A Review of Current Knowledge

Cyanobacterial Toxins (Cyanotoxins) in Water

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Cyanobacterial Toxins (Cyanotoxins) in Water

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CONTENTS

1. Introduction

Cyanobacteria, also termed "blue-green algae" are common, naturally-occurring microbial components of fresh-, marine- and brackish waters worldwide (Fogg *et al*., 1973). The term "blue-green algae" can be a misleading descriptor for mass populations of cyanobacteria in water: such accumulations of cells may range in colour from blue-green, to sky-blue, grass-green, khaki, chocolate brown to black, and when decaying in strong sunlight, to grey and white.

Cyanobacteria are ancient, photosynthetic bacteria, with a simple sub-cellular structure and lack a nucleus, consistent with their definition as bacteria. As ancient organisms, with fossil evidence dating back about 3.5 billion years, they are considered to be the original producers of the oxygen atmosphere which supports life on Earth today.

Cyanobacteria produce oxygen via the splitting of water in photosynthesis, the mechanism that is present today in algae and higher plants. In addition to their presence in water, cyanobacteria are also found in and on rocks, on soils and in extreme environments, including hot springs, deserts and in polar regions.

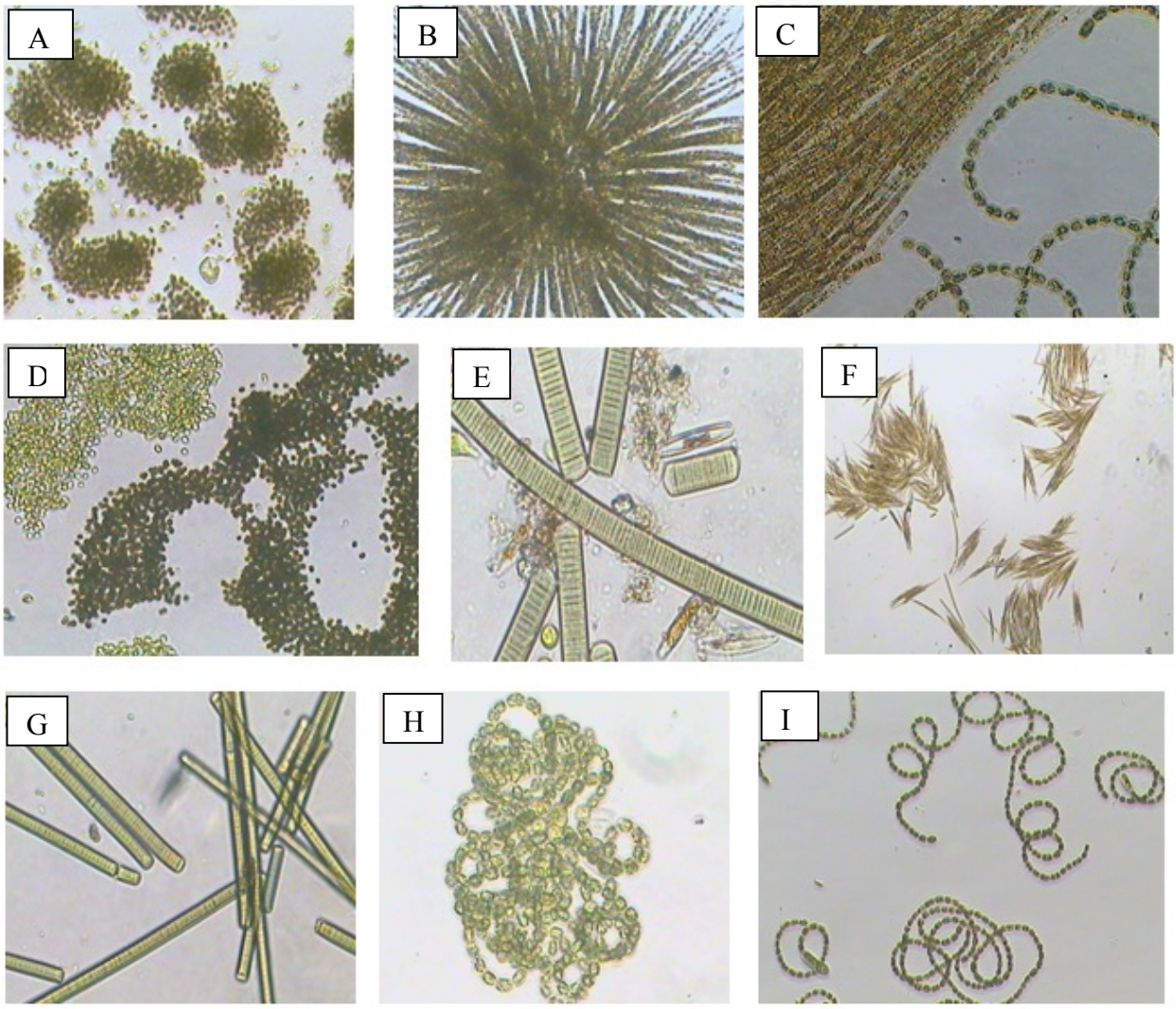
Microscopically, cyanobacteria can be observed as unicellular organisms or chains of cells and the individual cells are invisible to the naked eye (Fig. 1). Some unicellular cyanobacteria, such as *Microcystis*, can grow and exist as clumps (colonies) of identical cells. The colonies may be irregular in shape, bounded or not by a mucilaginous envelope and permeated by holes rather like a Swiss cheese. Such colonies can be several millimetres in diameter and thus readily visible to the naked eye.

Filamentous cyanobacteria can exist as single non-branched, or branched filaments, again not visible individually to the naked eye. However, some aquatic filamentous cyanobacteria such as *Aphanizomenon* and *Trichodesmium* can grow as bundles and be observed as macroscopic structures resembling greenish flakes of sawdust. The filaments of *Gloeotrichia* grow in a spherical radiating manner to produce balls, the size of pinheads. Whilst the filaments of some cyanobacteria, e.g. *Planktothrix* and *Phormidium* consist of apparently identical cells, other filamentous forms (e.g. of *Nostoc, Aphanizomenon, Cylindrospermopsis* and *Anabaena*) can be highly differentiated into structurally- and physiologically-specialised cells. These can include photosynthetically active vegetative cells, and specialised cells called heterocysts (or heterocytes). Heterocysts do not perform photosynthesis but fix atmospheric nitrogen (N_2) into ammonia and thence into organic nitrogen compounds which can then be used to support cyanobacterial

growth, negating the requirement to take up ammonia or nitrate for this purpose. A further adaptation in the differentiated filamentous cyanobacteria is the development of spore-like cells (akinetes). These cells, laden with nutrient reserves, allow the cyanobacteria to survive periods of environmental stress or starvation, and can then germinate again when conditions become favourable for growth.

Figure 1. Examples of freshwater cyanobacterial genera from UK freshwaters

A, *Microcystis*; B; *Gloeotrichia*; C, *Aphanizomenon* (left) with *Anabaena*; D, *Microcystis*; E, *Planktothrix*; F, *Aphanizomenon*; G, *Planktothrix*; H, *Anabaena*; I, *Anabaena*.



(courtesy of Dr. L.F. Meikleham).

Review of Current Knowledge

Cyanobacteria fulfill key roles in natural environments as part of the photosynthetic community of primary producers, forming the basis of aquatic food chains, and in the cycling of matter. However, the real issue with cyanobacteria, in terms of negative impacts on biodiversity, water quality and safety is when nutrient conditions in waterbodies become excessive (FWR, 2000). Eutrophic (high nutrient-containing) waterbodies allow the proliferation of algae and particularly cyanobacteria. Often in the Spring, diatoms will bloom before cyanobacteria, thriving at relatively low temperature and using excess available silicon compounds in the water to make their hard and porous cell walls (frustules).

However, as silicon availability becomes limiting, the diatom populations can decline and, with or without an intervening green algal bloom period, die off. Cyanobacteria without a high requirement for silicon can outcompete most other photosynthetic organisms to become the dominant phytoplankton in the water column, or in the populations of attached algae on sediments, rocks and water plants in shallow water. Under such conditions, cyanobacterial populations in open waterbodies can increase massively to form blooms in which the water is coloured and light penetration is greatly reduced, from perhaps metres to centimetres. Under calm conditions, many species of bloom-forming cyanobacteria can rise to the water surface, to form semi-solid scums. This vertical telescoping of the cyanobacterial population can result in at least a 100-fold concentration of cyanobacterial biomass in the surface layers. If, then, a gentle on-shore breeze occurs (less than about 4 metres per second), the surface scum may be further concentrated to form a thicker shoreline scum. The additional effect of horizontal concentration by gentle wind can result in an overall cyanobacterial concentration of at least 1000-fold above that of the original population dispersed in the open water column. Such scums commonly present aesthetic problems, engineering challenges if occurring at abstraction points for water treatment and supply, and acute health hazards to humans and animals. Attached, mat-forming cyanobacteria can also present health hazards since they can detach and, buoyed by trapped bubbles of oxygen, thereby float to the lake or reservoir surface and shoreline where they may also accumulate. Shoreline mats of cyanobacteria can also occur in slow-flowing rivers in the UK, for example. Cyanobacterial blooms, scums and mats can be aesthetically undesirable, largely due to the fact that waters are discoloured, turbidity is increased in recreational resources and offensive odours are generated from cyanobacterial accumulation and decay.

Cyanobacteria can produce a number of low molecular weight, earthy-, musty-smelling compounds including geosmin and 2-methylisoborneol. These do not present health hazards but can affect the quality of raw and treated drinking water

and lead to complaints from users of recreational waterbodies and treated drinking water-consumers (Juttner and Watson, 2007).

Concerning the potential of cyanobacteria to adversely affect water quality and safety of water with respect to animal and human health, their ability to produce a wide range of low molecular weight toxic compounds is of the greatest priority (Codd, 1995; Carmichael, 1997; Falconer, 1998; Sivonen & Jones, 1999 and Metcalf & Codd, 2012). Tests with such compounds have shown that they can be highly toxic to invertebrates and vertebrates, including mammals. Being colourless, tasteless and odour-free, these toxins, termed cyanobacterial toxins or cyanotoxins are largely unnoticed by the users of waterbodies and consumers of water. When compared with toxins produced by higher plants, fungi and animals, as examples, cyanotoxins are of similar, if not higher toxicity (Table 1). Some cyanotoxins, such as the saxitoxins and microcystins are considered chemical weapons and are covered by conventions, such as The Australia Group (www.australiagroup.net) which affect their production, purification, export and use for research and analysis in certain countries (Metcalf *et al.*, 2006).

Table 1. Comparative toxicity of some biological toxins administered via i.p. injection in rodents relative to cyanide.

Source	Toxin	Relative toxicity (× more toxic)
	Cyanide	1
Fungi	Phallotoxin	6
	Muscarin	9
	Amatoxin	17
Plants	Strychnine	20
	Curare	20
Cyanobacteria	Microcystins	13-200
	Nodularin	200-230
	Anatoxin-a	50
	Saxitoxin	1100
Animals	Cobra venom	500
	Tetrodotoxin	1250
	Poison Arrow Frog toxin	3700
Bacteria (non cyanobacteria)	Tetanus toxin	1×10^7
	Botulinum toxin	3×10^7

End point: mortality.

Of the many cyanobacterial genera known to occur in aquatic environments, only a limited number of colonial and filamentous forms are currently of human health concern. However, these are typically genera which can develop to produce blooms, scums and mats in waterbodies required for human use. Cyanobacteria, such as *Microcystis*, *Planktothrix, Cylindrospermopsis, Aphanizomenon* and *Anabaena* are known to produce an array of toxic compounds and examples are listed in Table 2. Of the toxins produced by cyanobacteria, as exemplified by the microcystins, these are largely retained within the cells of the cyanobacteria when the cells are healthy and intact and can be released when the cyanobacterial bloom/cells begin to break down and the cellular contents are released into the water. However, in the case of cylindrospermopsin, a large proportion of this toxin is typically found outside of the cell. If such water is to be used for human use and consumption, then the partitioning of cyanotoxins between the producer-cells and the surrounding water often can have important implications concerning water treatment. It is generally helpful in monitoring studies for risk management if the percentage distribution of cyanotoxins between the particulate (including intracellular) and dissolved fractions of water samples is determined, in addition to measuring the total cyanotoxin pool.

Table 2. Cyanobacteria known to produce the major classes of cyanotoxins (adapted from Metcalf & Codd, 2012).

Toxin	Published cyanotoxin- producers
Microcystins	*Microcystis* spp., *M. aeruginosa*, *M. viridis*, *Anabaena* sp., *Anabaena flos-aquae*, *A. subcylindrica*, *A. variabilis*, *Oscillatoria* (*Planktothrix*) *agardhii*, *Nostoc* sp., *Nostoc spongiaeforme*, *Anabaenopsis* sp., *Hapalosiphon* sp., *Gloeotrichia echinulata*, *Plectonema boryanum*, *Phormidium corium*, *Phormidium splendidum*, *Rivularia biasolettiana*, *R. haematites*, *Tolypothrix distorta*, *Arthrospira fusiformis*
Nodularins	*Nodularia spumigena*, *Nostoc sp.*
Anatoxin-a and homoanatoxin-a	*Arthrospira fusiformis*, *Anabaena* spp., *Aphanizomenon* sp., *Phormidium* sp., *Anabaena flos-aquae*, *Anabaena planktonica*, *Cylindrospermum* sp., *Oscillatoria* sp., *Raphidiopsis meditteranea*, *Phormidium formosum*
Anatoxin-a(*S*)	*Anabaena flos-aquae*, *Anabaena lemmermannii*
Saxitoxins	*Aphanizomenon flos-aquae*, *Anabaena circinalis*, *Lyngbya wollei*, *Cylindrospermopsis raciborskii*, *Planktothrix* sp.
Cylindrospermopsins	*Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum*, *Anabaena* sp., *Anabaena lapponica*, *Raphidiopsis curvata*, *Umezakia natans*

The cyanobacteria in Table 2 are capable of producing an extremely complex assortment of low molecular weight toxic compounds, sometimes with more than one class of toxin, or multiple examples from the same class of cyanotoxins, produced by an individual species or bloom. Mass populations of toxin-producing cyanobacteria are known to be produced worldwide, occurring in fresh, brackish and marine waters of various countries (e.g. Table 3). The production of these toxins by the cyanobacteria has been established as a result of the analysis of individual and contaminant-free laboratory cultures and correlated with cyanotoxin production in natural populations of cyanobacteria. In laboratory cultures, cyanotoxin production can result in high concentrations of the respective toxin, with concentrations of certain cyanotoxins accounting for at least 0.5% of the total dry weight of the culture.

Table 3: Geographic occurrence of toxic cyanobacterial blooms.

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

The use of the term "toxic" can have important implications with respect to the risk assessment of natural populations of cyanobacteria. The toxicity of natural populations of cyanobacteria can depend on many factors. These include, but are not limited to, the type of toxin, the dose (likely to be) received, the route of exposure and the body weight and health of the organism receiving the toxin. Certain cyanobacterial products, as in the case of microviridin J, are not toxic to mammals, but show high toxicity to aquatic invertebrates, including waterfleas, affecting their moulting. With respect to human consumption of drinking water, Guideline Values are based on the average consumption of water, often defined as 2 litres per day, which may change, depending on geographic location and the amount of physical exertion which may result in an increased consumption of water. Conversely, a small child would require much less of a dose of toxin to create a toxic effect. When cyanobacterial blooms are termed toxic, this is based on the assessment of the concentration of specific cyanotoxin(s) and the likely exposure route, in addition to known toxicities of the compound(s) present. However, without such risk assessment, cyanobacterial blooms may be labelled toxic, even though no risk assessment has been carried out. Furthermore, often when cyanobacterial blooms are present, water managers may adopt a precautionary principle and assume that the blooms are toxic, and advise that the water is avoided, not used, or that treatment measures are increased.

2. What toxic compounds are produced by cyanobacteria?

Although cyanobacteria have the potential to produce a wide variety of compounds showing toxicity, in general the cyanotoxins are divided into classes based on their primary modes of toxic action in mammalian systems. Although many such compounds are already recognised, scientific research into the toxicity of cyanobacteria is an ongoing endeavour, and with this intensive research, further compounds with adverse biological activities in a range of toxicity-based systems, involving mammals, fish, aquatic invertebrates, cultured animal cells and enzymes are being identified. An understanding of the complete range of cyanotoxins, of their occurrence and health significance, certainly with respect to aquatic life, is incomplete.

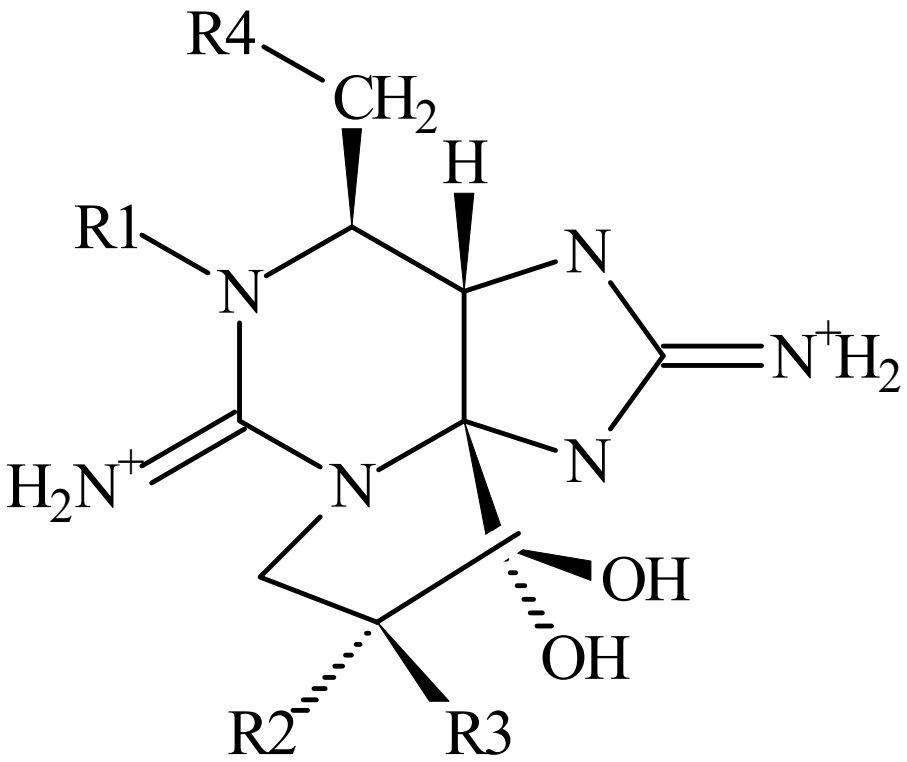
A further issue, with respect to cyanotoxins is the distinction between short- (acute) and long-term (chronic) toxicity. With most of the recognised cyanotoxins, much of the concern is with consequences of short-term exposure, as many of these compounds are of high toxicity. However, of increasing concern is the likelihood that repeated exposure to certain cyanotoxins, including microcystins, may affect long-term health. Further research will help to elucidate the potential

risk of repeated exposure to low, environmentally-relevant concentrations of cyanotoxins.

2.1 Neurotoxins

Of the cyanobacterial neurotoxins, one of the most acutely toxic classes is that of the saxitoxins, potent carbamate alkaloids (Fig 2). They are more commonly known as products of marine dinoflagellates, common components of red tides and are responsible for Paralytic Shellfish Poisoning (PSP). The saxitoxins comprise a group of over 20 structurally related compounds. Similar to pufferfish toxin, saxitoxins act by blocking sodium channels in nerves, leading to blocking of the neuron, with the end result that the nerve is unable to fire which can lead to paralysis, and in high doses, death.

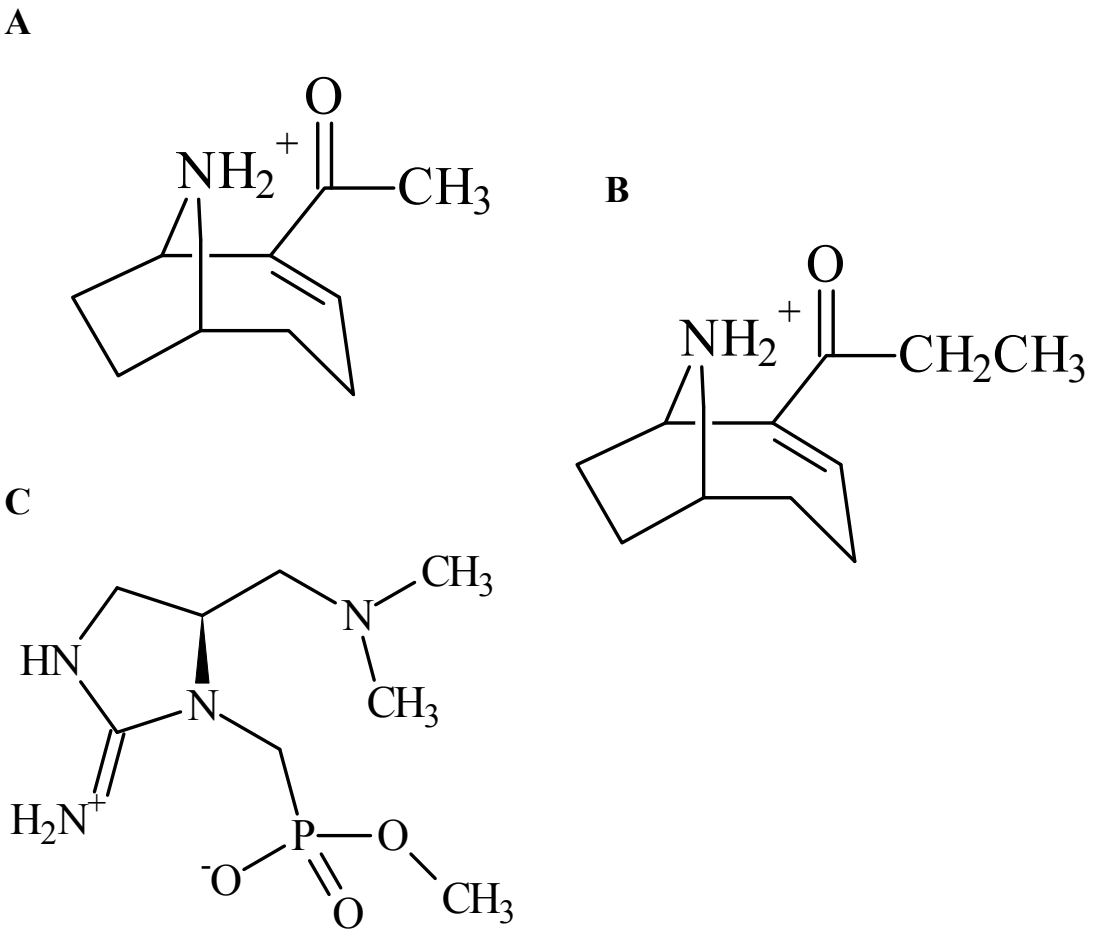
Figure 2. General structure of the saxitoxins with variable groups (R) shown.



A second class of cyanotoxins is named after anatoxin-a, the first toxin to be characterised from *Anabaena*. Anatoxin-a, unlike the saxitoxins, is an alkaloid secondary amine and is only known to be produced by cyanobacteria. Its neurotoxicity is due to its ability to mimic acetylcholine, a common mammalian

neurotransmitter. Consequently, anatoxin-a is able to bind to the acetylcholine receptor where the machinery of the cell is unable to remove it. As a result, the nerve continues to fire until it becomes exhausted and stops working. In high enough doses, anatoxin-a and related compounds can result in death. Although anatoxin-a and homoanatoxin-a (Fig. 3) are among the most commonly-occurring anatoxins known, currently five structurally-related compounds of anatoxin-a and congeners have been characterised, although some are considered to be degradation products of the parent toxin(s).

Figure 3. The cyanobacterial neurotoxins, anatoxin-a (A), homoanatoxin-a (B) and anatoxin-a(*S*) (C)



Anatoxin-a(*S*), not frequently found in cyanobacteria and so far only characterised in *Anabaena*, is a potent organophosphate toxin (Fig. 3). Similar to synthetic organophosphorus insecticides and pesticides, this natural product has the same toxic action as its synthetic counterparts. Toxicity occurs through inactivating the enzyme system (acetylcholine esterase) which removes acetylcholine from the

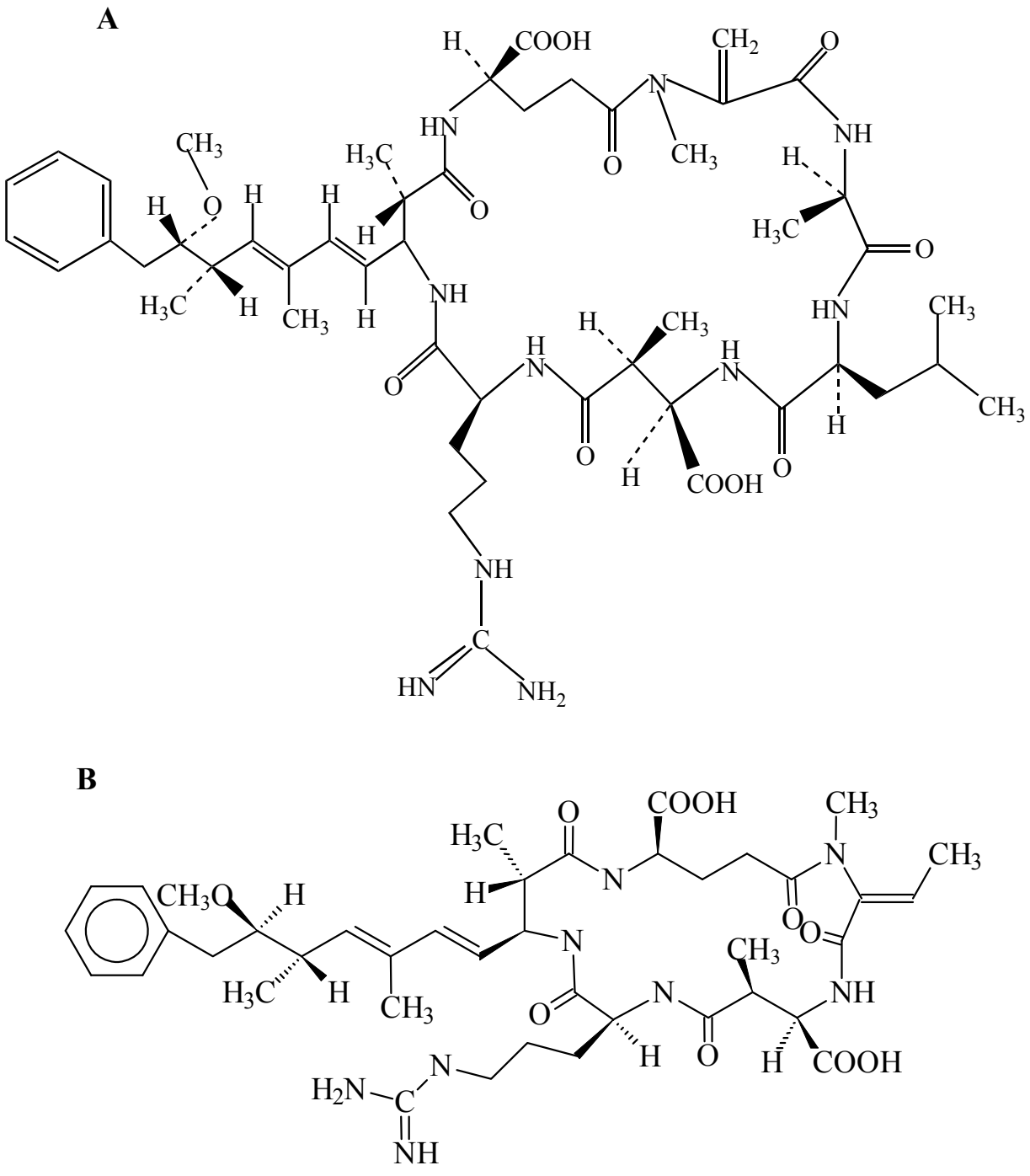
neuron, allowing it to continue to fire. Consequently, the neuron becomes exhausted and stops working, and ultimately in high doses, potentially leading to paralysis and death. The suffix (*S*) indicates that, similar to synthetic organophosphate poisonings, salivation and lachrymation (tears) are common visual symptoms of anatoxin-a(*S*) intoxication in mammals. So far, no further structural variants of anatoxin-a(*S*) are known.

2.2 Hepatotoxins

The most commonly reported potent cyanotoxins in freshwaters are members of the cyanobacterial hepatotoxins. Of these, the most extensively studied and detected are the microcystins (Fig. 4). These are cyclic, 7 amino acid-containing peptides including the novel amino acid, Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). Over 200 structural variants of the microcystins have been identified due to determination of minor modifications to the peptide ring, of which thousands are theoretically possible. The naming of individual microcystins is performed through identification of amino acid substitutions at positions 2 and 4 of the cyclic peptide ring and using the one-letter abbreviations for these two amino acids as a suffix. For example, one of the most commonly-occurring and most toxic microcystins is microcystin-LR, which contains L-leucine (L) and L-arginine (R) at positions 2 and 4 of the cyclic structure, respectively. In high enough doses, microcystins cause irreversible damage to the mammalian liver. After rapid uptake into the liver cells, microcystins cause a contraction of their internal cytoskeleton causing the liver cells and the cells of the liver's component blood vessels to separate, such that blood arriving in the liver becomes trapped. Death due to acute microcystin poisoning is due to pooling of the blood in the liver, with the mammalian organ doubling in weight, such that hypovolaemic shock, or a lack of blood supply to the other organs results.

At the molecular level, the microcystins are potent and irreversible inhibitors of protein phosphatases and phosphoprotein phosphatases, including PP1, PP2A, PPP4 and PPP5 (Hastie, *et al*., 2005)**.** These enzymes are among the most highly conserved in the living world and regulate many vital activities, including developmental, energetic and physiological processes in plants and animals, including humans. Methyldehydroalanine, one of the amino acids in the microcystin ring, is able to bind to the protein phosphatases, deactivating the enzyme. Additional harm to animal cells and whole animals by microcystins includes: kidney damage, male reproductive cell damage, cognitive dysfunction and tumour promotion in the development of primary liver cancer (Metcalf & Codd, 2012; Wang, L. *et al.,* 2013; Wang, X. *et al*., 2013; Wang, J. *et al.,* 2013).

Figure 4. Examples of microcystin and nodularin cyanobacterial hepatotoxins; microcystin-LR (A) and nodularin (B).

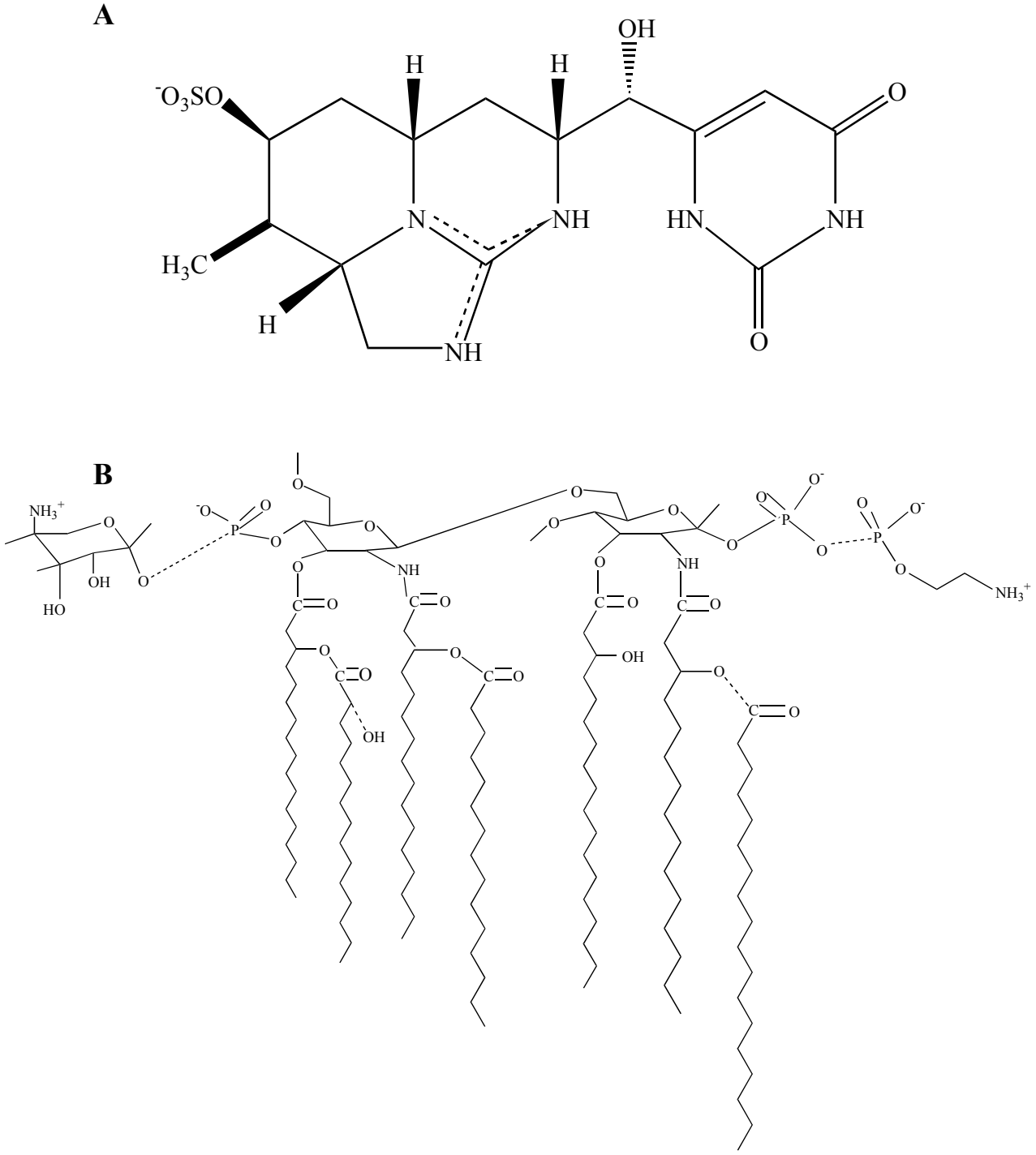


The nodularins (Fig. 4), like microcystins, are cyclic peptides containing the Adda moiety. Nodularins are also cyclic peptides but are composed of five amino acids. Although multiple cyanobacterial genera have been documented as producing microcystins, nodularins have only been identified in members of two genera: in the brackish water species, *Nodularia spumigena* and in a *Nostoc* which lives

symbiotically with a tree fern. Whilst also hepatotoxic like microcystins, and with the ability to inhibit protein phosphatases, nodularins are unable to chemically attach to these enzymes as they lack the methyldehydroalanine moiety. This ability to inhibit, but not attach to the enzyme, results in different potential toxicity outcomes during long-term exposure. Whilst microcystins are tumour promoters but not primary carcinogens, nodularins are considered to be both tumour promoters and carcinogens, as demonstrated in laboratory animals. Once tumour development has been initiated by a carcinogen, both microcystins and nodularins are able to serve as tumour promoters, stimulating tumour growth. In large doses, nodularins act like microcystins, with the end result of death principally from haemorrhage into the liver.

Not all cyanobacterial hepatotoxins are peptides: cylindrospermopsin (Fig. 5), its toxic stereoisomer 7-epicylindrospermopsin**,** and the non-toxic relative deoxy-cylindrospermopsin, are guanidine alkaloid toxins. Following a human poisoning incident in Australia in 1979, linked to a cyanobacterial bloom in a drinking water reservoir, a culture of the cyanobacterium *Cylindrospermopsis raciborskii* was isolated. Toxicity testing with this culture led to the discovery of cylindrospermopsin. The toxic action of cylindrospermopsin is slower than that of microcystins, or the anatoxins or saxitoxins (days *versus* hours or minutes), and although the liver is considered to be the main target organ affected, this hepatotoxin is also able to affect a wide variety of organs and tissues. At the cellular level, cylindrospermopsin inhibits protein synthesis and, based on its chemical and physical structure, it may be a carcinogen. Further actions of cylindrospermopsin of health significance include dermatotoxicity and genotoxicity, including the breakage of double-stranded DNA (de la Cruz *et al*., 2013). Although considered earlier to be a cyanotoxin of tropical/subtropical latitudes, cylindrospermopsin production is now reported to occur more widely and increasingly in European freshwaters.

Figure 5. The cyanobacterial hepatotoxin cylindrospermopsin (A) and the lipid A region of lipopolysaccharide endotoxin (B).



2.3 Lipopolysaccharides

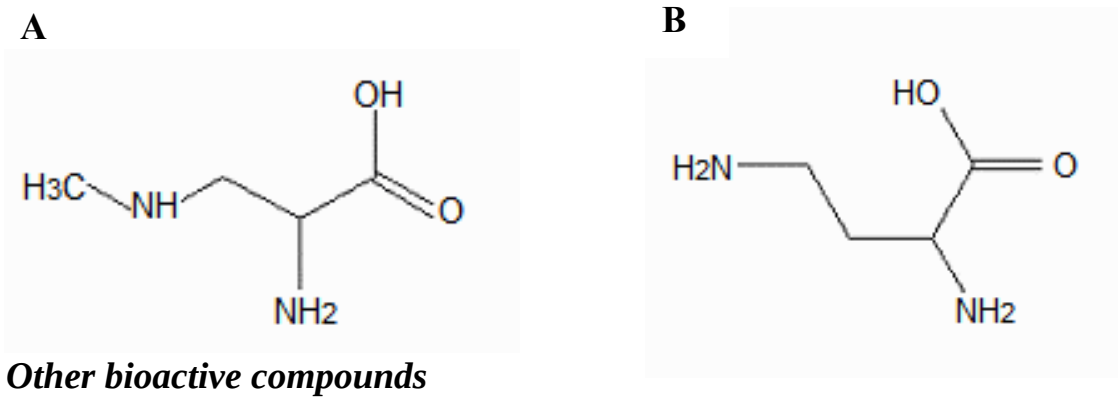
A standard feature of Gram negative bacteria, including coliforms, is the potential to produce lipopolysaccharide toxins (LPS). As Gram negative bacteria themselves, cyanobacteria also produce LPS as a standard component of their outer

cell wall. LPS consists of three main parts: O antigens, core polysaccharides and lipid A moieties. Bacterial LPS is heat-stable and shows toxicity to mammals. The lipid A region (Fig. 5) is responsible for the toxic response in mammals, namely fever, diarrhoea, vomiting and hypotension. After LPS exposure and ingestion, pyrogenic compounds within the body are released resulting in fever. Although it is difficult to distinguish bacterial from cyanobacterial LPS exposure (and indeed both may occur simultaneously), cyanobacterial LPS has been implicated in a number of human illness outbreaks linked to exposure to water. Purified cyanobacterial LPS is considered to be only about 10% as potent in fever generation as that of pathogenic coliform LPS. However, cyanobacterial LPS can be highly abundant in cyanobacterial blooms and scums, accounting, for example for about 0.5% of total cyanobacterial dry weight (Bláhová *et al*., 2013). This can result in high LPS toxin concentrations in raw water with risks to recreational activities.

2.4 Neurotoxic amino acids

Like plants, algae and fungi, cyanobacteria can produce a range of amino acids, different from the 20 standard amino acids that comprise proteins. One amino acid in particular, β-*N*-methylamino-L-alanine (BMAA, Fig. 6) discovered in the 1960s in ancient tree-like ferns called cycads which bear symbiotic cyanobacteria, has been shown to be produced by free-living cyanobacteria. Experiments performed *in vitro* and *in vivo* have shown that this amino acid is neurotoxic (Weiss *et al*., 1989). The ingestion of BMAA has been associated with the human neurological disease amyotrophic lateral sclerosis (ALS), also known as motor neuron disease, and the possibility of causality is under investigation (Metcalf & Codd, 2009). BMAA production appears to be widespread among cyanobacteria as evidenced by laboratory studies with pure, bacteria-free cyanobacterial cultures and with environmental samples from freshwater, brackish, marine and terrestrial sources. BMAA is acutely toxic to a range of aquatic organisms in laboratory exposures: to protozoa, brine shrimp and fish larvae, at environmentally-relevant concentrations (Purdie *et al*., 2009) and the impact of BMAA in natural and controlled waterbodies requires investigation. A second neurotoxic amino acid, 2,4-diaminobutyric acid (DAB, Fig. 6), with the same molecular weight and elemental composition as BMAA, but with a different structural arrangement, has also been shown to be present in cyanobacteria. Although not confined to cyanobacteria, DAB is often present when BMAA occurs in cyanobacteria.

Figure 6. The neurotoxic amino acids β-N-methylamino-L-alanine (BMAA, A) and L-2,4-diaminobutyric acid (DAB, B).



2.5 Other bioactive compounds

Cyanobacteria have the potential to produce a wide range of further compounds with biological activity. Such activity can range from the inhibition of enzymes through to toxic effects including skin and gastrointestinal irritation. Some marine cyanotoxins, including aplysiatoxin and lyngbyatoxins are skin irritants and tumour promoters due to their inhibition of protein kinase enzymes. Other bioactive compounds produced by cyanobacteria, such as the microviridins are not overtly toxic to mammals. However, they are toxic to aquatic invertebrates in that they prevent them from moulting (Rohrlack *et al*., 2004). Further bioactive products of cyanobacteria include anabaenopeptins, cyanobacterins, fischerellins and nostocyclamides. Further research into the production of such compounds, produced by large synthetase and synthase enzymes in the cyanobacterial cell is likely to lead to the identification and discovery of further cyanobacterial compounds with toxic potential. For example, new and emerging cyanotoxins include the production of retinoic acid-like compounds from cyanobacteria with the potential to be teratogenic.

3. How are cyanotoxins detected and quantified?

In order to provide accurate and timely data concerning the types, concentration and location of cyanotoxins, appropriate analytical methods are required. These are necessary to: (i) provide an understanding of the occurrence and abundance of cyanotoxins; (ii) investigate the potential toxicity of cyanobacterial populations during waterborne health incidents; (iii) provide data for the appropriate risk assessment and effective risk management of waterbodies required for human use and where cyanobacteria occur. The derivation of guideline values for acceptable cyanotoxin concentrations in water intended for human use has been and continues to be influenced by knowledge of cyanotoxin toxicity, case studies involving human and animal intoxications and mortalities, and the health risks associated

with short- and long-term exposure to the cyanotoxins. As a greater understanding of the potential health risks associated with long-term exposure to low concentrations of cyanotoxins develops, so guideline values, or maximum admissible concentrations, may be revised downwards. Many of the analytical methods for cyanotoxins currently in use have been developed to ensure that they are capable of detecting and quantifying cyanotoxins at and below the emerging health guidelines and this requirement is likely to continue. In addition, requirements for specificity, accuracy, precision, repeatability, linearity and assay range should be amenable to testing and approval via intra- and inter-laboratory validation. Ideally, more than one analytical method should be employed to determine the presence and concentration(s) of cyanotoxins, with, for example one method being based on physical and chemical principles and the complementary method being biologically- or toxicologically-based. Such methods should also be capable of providing warning of the potential to produce or respond to changes in the concentration of the cyanotoxin(s), so that appropriate avoidance or remediation measures can be implemented. Due to the nature of cyanotoxin detection, the development and validation of analytical methods continue to be intensive research topics to contribute to the protection of human and animal health.

Analytical methods are largely divided into groups, depending on the scientific basis of the technique and the information generated by the method. Techniques, involving complex analytical equipment, including liquid or gas chromatography, spectroscopy and mass spectrometry are defined as physicochemical techniques. Analytical methods with a biological basis, such as organism-based bioassays or those using antibodies or enzymes to measure inhibition, are a second group of methods and finally, those which measure the potential for cyanotoxin production, usually by detecting and quantifying essential genes involved in their biosynthesis. In addition to the many analytical methods for the detection and quantification of the toxins and their genes, methods of sample extraction and preparation are necessary and these may require modification. For example, with physicochemical methods, appropriate organic solvents used for cyanotoxin extraction or concentration should allow good resolution of the toxins of interest, whereas with enzyme inhibition and immunological assays, aqueous extracts, or the presence of low solvent concentrations should permit detection of the toxin without interfering with the analytical test. Other, less sensitive methods may require concentration of samples so that an adequate concentration of cyanotoxin can be presented for measurement.

3.1 Physicochemical methods

For the most commonly measured cyanotoxins, e.g. microcystins, nodularins, anatoxins, cylindrospermopsins and saxitoxins, chromatographic methods are available which allow their separation from other compounds with good resolution. Often such methods use liquid chromatography (LC) and this has largely been chosen over methods employing gas chromatography. Although LC methods have been used for cyanotoxins since the 1980s and the basis of these methods has not changed, periodic improvements to their performance and to the components that they use, such as column technologies, have continued apace.

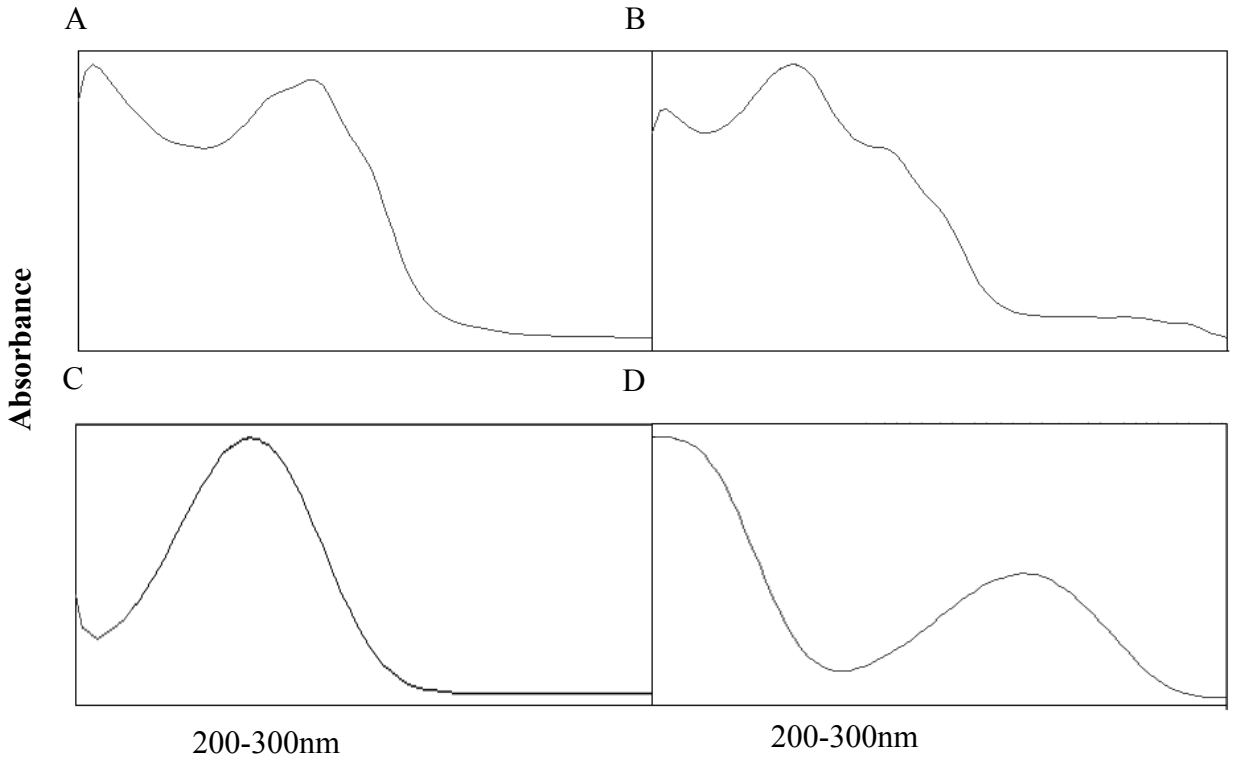
With the majority of cyanotoxins being analysed by high performance liquid chromatography (HPLC), run times of about 45 minutes to 1 hour have been reported. One change to LC is the introduction of ultra-high performance liquid chromatography (UHPLC or UPLC; Spoof *et al*., 2009). This allows LC methods to be run at approximately 5 to 10 times higher pressure than with conventional HPLC. With specially designed columns and equipment, run times with UPLC can be 10 minutes or less, allowing for the analysis of a much greater number of samples, and also potentially allowing for more rapid reporting and risk assessment. The majority of the LC methods for cyanotoxins employ "reversed phase" HPLC, employing a gradient from aqueous- to organic solvent conditions, often with C18-silica stationary phases (chromatography columns).

A recent advance called Hydrophilic Interaction Liquid Chromatography (HILIC) is now being applied to some cyanotoxins which may have poor retention on conventional C18 phases. However, with the new HILIC systems, samples are run from organic to aqueous phase (normal phase), allowing resolution of cyanotoxins such as anatoxin-a(*S*) which have previously been difficult to measure (Dörr *et al*., 2010).

Although the LC system is an essential component of the analytical machinery, without an appropriate detector system the cyanotoxins cannot be measured. The initial detection of cyanotoxins used ultraviolet (UV) detection, which when calibrated against a known standard cyanotoxin was very useful for the assessment of cyanotoxins which have good UV-absorbance characteristics. Indeed, UV absorbance of purified cyanotoxins is the most accurate method of measuring cyanotoxin concentration in solution when the molar extinction coefficient is known. Furthermore, for the calibration of all analytical machinery, spectroscopically-defined solutions of cyanotoxins are the most accurate standards for use. Although UV detectors are good for known defined cyanotoxins, as with many of the cyanotoxins, a number of congeners for each class, as in the

microcystins exist. In addition, the potential for interference by other compounds may affect the subsequent determined cyanotoxin concentration. As a result of this, photodiode array (PDA) detectors have been widely applied, as certain cyanotoxins, such as microcystins, anatoxin-a and cylindrospermopsin, have good UV spectra (Fig. 7). Using PDA, a putative cyanotoxin peak is scanned between known wavelengths (e.g. 200-300nm) after detection at the lambda max (λ_{max}) wavelength, and the spectrum can be compared to that of known cyanotoxin standards. Consequently, using PDA detectors, unknown cyanotoxin structural variants or congeners within a toxin class can be identified as members of that particular cyanotoxin family.

Figure 7. Examples of UV spectra of cyanotoxins analysed by HPLC with diode array detection. A, microcystin-LR; B, microcystin-WR; C, anatoxin-a; D, cylindrospermopsin.



Even when cyanotoxins cannot be detected by UV absorbance, due to their having no chromaphore or specific absorbance characteristics, LC methods can still be employed for their detection. In most cases, detection is through the use of fluorescence. For example, saxitoxins and BMAA cannot be observed by conventional UV detectors, but through chemical modification with fluorescent tags, they can be changed into a different, but unique compound which has added, inbuilt fluorescence. Furthermore, often when analysing compounds using

fluorescence detection, because of the fluorescent tag, there is an increase in sensitivity when compared to UV detection. Although compounds such as BMAA can be modified using commercially available kits for fluorescent labelling, with saxitoxins LC-fluorescence detection is traditionally achieved through oxidation of the compound making a fluorescent derivative, either pre- or post- analytical column, with the potential to subsequently resolve the 20-plus currently known saxitoxin variants. Other physicochemical methods such as electrochemical techniques with cyclic voltammetry and capillary electrophoresis also allow spectroscopic detection of cyanotoxins.

Of the physicochemical methods used in recent years for cyanotoxin detection, mass spectrometry (MS) has become the method of choice. This is largely due to the fact that MS methods are sensitive, specific, and in the case of analysing for cyanotoxin classes, MS has the potential to largely identify the specific variant of interest. With the exception of LPS, all of the known cyanotoxins can be detected and identified by MS. Further advances in the MS of cyanotoxins are through the application of triple quadrupole analysis. With this method, the mass spectrometer is able to isolate compounds of a known mass, detect them and measure fragments (daughter ions) of the compounds to confirm the presence of the parent cyanotoxin. For example, with microcystins and nodularins, although the parent ions have masses of between 800 and 1200 Da, during triple quadrupole MS, a daughter ion of m/z 135 is produced from the Adda moiety of these toxins and this daughter ion, in comparison with the parent ion, can be used as a diagnostic marker ion. Triple quadrupole MS has also been successfully applied to the detection of very difficult–to-analyse cyanotoxins, such as anatoxin-a(*S*) where the retention time, parent ion and ratio of daughter ions can allow for accurate quantification and detection of this toxin (Dörr *et al*., 2010).

Although MS can be used to measure the free parent ion, occasionally the toxin molecule may not be in the free form but bound to a cellular target. In the case of microcystins, the toxin molecule can be bound to protein phosphatases and other proteins. When this association is covalent (tightly bound) and the microcystin is chemically linked to the target, an oxidation product of Adda, erythro-2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) can be produced. Once created, this product can be analysed by liquid chromatography- fluorescence or gas chromatography- flame ionisation detection.

Commonly, MS determination of cyanotoxins generally examines one particular class of compound, due to the mass spectrometer requiring different settings for each toxin class. However, MS methods such as matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) and surface enhanced laser desorption

ionisation-time of flight (SELDI-TOF) MS can accommodate whole colonies and filaments of cyanobacteria, with the potential to analyse a wide variety of bioactive compounds from the material. Although with some cyanotoxins, such techniques do not provide a complete analysis, MALDI-TOF and SELDI-TOF are able to provide a chemical fingerprint of cyanobacterial blooms. Although not quantitative, data from such analyses have generated peptide toxin mass libraries which can be useful for cyanotoxin identification.

3.2 Bioassays, enzyme-, antibody- and molecular biological methods

The existence of cyanotoxins was originally inferred from cases of intoxication that occurred in wild animals, birds and domestic livestock. As a logical development, the original isolation and to some extent, quantification of cyanotoxins in cyanobacterial bloom material originally employed animal bioassays. The majority of these assessments were performed using the mouse bioassay. This has been extremely important in the initial work in purifying and characterising the toxins and is still specified in EC legislation concerning the permissible concentration of saxitoxins in shellfish intended for human consumption. However, due to intensive research into the toxicity, targets and fates of cyanotoxins, a large amount of molecular-based knowledge on their modes of action is now available. Bioassays using whole organisms are still used for the assessment of cyanotoxins, although mice have been increasingly replaced by aquatic invertebrates (e.g. brine shrimp, water fleas), plants (e.g. mustard seed germination and development) and microbial bioassays using protozoa. This move from mammalian bioassays has been partly due to ethical considerations. Although the relevance of the findings to mammalian susceptibility can be thereby reduced, such bioassay alternatives can increase detection sensitivity and statistical power employing larger numbers of test organisms when assessing the toxicity of cyanobacterial environmental material. Bioassays are useful, although they have advantages and disadvantages. For example, when assessing the toxicity of unknown material, although the organism can respond to a number of (toxic) factors present in the material, an accurate assessment of the toxicity of the sample is possible. However, this toxic response may be relatively non-specific in that the outcome is usually death with little or no indication of causation or of the toxic principle(s) involved.

As a result, whole organism bioassays have to some extent been replaced by more specific *in vitro* assays. These largely employ specific enzymes such as protein phosphatases and acetylcholine esterases to detect microcystins and anatoxin-a(*S*), respectively. Other systems may employ protein binding as in the saxiphilin assay or the acetylcholine-binding assay for saxitoxins and anatoxin-a, respectively.

Further biological methods involve more generic techniques: for the bioassay of cylindrospermopsin, as this toxin inhibits protein biosynthesis, protein translation assays can be used successfully.

In addition to the use of biochemical and enzymic responses, antibodies have been generated against a number of cyanotoxins and successfully applied to their detection and quantification (Metcalf & Codd, 2003). The major hurdle to producing antibodies against cyanotoxins is their small size. When presented to a mammalian immune system, as the majority of cyanotoxins are small molecules they go unrecognised and do not promote antibody formation. Consequently, to produce antibodies against such small molecules (haptens), it is necessary to chemically link the toxins to large proteins. This reduces the toxicity of the bound cyanotoxin and also produces a complex which is recognised by the immune system. As a result, a pool of antibodies will be produced against the entire complex, with a proportion recognising the cyanotoxin molecule. Although not always necessary, the toxin-specific antibodies can be purified from the general pool of antibodies for further use and application. Cyanotoxin antibodies have been produced against microcystins, saxitoxins and nodularins as examples, either for their use in research or for environmental monitoring, including water resources.

Once produced, antibodies can be used for a wide range of purposes. The main application has been in the production of immunoassays or enzyme-linked immunoassays (ELISAs), often based upon the standard 96-well plastic plate, where the concentration of the cyanotoxin can be compared with known standards to determine its concentration in the sample. Although plate-based assays have proven very useful for the determination of a wide range of cyanotoxins and have been produced as commercial kits, they suffer from the need to be carried out in a laboratory. More recent developments have included the production of "pregnancy kit"-style lateral flow immunoassays for cyanotoxins (Metcalf & Codd, 2012 and Tippkötter *et al*., 2009). Although not as accurate as plate-based assays, they are semi-quantitative, can be formatted with a minimum limit of detection below cyanotoxin guideline values for drinking water, and can thereby provide an initial estimate of cyanotoxin concentration. However, their real advantage is that they can be performed in the field next to a waterbody, or in the water treatment plant, allowing rapid and straightforward cyanotoxin detection.

There are many ways to produce antibodies against cyanotoxins and one major issue is the difference in reactivity between different congeners or variants within a toxin class. With microcystins, for example, there are over 200 different structural variants known of widely different toxicities. Structural variations give rise to differences in cross-reactivity between cyanotoxin variants and antibodies which

have invariably been raised against a particular single variant to date. Therefore, the testing of antibodies versus different toxin variants is an essential requirement for evaluating their performance including their applicability for use in the field where multiple cyanotoxin variants can occur (Metcalf *et al*., 2000 and Young *et al*., 2006). Microcystin immunoassay formats are generally aimed to permit microcystin detection at below the provisional guideline value of 1µg/l for microcystin-LR in drinking water derived by the World Health Organisation.

Although immunoassays using traditional polyclonal and monoclonal antibodies are well established, new techniques which may one day replace antibodies are being developed. These range from naïve phage display libraries and molecularly imprinted polymers, through to DNA-binding systems termed aptamers which can be rapidly produced using polymerase chain reaction (PCR) techniques.

Cyanobacterial LPS can be quantified by haemagglutination assays. The most common method is the *Limulus* amoebocyte lysate (LAL) assay (Anderson *et al*., 2002 and Rapala *et al*., 2002). Although not specific for LPS, this works on the principle that aqueous extracts from blood cells of the Horseshoe Crab *Limulus polyphemus* will react with the lipid component of LPS, resulting in gel clotting. The method originally determined turbidity to measure LPS concentration, to which this is proportional. Now a range of newly developed systems, such as the use of chromogenic substrates, is allowing more accurate LPS quantification. One of the challenges to the risk management of LPS problems associated with cyanobacterial blooms is to determine the relative contributions of the cyanobacterial LPS, versus that of the co-occurring bacteria, to overall LPS toxicity.

An approach to determining the potential of a cyanobacterial population to produce cyanotoxins is possible via the detection and quantification of key genes responsible for cyanotoxin production. Although not a quantitative system for measuring the toxins themselves, the genes can be recovered from single filaments and colonies of cyanobacteria in water samples, and amplified quantitatively by the polymerase chain reaction (PCR) (Dittmann *et al*., 2013). Furthermore, using PCR and/or ELISA, when a mixed bloom of cyanobacteria is present, the individual genera or species can be isolated by micromanipulation and each then measured for cyanotoxin production potential and/or actual cyanotoxin content. This can contribute to risk assessment as the “toxic” genera or species can be monitored to determine whether their abundance or percentage of the bloom for which they account is increasing or decreasing.

Specific gene clusters for a number of cyanotoxin classes have been determined, including microcystins, cylindrospermopsins, saxitoxins and anatoxin-a. Such gene clusters are large, as although the toxins are small molecules, they are not single gene products but are synthesised by multi-enzyme peptide synthetases and polyketide synthases, encoded by multiple genes in the cyanobacterium. Although genetic methods can provide valuable information, they are not a replacement for cyanotoxin analytical methods as they only provide an indication of the potential to produce cyanotoxins, and even when the transcription of the genes is known to be occurring within the cyanobacterial cells, translating such information to a toxin concentration per cell is problematic.

3.3 Concentration of samples for cyanotoxin analysis

Although some analytical methods can detect cyanotoxins from naturally-occurring cyanobacterial material and water samples, sample concentration may need to be carried out when the concentration of cyanobacteria is low or specific information regarding the types and quantities of cyanotoxin variants is required. Standard glass fibre filters have been successfully used to partition the cellular material from water, with material from litres of water being concentrated on a disk, allowing subsequent extraction of the material into a small volume for cyanotoxin analysis after lyophilisation. The inherent property of some cyanobacteria which produce internal, buoyancy-conferring gas vesicles can be used to quantitatively concentrate bloom samples. If the sample containing buoyant cells is allowed to stand in an illuminated glass vessel, the cyanobacteria will float to the surface from where they can be removed as a layer for microscopy and cyanotoxin analysis. Alternatively, the gas vesicles can be collapsed by a simple pressure shock to the sample, allowing the cells to be quantitatively concentrated as a pellet by bench centrifugation for microscopy and cyanotoxin analysis. Microscopic examination of the cyanobacteria in environmental samples and during sample concentration and preparation for analysis is an important requirement since:

(i) cyanotoxin partitioning between producer-cells and the surrounding water varies. Some indication is needed of whether the cell population consists of intact cells (when most of the total cyanotoxin pool, except for cylindrospermopsins, can be expected to be intracellular) or whether the cell population is rupturing with likely release of cyanotoxin into the water;

(ii) identification of the cyanobacteria to genus or species level can provide an initial pointer as to which cyanotoxins may potentially be present in the sample.

Reliance should not be made, however, on microscopy alone to indicate actual cyanotoxin presence.

It is particularly necessary to determine cyanotoxins in the water phase (the extracellular or soluble cyanotoxin fraction) in waterbodies, and in water treatment trains, if there is a recent history of cyanobacterial cells in the waterbody, even though the cells may no longer be apparent. Extracellular cyanotoxins, although susceptible to natural removal processes in a waterbody (bio- and photo-degradation, sorption) can persist for some weeks. Furthermore, extracellular cyanotoxins, if present, are potentially capable of passing through the standard filter processes designed to remove particulate matter, including cyanobacteria, during drinking water treatment.

In most cases, analysis of the extracellular cyanotoxin fraction is carried out by solid phase extraction (SPE), using commercially available cartridges containing different solid phases which, based on the chemistry of the cyanotoxin, will interact with the phase and stick. Once all of the water sample has been passed through the cartridge, with litre volumes being not uncommon, the cartridge can then be washed and the cyanotoxin(s) eluted, usually using an organic solvent, releasing and concentrating the cyanotoxin(s) potentially present for analysis. SPE methods have been developed for a number of cyanotoxins, including microcystins using C18 cartridges, cylindrospermopsin using graphitised carbon, and BMAA using cation exchange or mixed mode cartridges. Such methods, especially when combined with detection methods can be validated, adopted and published, such as with the British "Blue Book" method and the International Organisation for Standardisation (ISO), examining methods for microcystins.

Although SPE methods allow the concentration of cyanotoxins from water, they are not specific as other small molecules, such as pesticides and amino acids may also stick to the cartridge. Consequently, methods have been used to attach cyanotoxin-specific antibodies to the cartridge, creating immunoaffinity columns which, in the case of microcystins, have proven useful for the retention of a range of microcystin variants. Further research to produce cyanotoxin-specific cartridges is likely to occur and provide straightforward methods for toxin recovery from water.

3.4 Extraction methods

After collecting cyanobacterial samples for cyanotoxin analysis, appropriate methods are required to extract the toxins from the cells. With most cyanotoxins, including microcystins, this is usually achieved with organic solvents such as

methanol. However, some cyanotoxins, such as anatoxin-a can be quantitatively extracted from cyanobacterial cells using water. With extraction methods, attention should be given to the compatability of the solvents used with the subsequent analytical method. With physicochemical methods, organic solvents are usually fully compatible with the analytical equipment. However, high concentrations of organic solvents are usually inhibitory in bioassays and can cause false positives in immunoassays. They should thus be avoided or diluted out in the subsequent assays.

Some extraction methods use acids, such as for saxitoxins and BMAA. BMAA appears to exist in at least 2 pools in the cyanobacterial producer-cells and in the cells of organisms which have been exposed to and have sequestered BMAA in their tissues. These are a readily-extractable pool of soluble BMAA and a pool of BMAA which is associated with proteins. The protein-associated BMAA is typically sedimented by the centrifugation of extracts of cyanobacterial cells or other organic matrices, or retained during filtration. This particulate BMAA pool, which may range from a minor to the major fraction in cells, requires prolonged hydrolysis with 6M HCl to release the toxin for analysis.

4. Cyanotoxins as hazards to health

4.1 Human and animal poisoning episodes: actual and potential

Associations between cyanobacterial scums and blooms, and the deaths of wild and domestic animals and human illnesses at waterbodies, were made by indigenous peoples in Australia and North America and by naturalists, fishermen and physicians in Europe in the pre-industrial era and before the application of scientific method (Codd *et al.,* 1999). The occurrence of cyanotoxins was inferred from the deaths of sheep which had drunk water from Lake Alexandrina, South Australia in the 1870s. External and internal signs of poisoning, and survival times leading to sheep deaths after drinking *Nodularia* scum were reproduced by experimental oral-dosing with this material. It is now well established that cyanotoxins present hazards to animal and human health. Mortalities of sheep, cows, horses, pigs, dogs, poultry, wild birds, and fish have occurred as a result of the ingestion of cyanobacterial scum, mat and/or bloom material (Plate 1). Microcystins, anatoxin-a, homoanatoxin-a, saxitoxins, nodularin, cylindrospermopsin and anatoxin-a(*S*) have been identified as causative agents, either alone or in combinations.

Plate 1. Left: microcystin-containing scum of *Anabaena* spp. at a recreational freshwater (former reservoir). **Right:** shoreline accumulation of brown mat-forming cyanobacteria (*Phormidium/Oscillatoria*), containing anatoxin-a, at a freshwater site of associated dog deaths.

Although not as commonly reported as animal poisonings, human individuals and populations have been, and continue to be, adversely affected by the ingestion of water containing cyanobacterial cells and their toxins and by recreational and occupational skin contact (Codd, 1995; Carmichael, 1997; Codd *et al*., 1999; Duy *et al*., 2000 and WHO, 2003).

A serious human poisoning episode attributed to cyanotoxins occurred at Caruaru, Brazil in 1996. Water from a drinking water reservoir, with a history of cyanobacterial blooms and which had recently experienced a bloom of *Microcystis, Anabaena* and *Cylindrospermopsis*, was tankered to a haemodialysis clinic where it was ineffectively treated and then used in the treatment of patients using haemodialysis. As a result, 126 patients were severely affected and 60 patients eventually died over a number of months. Of the patients, 86% experienced toxic symptoms, including tender, swollen livers and biochemical evidence of liver injury. Severely affected patients also showed a range of neurological impairments. Further cases of human illness among haemodialysis patients exposed to water treated from source water with cyanobacteria have occurred in Brazil after 1996. Other cyanobacterial poisoning episodes, although not resulting in human mortalities, have resulted in the hospitalisation of people. In the UK, these have included soldiers who had been "barrel rolling" and swimming during canoeing and training exercises in water containing *Microcystis* scums. Symptoms included gastrointestinal illness and mucosal membrane blistering, with severe atypical pneumonia and indications of liver damage requiring hospitalization.

Poisoning episodes from the ingestion of reservoir water supporting cyanobacterial blooms have also been reported. In 1979, on Palm Island, Queensland, Australia, indigenous people were hospitalised after drinking water from a source which contained a bloom of *Cylindrospermopsis raciborskii*. Over a 21-day period, 139 children and 10 adults were affected, with approximately 70% receiving intravenous therapy. The syndrome included vomiting, headache, abdominal pain and diarrhoea with injury to the liver, kidneys, lungs, adrenals and intestines. The cyanobacterial bloom contained cylindrospermopsin, which remains as the primary suspected cause.

Other episodes of human illness associated with cyanobacteria included a statistically significant correlation between the drinking of treated water from a reservoir supplying Armidale, New South Wales, Australia, with a bloom of hepatotoxic *Microcystis aeruginosa* and indications of liver damage. Eutrophication of waterbodies, followed by the development of cyanobacterial blooms within two or three years can occur due to disturbance of the catchment surrounding lakes and reservoirs, or the drastic reduction of river flows. The construction of the Itaparica Dam Reservoir in Brazil resulted in increased eutrophication and the development of blooms of *Microcystis* and *Anabaena*. The human population which drank water treated from this reservoir reported cases of gastrointestinal upset and 88 deaths were recorded over a 42-day period, with cyanotoxins suspected as causatory agents.

Within the past 25 years, inland areas of South East China have shown an above average incidence of human primary liver cancer. Microcystins, via their actions as tumour promoters, are thought to have been partly responsible. Although fungal toxin (aflatoxin, a carcinogen) in food and endemic hepatitis B, both known to result in tumour production, were common in these areas, comparison of the microcystin content of different water sources versus the incidence of primary liver cancer indicated that people who drank from water sources with concentrations of microcystins were more likely to develop primary liver cancer, although the findings were not statistically significant. More recently, a significant correlation has been found between an increased incidence of primary liver cancer among communities taking drinking water from reservoirs with microcystin-producing cyanobacterial blooms in Serbia over a 10-year study period (1999-2008). No correlation with other potential risk factors (cirrhosis, hepatitis viruses) was found. Communities in the Serbian studies with the lowest incidence of primary liver cancer were using drinking water after ozonation (capable of removing microcystins), which had been introduced in 1985 and 1990 (Svirčev *et al*., 2013).

In addition to the possibility of contaminated finfish, shellfish and prawns from water containing cyanotoxins, water used for spray-irrigation of crop plants also can be contaminated with cyanobacterial blooms. At a commercial crop facility in the UK, a brick-lined pond used for storing water for crop-spray irrigation became contaminated by a bloom of *Microcystis aeruginosa*. Containing high concentrations of microcystins, this material was aerially spread over a lettuce crop. Subsequent microscopic examination of the lettuce and toxin analysis of the salad crop revealed contamination by *Microcystis* and microcystins, at concentrations deemed to be a health risk if consumed and the crop was withdrawn from sale as a precaution.

Reports on the effects of human exposure to cyanobacterial LPS are limited, although two exposure routes, via inhalation and haemodialysis, have been inferred to be significant. Concerning haemodialysis, an endotoxaemia incident was reported in 1974 among patients in a Washington DC, USA clinic, coincident with the detection of LPS in the local drinking water and a cyanobacterial bloom in the supply reservoir. The toxins of marine cyanobacteria, the inflammatory and tumour-promoting dermatoxins, aplysiatoxin, debromoaplysiatoxin and lyngbyatoxin have been reported in Florida USA, Okinawa Japan, and Queensland Australia, particularly in the summers between 1996 and 1998 (Codd *et al*., 1999). The dermal route of cyanobacterial intoxication is responsible for severe contact dermatitis conditions such as "swimmers itch" and can occur when people swim in the sea in contact with cyanobacteria. These allergic and irritative effects, not confined to marine environments, have been attributed to several cyanobacterial genera including *Lyngbya, Anabaena*, *Aphanizomenon*, *Nodularia*, *Planktothrix*, and *Gloeotrichia*. The irritative effects caused by these and other genera are often exacerbated by swim suits and wet suits which can trap cyanobacterial cells next to the skin. The abrasive nature and disruption caused by movement in such suits may help to breakdown cyanobacterial cells, releasing the toxins, whilst retaining the material next to the skin.

Other risk activities include showering in ineffectively-treated water and work practices where dermal and respiratory exposure to cyanobacterial scums, blooms and toxins may occur. Although initially investigated using nasal sprays in animal studies 20 years ago, the likelihood of exposure to cyanobacterial cells, cell débris and cyanotoxins, and cyanotoxin toxicity via this route have been largely neglected. However, microalgae and cyanobacteria are common components of airborne microbial communities, and with wind and wave action on lakes with cyanobacterial mass populations, cyanobacteria can be released into the air with the potential to contain toxins. Microcystins have been detected in air samples collected during recreational activities near waterbodies. Desert dust containing

microcystins may also be a health risk in desert regions where microbal crusts containing cyanobacteria are common.

The identification of scenarios leading to long-term exposure to cyanotoxins has initially focused on potential lifetime exposure to microcystins via drinking water. The health significance of long-term/lifetime exposure to BMAA is also of particular current interest. This neurotoxic amino acid is known to have been consumed over many years by indigenous people on the island of Guam via their diet. In addition to being acutely neurotoxic, long-term exposure among the Guam population has been associated with the development of human neurodegenerative disease (amyotrophic lateral sclerosis/Parkinsonism dementia complex: ALS/PDC, a form of motor neurone disease). The significance of BMAA in the brains of ALS/PDC victims from Guam and also in the brains of Alzheimer's victims in Canada and ALS sufferers in the USA, in contrast to brains of humans not dying of non-genetic neurological disease, is the subject of intensive current research to determine whether a causal relationship between exposure to BMAA and the development of human neurodegenerative disease exists. If so, then further research will be required on other potential exposure media and routes for this cyanotoxin, in addition to food intake. Exposure to BMAA via the drinking of raw water and of ineffectively-treated drinking water are candidates. Both short- and long-term respiratory exposure to cyanotoxins is also possible. Although not themselves volatile molecules, microcystins and BMAA can also be airborne by several means:

(i) as dissolved, free cyanotoxins in aerosols;

(ii) in water droplets and spray containing cyanotoxin-producer-cells;

(iii) as wind-blown dust containing particles of cyanotoxin-containing cyanobacterial desert crust.

Potentially acting over decades, cyanotoxins such as microcystins and BMAA are priorities for further research on potential long-term exposure. Such studies may have the potential to influence the development of risk management policies and the reduction of human disease.

4.2 Animal-dosing studies and risk assessment for the protection of human health

Many of the data concerning the uptake, sequestration, toxicity and fates of cyanotoxins have been obtained by laboratory animal-dosing studies (Table 4; Kuiper-Goodman, *et al*., 1999). Lethal Dose (LD) concentrations, including those which kill half of a test population (LD_{50}) have been derived for most of the major

cyanotoxins. Of further value for risk assessment are the doses of toxin which cause the lowest observed adverse effect level (LOAEL) and the no observed adverse effect level (NOAEL). Oral toxicity determinations and long-term-, ideally lifetime-exposure studies are necessary for risk assessments of long-term exposure, to minimise uncertainties, and to derive guidelines for long-term /lifetime health protection. Unfortunately adequate long-term/lifetime oral toxicity studies have only been determined for microcystin-LR and cylindrospermopsin (Humpage and Falconer, 2003).

Table 4. Toxicity of known cyanotoxins by various administration routes

Cyanotoxin	LD_{50} (µg per kg)				LOAEL	NOAEL
	i.v.	i.p.	i.n.	Oral		
Microcystins		25-150	36-122	5000-10900	100	40
Nodularin		50				
Anatoxin-a	<100	375	2000	>5000		100
Homoanatoxin-a		250				
Anatoxin-a(s)		20				
Saxitoxins	3.2-3.6	7.6-10.5		251-267		
Cylindrospermopsin		200-2000		4400-6900		30

i.v., intravenous; i.p., intraperitoneal ; i.n., intranasal; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level.

The information from these studies permits the estimation of Tolerable Daily Intakes (TDI) for individual toxins. From these, Guideline Values (GV) for cyanotoxins in drinking and recreational waters can be derived. For example, the WHO has produced a provisional GV for microcystin-LR in drinking water of 1µg per litre (Kuiper-Goodman, *et al.*, 1999). A similar GV for cylindrosermopsin has also been proposed. Such GVs take into account uncertainty factors and are designed to afford health protection during potential lifetime exposure via drinking water, and versus occasional recreational exposure. Data from poisoning cases, the dynamics of cyanobacterial populations and cyanotoxins in waterbodies and drinking water GVs, can be used to derive guideline levels (GL, or warning thresholds) for cyanobacterial cell and toxin concentrations in recreational waters. Further quantitative toxicity data via oral, and lifetime exposure in animals are needed to derive GVs and GLs for drinking water and recreational waters for the remaining known cyanotoxins.

Although there is a large amount of information on the toxicity of individual purified cyanotoxins in laboratory tests, little is known about the effects of exposure to multiple classes of cyanotoxins, either simultaneously or in sequence, or to exposure to cyanotoxins plus other toxicants, e.g. pesticides or metals, or health hazards such as waterborne microbial pathogens. For example, intranasal

exposure of mice to microcystin-LR was found to have a toxicity 10 times greater than that of oral administration of the toxin by gavage. Examination of the nasal cavities revealed extensive necrosis of the olfactory and respiratory zone epithelium. In the same study, anatoxin-a was also co-administered with microcystin-LR intranasally and synergistic effects were noted (Fitzgeorge *et al*., 1994). The need for toxicity assessments of cyanotoxins in combination with other stressors and health hazards may more closely reflect real-life potential exposure scenarios, since individual cyanotoxins do not typically occur in isolation in aquatic or other environments.

5. Effects of cyanotoxins on wild animals and plants

Cyanobacterial mass populations can have adverse effects on wildlife in addition to humans and domestic livestock (Codd, 1995; Carmichael, 1997; Falconer, 1998; Sivonen & Jones, 1999 and Metcalf & Codd, 2012). A wide range of wild animals (mammals, amphibians, fish, invertebrates) and birds have been affected, with consequences ranging from non-fatal (inhibition of invertebrate feeding, delayed fish egg-hatching) to fatal (mass mortalities). Wild animal poisonings can occur after incidental ingestion of cyanobacterial biomass and toxins during drinking or feeding. In the case of fish, it is possible that additional exposure can occur via the gill surfaces.

Knowledge is increasing about the transfer of cyanotoxins along aquatic and terrestrial food chains. In the Baltic Sea, nodularin, produced by blooms of *Nodularia,* has been found in the primary consumers of the cyanobacteria (juvenile blue mussels) and also in the livers of eider ducks which feed on the mussels. BMAA has been found in a wide range of marine invertebrates, including blue crabs and oysters, and in top predators such as sharks (Mondo *et al*., 2012). In some cases, the deaths of aquatic animals have been recorded in which the mortalities were associated with consumption of the natural food which contained high concentrations of cyanotoxins. For example, muskrat deaths have been ascribed to feeding on mussels which had accumulated microcystins. Certainly, cyanobacterial blooms containing µg to mg per litre concentrations of cyanotoxins pose health risks to adults and juveniles of aquatic vertebrates and invertebrates. Periodic mass mortalities of many thousands of Lesser Flamingos have occurred over recent decades at Rift Valley lakes in Kenya and Tanzania. Flamingos are filter-feeders and can feed on cyanobacteria as a major, or sole food-source. Their pink plumage is a result of the ingestion of cyanobacterial pigments. Analyses of dead flamingos have shown significant concentrations of microcystins and anatoxin-a in the liver, stomach, intestines and faecal pellets. These findings, plus signs of poisoning and the presence of these hepato- and neurotoxins in

environmental cyanobacterial samples indicate that cyanotoxins in the diet, alongside additional toxicants and stressors, are significant contributors to the flamingo mass mortalities. Microcystins have also been identified as the most likely causes of mass deaths of Greater and Chilean Flamingos in a Spanish national park wetland and a Florida zoo pond (Codd *et al*., 2003). A valuable and facile indicator of potential dietary exposure to cyanotoxins in birds has emerged through the findings of significant quantities of microcystins and BMAA in the feathers of dead flamingos from the Kenyan lakes and of nodularin in the Baltic Sea eider ducks.

Cyanobacterial bloom development in waterbodies is often associated with a significant decline in biodiversity including the loss of water plants (macrophytes). Competition between the cyanobacteria and macrophytes for nutrients, and the shading of submerged macrophytes by turbid cyanobacterial suspensions and scums are recognized as causative factors. The effects of cyanotoxins on macrophytes are less clear. However, submerged and emergent pondweeds and reeds can take up microcystin at environmentally-encountered concentrations. The uptake of anatoxin-a and BMAA by macrophytes also occurs and in these cases the cyanotoxins are assimilated from the surrounding water. Whilst all plant systems tested have shown some ability to metabolise and detoxify the cyanotoxins, the inhibition of whole leaf photosynthesis in French bean plants and in the pondweed *Ceratophyllum* by microcystin suggests that cyanotoxins, after release into the water, may contribute to the adverse effects of cyanobacterial mass populations on plants in waterbodies.

6. Multiple fates of cyanotoxins and their persistence in waterbodies

Cyanotoxins undergo multiple fates after biosynthesis (Sivonen & Jones, 1999). These fates are not only of biological interest, but offer potential challenges and opportunities. When cyanobacterial cells are intact and actively growing, microcystins and most of the other known cyanotoxins except cylindrospermopsin are largely retained within the producer-cells. Extracellular release of the bulk of the total cyanotoxin pool occurs when the cyanobacterial cells are disrupted. Cell disruption can occur via natural processes including senescence due to nutrient starvation and lysis by naturally occurring viruses, bacteria and fungi. Some cyanotoxin-producing cyanobacteria, especially *Microcystis*, are also readily broken by mechanical shear forces, for example as encountered in pumping operations for raw water abstraction and delivery to treatment plants. The possibility therefore arises that raw water delivered to a water treatment works may

contain a higher proportion of extracellular cyanotoxin than occurred in the raw water before abstraction and pumping.

The stability and persistence of cyanotoxins after extracellular release or cell disruption are important factors in influencing raw water quality and the ability of water treatment processes to remove and/or detoxify the toxins. The known cyanotoxins are non-volatile and relatively stable. For example, purified microcystins persist without significant loss if stored in the dark at 4°C, in sterile water, for at least one year. Microcystins can withstand boiling and extremes of pH from 1 to 12. However, in natural environments, they are subject to photodegradation by UV in sunlight. Photodegradation of microcystins by visible wavelengths in sunlight can also occur but this requires the presence of a suitable photosensitizer such as the cyanobacterial blue pigment, phycocyanin, since the toxins themselves only absorb UV light. Microcystins are also susceptible to biodegradation by naturally occurring aquatic and terrestrial bacteria and fungi. Cylindrospermopsin biodegradation has also been identified and it is likely that biodegradative microbes can be isolated for other cyanotoxins. Further loss processes, which include sorption onto mineral matter and cyanotoxin detoxification are common through naturally-occurring, multispecific enzymes in animals, from zooplankton to higher animals, and plants, including glutathione-*S*-transferases (Pflugmacher *et al*., 2001) and cytochrome P450. These processes are assumed to influence the persistence of cyanotoxins in the water phase of lakes and reservoirs after a cyanobacterial bloom has declined. When rates of loss of dissolved cyanotoxins are expressed as half-lives, these may range from days to weeks.

7. What is being done about cyanotoxins?

Raising awareness of the properties and production of cyanobacteria and their toxins is a necessary part of the risk management of cyanotoxin problems in the health, recreation, amenity, agriculture, aquaculture and drinking water supply sectors (NRA, 1990). This is being achieved by workshops, seminars, and handbooks for health, environmental and water industry professionals. Recent and current European Union (e.g. EC CYANOCOST www.cyanocost.com) and other international agency programmes (e.g. the Water Research Foundation, www.waterrf.org) are addressing these needs by facilitating technology transfer and the dissemination of best practice. These actions are also needed in developing countries to help to avoid the health problems with cyanotoxins encountered in e.g. Europe, Australia, the USA and Brazil over recent years.

The risk management of cyanotoxins includes reactive and proactive measures (NRA, 1990; NSWBGATF, 1992; Yoo *et al*., 1995). Regarding reactive actions, the removal/destruction of cyanobacteria and cyanotoxins by traditional and advanced drinking water treatment processes continues to be investigated and optimized (EU Project Toxic EVK1-CT-2002-00107 and Ho *et al*., 2012) with a focus on microcystins, anatoxin-a, cylindrospermopsin and saxitoxins. The removal of cyanobacterial LPS (Rapala *et al*., 2002) has been demonstrated with a 59 to 97% reduction achieved by conventional treatments including coagulation, settling and sand filtration. Reactive measures also include the formulation of decision-making systems, including emergency measures for access to, and use of waterbodies in the event of cyanobacterial populations and toxins having already developed to unacceptable concentrations (NRA, 1990; NSWBGATF, 1992; and Yoo*, et al*., 1995).

When blooms do occur and there is a risk of exposure through drinking water, a number of measures can be introduced to mitigate potential risks and concerns. These can include verifying and upgrading treatment processes and facilities, or using an additional or alternative water source with a lower cyanobacterial/cyanotoxin burden to reduce the overall cyanotoxin concentration. Bottled drinking water has been supplied to communities in several countries when unacceptable cyanotoxin concentrations have occurred for short periods.

Although considerable basic and applied research on the production, properties and control of cyanotoxins has been carried out and much of the knowledge required is now in place, several uncertainties remain. To a large extent, research is constrained by the limited availability of purified, quantitative analytical standards for the cyanotoxins and of certified reference materials (Wang, L. *et al.,* 2013). Such materials are needed for the further development of analytical methods, with an increasing focus on techniques for lake-side, or treatment works usage, and for the further evaluation and optimization of drinking water treatment processes. A great deal of research effort has been placed on understanding the genetic basis of cyanotoxin production and for the established cyanotoxins, gene probing of natural and controlled waters is making a useful contribution to the estimation of cyanotoxin-producing potential (Dittmann *et al*., 2013). Research is also focusing on the newly-emerging cyanotoxins, in particular BMAA and on its association with human neurodegenerative diseases. Research concerning the co-occurrence of different classes of cyanotoxins, and into their toxicities, both in combination and together with other waterborne health hazards is at an early stage but may influence the refinement of existing guidelines and lead to further recommendations.

Proactive measures need to include increasing basic knowledge of cyanotoxin toxicity via oral, intranasal and dermal exposure routes, and on the production and persistence of the toxins. This is necessary to permit the further, confident derivation of drinking water GVs for additional cyanotoxins.

It is notable that over ten years after the derivation of the GLs for cyanobacterial cells in recreational waters and the GV for microcystin-LR in drinking water, these remain provisional values of the WHO. These guidelines include thresholds for acceptable concentrations of cyanobacterial cells and respective chlorophyll *a* and equivalent microcystin concentrations (Kuiper-Goodman *et al.*, 1999). These concentrations, corresponding to risks of high-, medium- and low-severity adverse health outcomes, refer to cyanobacterial scums (high risk), followed by about 100,000 cells per ml and 20,000 cells per ml, respectively. At the lowest level, 20,000 cells per ml, the concentration of cyanobacteria is equivalent to about 10 µg per litre chlorophyll *a* and potentially to 1 µg per litre microcystin-LR, the WHO provisional GV for microcystin-LR in drinking water. Guidelines for acceptable concentrations of cyanobacterial cells and microcystin, either taken directly from WHO recommendations or modified nationally, continue to be introduced at national level throughout Europe and beyond for potable and recreational waters (Chorus (ed.), 2012). In Canada, for example, a maximum admissible concentration of 1.5 µg microcystin-LR per litre is applied. The guidelines, for inclusion in decision-making systems and action plans to permit better risk management of water resources which are sensitive to the occurrence of cyanotoxins, are aimed to help to ensure the provision of safe drinking waters and the protection of healthy working, aesthetically pleasing and recreational aquatic environments.

Multidisciplinary working groups have been established at national level to specifically assess the occurrence of cyanobacterial blooms and toxins and the potential of waterbodies to support cyanobacterial mass development. Those established in the UK and Australia (NRA, 1990 and NSWBGATF, 1992) and subsequently disbanded after accomplishing their tasks, have served as a model. Further national working groups have continued with this work in e.g. the USA, Norway, South Africa, the Netherlands, Germany, Poland, Spain and France. These groups continue to formulate and implement policies for cyanobacterial bloom and toxin risk management. The procedures include exposure and risk assessments, taking into account the WHO-derived GVs and GLs, alongside the national experience. Several countries e.g. Spain and Brazil have established these-, or similar GVs and GLs in national legislation. Others, including the UK, use them as guidelines in an advisory mode. Cyanotoxin concentrations are not specified in EU legislation so far.

Since policy development for cyanotoxin risk management follows closely behind primary research and the developing awareness of the health significance and impacts of the toxins, it is necessary for the effectiveness and suitability of action plans to be reviewed periodically, and if necessary modified. This rolling approach is presently being taken by e.g. the Environment Agency for England and Wales and the Scottish Government (Scottish Executive, 2012).

Finally, cyanotoxins and their undesirable effects are being increasingly seen as part of the consequences of eutrophication. Measures to reduce the latter by e.g. restricting the excessive enrichment of water resources due to agricultural runoff and inadequately-treated sewage, now being addressed from local to catchment level, are seen as important longer-term actions which will contribute to cyanobacterial bloom and cyanotoxin risk management.

8. Summary

Cyanobacteria (blue-green algae) are natural inhabitants of fresh, brackish and marine waters and are of worldwide distribution. They produce a diverse range of small molecules (cyanobacterial toxins: cyanotoxins) which are hazardous to human and animal health. The sources and properties of these toxins are briefly reviewed. Their harmful effects range from mild to serious, and include gastrointestinal upsets, skin irritations, liver and neurological damage. Examples of the adverse effects on human health, domestic animals and wildlife are given. Risk assessments for health protection against some of the most common and potent cyanotoxins have been made and included in emerging schemes for the risk management of cyanotoxin problems which can occur in potable and recreational waters. Reactive and proactive measures and further needs in this context are presented. The reduction of cyanotoxin problems in natural and controlled waters as a potential benefit of eutrophication control is also discussed.

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