate information is available on long-term single-species ecotoxicity and environmental fate. Hence, for 99.99% of all existing chemicals no adequate information on sources, effects and concentrations is available, which implies the importance of biomarker assessment on chemicals like coal tar residue (Van Leewen 1992).

2.6.2 Methods for detection of microsytin

The most common methods for monitoring microcystin concentrations have been high-performance liquid chromatography combined with a UV-visible light diode array detector, protein phosphatase inhibition and enzyme-linked immunosorbent assays (ELISA) (Harada *et al*. 1999). The intraperitoneal mouse bioassay, which has been the most extensively used biotest to determine the toxicity of cyanobacterial blooms, is useful as an initial screening test, but requires expensive husbandry, is strictly regulated in some countries, and is opposed on moral grounds (Bell & Codd 1996). However, such analysis does not indicate which cyanobacterial strains produce the toxins. Microcystin concentration in a body of water seems to be mostly dependent on the density of the hepatotoxic cells (Sivonen & Jones 1999). It has also been demonstrated that some strains may produce higher concentrations of microcystins than other strains under the same laboratory conditions. In addition, environmental factors, such as nutrient concentrations, light, and temperature, may also affect the intracellular microcystin concentration (Sivonen & Jones 1999). Since it is not possible to distinguish toxic and nontoxic strains with a microscope, microscopic analysis cannot be useful in estimating toxicity of cyanobacterial strains.

2.6.2.1 Alternative Bioassays

Under cyanobacterial bloom conditions the amount of zooplanton is known to decrease (Lightner 1978), consequently, bioassays have been devised using *Daphnia* sp. and *Artemia salina* (Kiviranta *et al.* 1991). Major difficulties in the use of alternative bioassays, such as those based on *Artemia salina* are highlighted by Kiviranta *et al.* (1991). High to moderate concentrations of neuro-and hepatotoxins

can be detected in bloom samples using this organism, but mouse bioassay is more reliable. In contrast, a laboratory-grown strain of *Oscillatora agardhii* was toxic to larvae of mosquito *Aedes aegyptii* (Kiviranta & Abdelhameed 1994), *Artemia salina* and *Daphnia pulex*, but non-toxic to mice (Reinikainen *et al.* 1995). Also, toxicity differs for the larval and adult stages of the shrimp (*Artemia salina*), particularly for neurotoxins. It is possible that some compounds may enable the toxin to enter the larvae by affecting the biochemistry of the crustacean or by hydrogen bonding to the chelates, which are more easily absorbed. Therefore the use of alternative bioassays must be approached with caution (Kiviranta & Abdelhameed 1994).

2.6.2.2 Use of animal and/or cell bioassays

Fish health assessment or pathology usually deals with the causes processes and effects of disease, which is a function of the general health of the associated environment. Pathological investigations include procedures such as necropsis, histological examinations, parasitological examinations and liver enzyme assays (Albert &Washuta 1992).

Worldwide the mouse bioassay is the recognized standard in terms of establishing the LD_{50}, symptoms and effects of cyanotoxins, and thus by mouse bioassay the toxicity of cyanobacterial may be assessed (Chaivimol *et al.* 1994). Toxic symptoms vary depending upon material and concentration, but are in general distinct for a category of toxins. The symptoms of death of the animal allow for a clear distinction to be made as to whether the toxicity is due to a neuro or hepatotoxin. Neurotoxicosis is rapid compared to hepatotoxicosis and does not cause liver damage to animals (Falconer & Yueng 1992). A major disadvantage of the mouse bioassay is that it

cannot detect low amounts of toxins and can therefore only be used for concentrated cell samples or concentrated toxin. In addition, it cannot distinguish between different types of neuro- or hepatotoxins, particularly when several are present in the same sample (Carmichael 1992).

Hepatotoxins cause deformation and ultrastructural changes of the cytoskeleton of mouse liver hepatocytes primarily due to the inhibition of phosphatase activity (Eriksson *et al.* 1990). Aune and Berg (1986) observed these changes and proposed that freshly prepared rat hepatocytes can be used to study the toxicity of cyanobacterial blooms. They have shown that the *in vitro* toxicity to cells can be correlated with control animal experiments when crude cyanobacterial biomass is used. Similar effects have been observed with permanent cell lines and erythrocytes (Grabow *et al.* 1982). However, despite the remarkable toxic potential of hepatotoxins *in vivo*, no cell lysis, liberation of lactate dehydrogenase or haemolysis has been observed after application of pure hepatotoxin to primary or permanent cell lines (Eriksson *et al.* 1987). Furthermore, mouse bioassays is also not always feasible, since standardized laboratory facilities with specific ratings is required by law, and thus, not suitable for testing by water purification plants.

2.6.2.3 Enzyme-linked assays

The discovery that the hepatotoxic cyanobacterial toxins produce their toxic effects through the inhibition of protein phosphatases 1 and 2A has laid the foundation for toxin assays based on the inhibition of these enzymes (Sim & Mudge 1994). This is a cheap, effective assay and reliable means of detecting all hepatotoxic cyanobacterial

toxins and has been enhanced by the development of a colorimetric procedure (Ward *et al*. 1997), provided the measurement of toxicity is the only requirement. However, it does not provide for high sensitivity and the specificity with regard to toxic peptides as with the enzyme-linked immunosorbent assay (ELISA) and reversed-phase high performance liquid chromatography (RP-HPLC) analysis.

An ELISA assay has been developed by Chu *et al*. (1989), to the point where ng/mL of microcystin can be quantitated in supplies of domestic water. This immunoassay is based on polyclonal antisera raised in rabbits against bovine serum albumin conjugated to microcystin-LR (Chu *et al*. 1989). The antisera showed good cross-activity with microcystin-RR, -YR, -LR and nodularin, but less with -LA and -LY. For detection of binding to the antisera the enzyme horseradish peroxidase was conjugated to microcystin-LR. The sensitivity of the immunoassay showed approximately 50% binding of the enzyme at a toxin concentration of 1 ng/mL, which is ideal for normal water quality testing. Although ELISA is easy, relative robust to use and provide for fast results indicating trends, it lacks sensitivity with regard to specificity of toxins (Echols & Jones 2005). The assay is also relatively expensive and requires a reasonablely well equipted laboratory (e.g., plate reader), which make it less suitable for small-scale water purification plants.

2.6.2.4 Chromatographic Analysis

Methods based on the chemical structure of hepatotoxin were developed for high sensitivity and specificity during analysis, these include chromatographic methods and atom bombardment and proton nuclear magnetic resonance technology. Initially

to purify the hepatotoxins, thin-layer chromatography was used, however, the technique lacked sensitivity and specificity, and thus, it was superceded by high performance liquid chromatography (HPLC). Since then HPLC was shown to be the method of choice for detection of toxins, hepatotoxins from several cyanobacterial species and environmental biomass with high sensitivity (Harada *et al*. 1996a,b). Resolution of the toxic fraction from *Microcystis aeruginosa* PCC7806 into a closely related peptide has been achieved by reversed-phase (RP) HPLC (Cremer & Henning 1991). Integration of diode array spectroscopy with RP-HPLC has provided a rapid and sensitive method of assessing the nature and concentration of toxic peptides from cyanobacterial blooms, and is probably the method of choice when high sensitivity and resolution is required (Echols & Jones, 2005).

The characteristics of the structure of hepatotoxins have been ascertained primarily by fast atom bombardment mass spectrometry (MS) and proton nuclear magnetic resonance (Kusumi 1996). More recently, matrix assisted laser desorption ionisation mass spectrometry has permitted procedurally easier analysis of cyanobacterial biomass. This presents the possibility of rapid, sensitive analysis of environmental biomass for the presence of cyanotoxins (Chaivimol *et al*. 1994). Although RP-HPLC and MS provide for high resolution distinction of toxic peptides, it is costly, time consuming and usssually applied for single preparative scale runs.

2.6.2.5 Monitoring toxigenicity of cyanobacterial strains by molecular assay

Monitoring the quality of water destined to public supply includes identification of potentially toxic cyanobacteria and their population density. Identification of such

microorganisms based on morphological features only, though widespread, has proven problematic, mainly for the genus *Microcystis*, due to its extensive phenotypic plasticity (Kondo *et al*. 2000).

Identification of a cyanobacterial genus by microscopic morphology or molecular analysis does not indicate the potential for toxin production. Different strains of one species can be morphologically identical but differ in toxigenicity. *Microcystis aeruginosa* for example has both toxic and nontoxic strains (Meiβner *et al*. 1996). There have been numerous attempts to refine the identification of strains by using specific gene analysis. Examples include the use of PCR-based methods for amplification of the phycocyanin intergenic spacer (PC-IGS) between the α- and β-subunits of the phycocyanin operon in environmental samples (Baker *et al*. 2001) the 16S-23S rRNA internally transcribed spacer region (Otsuka *et al*. 1999) and the DNA-dependent RNA polymerase (*rpoC*I) gene (Fergusson & Saint 2000). Although these molecular techniques have improved the accuracy of strain identification, they have not been able to distinguish toxigenic from nontoxigenic strains of the same species.

The biosynthetic pathway for production of microcystin has now been elucidated (Tillett *et al*. 2001) and this has enabled the development of specific oligonucleotide primers for gene common to production of microcystins (Tillett *et al*. 2001). To better detect microcystin-producing cyanobacterial strains, Neilan *et al*. (1999) and Nishizawa *et al*. (1999) have developed genetic probes directed, respectively, to the *mcyB* gene and to adenylation domains within the microcystin synthetase gene cluster. The *mcy* gene cluster contains 55kb of DNA encoding six large open reading frames,

mcyA-E and -*G*, together with a further four small open reading frames *mcyF* and *H-J*, placed in the chromosome (Tillet *et al*. 2001). The insertional inactivation of microcystin peptide synthetase gene *mcyB* of a *Microcystis aeruginosa* strain (PCC 7806) resulted in loss of microcystin production, showing their involvement in microcystin synthesis. It was also observed by Dittmann *et al*. (1997) that all isoforms of the cyclic heptapeptide were disrupted by inactivation of the microcystin synthetase gene sequence *mcyB*.

Several reports on the utility of the *mcyB* gene sequence for identification of toxic potential in environmental strains was published (do Carmo Bittencourt-Oliveira, 2003; Oberholster 2004; Grobbelaar *et al*. 2004; Grobbelaar 2005; Ouahid *et al*. 2005). Ouahid *et al*. (2005) applied PCR primers that were designed from the six characteristic segments of the microcystin synthetase *mcy* cluster to decern between toxin and non-toxin producing strains using ~2 000 cells as template in a multiplex reaction procedure. In a study by Oberholster (2004) and Grobbelaar *et al*. (2004), the *mcyB* gene from PCC7813 and UV027 were sequenced, resulting in fragments of 2174 and 2170 base pairs in size, respectively. The obtained sequences showed homology to other published sequences in GenBank (AY034601 for PCC7813 and AY034602 for UV027; e-value = 0.0). Upon further analysis of the sequences, it was obvious that there are several base differences between the sequences of the two strains, which suggested the potential of using differences in restriction sites, and thus insertions/deletions (indels) in nucleotide sequence to discriminate between the other *M. aeruginosa* strains, as well as using the *mcyB* gene sequence (e.g., primer pairs Tox 3P/2M, Tox 1P/1M, Tox 7P/3M and Tox 10P/4M), to discern between *M. aeruginosa* and *M. wesenbergii* in raw water samples (Fig. 2.1). The presence of the

gene *mcyB* in three of the four environmental strains was indicative of the strains' potential to produce microcystin (Oberholster 2004).

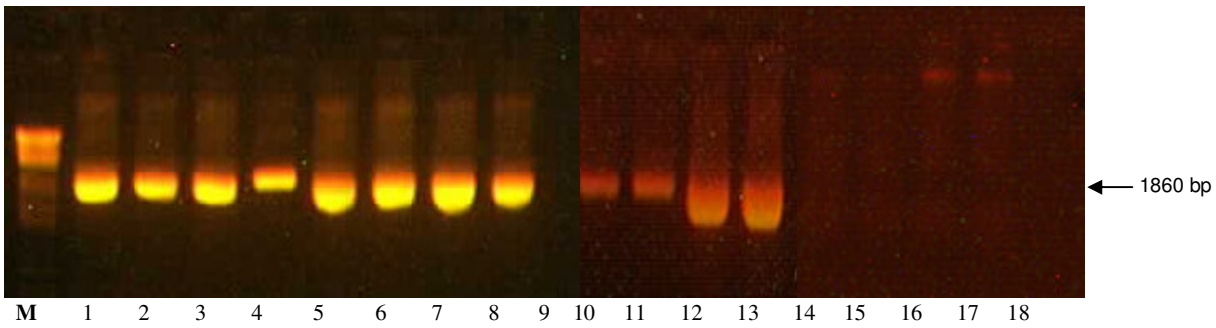

Figure 2.1 PCR fragments obtained after amplification of *Microcystis* strains with primer pairs specific for *mcyB*. Lanes 1-13 represents *Microcystis aeruginosa* strains, and lanes 14-17 represents *Microcystis wesenbergii*. M = Marker III (lambda DNA restricted with *Eco*RI and *Hind*III), 1 = PCC7813 (0.2 µl DNA); 2 = SAG1 (0.2 µl DNA); 3 = CCAP1450/1 (0.2 µl DNA), 4 = UV027 (0.2 µl DNA), 5 = PCC7813 (0.4 µl DNA); 6 = PCC7813 (0.5 µl DNA); 7 = SAG1 (1 µl DNA), 8 = SAG1 (0.8 µl DNA); 9 = water control; 10 = UP01 (0.2 µl DNA); 11 = UP03 (0.2 µl DNA); 12 = UP03 (1 µl DNA); 13 = UP04 (1 µl DNA); 14 = UP02 (0.2 µl); 15 = UP02 (0.2 µl); 16 = UP02 (1 µl); 17 = UP02 (1 µl); 18 = water control (Oberholster 2004).

A vast number of restriction sites were identified with differences followed by restriction digest of the specific polymerase chain reaction (PCR) *mcyB* gene fragment (Oberholster 2004, Figure 2.2; Grobbelaar *et al*. 2004). These reports demonstrate that PCR assays provide a useful indicator of toxicity, as well as the identification of taxonomical characteristics between laboratory cultures and environmental isolates.

2.7 Raw water treatment processes

Traditionally raw water treatment aimed to reduce the concentration of organic matter in drinking water. However, primary and secondary treatments cannot meet anymore the increasingly stringent requirements of water pollution laws and the pressures due to deteriorating raw water quality. Pietsch *et al*. (2002) reported that flocculation and

filtration resulted in an increase of extracellar toxin after experiments with *Microcystis aeruginosa* and *Planktothrix rubescens*. The researchers suggested turbulences in pipes and pressure gradients in the filter as reasons for the increase of the toxin level. The efficacy of chlorine (0.5 mg/L) to eliminate microcystin is also doubtful (Hitzfeld *et al*. 2000). Water treatment studies conducted at the laboratory and pilot plant-scale have concluded that granular activated carbon filtration is effective in removing the cyanobacterial toxins from water (Newcombe *et al*. 2001). This treatment add considerably to the expenses of water treatment and only a few purification water treatment plants in the world is equipped with granular activated carbon systems, the rest make use of conventional water treatment practices that remove live cyanobacterial cells and debris but not biotoxins in solution. In rural areas the choice of water supply may be limited, depending on the stage of development of the country. Similarly, in urban areas if the reticulated drinking water is of doubtful quality, the only choice may be bottled water, which is financially out of reach for the poorer majority of the population. Thus, the potential for injury from biotoxins in water supplies will to some extent depend on the level of development of the country and to some extent on the socio-economic status of the family (Falconer 1999).

Survey analysis of utility waters in the United States and Canada were confirmed to contain microcystin during the sampling period of June 1996 to January 1997. Of the 677 samples collected, 539 (80 %) were positive for microcystin when tested using ELISA. Of the positive samples, 4.3 percent were higher than the WHO drinking water guideline levels of 1µg/L. Only two of the plant outlet samples submitted exceeded the 1-µg/L WHO drinking water guideline. This indicates that, although almost all water treatment plants had adequate procedure to reduce microcystin to safe

levels in the finished water during the test period, the majority of source waters with cyanobacteria do contain microcystin (Carmichael 2001).

2.8 Conclusion

By reviewing the existing information on (i) the impact of anthropogenic activity on water resources, which result in the presence of toxic compounds in water resources, and thus deterioration of water resource quality in general and specific to the US; (ii) toxic chemical pollutants and cyanobacterial toxins and the risk thereof to human health; and (iii) detection methods, my goal was to introduce to the water management professionals the risks involve in poor management strategies. It is important to note that it is not merely high dosage exposures to toxic compounds, but also long-term intakes and exposures that have major detrimental health effects, thus making monitoring and early detection of toxic compounds a high priority. By using biological organisms (i.e., benthic phytoplankton, macroinvertebrate, fish, etc.) as bioindicators of aqautic ecosystem health, an inexpensive tool for assessment of the presence of chemical and biological toxic compounds is provided. For specific biotoxins (i.e., microcystins) the *mcy* gene cluster in PCR assays, applied directly to environmental samples, provide a useful indicator that mixed-species phytoplankton samples may have the genetic potential to produce microcystin. Although HPLC provides a direct measure of toxins present, it does require a large capital investment and considerable sample preparation. The PCR-based assays detect toxigenic cells rather than toxins and require little sample preparation and modest capital costs. Detection of toxic *Microcystis aeruginosa* strains through molecular markers for microcystin may have great use-potential in routine analysis of aquatic ecosystems.

Thus, it may make water monitoring more feasible and allow the early application of corrective action before cyanobacteria blooms start to die or disintegrate. The PCR-based assay is effective at a level of 10 cells/mL and can indicate a possible toxic bloom well before the cell count reaches the action alert at a cell density of 2 000 /mL, as recommended by the Australian Drinking Water Guideline (National Health and Medical Research Council/Agriculture and Resource Management Council of Australia and New Zealand 1996) and a high alert level of 20 000 cell/mL, where blooms may contain sufficient toxin to be of concern for human health (Fitzgerald *et al*. 1999).

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