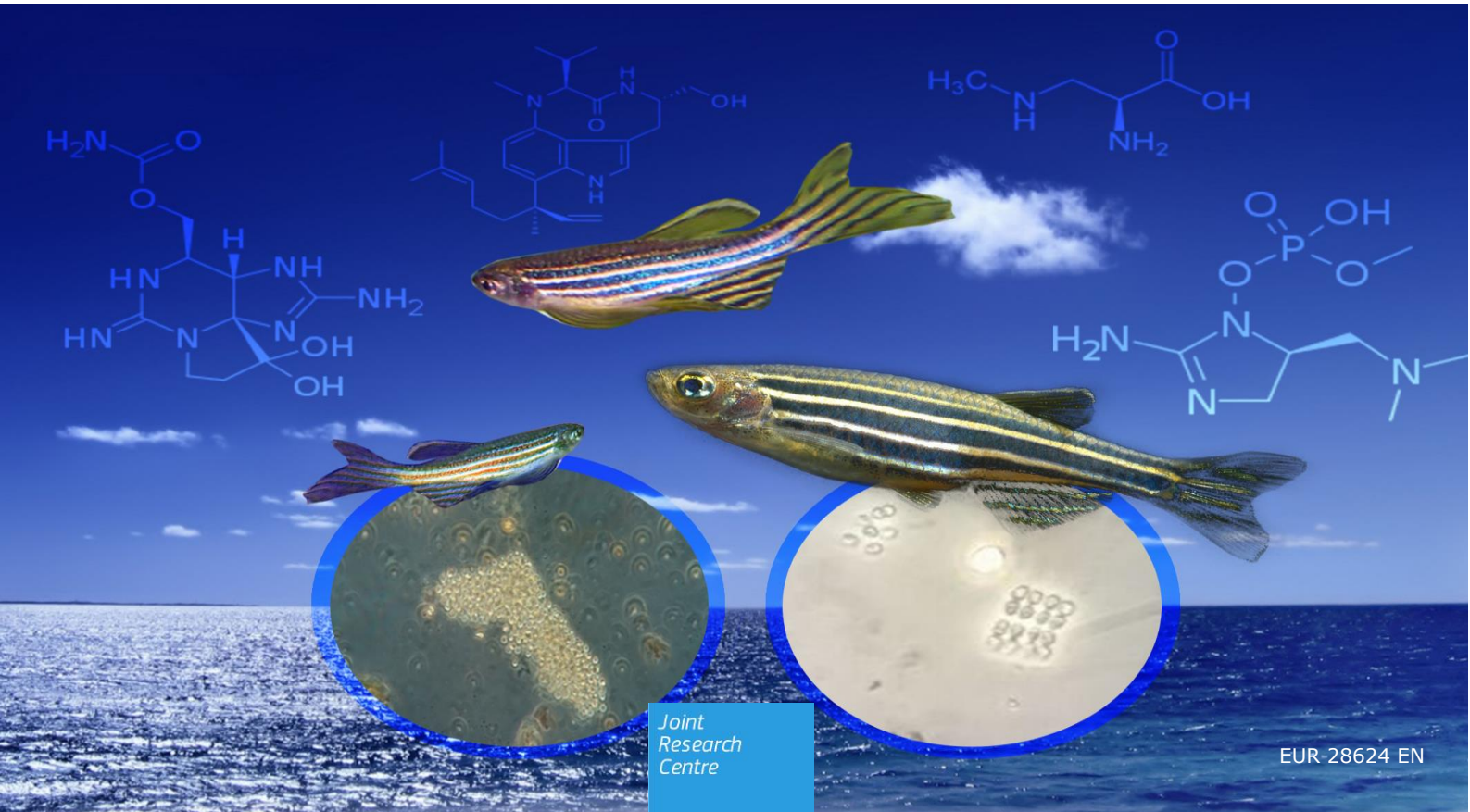
European Commission

JRC TECHNICAL REPORTS

Cyanotoxins: methods and approaches for their analysis and detection

Isabella Sanseverino, Diana Conduto António, Robert Loos and Teresa Lettieri

2017



EUR 28624 EN

Cyanotoxins: methods and approaches for their analysis and detection

Contact information
Name: Teresa Lettieri
Address: Joint Research Centre (JRC), Directorate D-Sustainable Resources, Unit D.02 Water and Marine Resources, TP 121, Via Enrico Fermi, 2749, 21027 Ispra (VA), Italy
Email: teresa.lettieri@ec.europa.eu
Tel.: +39-0332789868

JRC Science Hub
https://ec.europa.eu/jrc

JRC106478

EUR 28624 EN

PDF	ISBN 978-92-79-69099-0	ISSN 1831-9424	doi:10.2760/36186
Print	ISBN 978-92-79-69100-3	ISSN 1018-5593	doi:10.2760/630736

Luxembourg: Publications Office of the European Union, 2017

How to cite this report: Isabella Sanseverino, Diana Conduto António, Robert Loos and Teresa Lettieri; *Cyanotoxins: methods and approaches for their analysis and detection*; EUR 28624; doi:10.2760/36186

Table of contents

Acknowledgements

We thank Dorota Napierska and Raquel Negrão Carvalho for critically reading the report and Úna Cullinan for the bibliographic support. We also thank Blasco Muñoz Jose and Laura Spirito for the realisation of the cover picture, and Chiara Facca for the microscopy images of cyanobacteria.

Abstract

Cyanotoxins are secondary metabolites produced by cyanobacteria, a group of photosynthetic prokaryota especially found in freshwater. In favourable conditions (i.e. high nutrient levels, light intensity, water temperature), cyanobacteria can form blooms, a natural phenomenon characterised by an algal biomass accumulation and the possible release of cyanotoxins in water ecosystems. Toxins represent an emerging threats for the aquatic organisms which can bioaccumulate these compounds and transfer them throughout the food chain to wildlife and humans. Other ways of exposure for humans include the oral, dermal and inhalation route.

The consumption of contaminated drinking water, skin contact and swallowing water during recreational activities are among the most frequently reasons for human poisonings caused by cyanotoxins. The associated symptoms usually range from severe headache to fever, respiratory paralysis and in rare case, death. The World Health Organization (WHO) has issued a provisional guideline value of 1 µg/L in drinking water for Microcystin-LR (MC-LR), the most toxic, widespread and common toxin in water supplies. Due to the lack of complete toxicological data for a range of cyanotoxins, their concentration in drinking water is not yet well regulated even in countries belonging to the European Union (EU).

In this report, attention is focused on the methodologies commonly used to detect cyanotoxins in water environments. These applications can be grouped in: I) microscopy analysis II) physicochemical methods III) molecular-based methods IV) biochemical-based methods V) chemical methods. Each technique shows specific limitations in terms of sensitivity, reliability and limit of detection. The choice of the best one to use is determined in accordance with the information they provide, the availability of facilities and the technical expertise of the operators. Most of the research about cyanotoxins has been mainly focused on microcystins (MCs). The other cyanotoxins have been much less investigated and more tools need to be developed to overcome this problem. Notwithstanding there is no a single analytical application able to detect all cyanotoxin variants in an environmental sample. Some current methods described in this report show great promise in terms of being simple, cost-effective, specific and sensitive for the analysis of a defined toxin.

1. Introduction

Cyanobacteria are photosynthetic bacteria which are mostly found in freshwater systems. Due to their long evolutionary story, they have adapted to climatic, geochemical and anthropogenic changes. Indeed, they can also colonise water that is salty or brackish and are capable to survive extremely low and high temperatures. Cyanobacteria have a key role in maintaining the environmental balance as well as the biodiversity of microorganisms and higher organism communities. They use the energy of sunlight to perform photosynthesis and are specialised in converting the inert atmospheric nitrogen (dinitrogen) into inorganic forms (nitrate or ammonium). Aquatic plants and algae need all these forms of nitrogen for their growth. Nevertheless, the anthropogenic eutrophication and climate changes are contributing to the intense proliferation of cyanobacteria in waterbodies, so causing a phenomenon knowns as bloom which may compromise the quality of drinking and recreational water. It has been observed that when a bloom occurs, secondary metabolites with high levels of toxicity, known as cyanotoxins, are produced by cyanobacteria. Cyanotoxins can impact on humans, animals and plants and they are classified according to their toxicological target in hepatotoxins, cytotoxins, neurotoxins and dermatoxins. The global occurrence of cyanotoxins in natural water reservoirs, in surface waters used for potable or recreational aims or in seafoods, promoted the development of methods and approaches for detection of toxins in environmental samples. A good policy of water management is in fact necessary not only to detect cyanobacteria causing the blooms but especially to assess the presence of cyanotoxins produced in waterbodies. Nowadays, molecular, biochemical and chemical techniques are the most frequent methodological approaches to detect toxic cyanobacteria and their cyanotoxins. Microscopic counting has traditionally been employed in monitoring programs of waterbodies but this method is not able to distinguish between toxic and nontoxic strains. During a bloom, some cyanobacteria known to be toxic, and some others which do not produce toxins, may be present together. Since they not show any morphological difference, the microscopic identification is not a suitable approach to detect their distribution. Molecular-based methods have thus supplemented biological methods because of their potential to specifically identify and quantify cyanotoxins-coding genes. However Desoxyribonucleic Acid (DNA)-based detection approaches are not informative about the presence of toxicity in terms of expression at protein level. To overcome this limit, other methods like biochemical and chemical techniques have been developed to quantify which toxin is present in a specific environmental sample. The currently detection approaches are different in term of sensitivity and specificity and the choice of the best method to use is influenced by the information they provide, the availability of facilities (when required) and the technical expertise of the operators.

Considering the risks posed by cyanotoxins to human health and the environment, many countries have adopted the provisional value of 1µg/l in drinking water for Microcystin-LR (MC-LR), the most toxic and widespread hepatotoxin produced by cyanobacteria. Specific regulatory approaches adopted by different countries are included in the booklet published in 2012 by the Umweltbundesamt (UBA). Only few European countries are mentioned in this document underlying the need of implementing the assessment and management of cyanotoxins risks on the broad European scale. National regulations and recommendations aimed to limit cyanobacterial biomass in our waterways are therefore necessary to reduce health risks and frequencies of blooms. The improvement of early warning methods to detect cyanobacteria is at the same time required in order to support the regulatory policies and prevent additional human and ecosystem health hazards.

2. Cyanotoxins

The word cyanotoxins refers to diverse groups of secondary metabolites produced by various genera of cyanobacteria which are highly toxic to many organisms including animals, plants, algae and humans. Cyanotoxins are produced by bloom-forming cyanobacteria whose rapid proliferation is regulated by a combination of environmental and anthropogenic factors. A bloom is a natural phenomenon caused by a significant production of biomass and is often characterised by the formation of a dense layer of cells at the surface of the water[1]. The massive growth of cyanobacteria can be induced by different physical, chemical and biological factors among which the warmer water temperature (25°C or above), the light intensity (a species-specific necessity) and the trophic status of the water (understood as the increased input of nutrients in aquatic systems, mainly phosphorous and nitrogen)[2,3]. Under these increasingly recurring circumstances, both planktonic and benthic cyanobacteria can reach high concentrations and have severe impacts on the ecosystem so that the blooms formation process has become a worldwide environmental problem affecting aquatic ecosystems including freshwater and brackish water.

Blooms are not necessary related to toxicity since not all cyanobacteria strains are toxic[4]. Each toxin is produced by cyanobacteria only when the appropriate toxin gene is carried by a particular strain and if its expression is activated by environmental conditions. In most cases, toxic and nontoxic species coexist during a bloom but the amount of toxins in the waterbody is not always directly correlated to the presence of toxin-producing cyanobacteria. A specific toxin, indeed, can be produced by different species and a single species is able to produce multiple types and variants of toxins[5].

The majority of cyanotoxins are found intracellularly, in the cytoplasm of the cells. Cyanobacteria usually release their intracellular content of toxins in the water when an algal bloom decays but in some species, toxins can be also secreted by live cells (extracellular toxins)[5]. All the toxins released into the water can bioaccumulate in the environment and in waterborne organisms which can transfer them to aquatic fauna and humans. When toxins accumulate in shellfish, their consumption by human populations may cause symptoms ranging from severe illnesses to death. Several studies are ongoing to understand the broad spectrum effects of cyanotoxins and more attention has been recently paid to investigation of their toxicity and possible impacts on human health.

2.1 Classification of cyanotoxins

Cyanotoxins are usually classified in four classes according to their toxicological target: **i) hepatotoxins** that act on liver (*Microcystins and Nodularin*) **ii) cytotoxins** that produce both hepatotoxic and neurotoxic effects (*Cylindrospermopsin*) **iii) neurotoxins** that cause injury on the nervous system (*Anatoxins, Saxitoxins and β-Methylamino-L-Alanine –BMAA-*) and **iv) dermatoxins** that cause irritant responses on contact (*Lypopolysaccharide, Lyngbyatoxins and Aplysiatoxin*) (Table 1).

In terms of their chemical structures, cyanotoxins fall into three groups: **cyclic peptides** (*Microcystins and Nodularin*), **heterocyclin compounds** (alkaloids) (*Cylindrospermopsin, Anatoxins, Saxitoxins, Lyngbyatoxins*, *Aplysiatoxin)* and **lipidic compounds** *(Lypopolysaccharide)*.

Table 1: **Toxins produced by cyanobacteria: their effects and primary targets**
(Table taken from the Report "Algal Bloom and its economic impact", JRC Technical Report, 2016[1])

Toxin classification	Toxins	Most common cyanobacteria genera producing toxins	Main organ affected	Effects	Main targets
Hepatotoxins	Microcystins	*Microcystis, Anabaena, Anabaenopsis, Aphanizomenon, Planktothrix, Oscillatoria, Phormidium*	Liver	Diarrhea, vomiting, weakness liver inflammation, liver hemorrhage, pneumonia, dermatitis	Serine/ threonine protein phosphatases
	Nodularin	*Nodularia, Nostoc*	Liver	Diarrhea, vomiting, weakness liver inflammation, liver hemorrhage, pneumonia, dermatitis	Serine/ threonine protein phosphatases
Cytotoxins	Cylindrospermopsin	*Cylindrospermopsis, Anabaena, Aphanizomenon, Raphidiopsis, Oscillatoria, Lyngbya, Umezakia*	Liver	Gastroenteritis, liver inflammation, liver hemorrhage, pneumonia, dermatitis	Protein synthesis
Neurotoxins	Anatoxins	*Anabaena, Aphanizomenon, Planktothrix, Cylindrospermopsis, Oscillatoria*	Nervous system	Muscle twitching, burning, numbness, drowniess, salivation, respiratory paralysis leading to death	Nicotinic receptors or acetylcholinesterase
	Saxitoxins	*Anabaena, Aphanizomenon, Cylindrospermopsis Lyngbya, Planktothrix, Rhaphidiopsis*	Nervous system	Muscle twitching, burning, numbness, drowniess, headache, vertigo, respiratory paralysis leading to death	Sodium channels
	BMAA*	*Nostoc, Microcystis, Anabaena, Aphanizomenon, Nodularia*	Nervous system	No specific clinical symptoms, ALS/PDC with long-term consistent exposure	NMDA* excitotoxicity, ROS production
Dermatoxins	Lypopolysaccharide	*Synechococcus, Microcystis Anacystis, Oscillatoria Schizothrix, Anabaena*	Skin	Skin irritation, eye irritation, headache, allergy, asthma, fever	Toll-like receptors
	Lyngbyatoxins	*Lyngbya*	Skin	Skin and eye irritation, respiratory problems	Protein kinase C
	Aplysiatoxin	*Lyngbya, Schizothrix, Oscillatoria*	Skin	Skin irritation, asthma	Protein kinase C

* **BMAA** stands for β-Methylamino-L-Alanine; **NMDA** stands for N-Methyl-D-Aspartate

2.1.1 Hepatotoxins: Microcystins and Nodularins

The hepatotoxins Microcystins (MCs) and Nodularins (NODs) are cyclic heptapeptides and pentapeptides with similar structures and mechanisms of action. These toxins possess in their molecules the unusual β-amino acid Adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E dienoic acid), which is found only in cyanobacterial peptides and is often associated with the toxicity of these compounds[3]. To date, more than 80 variants of MCs and 9 congeners of NODs have been identified. In MCs, each congener is determined by multiple combinations of the variable amino acids X and Z, while the NODs structure (Figure 1) shows only one variable amino acid Z[6]. Modification at the level of functional groups within the molecules are also involved in determining the hepatotoxins variants.

Microcystins are the most widespread and well studied cyanotoxins. Among the MCs, the congener Microcystin-LR (MC-LR) (Figure 2), characterised by having the amino acid Leucine and Alanine respectively in the position X and Z, is the most toxic MCs variant and is also the most prevalent in brackish and freshwater blooms[7]. Considering its high toxicity, the World Health Organization (WHO) has set a provisional guidance value, equal to 1μg/L, for the maximal acceptable concentration of MC-LR in drinking water[8]. This value has been adopted by different countries: Brazil, Czech Republic, France, New Zeeland, Singapore, Spain, Uruguay and South Africa. In Australia the guidance value for this hepatotoxin in drinking water is equivalent at 1.3 μg/L, and in Canada the provisional maximum value (PMAV) for MC-LR has been fixed at 1.5 μg/L[8]. In four countries belonging to the European Union (EU) (Denmark, Germany, Netherland and Italy) there are no cyanotoxin drinking water specific regulations. In Denmark and Germany, in particular, this is because almost all drinking water originates from ground water. In Italy, MCs are monitored only if a contamination risk is expected. When this phenomenon happens in waterbodies used as a source of drinkable water, the local authorities follow the provisional WHO guideline for MC-LR of 1μg/L. Finally, in Finland, the national regulation has fixed the value for MCs in raw water as low as 1μg/L.

Microcystins take their name from Microcystis, the first genera of cyanobacteria which has been identified to be responsible for their biosynthesis. Today, however, it is known that MCs are produced by many other species of cyanobacteria such as Oscillatoria, Aphanizomenon, Anabaena, Planktothrix and Anabaenopsis; the production of NODs, on the contrary, has been reported only for species *Nodularia spumigena*, *Nodularia sphaerocarpa* and for the genera Nostoc[2]. Toxins synthesis is a non-ribosomal process regulated by genes coding for Non-Ribosomal Peptide Synthetase (NRPS) and Poliketide Synthase (PKS)[9]. The regulation of both MCs and NODs synthesis is influenced by environmental conditions but factors like nutrients concentration and light intensity are also deeply involved in their production.

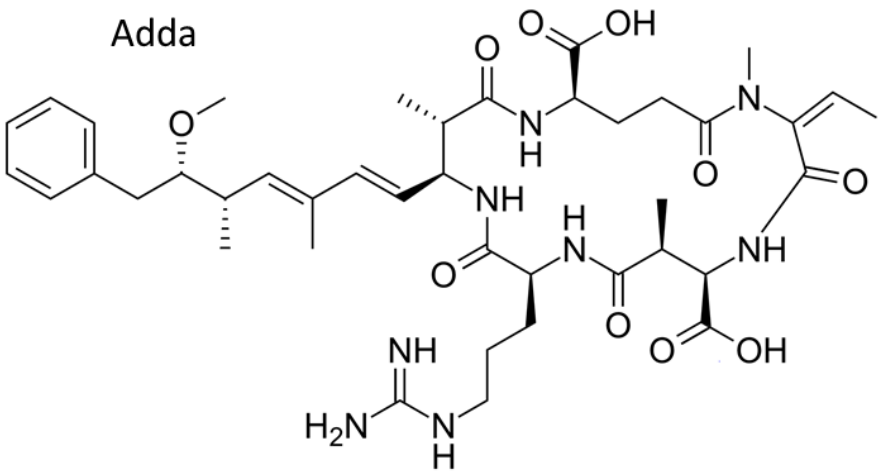


Figure 1: Chemical structure of Nodularin (NOD).

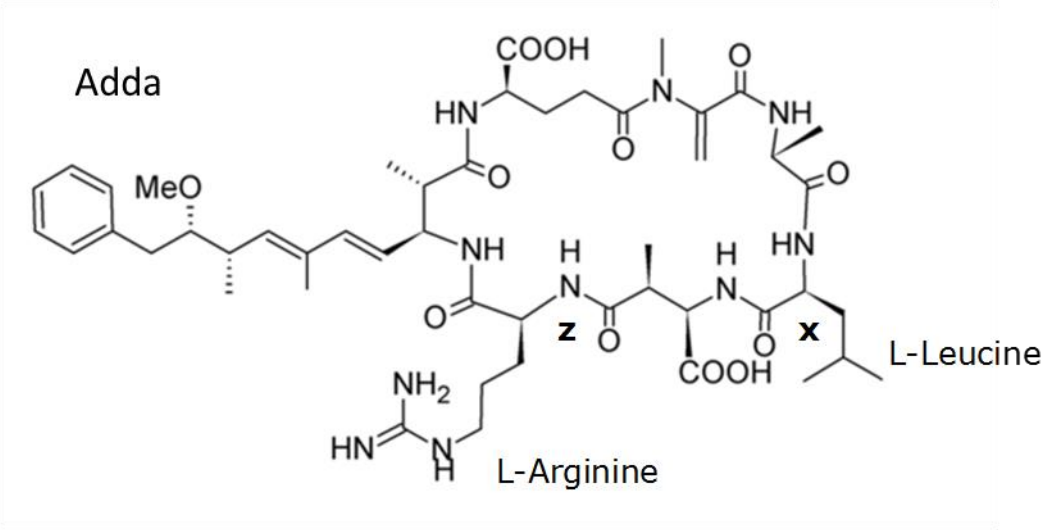


Figure 2: Chemical structure of Microcystin-LR (MC-LR). Structure of MC-LR with the aminoacids Leucine and Arginine respectively in the position X and Z of the molecule. The amino acid Adda is also indicated. (Source: DrJohn1100 - Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=34824794)

Microcystins are hydrophilic molecules and they are incapable of crossing passively the cell membrane but rather require active transport via specific transporters. The hepatocytes, the main target cells of MCs, are responsive to these toxins via the bile acid transportation system and the members of the Organic Anion Transporting Polypeptides (OATP)[10]. Microcystins toxicity is not restricted to the liver, indeed OATP are also localised in the kidney, gastrointestinal tract and they can also pass through the bloom-brain barrier. The discovery of these new targets explains the damage caused by MCs on both renal and gastrointestinal functions and its hypothetical role in certain neurodegenerative diseases[11].

The toxicological effect of MC-LR in mice after intra peritoneal injection corresponds to an LD_{50} (the Lethal Dose defined as the amount of a substance that kills 50 percent of treated animals) of about 50 µg/kg body weight[12]. On the contrary, the oral administration of MC-LR is less toxic and the LD_{50} is equal to 5000 µg/kg body weight, a value maybe due to a slow gastrointestinal uptake of toxins in mice[13]. An estimated No Observed Adverse Effects Level (NOAEL), the level of exposure at which there are no

reported adverse effects in treated animals, was derived following 90 days exposure of mice to MC-LR through gavage and corresponds to a value of 40 µg/kg body weight/day; while the Lowest Adverse Effect Level (LOAEL), the level of exposure at which minor hepatic damages have been observed in mice, has been calculated to be 200 µg/kg body weight/day[11]. Like MC-LR, NODs have a similar value of intraperitoneal LD_{50} in mice (50-70 µg/kg body weight) but no data are available for the oral LD_{50}, the NOAEL and LOAEL even if it is expected the values would be comparable to MC-LR.

With regard to the mechanism of action, MC-LR and NODs are inhibitors of serine/threonine-specific Protein Phosphatases 1 and 2A (PP1 and PP2A)[14]. The inhibition results in the disruption of the cytoskeleton and the subsequent cytolysis and apoptosis involving mainly the hepatocytes. All MCs congeners are able to induce a comparable inhibition of PPs *in vitro* while they differ among themselves in terms of selectivity for the OATP members resulting in a different absorption and tissue distribution of the MCs variants. Unlike the MCs which can covalently bound to the PP1 and PP2A, NODs do not, but this difference doesn't compromise their inhibitory effect. NODs, moreover, have a smaller ring-structure relative to the larger ring- structure of MC-LR which enables it to easily enter the hepatocytes and cause significant effects on the liver[15].

It has been reported that MCs and NODs can also induce the formation of Reactive Oxygen Species (ROS), reactive products belonging to the partial reduction of oxygen and involved in the induction of serious cellular damages such as genotoxicity and apoptosis. Studies regarding the mechanism leading to the MC-LR-induced ROS generation are very limited but it has been demonstrated that MC-LR may induce the oxidative stress via its link with the human liver Aldehyde Dehydrogenase 2 (ALDH2)[16]. It is also likely that this biochemical mechanism of toxicity is responsible for genotoxic and clastogenic effects of MCs.

Genotoxicity of cyanobacteria has been investigated both in bacterial and mammalian test systems. In particular, studies have been conducted with microcystic cyanobacterial extracts from field samples and/or with pure MCs extracts and the results have indicated that MCs are probably not bacterial mutagens[14]. It has been observed a huge discrepancy between data and, when positive results were obtained, the extracts showed higher cytotoxicity then the pure toxins proving that environmental contaminants could contribute to the genotoxicity of the extracts. Experiments performed on mammalian cells displayed that MCs-induced oxidative stress results in a significant increase of micronuclei formation and Desoxyribonucleic Acid (DNA) damage both *in vivo* and *in vitro*[14]. In addition, MC-LR has been reported to attenuate the Nucleotide Excision Repair (NER) and the double-stranded breaks in DNA by the Non-Homologous End Joining (NHEJ)[17]. It can therefore be concluded that the combination of the oxidative stress induction and the inhibition of pathways involved in DNA repair are two cellular mechanisms contributing to the genomic instability caused by the exposure to MCs.

Some studies suggest that MCs and NODs might act as tumor promoters. There has been evidence for tumor promotion properties of MCs from numerous animal experiments and in 2010, a working group of WHO, the International Agency for Research on Cancer (IARC), classified MC-LR as "possible human carcinogen"[18]. One of the key mechanism implied in MCs tumor promotion is the induction of hyperphosphorylated cellular proteins following the inhibition of PP1 and PP2A. In addition, the strong induction of Tumor Necrosis Factor-α (TNF- α) and proto-oncogenes such ad c-jun, c-fos, c-myc might be another possible way through which MC-LR induces carcinogenicity.

Nodularins exert effects comparable to those induced by MC-LR: it induces the expression of TNF-α, proto-oncogenes, oxidative stress, DNA damages and interferes with the DNA damage repair pathway NER[14]. However the hepatotoxin NOD has not been studied so deeply as the MC-LR and for this reason it has been classified by IARC as "animal carcinogen but not classifiable as to its carcinogenicity to humans"[18]. The New Zeeland is the only country to have set a PMAV for NODs in drinking water (1ug/L)[8].

2.1.2 Cytotoxins: Cylindrospermopsin

Cylindrospermopsin (CYN) (Figure 3) is a cyanobacterial cytotoxin enclosing a tricyclic guanidine group which is linked through a hydroxyl bridge to a uracil moiety potentially responsible for the toxicity. In addition to this chemical structure, two other variants of the toxin are known; 7-epi-CYN which is characterised by having a different orientation of the hydroxyl group close to the uracil ring and 7-deoxy-CYN which is missing of the oxygen adjacent to the uracil moiety[19]. The congener 7-epi-CYN shows severe toxicity similar to CYN, while 7-deoxy-CYN seems to be non-toxic[20-22].

Isolated for the first time from the cyanobacterium *Cylindrospermopsis raciborskii* in 1992, CYN is also biosynthesised by other cyanobacteria including *Anabaena bergii*, *Aphanizomenon ovalisporum* and *Raphidopsis curvata*[19]. CYN is classified as cytotoxin because it can affect both the liver (hepatotoxic) and the nervous system (neurotoxic). Similarly to Microcystins (MCs) and Nodularins (NODs), CYN is synthetised non-ribosonally by the Polyketide Synthase (PKS) and the Non-Ribosomal Peptide Synthase (NRPS) while its route of elimination is not only via the urine, but also via feces[23]. Cell membranes are impermeable to the molecule because of its high polarity even if the small size of the toxin promotes a low passive diffusion through the membranes[24]. The half-life of CYN in high purity water is more than 10 days. Furthermore, when bacterial communities were exposed to the toxin, the biodegradation of CYN was not observed even after 40 days, suggesting that this event may concur to promote the accumulation of the toxin in waterbodies.

Once in the organism, the prime target of CYN is the liver, but other organs including the kidney, spleen and lungs might be affected. Cylindrospermopsin has a late and acute toxicity as demonstrated in a study where the Lethal Dose 50 (LD_{50}) of pure CYN after intraperitoneal (i.p.) administration to mice was 2.1 mg/kg bw after 24 hours and decreased ten times after 120-144 hours[11]. When extracts were intraperitoneally injected to mice, the LD_{50} was 0.18 mg/kg alkaloid equivalent after 7 days while its oral injection showed a lower toxicity in the range of 4.4-6.9 mg/kg alkaloid equivalent maybe caused by a different toxicokinetic[11].

The No Observed Adverse Effect Level (NOAEL) and the Lowest Observed Adverse Effect Level (LOAEL) values for the pure CYN were determined in a study where the toxin was administrated at different doses to mice by daily gavage for 11 weeks. The NOAEL value of 30 μg/kg bw per day and the LOAEL of 60 μg/kg bw per day were based on kidney toxicity and on the dose-related and statistically significant increase in relative kidney weight[25]. A dose dependent increase of the liver weight was also observed even if the kidney was the more sensitive organ to the toxin. Based on this NOAEL, a value of 1 μg/L was proposed in Australia and New Zeeland as a guideline for maximum concentration of CYN in drinking water while in Brazil the guidance value has been fixed at 15 μg/L[8].

Cylindrospermopsin exerts its main action by inhibiting the protein synthesis, a mechanism which may lead to cell death[26]. A genotoxic, clastrogenic and aneugenic activity for CYN has been also observed after the metabolic activation of the toxin by cytochrome P-450 enzymes. Indeed, in metabolically competent cells and also in *in vivo* experiments, CYN exposure may leads to micronucleus induction, tumor initiation, fetal toxicity, Desoxyribonucleic Acid (DNA) strand breaks and chromosome loss. In addition, CYN can induce stress responses in human cell lines, presumably due to the damage to cellular components, causing the activation of p53 target genes[27]. The involvement of p53 in CYN toxicity needs to be investigated mainly because of the important role the protein has in growth arrest, DNA repair processes and apoptotic cell death. The exact mechanism through which CYN promotes carcinogenity is not well understood and the toxin was not evaluated by the World Health Organization (WHO); moreover in the Environmental Protection Agency's (EPA) guidelines for carcinogen risk assessment there is inadequate information to assess carcinogenic potential of CYN. All the experimental results underline the potential danger of this molecule and more studies should be a

priority for future research in order to understand its genotoxic and carcinogenic potential.

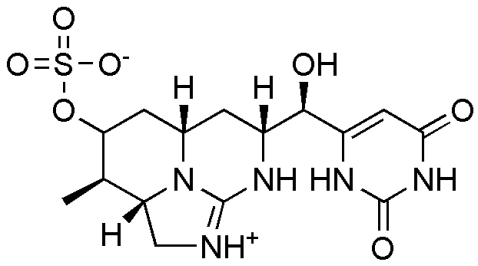


Figure 3: Chemical structure of Cylindrospermopsin (CYN).

2.1.3 Neurotoxins: Anatoxin-a, Anatoxin-a(s), Saxitoxins and β-N-methylamino-L-alanine

Anatoxin-a (ATX-a) (Figure 4) is a bicyclic alkaloid whose variant, the homoanatoxin-a (hATX-a) has a chemical structure varying from ATX-a by the methylation of the carbon at the ketone structure. These two compounds show the same toxicological properties and are considered two potent neuromuscular blocking agents. ATX-a, in particular, acts by binding the acetylcholine receptors at the same position as acetylcholine and affects muscle junctions causing uncoordinated muscle contractions, respiratory dysfunctions and paralysis[28,29]. ATX-a is produced by various genera of cyanobacteria including *Anabaena*, *Planktothrix* and *Aphanizomenon*[30,31]. The toxin is water-soluble and not stable at pH>10 and under sunlight. After ingestion, the toxin is rapidly absorbed from the gastrointestinal tract, distributed to various body tissues including the brain and subsequently degraded.

The toxicity of ATX-a has been estimated at 250–375 μg/kg bw by intraperitoneal (i.p.) injection into mice, while the oral Lethal Dose 50 (LD_{50}) is >5000 μg/kg bw. Moreover a higher toxiticity has been obtained through intravenous injection (i.v.) of the toxin (LD_{50}<60 μg/kg bw)[32,33]. The neurotoxin hATX-a shows a similar toxicity to ATX-a with a LD_{50} value around 330 μg/kg bw[34]. At present, no No Observed Adverse Effects Level (NOAEL) value has been proposed for these two toxins even if a research by Fawell et al. (1999) suggested a guide value of 1 μg/L for ATX-a in drinking water[29]. In Canada, the Provisional Maximum Concentration (PMAC) for ATX-a in drinking water is 3.7 μg/L, while in New Zeeland the Ministry of Health has set a Provisional Maximum Value (PMAV) of 6 μg/L[8].

Concerning the teratogen effects exerted by ATX-a, there are no evidences of fetal abnormalities or maternal toxicity after treatment of gravid mice with the toxin. Experiments performed on male mice treated intraperitoneally with ATX-a for 7 days indicate a toxic effect on testes and sperm counts[35]. No genotoxic data are available for these two toxins.

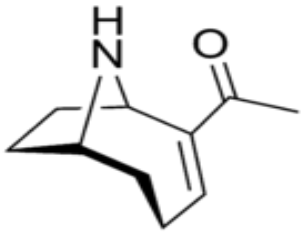


Figure 4: Chemical structure of Anatoxin-a (ATX-a).

Anatoxin-a(s) (ATX-a(s)) (Figure 5) is a guanidinemethyl phosphate ester that is similar in structure to synthetically produced organophosphate-based insecticides. This toxin was isolated from the freshwater cyanobacteria *Anabaena flos-aquae* and its production has been associated only with *Anabaena* strains. The mechanism of ATX-a(s) toxicity is the inhibition of acetylcholinesterase activity at the neuromuscular junction resulting in a persistent muscle stimulation, paralysis and death due to respiratory arrest[36].

There are no structural variants of ATX-a(s). The suffix "(s)" stands for salivation factor and refers to a rapid onset of excessive salivation in animals poisoned with ATX-a(s). Its high affinity for the human erythrocyte acethylcholinesterase indicates that this toxin may represent a possible risk for human health. The ATX-a(s) shows high instability: it is inactivated at temperature higher than 40°C and at pH>7. The Lethal Dose 50 (LD_{50}) of this molecule is 20-40 µg/kg bw in intraperitoneally injected mice and there are no data about oral administration[37-39]. No mutagenic and genotoxic activities have been observed for the ATX-a(s) and only in one study it has been demonstrated to cause chromosomal disruptions in human lymphocytes at the high concentration of 0.8 µg/mL. There are no guidelines for ATX-a(s) in drinking water but the New Zeeland has established a Provisional Maximum Value (PMAV) of 1ug/L for this neurotoxin[8].

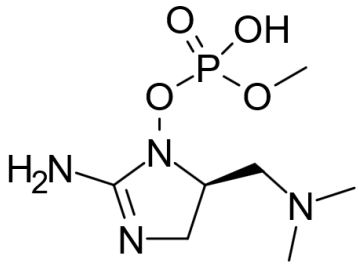


Figure 5: Chemical structure of Anatoxin-a(s) (ATX-a(s)). (Source: National Library of Medicine)

Saxitoxins (STXs) (Figure 6) are a family of potent neurotoxins with a chemical structure which share tricyclic backbone with different chemical side-chains. These molecules have been identified and characterised in both freshwater cyanobacteria and marine dinoflagellates.

More than 57 congeners have been described. All STXs share the same mechanism of action but differ in toxicity, with STX resulting the most toxic[40]. This molecule binds to the voltage sodium channel in neuronal cells with great potency blocking the nervous transmission and causing nerve dysfunction with death occurring from paralysis of respiratory muscles[41,42]. It can also inhibit calcium and potassium channels in excitable cells thereby affecting the production of action potentials which can cause fatal cardiac arrhythmias[43,44].

Evidences of the bioaccumulation of this toxin in shellfish and molluscs make STXs very dangerous especially for animals and human health. The Lethal Dose 50 (LD_{50}) of STXs is 10 µg/kg after intraperitoneal injection[45]. No directives for STXs are reported for drinking water but a value of 3 µg STX eq/L is recommended in Australia, Brazil and New Zeeland[8].

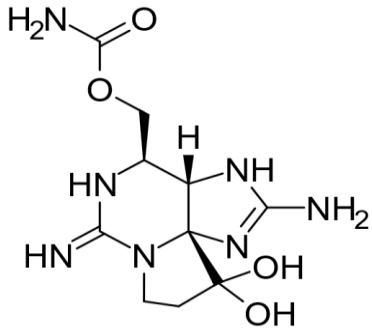


Figure 6: Chemical structure of Saxitoxins (STXs).

β-N-methylamino-L-alanine (BMAA) (Figure 7) is a neurotoxic non-protein amino acid whose occurrence has been reported in a wide variety of cyanobacterial strains. Once in the organism, BMAA acts on motor neurons by fixation on glutamate receptor and it is involved in mechanisms inducing oxidative stress[46]. This toxin has been proposed to contribute to neurodegenerative diseases such as Parkinson's and Alzheimer's because of its ability to cause intraneuronal accumulation of misfolded proteins, a characteristic of neurodegenerative disorders[3]. Experiments in mice suggest that the administration of BMAA during the neonatal period causes a cognitive injury in adult mice while impaired locomotor ability and hyperactivity have been observed in the behaviour of neonatal rats in which the BMAA has been mainly localised in the brain, hippocampus, striatum, brainstem, spinal cord and cerebellum[47,48].

The BMAA toxicity is controversial; data obtained in *in vivo* and *in vitro* studies underline a low toxic potential of BMAA even if this toxin can potentiate neuronal injury induced by other insults at concentrations as low as 10 µM[49]. Overall, however, the paucity of available data does not allow to derive a Lethal Dose 50 (LD_{50}) or a No Observed Adverse Effects Level (NOAEL) value for this toxin. No guidelines have been suggested for BMAA in drinking water.

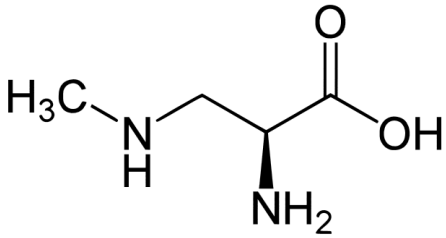


Figure 7: Chemical structure of β-N-methylamino-L-alanine (BMAA).

2.1.4 Dermatoxins: Lypopolysaccharide, Lyngbyatoxin, Aplysiatoxin

Lypopolysaccharide (LPS) is the main molecular component of the outer membrane layer of Gram-negative bacteria where it can act as a first line defence barrier. LPS consists of three different domains: a glycolipid portion called Lipid A connected by a polysaccharide core region to a glycan with an O-specific polysaccharide. The LPS molecules are also part of the outer membrane of cyanobacteria and their structural differences respect to the classic Gram-negative LPS seem to be responsible of their lower toxicity in comparison to common LPS as demonstrated in experiments both *in vitro* and *in vivo*[50].

When a pathogen infect the host, the binding between the Gram-negative LPS and the Toll-like Receptor 4 (TLR4) which is present on the surface of many immune cells, is responsible for eliciting an immune response characterised by the release of cytokines and the activation of various cells like monocytes and macrophages[51]. At present, the exact mechanism of action of the cyanobacteria LPS is not known. Many studies have tried to understand its biological activity; in addition to the studies suggesting an involvement of this molecule in causing allergy or respiratory and skin diseases, the LPS isolated from the *Oscillatoria planktothrix* has shown an antagonist effect on the TLR4-mediated immune activation[51].

Further studies will be necessary to better understand the biological properties of the several cyanobacterial LPSs in order to understand the particular hazards for human and environment but similar efforts are also required to better investigate the possible use of the LPS isolated from selected cyanobacterial strains in the prevention of anti-inflammatory diseases.

There is no much information about the Lethal Dose 50 (LD_{50}) value in mice injected with LPS extracts and the few data available indicate a variation between 40 and 190 mg/kg bw even if in some experiments the mortality of some animals has been reached with a value of 250 mg/kg bw[52].

Lyngbyatoxin (LT) is an indole alkaloid first isolated in 1912 in Hawaii from the benthic cyanobacterium *Lyngbya majuscula* during an epidemic episode of acute contact dermatitis. This cyanotoxin is slightly lipophilic and its penetration as a percentage dose in guinea pig and human skin was respectively 23% and 6.2% after one hour of topical exposure. This observation indicates that during bathing activity, the body's exposure to the water contaminated with *Lyngbya majuscula* might cause serious concerns.

There are three different isoforms of Lyngbyatoxin: A (LTA), B (LTB) and C (LTC)[53]. The isoforms LTB and LTC have 1/200th and 1/20th the activity of LTA (Figure 8) in inhibiting the Tumor Promoter ^{3}H-12-*O*-Tetradecanoylphorbol-13-Acetate (TPA). Lyngbyatoxins also act like a tumor promoter by binding to Protein Kinase C (PKC), a cellular kinase involved in a wide variety of cellular processes including carcinogenesis, differentiation and development[54-56].

The intraperitoneal (i.p.) Lethal Dose 50 (LD_{50}) value in mice for LTA is 250 µg/kg bw. No environmental concentration of LT have been assessed yet[53].

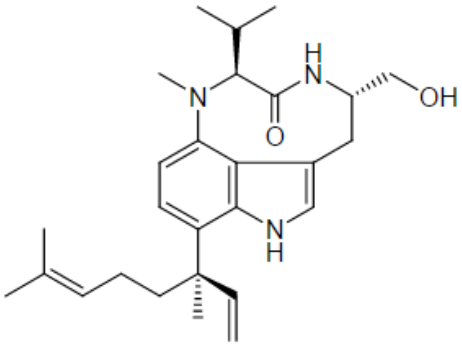


Figure 8: Chemical structure of Lyngbyatoxin A (LTA).

Aplysiatoxin (APX) (Figure 9) is a phenolic bislactone whose intraperitoneal (i.p.) Lethal Dose (LD_{50}) value in mice has been reported as 100-120 µg/kg bw[19]. It was identified in strains of *Lyngbya majuscula,* in the sea hare *Stylocheilus longicauda* which is known to feed on *Lyngbya majuscula* and in the red alga *Gracularia coronopifolia.* Aplysiatoxin*, as with* Lyngbyatoxin (LT), is the causative agent of severe contact dermatitis and is a potent tumor promoter which exerts its effects through the activation of Protein Kinase C (PKC)[57]. Environmental concentration of APX have not been explored yet.

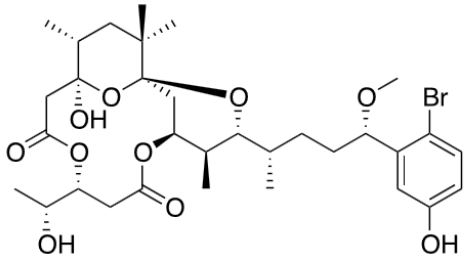


Figure 9: Chemical structure of Aplysiatoxin (APX).

3. Cyanotoxins: effects on human health

Cyanotoxins have been associated to adverse health effects in humans, which may be exposed to these compounds through different routes: oral, inhalation or dermal.

In a recent review, data collected from year 1800 to present and regarding the incidents in humans following the exposure to cyanotoxins, have shown that recreational activities are responsible for half of the reported episodes[58]. The exposure modes to different cyanotoxins in recreational settings include the accidental ingestion of contaminated water (in particular when cyanobacteria bloom or form scum), the skin-contact with the infected waterbodies and the inhalation of aerosolised cyanotoxins.

Sometimes these exposures have resulted in severe headache, fever, pneumonia, vertigo and myalgia[11,59]. A unique case of death was reported in United States of America (USA) where a teenager accidently ingested the water from a golf-course pond contaminated with the Anatoxin-a (ATX-a). Although the toxin was detected in his blood,

the unusually long time between exposure and death (48h) raised doubts about this event[60]. A recent incidents caused by the exposure to cyanotoxins through recreational activities have been described in Argentina; a young jet-skier accidentally had a prolonged exposure (approximately two hours) to water contaminated with cyanobacteria and few hours later reported nausea, abdominal pain and fever while respiratory distress and liver damage were diagnosed some days after the exposure[61].

Inhalation of aerosolised cyanotoxins is another risk for humans. Even if a study performed in New Zealand has shown that aerosols collected for 4, 12 and 24 hours close to two lakes did not constitute a direct hazard to humans, the aerosolised toxins could represent a risk if combined with other exposure sources such as the oral one[62].

Among all the existing cyanobacteria, the marine *Lyngbya majuscula* has been shown to be highly toxic to human health. The toxins released by some harmful varieties of *L. majuscula* have been reported to cause symptoms of rashes, blistering, skin and eye irritation not only on exposed area of the body but also on the part covered by the swimsuit[63]. In addition, burning of the upper gastrointestinal tract after ingestion was documented[64]. Some description of allergic responses due to cyanobacteria have also been published and they have been associated to the action of Lipopolysaccharide (LPS) endotoxins and in particular to the Lipid A, the LPS component considered responsible for toxic effects[65,66]. However, as the Lipid A moiety has not been clarified, at present it is not possible to confirm its toxic potential.

In temperate zones, the chronic exposure to cyanotoxins is improbable while it is higher in areas often affected by cyanobacterial blooms. Considering the incidents associated with the recreational activities, the World Health Organization (WHO) has defined some guidelines in order to protect human health form cyanobacterial blooms. The value of 200.000 cells/ml has been fixed as a cyanobacterial density corresponding to a low probability of adverse effects while the probability of a serious danger for bathers ingesting a small volume of water or the cyanobacterial scum has been considered very high at densities of about 100.000 cells/ml[67].

Exposure to cyanobacterial toxins through consumption of drinking water has also caused human poisonings. In 1990, in Australia, after the addition of copper sulphate to cyanobacteria-infested water, 140 children and 10 adults were hospitalised for liver and kidney damage[68]. The etiological agent subsequently identified was the cyanobacterium *Cylindrospermopsis raciborskii*[69]. Some years later, in Brazil, 2000 cases of gastroenteritis and 88 deaths were reported and attributed to a bloom of Anabaena and Microcystis in the water supply[70]. Another study documented 100 human deaths at Lake Embu, in Kenya, attributable to cyanotoxins[71]. Although there have been reported several episodes of human poisonings associated with drinking water contaminated by cyanotoxins, at present, the first confirmed and most severe episode in human occurred in Brazil when surface waters contaminated with cyanobacteria were used for hemodialysis, causing the death of at least 50 people[72].

Most of the studies have not sufficient data to directly correlate harmful effects in human with exposure to cyanobacteria, making difficult to evaluate the risk associated to cytotoxins. This is also the case of episodes of hepatocellular carcinoma and colorectal cancers described in nations like China, United States and Serbia, where the consumption of potentially contaminated water with cyanobacteria has been hypothesised to be the main cause of the high incidence of tumors in those countries[73-75]. In 2006, the International Agency for Research on Cancer (IARC) established that there were inadequate epidemiological data in humans for the carcinogenicity of Microcystin-LR (MC-LR). However, since there are strong evidences suggesting an

involvement of cyanotoxins in tumor promotion, the IARC concluded that MC-LR is a possible carcinogen for humans, classifying it as a group 2B carcinogen.

Cyanotoxins require additional attention also because of the high levels of Microcystins (MCs) (sometimes higher than the provisional guidance value) reported in Blue Green Algae Supplements (BGAS), food supplements containing blue-green algae and generally used as natural products for their supposed beneficial effects such as losing weight during hypocaloric diets or elevating mood for people with depression. These BGAS are mainly obtained from *Spirulina* spp. and *Aphanizomenon flos-aquae*, usually collected from the natural environment or artificial ponds where potentially toxic cyanobacteria can be present and cause BGAS contamination. A human fatality regarded a woman who died after the chronic consumption of BGAS and the death was probably caused by the contamination of BGAS by MCs even if a direct link was not demonstrated in the study[76].

Humans can also be exposed to cyanobacteria through the consumption of aquatic organisms which can bioaccumulate the toxins in their tissues and transfer them through the food web to wildlife and humans. Microcystins are mainly accumulated in the hepatopancreas of the shellfish and molluscs while, in fish, they can be predominantly detected in viscera. The maximum concentration of MCs in the edible parts of fish, crustaceans and mussels has been reported to reach levels of 300, 2700 and 16,000 µg/kg, respectively[11]. The European Regulation No. 853/2004[*] indicates specific hygiene rules for the monitoring of toxins (Saxitoxins – STXs-, Amnesic Shellfish Poison –ASP-, okadaic acid, yessotoxins and azaspiracids) presence in the bivalve molluscs before their placing on the market. The limit value for STX has been set at 800 µg/kg.

Many cases of intoxication and deaths in humans have been attributed to the ingestion of marine organisms infected with STXs, a neurotoxin associated with the disease known as Paralytic Shellfish Poisoning (PSP). Every year, more than 2000 cases of human poisoning through fish or shellfish consumption are reported worldwide with a mortality rate of 15%[77]. Saxitoxin is one of the most potent marine biotoxin and when ingested in high concentration this toxin can induce rapid symptoms ranging from tingling of the extremities to respiratory paralysis and death[78]. The disease outcome depends on the timeliness of medical cares including artificial respiration, gastric lavage and fluid therapy. When an individual survive 12 hours post-ingestion, all symptoms resolve rapidly although it generally takes 24 hours to completely depurate the blood from the chemical.

Consumption of meat could represent an additional way of exposure to cyanotoxins, however, data on the accumulation of MCs in livestock (sheep, cow etc.) after ingestion of contaminated water reveal that no trace of cyanotoxins are present into milk or meat[79,80]. Instead, with regard to vegetables, studies conducted with water infested with blooms or scums and used to water rice, rape and lettuce have showed the presence of cyanotoxins in plant extracts. It is important to point out that cyanobacterial cells can be removed from vegetables following the washing procedures and that when broccoli were irrigated with water containing MCs at a concentration of 1-10 µg/L, corresponding to levels typically found in surface water, the toxins were detected only in the roots, not therefore endangering human health[81].

[*] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs.

4. Methods for detection of cyanobacteria and cyanotoxins in environmental samples

4.1 Microscopy analysis

Microscopy analysis have been traditionally employed for the monitoring of cyanobacterial communities in waterbodies. Their use enables the taxonomic analysis of microorganisms present in a cyanobacterial bloom and the assessment of cyanobacterial relative abundance in a water ecosystem. The main disadvantage of this technique is the impossibility to distinguish between toxic and non-toxic cyanobacteria, not allowing the risk assessment of a bloom.

4.1.1 Cell counting

Cyanobacteria bloom monitoring is often performed by microscopy analysis. Epifluorescence and inverted light microscopy (Figure 10), using sedimentation chamber, are the most recurrent methods. Epifluorescence analysis of samples stained with selective dyes allows identification and quantification of cyanobacteria species and discrimination between autotrophic and heterotrophic cells. Autotrophic cells, namely cyanobacteria, can be analysed according to Haas method, using proflavine dye at 0.25% (w/v)[82]. Heterotrophic cells can be analysed using Hobbie's method, where orange acridine orange at 1% (w/v) is the suggested dye[83]. Epifluorescence based analysis requires immediate sample fixation, with glutaraldehyde at 2% (v/v), and processing within 24 hours. Sample preparation comprises staining of samples directly in dark membrane filters. Samples must be treated and preserved in dark, having a life spam quite short when exposed to light sources, but can be stored at -20°C, for long periods, until analysis. Autotrophs are counted using green light source (515 to 560 nm) while for heterotrophs is required a blue light source (420 a 490 nm). Assuming a homogeneous distribution of the cells, a total of 20 fields must be analysed, including a minimum of 200 cells, to warrant representativeness[84]. The density of each organism (cell.L^{-1}) is calculated based on the number of specific cells counted per microscope field area and sampled volume. Inverted light microscopy, using sedimentation chamber, allows determination of phytoplankton community relative abundance. This method is used for analysis of phytoplanktonic communities both in fresh and seawaters. The standard method for identification of phytoplankton using sedimentation chambers was presented in 1958 by Utermöhl and is still part of national monitoring programs[85,86]. Samples used for inversion microscopy analysis can be long preserved in dark conditions using Lugol-based solutions, such as acid Lugol[87], in dark conditions. Samples are allowed to sediment for 24 hours before analysis. Similarly to the quantification by epifluorescence microscopy, 20 fields or a minimum of 200 cells should be counted for representativeness. This method allows identification of organisms to the genus, or even species, by specialised technicians. Relative abundance of specific cell groups or species, in cells per mL, is also determined according to the target organisms' amount per analysed area and sample volume. The preferential magnification used is not suitable for analysis of small cells, reason for which epifluorescence methods are more accurate for identification and quantification of cyanobacteria. On the other hand, inversion microscopy uses higher sample volumes being representative of the total phytoplanktonic community.

Both techniques have the disadvantage of inaccuracy. Optical microscopy has a resolution limit of 200 nm, which difficult the identification of small cells. The presence of dense colonies may give inaccurate cell estimations as well as non-homogeneous distribution of the cells. Heterogeneous distribution of cells promotes biased estimation

of cell abundance. On the other hand, aggregation state of some cyanobacterial colonies, namely Microcystis, does allow its identification at species level, based on morphology[88]. Moreover, microscopy analysis does not allow identification of toxic species or prediction of blooms toxic potential.

Other methods, such as flow cytometry, can be used for phytoplankton analysis. Flow cytometry has higher sensitivity than the microscopy approach. This application allows the identification of smaller cells without, however, giving any taxonomic information about the organisms present in a cyanobacterial bloom[89].

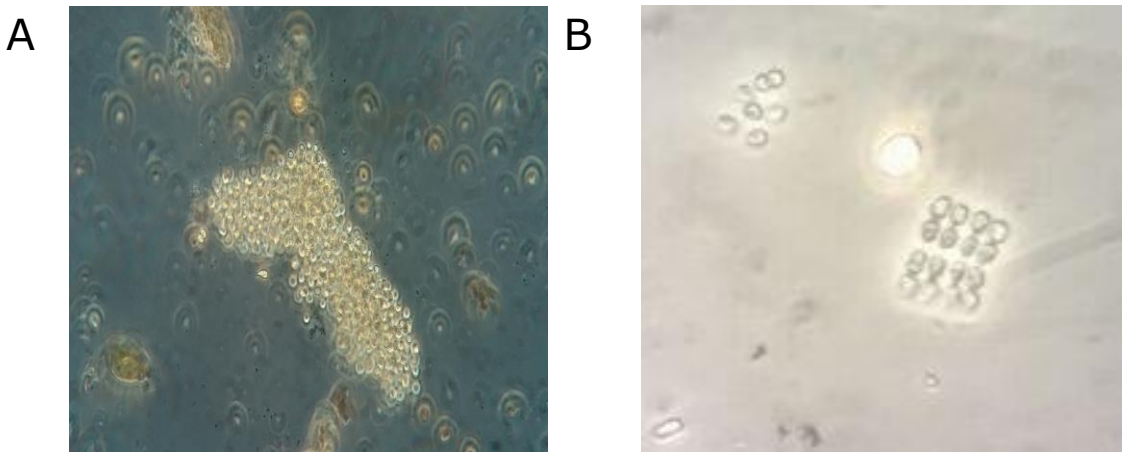


Figure 10. Microcystis and Merismopedia colonies. Microcystis (A) and Merismopedia (B) colonies present in a water sample and visualised with an inverted light microscope. (Image courtesy of Chiara Facca)

4.2 Physicochemical Methods

Physicochemical parameters including weather, nutrients availability or presence of photopigments can be used to evaluate the growth condition of phytoplankton in waterbodies[90]. The weather seems to have an impact on blooms promoting their manifestation through the modification of the seasonal patterns. The effects of climate variations include warming of air and water which added to the input of nutrients in waterbodies concur to increase the incidence of blooms. Photopigments are differently distributed among the planktonic community providing valuable information on the relative abundance of different organisms in an ecosystem (Table 2). Phytoplankton community dynamics can be assessed by pigments quantification both through spectrophotometry or fluorometry. Bloom-driven parameters including radiation, water temperature, pH, salinity and dissolved oxygen can be assessed *in situ* by multi-parametric probes.

Table 2: **Photosynthetic pigments distribution among potentially toxic phytoplanktonic phyla** (adapted from Dring, 1992 and Fietz et al., 2004[91,92])

Phylum	Accessory pigments
Cyanobacteria	Chlorophyll *a*, phycocyanin, phycoerythrin
Bacillariophyta	Chlorophyll *a*, chlorophyll *c*, fucoxanthin
Dynophyta	Chlorophyll *a*, chlorophyll *c*, diatoxanthin, diadinoxanthin, peridinin

4.2.1 Pigments quantification by fluorometry and spectrophotometry

Pigments quantification can be used to determine phytoplankton abundance in water samples by chlorophyll *a* analysis. Chlorophyll *a* is frequently analysed in limnology. This pigment is present in many organisms and therefore is a good indicator of total autotrophic phytoplankton[91,92]. It can be extracted in methanol or acetone and quantified by fluorometry. Chlorophyll *a* quantification by fluorometry, usually expressed in µg/L, is determined by the signal frequency at a specific emission wavelength. Quantification is based on a calibration curve built at different concentration of chlorophyll *a*, previously quantified by spectrophotometry[93]. Incomplete extraction, presence of other pigments or degradation products are some of the possible technical errors to account for in this method. Quantification of chlorophyll *a* based on acidification methods is also known to be misleading in the presence of chlorophyll *b*, requiring exhaustive sample preparation in order to remove the contaminant pigment[94]. Furthermore, the analysis is constrain by the amount of collected samples.

Pigments quantification can be assessed *in situ* using multi-parametric probe analysis and compared to benchtop analysis, allows to obtain a more exhaustive set of data along the entire water column. Such methods are based on the on-line analysis of pigments achieved through fluorometric sensors which are available for quantification of chlorophyll *a* and cyanobacteria-specific pigments (phycocyanin and phycoerythrin). Phycocyanin is the most dominant accessory pigment of freshwater cyanobacteria species while phycoerythrin dominates in marine ecosystems. Such pigments can be analysed in real time allowing the estimation of total cyanobacteria biomass[95,96,97]. Real-time quantification of pigments can overcome the false low quantification due to vertical migration of cyanobacteria on the water column reducing as well the length of analysis, the use of chemical substances and the improvement of detection specificity for cyanobacteria, both in freshwater and drinking water. Similarly to the determination of chlorophyll *a*, the pigment concentration per cell is variable and linked to the species and stage of the cell cycle. *In situ* analysis also poses limitations, like the presence of suspended material or the lack of calibration standards[98].

Spectrophotometric quantification of pigments can be performed according to different methodologies. It can be based on the relative absorbance intensity at a specific wavelength[93]. Similarly to fluorometry, this technique is influenced by the presence of other pigments[93]. Spectrophotometric analysis of pigments reveals lower sensitivity, however it is a fast and straightforward method.

4.2.2 Other parameters

Physicochemical parameters such as radiation, water temperature, pH, salinity and dissolved oxygen are used to evaluate the growth conditions of phytoplankton in waterbodies[90].

Water temperature and oxygen give an indication of the water column stratification which were shown to be correlated with the cyanobacteria blooms[99,100]. Limiting nutrients for phytoplankton growth such as nitrogen and phosphate could be an indicator of blooms. These parameters can influence the eutrophication process, an important problem in aquatic ecosystems caused by an excess input of nutrients which have been shown to contribute to the bloom events[3,101].

Multi-parametric probes represent the most frequent way to analyse the series of physicochemical parameters. These probes, when regularly maintained, allow accurate long-term analysis as well as data reliability. For a deeper investigation, additional sensors for chemicals (chloride, nitrate and ammonia) and pigments detection can be coupled.

4.3 Molecular-based methods

Microscopy techniques have always allowed to identify and quantify cyanobacterial species, however their main disadvantage is the impossibility to distinguish toxic from nontoxic cyanobacteria. In addition, these approaches require a significant expertise in taxonomy. The specificity, reliability and speed of molecular methods could overcome these limitations providing data about the detection and quantification of cyanobacteria and the toxins encoding genes. Due to the high sensitivity of molecular based techniques, they enable the early warning of toxic cyanobacteria in water which can be detected long time before the manifestation of cyanobacterial blooms, so representing a useful monitoring method. These molecular approaches include the Polymerase-Chain Reaction (PCR), the quantitative Real-time Polymerase Chain Reaction (qPCR) and the Desoxyribonucleic Acid (DNA) Microarray (DNA chips).

4.3.1 Polymerase-Chain Reaction (PCR)

The conventional Polymerase-Chain Reaction (PCR) is a widespread technique usually used to detect the expression of a gene by using primer pairs that target a specific sequence (Figure 11). Concerning the cyanobacteria, a first reaction is usually performed to identify their presence in the water samples. This is commonly achieved by amplifying the 16S ribosomal Ribonucleic Acid (rRNA), a gene generally used for the identification of prokaryotes[102]. In the case of cyanobacteria, the primers have been designed to target properly the 16S conserved region and the result is the exponential amplification of the gene of interest that can be easily visualised on the agarose gel. The PCR is also widely used to identify toxins-producing cyanobacterial genera. Most of the primers developed so far have been designed for targeting the Microcystin (MC) gene cluster (*mcy*) but the toxicological evaluation of these microorganisms includes all the main cyanotoxins, nodularins, cytotoxins and neurotoxins thus making the PCR a technique broadly used in toxins detection studies[102,103]. In addition to the conventional PCR which analyse one single gene per reaction, the most recent multiplex PCR enables to amplify more than one sequence in a single test run[104]. This PCR approach has been used to simultaneously identify different genes associated with Cylindrospermopsin (CYN) production or to distinguish MC-producing from non-producing *Microcystis* strains[105,106]. For this purpose, specific primers have been used to amplify different segments of the MC synthetase *mcy* cluster during the same reaction[106]. The multiplex PCR has been also used to target the 16S rRNA and the locus *mcy*A in order to identify the contamination of dietary supplements with the toxic *Microcystis*[107]. In the light of this, the multiplex PCR may be considered a valid method to give an immediate indication on the toxic potential of

cyanobacterial blooms which may represent a useful information in monitoring waterbodies by local authorities.

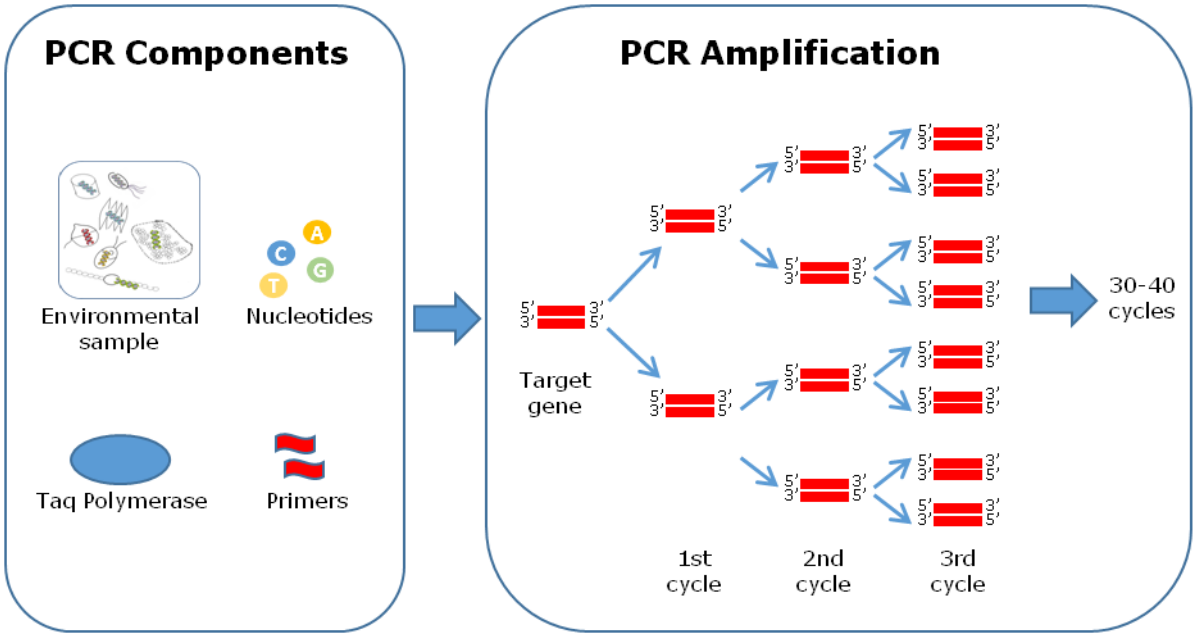


Figure 11. Schematic representation of the Polymerase-Chain Reaction (PCR) technique. During a PCR reaction, the Desoxyribonucleic Acid (DNA) is first denatured and subsequently, if the gene of interest is present in the environmental sample, the primers can bind to the end of the target sequence and act as a trigger to enable the Taq Polymerase to syntethise the complementary strand. Each PCR cycle doubles the quantity of the specific sequence and after 30-40 cycles the total amount of the amplified product can be analysed and used for other experimental procedures.

4.3.2 Quantitative Real-time Polymerase Chain Reaction (qPCR)

The quantitative Real-time Polymerase Chain Reaction (qPCR) is a technique based on the amplification of a specific gene sequence and the quantification of the reaction product in gene copy numbers. Compared to the conventional Polymerase-Chain Reaction (PCR), qPCR is a quantitative method and is much more sensitive with a detection limit of 8.8 cells per millilitre of *Microcystis* spp. and 258 cells per millilitre of *Cylindrospermopsis raciborskii*[108,109]. This technique allows the detection and quantification of cyanobacteria-specific 16S Ribosomal Ribonucleic Acid (rRNA) genes, such as *Mycrocystis*-specific rRNA gene. The presence of toxic cyanobacteria cells in water samples has been reported in a study where the abundance of toxic Microcystis has been investigated in western Lake Erie using primers highly specific for that conserved gene region[110]. The qPCR is also used to detect toxins produced by cyanobacteria, even if most of the protocols are just related to a restricted number of toxins such as Microcystins (MCs)[102,111,112]. Using the qPCR approach, the detection of the genetic fragment of interest is determined by the increasing amount of the specific sequence, amplified exponentially every cycle while the quantification of fluorescent molecules allows indirect determination of the amount of target sequences (Figure 12). Notwithstanding the considerable advantages, the qPCR has some limitations among which the reduction of the amplification efficiency with the increase in length of the reaction product. In addition, the presence of an amplified toxin-encoding gene does not

always correspond to the transcription and translation of the product by cyanobacteria. For this reason, qPCR data need to be integrated and validated with other methods like chemical and biochemical techniques.

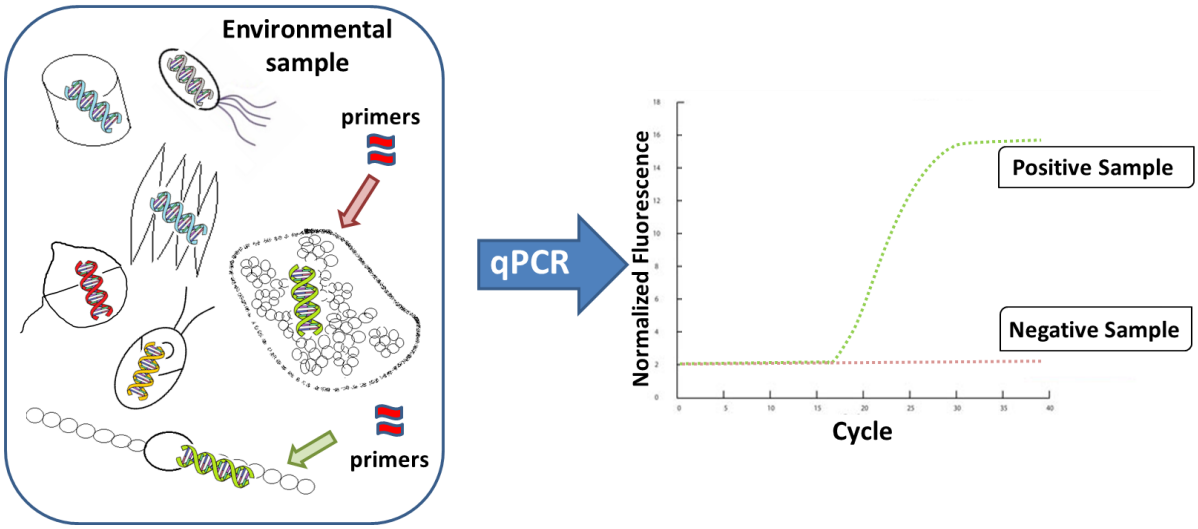


Figure 12. Schematic representation of the quantitative Real-Time Polymerase Chain Reaction (qPCR) method. The qPCR implies the use of short sequences (primers) which target specific microorganisms or group of microorganisms. In the left panel, the Desoxyribonucleic Acid (DNA) sequences of cyanobacteria are represented in green. Universal primers targeting the cyanobacteria-specific 16S Ribosomal Ribonucleic Acid (rRNA) will anneal exclusively with that specific sequence, giving a positive (fluorescent) signal during the analysis. On the other hand, DNA from other organisms, such as bacteria (in purple), diatoms (in blue), green algae (in orange) or dinoflagellates (in red), will not anneal and, consequently, no fluorescence signal will be recorded upon qPCR analysis (negative sample). (Image courtesy of Teresa Lettieri)

Two different methods are usually used for PCR quantification: SYBR Green and TaqMan (Figure 13)[113,114].

SYBR Green was the first method developed for quantitative PCR analysis. It consists of a fluorescent molecule able to intercalate double stranded Desoxyribonucleic Acid (DNA). In this case the primer sets are designed as the ones for classic PCR analysis, amplifying regions higher than 100 nucleotides. A disadvantage of this process could be the unspecific binding which may occur during amplification.

In the TaqMan system a third short sequence, called probe, is added to the sample. This probe is complementary to a sequence located within the primers and contains a fluorophore at the 5' and a quencher at 3' ends. The quencher acts as a fluorescent suppressor when near the dye. Once the polymerase hydrolyses the probe sequence, during *de novo* synthesis, the quencher-fluorophore distance increases and the fluorescent signal can be detected. The amplicon is shorter in this system, below 100 nucleotides, and is highly specific.

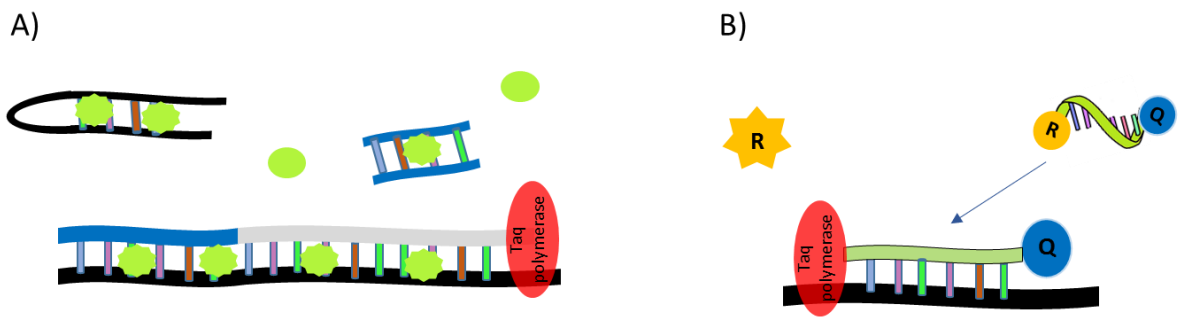


Figure 13. TaqMan and SYBR Green Real-time Polymerase Chain Reaction (qPCR)-based assays. A) *De novo* synthesis (in grey) occurs when the Taq Desoxyribonucleic Acid (DNA) polymerase (in red) binds to a double stranded region, made by the denaturated template sequence (in black) and the specific primer (in blue). SYBR Green I dye (in green) has low fluorescence signal in solution although it increases once it binds to a double stranded DNA sequence. Dimerization of primers or formation of loops can promote false readings. The signal intensity is directly proportional to the quantity of amplicons, increasing at each extension cycle. B) TaqMan probes (in green) are short nucleotide sequence with a reporter (R, in yellow) and a quencher (Q, in blue) placed in opposite extremities. The quencher masks the fluorescent signal of the proximal reporter. The Taq DNA polymerase (in red) extends the primer until it reaches the probe position. The polymerase has exonuclease activity which hydrolyses the probe (in green) from 5' to 3', releasing the reporter. The detachment of the reporter increases the fluorescence signal. (Image courtesy of Teresa Lettieri)

4.3.3 Microarray

Microarray is a technique used to make a screening of gene expression on a genomic scale. The gene profile is analysed in a quickly and efficient manner by using a chip, a solid surface on which are located many spots, each containing picomoles of a specific Desoxyribonucleic Acid (DNA) sequence, defined as probes. The hybridisation of labeled targets to the probes provide quantitative information on the relative abundance of nucleic acids in the sample (Figure 14)[115]. This technique can be adopted to perform gene expression analysis by using as target the Ribonucleic Acid (RNA) properly retrotranscribed into Complementary Desoxyribonucleic Acid (cDNA) or to identify genetic variations such as Single Nucleotide Polymorphisms (SNP) or mutations. In this case the starting sample is the genomic DNA. The advantage of the microarray approach is the possibility to simultaneously analyse the expression of a large number of genes thus making the microarray a good tool for high throughput analysis. The use of microarray for the study of cyanobacteria is reported in a paper where the authors have designed a chip containing 16S ribosomal Desoxyribonucleic Acid (rDNA) probes. This array has been useful to detect different strains of cyanobacteria: Phormidium, Microcystis, Planktothrix, Anabaena, Aphanizomenon and Nostoc in eight Norwegian lakes[116]. The DNA-chip method has been also developed to detect simultaneously the Microcystin (MCs) (*mcy*E) and Nodularin (NOD) synthetase genes (*nda*F) expressed by five hepatotoxin-producing cyanobacteria genera: *Anabaena, Microcystis, Planktothrix, Nostoc and Nodularia*[117]. In addition, a technology combining the immobilisation of 16S rDNA probes specific for five cyanobacterial genera (*Anabaena* spp., *Microcystis* spp., *Nostoc* spp., *Oscillatoria* spp., and *Synechococcus* spp.) with the magnetic-capture hybridisation technique (MAG-microarray) which uses Bacterial Magnetic Particles (BMP) has allowed identification of cyanobacteria above mentioned[118]. Even if the use of microarray for the detection of cyanobacteria diversity is quite recent, this method shows the great potential to rapidly identify toxic organisms, to perform a large-scale

screening of microbial communities and provide instantaneous information on cyanobacterial biomass. Despite high costs involved in the use of this method, it may be adopted as a great monitoring strategy to forecast cyanobacterial blooms.

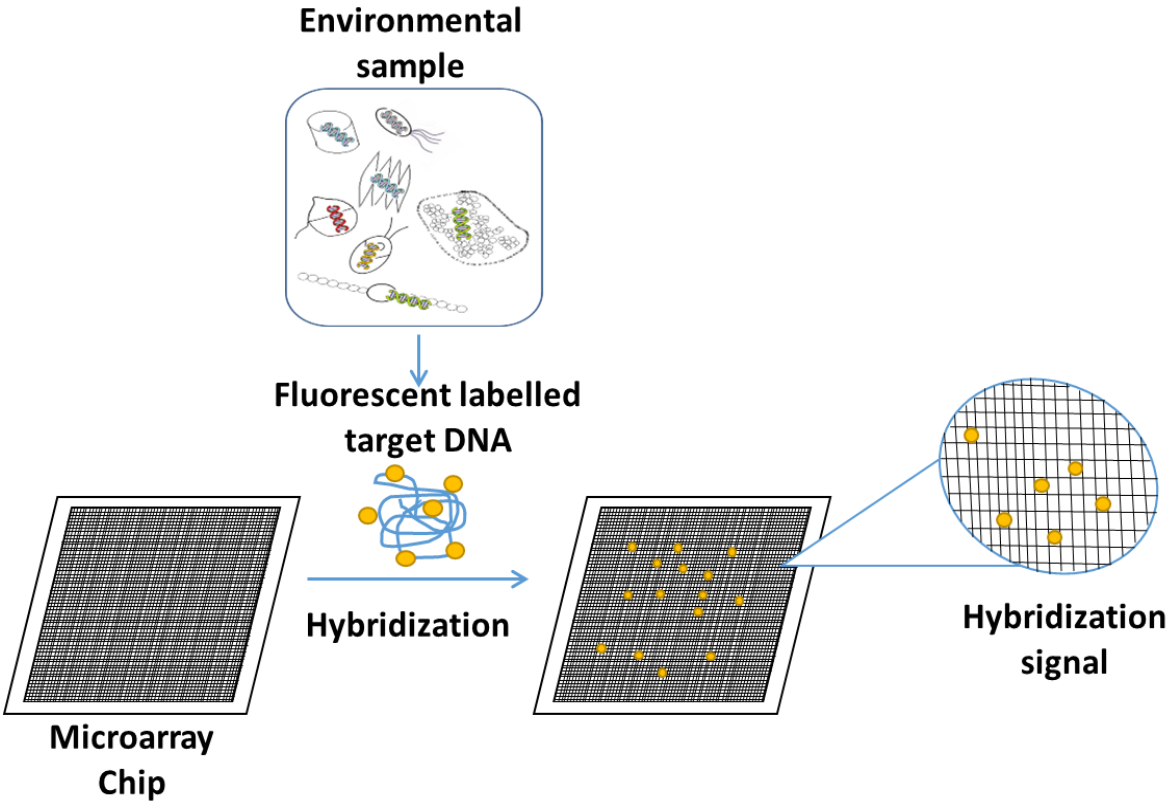


Figure 14. Schematic representation of the Microarray technique. Starting from an environmental sample, the Desoxyribonucleic Acid (DNA) of interest is amplified, fluorescently labelled and hybridised to a microarray chip. When the target sequence recognises the probes immobilised on the chip surface, a fluorescent hybridisation signal can be visualised, so providing information about the detected genes and their levels of expression.

4.4 Biochemical-based methods

Biochemical methods represent an easy and cost-effective approach for rapid monitoring of cyanotoxins concentration. Among these techniques, the two most commonly used are the Enzyme-Linked Immunosorbent Assay (ELISA) and the Protein Phosphatase Inhibitor Assay (PPIA). Their application in cyanotoxin detection is complementary to other approaches such as molecular based methods and provides a confirmation on the toxins presence in waterbodies.

4.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The Enzyme-Linked Immunosorbent Assay (ELISA) is an antibody based assay which can be either monoclonal, specific for a certain molecule variant, or polyclonal, recognising several types of molecules[119,120]. Enzyme-Linked Immunosorbent Assays are rapid, sensitive, and a low level of expertise is required. They are useful as primary quantitative screening tools with limits of detection around 0.1 µg/L, but they are susceptible to interferences that limit their use for quantitative analyses. Antibodies have been generated with variable cross-reactivities against one of the Microcystin (MC) variants, the Microcystin-LR (MC-LR) and successfully used to determine the MC content of environmental samples. Also commercial kits are available for determining MCs in water samples and to quantify other toxins like Nodularin (NOD), Cylindrospermopsin (CYN), Saxitoxin (STX) and β-N-methylamino-L-alanine (BMAA)[103,120-123]. Enzyme-Linked Immunosorbent Assay tests can be made both with freshly collected and preserved samples, allowing to detect both extracellular and intracellular toxins released in the supernatant and guaranteeing both flexibility and cost reduction. The Figure 15 shows in a schematic way one example of the ELISA assay: the competitive direct ELISA.

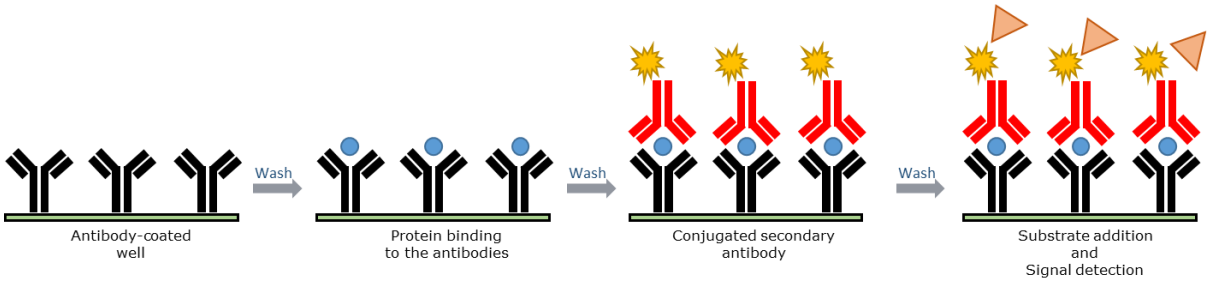


Figure 15. Schematic representation of the Enzyme-Linked Immunosorbent Assay (ELISA) technique. In the ELISA test, the antibodies are first immobilised on a 96-well plate. When the environmental sample is added to the plate, antigens present in the supernatant can bind to the specific antibodies. After the excess of protein is washed away, the enzyme-linked secondary antibody can itself recognise the protein. The subsequent addition of the substrate converts the enzyme into coloured product. The intensity of the colour formation is proportional to the amount of protein contained in the sample.

Table 3 summarises the ELISA methods for the detection of MCs, NODs and STXs

Table 3: Enzyme-Linked Immunosorbent Assay (ELISA) methods for the analysis of cyanotoxins

Cyanotoxin	Detection limit (µg/L)	Extraction / sample preparation	Location	Reference
Microcystins Nodularin	1	n.a.	Drinking water	124
Microcystins Nodularin	Not given	Filtration and sonication	Freshwater and brackish water (Finland)	125
Microcystin LR	Not given	Sonication; SPE	Lake water, China	126
Microcystins	Not given	Lyophilisation	Lake water, New Zealand	127
Saxitoxin	n.a.	Solid phase adsorption toxin tracking (SPATT)	Coastal waters (California, USA)	128
Saxitoxin	0.02 µg/L	n.a.	Arctic freshwater ecosystems	129
Microcystins	0.12	n.a.	Tap and drinking water (China)	122
Microcystins	Not given	Filtration	Constance Lake, Canada	130
Microcystins Nodularin	0.1	n.a.	Surface and drinking waters (Greece)	123
Microcystins in water and mussels	0.1 µg	Lyophilisation	Puget Sound estuary, USA	131
Microcystins in water, sediment, fish, and bloom	Not given	Water: SPE (C18) Fish: Lyophilisation Sediment: Lyophilisation	Lake Eğirdir (Turkey)	132
Microcystin LR in clams	0.1 ng/mL	SPE	Coastal ponds (Sardinia; Italy)	133
Microcystins	0.05	n.a.	River Ganga, India	134

4.4.2 Protein Phosphate Inhibition Assay (PPIA)

Since Microcystins (MCs) and Nodularins (NODs) are potent inhibitors of protein phosphatase, these toxins can be detected using a biochemical Protein Phosphatase Inhibition Assay (PPIA), which is a simple, rapid, sensitive and reproducible colorimetric method[135]. Inhibition of eukaryotic protein phosphatases is an established assay as an indicator of toxin concentration. Measurements are made using (a) radio-isotopic techniques based on 32P radiolabelled substrates and (b) colorimetric assays based on substrates such as p-nitrophenyl phosphate[121,136,137]. The former method is dependent on radiolabelled proteins and therefore, not suitable for routine monitoring[90]. Before incubation with the relevant substrate, the enzyme is exposed to an aliquot of the sample containing the toxin. Measuring the absorbance of the mixture at a specific wavelength allows the detection of the substrate (or its transformation product) and the assessment of the enzyme activity, which is inversely proportional to the concentration of the toxin. According to the method employed, PPIA can ensure toxin detection within a few hours for a large number of samples. Such procedure allows the quantification of Microcystin-LR (MC-LR) with a detection limit reaching 0.01 µg/L[135]. However, PPIA cannot distinguish co-occurring variants of MCs and cannot distinguish MCs and NODs. Therefore, results are often expressed as equivalent MC-LR/L. In addition, when analysing bloom-containing water, interferences with unknown compounds leading to overestimation or underestimation of toxin concentration should be considered. Moreover, since PPIA detects only MCs and NODs, further analysis should be undertaken to detect other cyanotoxins potentially occurring in the sample[3]. Depending on the class of MCs, the PPIA shows different sensitivities to different classes of toxin, it provides a measure of relative toxicity and an identification of the toxins is not possible[3,121,127,135,136,138]. A commercially available PPIA test in 96-well microplate format is available[139].

Table 4 shows the analytical PPIA methods for the determination of MCs and Nodularin.

Table 4: Protein Phosphatase Inhibition Assay (PPIA) methods for the analysis of Microcystins (MCs) and Nodularins (NODs)

Cyanotoxin	Detection	Extraction /	Location	Reference

	limit	sample preparation		
Microcystins	0.2	Freeze drying	Australia	136
Microcystins Nodularin	Not given	SPE with C18	Lake water (Ireland)	140
Microcystins	0.2	Lyophilisation	Lake water, New Zealand	127
Microcystins	0.01	n.a.	Water supply reservoirs in Belo Horizonte (Brazil)	135
Microcystins	0.2	n.a.	Surface and drinking waters (Greece)	123
Microcystins Nodularin	Not given	n.a.	Lake Marathonas (Greece)	139
Microcystins (variants)	Not given	Freeze drying	Lake des Oiseaux, Algeria	141

4.5 Chemical methods

The chemical methods used for determining cyanotoxins in waters are mostly liquid-based separations (High Performance Liquid Chromatography - HPLC) combined with an Ultraviolet (UV), fluorescence or mass spectrometric detector. However, identification based on UV absorbance or fluorescence alone is not sufficient because of the presence of other compounds with similar absorbance spectra (lack of specificity). Today, Nuclear Magnetic Resonance (NMR) and several different mass spectrometric techniques like Triple Quadrupole Mass Spectrometry (LC-MS/MS), Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) MS, and Liquid Chromatography - Time-of-Flight (LC-TOF) MS with high sensitivity and selectivity for the unequivocal identification and quantification of different cyanotoxins are available. The chemical techniques usually used to analyse cyanotoxins are described in the following paragraphs. Microcystins (MC) and Nodularin (NOD) are among the most studied cyanotoxins in the chemical field, while no publications about the chemical analysis of Lipopolysaccharide, and Aplysiatoxin could be found in the literature.

4.5.1 Chemical analysis in water

4.5.1.1 High-performance Liquid Chromatography (HPLC) – Ultraviolet (UV)

In Liquid Chromatography (LC) the cyanotoxin analytes are chromatographically separated by a liquid under high pressure in the LC columns which are packed with very small particles (2–50 µm in size). Ultrahigh Pressure (or performance) LC (UHPLC) uses even smaller particles (sub 2 µm) and smaller columns leading to faster analysis with higher separation efficiency. High Performance Liquid Chromatography coupled with a Photodiode-Array (PDA) detector for Ultraviolet (UV) absorbance was in the past the most commonly used technique for analysing cyanotoxins with the UV absorption maxima of Microcystins (MCs) between 222 and 238 nm[130,142,143].

Microcystins can be separated using both gradient elution and isocratic mobile phases; although a wider range of toxin variants can be resolved when a gradient is employed[144]. Usually Reversed-Phase (RP) HPLC is used but very polar substances such as Saxitoxins (STXs) or β-N-methylamino-L-alanine (BMAA) are better separated by Hydrophilic Interaction Liquid Chromatography (HILIC)[145-149]. Today, RP-HPLC is, in contrast to normal-phase HPLC, due to its wide applicability to many different substances the most often applied HPLC method.

In normal-phase liquid chromatography, the stationary phase is more polar than the mobile phase. The retention increases as the polarity of the mobile phase decreases, and thus polar analytes are more strongly retained than nonpolar ones. The opposite situation occurs in reversed-phase liquid chromatography where the separation is based on both adsorption and partition chromatography. The stationary phase (column) used in RP-HPLC is based on modified silica gel to which hydrophobic alkyl chains (mainly C18) are attached. In contrast, HILIC is a variant of normal-phase HPLC that partly overlaps with other chromatographic applications such as ion chromatography and reversed phase liquid chromatography. Hydrophilic Interaction Liquid Chromatography uses hydrophilic stationary phases with reversed-phase type eluents and provides an alternative approach to effectively separate small polar compounds on polar stationary phases[150].

However, identification based on UV absorbance alone is not sufficient because of the presence of other compounds with similar absorbance spectra. The diode-array detector lacks specificity for cyanotoxins and is prone to interferences from other analytes. In addition, the lack of standards for many MC variants makes identification difficult, therefore results are generally expressed as Microcystin-LR (MC-LR) equivalents[90,140,143,144].

Table 5 summarises the HPLC-UV/PDA methods for the analysis of cyanotoxins.

Table 5: High Performance Liquid Chromatography coupled with a Photodiode-Array (HPLC-UV/PDA) methods for the analysis of cyanotoxins

Cyanotoxin	Detection limit	Extraction / sample preparation	Location	Reference
Microcystins	n.a.	n.a.	No real samples	143
Microcystins Nodularin	n.a.	Filtration and sonication	Freshwater and brackish water (Finland)	125
Microcystin-LR	n.a.	Sonication; SPE	Lake water, China	126
Microcystins Nodularin	n.a.	SPE with C18	Lake water (Ireland)	140
Anatoxin-a	n.a.	Freeze drying	Biofilms in La Loue River (France)	151
Microcystins	n.a.	Filtration	Constance Lake, Canada	130
Microcystins in water, sediment, fish, and bloom	n.a.	Water: SPE (C18) Fish: Lyophilisation Sediment: Lyophilisation	Lake Eğirdir (Turkey)	132

4.5.1.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

Today Liquid Chromatography-Mass Spectrometry (LC-MS) with electrospray ionisation, which was developed 20 years ago, is due to its high sensitivity and selectivity the standard method for the detection, identification and confirmation of cyanotoxins in environmental samples. Liquid Chromatography - Mass Spectrometry is a powerful analytical chemistry technique that combines the physical separation capabilities of LC (or High-Performance Liquid Chromatography - HPLC) with the mass analysis capabilities of Mass Spectrometry (MS). Mass Spectrometry works by ionising chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios (m/z). The ions are separated according to their mass-to-charge ratio in an analyser by electromagnetic fields, and the ion signal is processed into a mass spectra. Liquid Chromatography - Mass Spectrometry enables the simultaneous separation and identification of cyanotoxins in a mixture. Single quadrupole MS analyses only the molecular ions (m/z) of the target molecules and therefore is less selective than triple quadrupole MS.

The analysis of Microcystins (MCs), Nodularin (NOD), and Cylindrospermopsin (CYN) by the different LC-MS techniques is relatively easy and straightforward. Many different studies and applications have been published (see Tables 6).

Saxitoxins (STXs) are highly polar compounds presenting numerous isomers, and therefore their analysis is difficult. The toxicity of the different analogues can vary depending on the chemical functional groups they present. Analysis has been performed by Enzyme-Linked Immunosorbent Assay (ELISA) (Table 3) or different LC-MS methods to detect, identify and differentiate the isomers[128,129] (see Tables 6).

Analysis of Anatoxin-a (ATX-a) is difficult due to its rapid decay. Chromatographic methods coupled with Ultraviolet (UV) spectroscopic detection are not sensitive enough, and necessitate derivatisation to improve their detection limits. The coupling with MS allows for better sensitivity and selectivity; however the essential amino acid phenylalanine (PHE) is an isobaric interference (with a similar molecular weight) in MS detection of ATX-a (m/z 166). Multiple options have been described by Furey et al. (2005) to remove this interference: (i) derivatisation of ATX-a and PHE with 4-fluoro-7-nitro-2,1,3-benzoxadiazole to alter significantly the compounds' chromatographic behaviour, (ii) selective methylation of PHE with diazomethane prior to LC-MS analysis, or (iii) use of other MS techniques such as hybrid Quadrupole Time-Of-Flight (QqTOF) or Quadrupole Ion-Trap (QIT) providing respectively higher mass resolution or intrinsic mass fragmentation pathway[152]. Otherwise, sufficient chromatographic resolution of PHE from ATX-a, without derivatisation, can be achieved with HPLC or Ultrahigh Pressure Liquid Chromatography (UHPLC)[30,151,153-155]. Single quadrupole LC-MS is not able to distinguish between ATX-a and PHE[30].

Analysis of β-N-methylamino-L-alanine (BMAA) is difficult because of its small molecular structure. Only few studies have addressed the methods necessary for an accurate determination of this cyanotoxin in environmental samples. Liquid chromatography is better performed by Hydrophilic Interaction Liquid Chromatography (HILIC)[156-159]. Banack et al. (2007) described five different analytical methods to detect BMAA in marine cyanobacteria including HPLC with fluorescence, UV, and MS detection (LC-MS and triple quadrupole LC-MS/MS)[160].

Table 6 gives some selected single-quadrupole LC-MS methods for the analysis of cyanotoxins.

Table 6: Liquid Chromatography single quadrupole Mass Spectrometry (LC-MS) methods for the analysis of cyanotoxins in water

Cyanotoxin	Detection limit	Extraction/ sample preparation	Location	Reference
Anatoxin-a	0.0021 µg/L	SPE disk; on-line pre-column derivatisation	Freshwaters (Japan)	161
Microcystins	1 µg/g	Matrix solid-phase dispersion	MCs in natural water blooms and cyanobacteria strain cultures (Spain)	162
Microcystins Anatoxin-a Cylindrospermopsin Saxitoxins	n.a.	Lyophilisation	Scum forming bloom samples from Coolmunda Dam (South-east Queensland; Australia)	145
BMAA (HILIC chromatography)	5 ng	Cation-exchange resin	n.a.	156

4.5.1.3 Liquid Chromatography-Triple Quadrupole Mass Spectrometry (LC-MS/MS)

Liquid Chromatography - Triple Quadrupole Mass Spectrometry (LC-MS/MS) allows due to its excellent sensitivity and selectivity the unequivocal identification and quantification of different and also unknown toxins in environmental samples. Specific daughterons are formed in a collision cell from the precursor molecular ion and these MS/MS transitions are detected.

A characteristic fragment ion common to most Microcystins (MCs) and Nodularin (NODs) is m/z 135 (derived from the side chain ADDA)[155,163-168].

Even though Cylindrospermopsin (CYN) has a different molecular structure than the MCs and NODs, it is usually analysed together with these cyanotoxins by LC-MS/MS by applying the specific MS-MS transitions m/z 416 > 336, 274, 194[155,165,169,170]. No other analytical methods were found in the literature for CYN.

The specific MS/MS transitions for the detection of Anatoxin-a (ATX-a) are m/z 166 > 149, 131, 107, and 91[153-155].

Saxitoxins (STXs) are detected with the specific MS/MS transitions m/z 300 > 282, 204[145].

Faassen et al. (2012) compared High Performance Liquid Chromatography (HPLC)-fluorescence analysis of β-N-methylamino-L-alanine (BMAA) with LC-MS/MS analysis of both derivatised and underivatised samples and found out that HPLC-fluorescence overestimated BMAA concentrations in some cyanobacterial samples due to its low selectivity[171]. Therefore, only selective and sensitive analytical methods like LC-MS/MS should be used for BMAA analysis. During analysis BMAA may be confused with its structural isomer 2,4-diaminobutyric acid (2,4-DAB) or other compounds (amino acids). Banack et al. (2010) managed to reliably separate BMAA from 2,4-diaminobutyric acid (2,4-DAB) by reversed-phase HPLC, and confidently distinguished BMAA from 2,4-DAB during triple quadrupole LC-MS/MS analysis[172]. In the following study, the authors also investigated the separation of BMAA from other diamino acids[173]. Metcalf et al. (2008) measured BMAA in environmental samples collected between 1990 and 2004 in the United Kingdom (UK) using HPLC-fluorescence and LC–MS/MS after derivatisation[174]. The specific MS/MS transition for the detection of underivatised BMAA is m/z 119 > 102[157-159,171]. Al-Sammak et al. (2013; 2014) and Faassen et al. (2012) analysed it by LC-MS/MS also in a derivatised form[175,176].

Table 7 gives several LC-MS/MS methods for the analysis of different cyanotoxins.

Table 7: Liquid Chromatography – Triple Quadrupole Mass Spectrometry (LC-MS/MS) methods for the analysis of cyanotoxins in environmental samples

Cyanotoxin	Detection limit (µg/L)	Extraction / sample preparation	Location	Reference
Microcystins Nodularin	n.a.	Filtration and sonication	Freshwater and brackish water (Finland)	125
Microcystins Nodularin	n.a.	n.a.	Water (Finland)	177
Microcystins Anatoxin-a Cylindrospermopsin Saxitoxins	n.a.	Lyophilisation	Scum forming bloom samples from Coolmunda Dam (South-east Queensland; Australia)	145
Microcystins	n.a.	Matrix solid phase dispersion	Natural waters and blooms	162
Microcystins	0.002	Sonication; SPE	Spiked surface water (Singapore)	178
Microcystins Nodularin	0.1	SPE with C18 (use of an ion-trap-MS instrument)	Lake water (Ireland)	140

Microcystins	0.04-2.0	SPE	Lake waters (China)	164
Anatoxin-a	0.013	n.a.	Lakes Albano, Nemi, Vico (Rome; Italy)	153
Microcystins Nodularin	0.25	n.a.	Lake and tap water (Ireland)	163
Microcystins	1.3-6.0	SPE with Oasis HLB	River and Lake waters (China)	167
BMAA	n.a.	Vortex	Marine cyanobacterium cultures	160
BMAA	n.a.	Freeze drying Derivatisation	Freshwater lakes (UK)	174
BMAA (HILIC chromatography)	1 µg/g	Sonication	Cyanobacterial samples of worldwide origin	158
BMAA (HILIC chromatography)	2 pg BMAA on column	Sonication and SPE with Oasis-MCX	Freshwaters (China)	157
BMAA (HILIC chromatography)	0.38	SPE with Oasis-MCX and Strata-X-C polymeric cation-exchange cartridges	Freshwaters (China)	159
BMAA	1 µg/g	Trichloroacetic acid extraction	Cyanobacterial samples (NL)	171
Lyngbya wollei toxins (neurotoxins)	n.a.	Lyophilisation; ultrasonication (Multi-toxins method)	Benthic macroalgae in fluvial lakes of the St. Lawrence River (Canada)	179
BMAA Anatoxin-a	0.8-3.2	Cation-exchange SPE (Oasis-MCX)	Water, fish and plants (Nebraska reservoirs; USA)	175,176
Microcystins Anatoxin-a Cylindrospermopsin	MC-LR: 1.0 Anatoxin	Freezing and thawing of cells	Surface waters in Wisconsin (USA)	169

	-a: 0.5 Cylindrospermopsin: 0.5			
Microcystins	0.06-0.2	SPE with C18	Lake waters (China)	180
Microcystins Anatoxin-a Cylindrospermopsin	Microcystins: 0.002-0.009 Cylindrospermopsin: 0.3	SPE	Lake waters (Italy)	181
Microcystins Anatoxin-a Cylindrospermopsin	n.a.	Sonication	Lake waters (USA)	155
Microcystins Nodularin	< 0.02	SPE	Surface and drinking waters (Greece)	123
Anatoxin-a Homoanatoxin-a	n.a.	Solid phase adsorption toxin tracking (SPATT)	Waipoua River (New Zealand)	182
Microcystins Nodularin	0.002	SPE with Oasis HLB	Lake Marathonas (Greece)	139
Microcystins Anatoxin-a Cylindrospermopsin	n.a.	Sonication	Fresh and brackish waters (Germany)	170
Microcystins Anatoxin-a Cylindrospermopsin	0.01-0.02	On-line SPE	Lakes in Canada	165
Microcystins (variants)	n.a.	Sonication	Lake des Oiseaux, Algeria	141
Microcystins (variants)	0.003-0.03	SPE	Lake Occhito, Italy	183

4.5.1.4 Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF)

Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) is a rapid, selective and sensitive technique, with high resolution, and allows exact mass measurements and detection of compounds based on molecular formula. Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight is a mass spectrometric method in which an ion's mass-to-charge ratio is determined via a time measurement. Modern software allows high-throughput, multiplexed analyses. Microcystins (MCs) are peptides and as such readily susceptible to detection with MALDI-TOF MS. It can provide considerable support to High Performance Liquid Chromatography (HPLC) by identifying cyanotoxins in very small samples such as single cyanobacteria colonies not available as purified standards, and it provides the molecular mass of all of the peptides and MC variants present. Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight is essentially an off-line technique, which in some ways is a disadvantage, as sample preparation in combination with chromatographic techniques can be automated only to a small degree. This method is usually utilised only for qualitative detection and identification of toxin congeners, and not for quantification of the substances[166,184-187].

Although the presence of cell components does suppress MC signals to some degree, the polar variants exhibit sufficiently low detection limits (0.023-0.071 µM) to permit use with field samples when a typical particulate collection protocol is used. Howard and Boyer (2007) filtered in their field work 2-20 L of water to collect the cyanobacterial cells, which were then extracted in 10 mL of solvent to obtain the MCs. This provides a 200- to 2000-fold concentration factor, sufficient to detect MCs at levels as low as 0.012 μg/L[184].

Table 8 gives few MALDI-TOF- methods for the analysis of cyanotoxins.

Table 8: Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) methods for the analysis of cyanotoxins

Cyanotoxin	Detection limit	Extraction / sample preparation	Location	Reference
Microcystins	n.a.	Lyophilisation; sonication	Tamega River (Portugal)	186
Microcystins Nodularin	0.015 µM	n.a.	n.a.	184
Microcystins	3 ng	HP thin layer chromatography	Spiked water samples	185

4.5.1.5 Liquid Chromatography – Time-of-Flight Mass Spectrometry (LC-TOF-MS)

Unlike single quadrupoles, ion traps or tandem mass spectrometers, Liquid Chromatography - Time-of-Flight Mass Spectrometry (LC-TOF-MS) has the capability and advantage to produce exact mass measurements thus providing good selectivity in complex sample matrices. Furthermore, the detection of known compounds based on molecular formula and exact mass chromatogram extraction are possible even if the standard reference is not available.

Table 9 gives few LC-TOF-MS methods for the analysis of different cyanotoxins.

Table 9: Liquid Chromatography - Time-of-Flight Mass Spectrometry (LC-TOF-MS) methods for the analysis of cyanotoxins in environmental samples

Cyanotoxin	Detection limit	Extraction / sample preparation	Location	Reference
Saxitoxin	0.1 µg/g	SPE (C18)	n.a.	188
Microcystins Nodularin Anatoxin-a	1 µg/L	SPE disks	Water samples from little River near Clermont (USA)	189
Anatoxin-a	2 µg/L	Lyophilisation; methanol	Cyanobacteria samples from Lake Caragh (Ireland)	190
Microcystins	Microalgae samples: 0.1 µg/g; Water: 0.1 µg/L	Microalgae capsules: ultrasonic bath; Water: no sample concentration	MCs in microalgae dietary supplement products and water (Switzerland)	191
Anatoxin-a	n.a.	Freeze drying	Biofilms in La Loue River (France)	151
Lyngbya wollei toxins (neurotoxins)	n.a.	Lyophilisation; ultrasonication (Multi-toxins method)	Benthic macroalgae in fluvial lakes of the St. Lawrence River (Canada)	179
Saxitoxin (Chromatography by RPLC and HILIC)	0.6 ng	Ultrasound-assisted extraction	Different lipophilic and hydrophilic toxins (China)	149

4.5.1.6 Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is an analytical technique that exploits the magnetic properties of certain atomic nuclei and provides detailed information about the structure of molecules. Nuclear Magnetic Resonance is not a useful analytical method for the quantitative analysis of cyanotoxins in environmental samples. Nevertheless, Moura et al. (2009) developed a Proton Nuclear Magnetic Resonance (1H-NMR) method for the determination of β-N-methylamino-L-alanine (BMAA) in environmental aqueous samples[192]. The BMAA was extracted from samples by means of Solid-Phase Extraction (SPE) and identified and quantified by 1H-NMR without further derivatisation steps. The Limit Of Quantification (LOQ) was 5 mg/L. Dagnino and Schripsema (2005) published a 1H-NMR method for the quantification of microgram quantities of Anatoxin-a (ATX-a) in standard solutions for the analysis of biological samples[193].

A new family of cyanobacterial peptides (Micropeptin 1106 and 1120) was discovered in 2014 by Isaacs et al. (2014) using Liquid Chromatography Mass Spectrometry (LC-MS) and NMR[194]. These new micropeptin cyanopeptides were discovered to be released *by Microcystis Aeruginosa* blooms in North Carolina's Cape Fear River and were discovered while carrying out a detailed chemical analysis of Microcystins (MCs) following two large algal blooms. Along with these new micropeptins, Microcystin-LR (MC-LR) and RR (MC-RR) were also present, with the RR form found at 3 times that of LR. Levels of the new micropeptins were comparable to the MCs. While their biological activity and potential environmental effects are not yet fully determined, their high levels suggest they should be further investigated.

4.6 Biota analysis

Many studies have shown that cyanotoxins accumulate in a wide range of aquatic organisms including seafood consumed by humans, indicating a potential threat to human health. Due to the high toxicity of cyanotoxins to humans, their analysis is very important in biota samples. Health hazards of Microcystins (MCs) lead the World Health Organization (WHO) to establish for Microcystin-LR (MC-LR) a provisional guidelines value of 1 µg/L in drinking water and a provisional tolerable daily intake for sea food of 0.04 µg/kg body weight/day[131-133,195]. Commission Regulation No. 853/2004 [†] laying down specific hygiene rules on the hygiene of foodstuffs gives for the control of the Saxitoxin (STX)-group toxins in the European Union (EU) a limit value for Paralytic Shellfish Poison (PSP) of 800 µg/kg in bivalve molluscs. This limit corresponds with most limits established in countries outside the EU (Australia; USA; Canada)[196]. Microcystins are not regulated in (sea) food.

Analysis of cyanotoxins in biota samples have been performed by biological methods such as the *in vivo* mouse bioassay, other assays, immunoassays (enzyme-linked immunosorbent assay-ELISA), and chemical methods[197,198].

Commission Regulation 2074/2005 [‡] lays down the mouse bioassay as the reference method for detecting STX in sea food. In addition to biological testing methods, alternative detection methods, such as chemical methods and *in vitro* assays, should be allowed if it is demonstrated that the performance of the chosen methods is at least as effective as the biological method and that their implementation provides an equivalent

[†] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for on the hygiene of foodstuffs.

[‡] Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004

level of public health protection. Provision should be made for the replacement of biological tests as soon as possible[199].

The PSP mouse bioassay corresponds to the Association of Official Analytical Chemists (AOAC) official method 959.08 and has been in use for more than fifty years in many Member States. Its detection limit is approximately 400 µg STX equivalents/kg shellfish tissue, which is 50% of the current Maximum Residue Limit (MRL), and it has a precision of 15–20%. The method states that, initially, one, but preferably two or three mice are injected intraperitoneally with 1mL of an acid extract (pH 2–4) of shellfish[198,200,201]. This method is painful to the animals, stipulates death as an endpoint, and requires large numbers of mice, and therefore should be avoided. Moreover, it only provides a total toxicity value and no information about the specific toxin profile of a sample, and underestimates the real STX concentrations[197,202].

Functional fluorescent methods based on the use of membrane potential dyes have also been developed for the detection of STX[203,204].

Moreover, antibody-based or receptor binding biochemical/immunological methods (e.g. ELISA) have been developed for the analysis of STX in biota (sea food) samples, which can be used by shellfish farmers and by processors in end-product testing[205-207]. The lowest limit of detection obtained by an ELISA test was 0.1 µg/kg of shellfish tissue[208,209]. Based on this principle, a number of test kits are commercially available.

ELISAs have also been applied for the screening of Microcystins in seafood tissues[132,133,209-212] (Table 10).

In addition, a receptor-binding assay, initially described by Vieytes et al. (1993) and further developed by Doucette et al. (1997), is now available in a radioactively-labelled isotope format, which shows very good precision and robustness, as well as an excellent comparability with the animal test[198,213-215].

Chemical analytical methods are based on Liquid Chromatography (LC) followed by fluorescence or Mass Spectrometric (MS) detection.

A pre-chromatographic oxidation LC-fluorescence method has been collaboratively studied, and was approved by AOAC International in June 2005 and as a European CEN standard[216-218]. The correlation with mouse bioassay data was high. Also a post-column oxidation method was developed, which became the AOAC International method 2011.02[197,219]. The two methods have been officially validated by the AOAC with interlaboratory studies and both are capable of detecting PSP toxins within the regulatory limit, 800 µg STX equivalents/kg[220].

During the last years Liquid Chromatography – Triple Quadrupole Mass Spectrometry (LC-MS/MS) techniques have been developed for the analysis of STX in biota (mainly mussel) samples. Boundy et al. (2015) reported an effective desalting pre-treatment cleanup step using inexpensive graphitised carbon Solid Phase Extraction (SPE) cartridges prior to Hydrophilic Interaction Liquid Chromatography (HILIC) separation and MS/MS detection[221]. This represents a major technical breakthrough and allows sensitive, selective and rapid analysis of paralytic shellfish toxins from a variety of sample types, including many commercially produced shellfish (mollusc) species. The procedure reduced matrix effects substantially and the method was subjected to an extensive single laboratory validation study on various shellfish species as reported by Turner et al. (2015)[222]. Method sensitivity was significantly increased and detection limits were found to be similar or below those reported for both pre- or post-column oxidation LC-fluorescence methods[197].

In order to guarantee uniformity in the test procedures for marine biotoxins within the European Union (EU), a Community Reference Laboratory (CRL) has been established. The laboratory of the "Ministerio de Sanidad y Consumo" in Spain was designated as the CRL in 1993. Since 2005, the CRL has been part of the Spanish Food Safety Agency of the Spanish Ministry of Health[198].

The analysis of Microcystins in biota samples is usually performed first by an ELISA screening assay followed by LC-MS/MS confirmation. Table 10 summaries these ELISA and LC−Triple Quadrupole Mass Spectrometry (LC-MS/MS) methods for the analysis of Microcystins (and Nodularin) in biota samples.

Table 11 gives LC−Triple Quadrupole Mass Spectrometry (LC-MS/MS) methods for the analysis of STX in biota samples.

In addition, Zastepa et al. (2015) analysed cyanotoxins in sediment and pore water by LC-MS/MS (Table 12)[168].

Table 10: Enzyme-Linked Immunosorbent Assay (ELISA) Liquid Chromatography – Triple Quadrupole Mass Spectrometry (LC-MS/MS) methods for the analysis of Microcystins (and Nodularin) in (aquatic) biota

Cyanotoxin	Species	Detection limit (µg/kg)	Extraction / sample preparation	Location	Reference
Microcystins	Silverside; carp	n.a.	n.a.	Lake Patzcuaro (Mexico)	210
Microcystins Nodularin	Catfish; Swai; basa	10	Acidified acetonitrile/water (3:1) followed by hexane partitioning cleanup	Food (USA)	212
Microcystins	Mussels	n.a.	Lyophilisation	Puget Sound estuary, USA	131
Microcystin LR	Clams	2	SPE	Coastal ponds (Sardinia; Italy)	133
Microcystins in water, sediment, fish, and bloom	Fish	n.a.	Lyophilisation	Lake Eğirdir (Turkey)	132

Table 11: Liquid Chromatography – Triple Quadrupole Mass Spectrometry (LC-MS/MS) methods for the analysis of Saxitoxins (STX) in biota

Cyanotoxin	Species	Detection limit (µg/kg)	Extraction / sample preparation	Location	Reference
Saxitoxins	Shellfish	89	Acidic acetonitrile/water; cleaned-up with Oasis HLB and Carbograph activated carbon SPE columns	Norway	223
Saxitoxins	Sea food	1-20	QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe)	China	224
Saxitoxins	Oyster; mussel	0.1-4	Acetic acid/water; graphitised carbon SPE cartridges	UK and New Zealand	221
Saxitoxins	Mollusc	n.a.	SPE (C18)	Arctic Chukchi and Bering Seas	225

Table 12: Analysis of cyanotoxins in sediment and pore water by Liquid Chromatography – Triple Quadrupole Mass Spectrometry (LC-MS/MS)

Cyanotoxin	Detection limit (µg/kg)	Extraction / sample preparation	Location	Reference
Microcystins Nodularin	1.1-2.5	Accelerated solvent extraction, hydrophilic–lipophilic balanced solid phase extraction	Lakes (Canada)	168

Conclusion

Cyanotoxins are secondary metabolites produced by cyanobacteria under eutrophic conditions and/or as a result of climate changes. These anthropogenic and environmental factors can induce the rapid growth of cyanobacteria with the consequent formation of blooms which may eventually represent a serious risk of toxicity for the environment. Due to the growing recurrence of toxic blooms, cyanotoxins are considered an emerging hazard to natural reservoirs and drinking water supply. In addition, their release in waterbodies has also important repercussion on human health. Harmful effects of cyanobacteria in humans may occur after the consumption of contaminated drinking water or via the consumption of contaminated seafood. The European Regulation No. 853/2004 stated the limit values of a range of toxins in marine seafood and fixed at 800 µg/kg the maximum quantity for Saxitoxins (STX). However, to our knowledge, there are no specific guidelines and regulations for cyanotoxins in freshwater seafood both in Europe and in all the other countries and states around the globe, with the exception of California[226]. Different countries in Europe have also adopted specific national guidelines for managing cyanobacteria in drinking water and it is not clear how there are much more regulations for the exposure of cyanotoxins through drinking water than *via* food.

The best goal for water quality monitoring programs and water management is the prediction and the consequent reduction of bloom events. To achieve these aims, specific technical methods need to be used to assure the identification and quantification of the toxins in an environmental sample. Among all the cyanotoxins, Microcystin-LR (MC-LR), the most toxic and widespread hepatotoxin, is also the most studied. As showed in this report, there are a variety of analytical detection techniques for cyanobacteria or cyanotoxins including bioassays and chemical methods. The first and simplest approach usually is cell counting and pigment monitoring which are useful for initial screening of bloom samples. Cyanobacteria and cyanotoxins can be however detected and quantified through biological, chemical or biochemical approaches, each with specific benefits and limitations. The chemical methods, although largely used in the research of the cyanotoxin-contaminated samples, are characterised by high costs and the requirement of highly qualified staff. Other methods such as molecular and biochemical approaches are therefore preferred for routine water monitoring but advantages and disadvantages should be carefully evaluated for each of them. Speed, costs, effectivity, selectivity, ease of use and detection limits are of great importance in selecting the technique to be used. The integration of genomic, transcriptomic and proteomic data represents a good strategy to identify the organisms associated with the blooms and the toxins released in the waterbodies. However, many efforts have still to be made in order to improve the existing available techniques and to develop new tools for detecting toxic cyanobacteria species and species-specific cyanotoxins which are still not well investigated. The lack of comprehensive toxicity data referred to all cyanotoxins is also an important gap to overcome so as to improve cyanobacteria regulations and to make a decisive step towards recognising cyanotoxins as a risk to human health and the environment.

References

1 Sanseverino, I., Conduto, D., Pozzoli, L., Dobricic, S. & Lettieri, T. Algal bloom and its economic impact. Report No. EUR 27905 EN, (2016).

2 Boopathi, T. & Ki, J. S. Impact of environmental factors on the regulation of cyanotoxin production. *Toxins (Basel)* **6**, 1951-1978, doi:10.3390/toxins6071951 (2014).

3 Merel, S. *et al.* State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environment International* **59**, 303-327, doi:http://dx.doi.org/10.1016/j.envint.2013.06.013 (2013).

4 Blaha, L., Babica, P. & Marsalek, B. Toxins produced in cyanobacterial water blooms - toxicity and risks. *Interdiscip Toxicol* **2**, 36-41, doi:10.2478/v10102-009-0006-2 (2009).

5 EPA. United States Environmental Protection Agency. Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems. EPA-810F11001. (2014).

6 Sivonen, K. & Jones, G. Cyanobacterial toxins. In: Chorus I, Bartram J, editors. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. London, UK: E & FN Spon. 41-111 (1999).

7 EPA. California Environmental Protection Agency. Microcystins. A brief overview of their toxicity and effects, with special reference to fish, wildlife, and livestock. (2009).

8 Chorus, I. Current Approaches to Cyanotoxin Risk Assessment, Risk Management and Regulations in Different Countries. (2012).

9 Pearson, L., Mihali, T., Moffitt, M., Kellmann, R. & Neilan, B. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Mar Drugs* **8**, 1650-1680, doi:10.3390/md8051650 (2010).

10 Fischer, W. J. *et al.* Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol Appl Pharmacol* **203**, 257-263, doi:10.1016/j.taap.2004.08.012 (2005).

11 Funari, E. & Testai, E. Human health risk assessment related to cyanotoxins exposure. *Crit Rev Toxicol* **38**, 97-125, doi:10.1080/10408440701749454 (2008).

12 Fawell, J. K., James, C. P. & James, H. A. Toxins from blue-green algae: toxicological assessment of microcystin-LR and a method for its determination in water. *Marlow, UK: Foundation of Water Research (Report No. FR 0359/2/DoE 3358/2)* (1994).

13 Fawell, J. K., Mitchell, R. E., Everett, D. J. & Hill, R. E. The toxicity of cyanobacterial toxins in the mouse: I Microcystin-LR . *Human Exp Toxicol* **18**, 162-167 (1999).

14 Zegura, B., Straser, A. & Filipic, M. Genotoxicity and potential carcinogenicity of cyanobacterial toxins - a review. *Mutat Res* **727**, 16-41, doi:10.1016/j.mrrev.2011.01.002 (2011).

15 Ohta, T. *et al.* Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Res* **54**, 6402-6406 (1994).

16 Chen, T. *et al.* Identification of human liver mitochondrial aldehyde dehydrogenase as a potential target for microcystin-LR. *Toxicology* **220**, 71-80, doi:10.1016/j.tox.2005.12.001 (2006).

17 Lankoff, A. *et al.* Inhibition of nucleotide excision repair (NER) by microcystin-LR in CHO-K1 cells. *Toxicon* **48**, 957-965, doi:10.1016/j.toxicon.2006.08.003 (2006).

18 IARC. Cyanobacterial peptide toxins (2006).

19 Dittmann, E., Fewer, D. P. & Neilan, B. A. Cyanobacterial toxins: biosynthetic routes and evolutionary roots. *FEMS Microbiol Rev* **37**, 23-43, doi:10.1111/j.1574-6976.2012.12000.x (2013).

20 Banker, R., Teltsch, B., Sukenik, A. & Carmeli, S. 7-Epicylindrospermopsin, a toxic minor metabolite of the cyanobacterium Aphanizomenon ovalisporum from lake Kinneret, Israel. *J Nat Prod* **63**, 387-389 (2000).

21 Runnegar, M. T. *et al.* In vitro hepatotoxicity of the cyanobacterial alkaloid cylindrospermopsin and related synthetic analogues. *Toxicol Sci* **67**, 81-87 (2002).
22 Neumann, C., Bain, P. & Shaw, G. Studies of the comparative in vitro toxicology of the cyanobacterial metabolite deoxycylindrospermopsin. *J Toxicol Environ Health A* **70**, 1679-1686, doi:10.1080/15287390701434869 (2007).
23 Schembri, M. A., Neilan, B. A. & Saint, C. P. Identification of genes implicated in toxin production in the cyanobacterium Cylindrospermopsis raciborskii. *Environ Toxicol* **16**, 413-421 (2001).
24 Chong, M. W., Wong, B. S., Lam, P. K., Shaw, G. R. & Seawright, A. A. Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. *Toxicon* **40**, 205-211 (2002).
25 Humpage, A. R. & Falconer, I. R. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. *Environ Toxicol* **18**, 94-103, doi:10.1002/tox.10104 (2003).
26 Terao, K. *et al.* Electron microscopic studies on experimental poisoning in mice induced by cylindrospermopsin isolated from blue-green alga Umezakia natans. *Toxicon* **32**, 833-843 (1994).
27 Bain, P., Shaw, G. & Patel, B. Induction of p53-regulated gene expression in human cell lines exposed to the cyanobacterial toxin cylindrospermopsin. *J Toxicol Environ Health A* **70**, 1687-1693, doi:10.1080/15287390701434877 (2007).
28 Swanson, K. L., Allen, C. N., Aronstam, R. S., Rapoport, H. & Albuquerque, E. X. Molecular mechanisms of the potent and stereospecific nicotinic receptor agonist (+)-anatoxin-a. *Mol Pharmacol* **29**, 250-257 (1986).
29 Fawell, J. K., Mitchell, R. E., Hill, R. E. & Everett, D. J. The toxicity of cyanobacterial toxins in the mouse: II anatoxin-a. *Hum Exp Toxicol* **18**, 168-173 (1999).
30 Osswald, J., Rellan, S., Gago, A. & Vasconcelos, V. Toxicology and detection methods of the alkaloid neurotoxin produced by cyanobacteria, anatoxin-a. *Environ Int* **33**, 1070-1089, doi:10.1016/j.envint.2007.06.003 (2007).
31 van Apeldoorn, M. E., van Egmond, H. P., Speijers, G. J. & Bakker, G. J. Toxins of cyanobacteria. *Mol Nutr Food Res* **51**, 7-60, doi:10.1002/mnfr.200600185 (2007).
32 Rogers, E. H. *et al.* Potential developmental toxicity of anatoxin-a, a cyanobacterial toxin. *J Appl Toxicol* **25**, 527-534, doi:10.1002/jat.1091 (2005).
33 Astrachan, N. B., Archer, B. G. & Hilbelink, D. R. Evaluation of the subacute toxicity and teratogenicity of anatoxin-a. *Toxicon* **18**, 684-688 (1980).
34 Namikoshi, M. *et al.* Simultaneous production of homoanatoxin-a, anatoxin-a, and a new non-toxic 4-hydroxyhomoanatoxin-a by the cyanobacterium Raphidiopsis mediterranea Skuja. *Toxicon* **42**, 533-538 (2003).
35 Yavasoglu, A. *et al.* Toxic effects of anatoxin-a on testes and sperm counts of male mice. *Exp Toxicol Pathol* **60**, 391-396, doi:10.1016/j.etp.2008.04.001 (2008).
36 Mahmood, N. A. & Carmichael, W. W. The pharmacology of anatoxin-a(s), a neurotoxin produced by the freshwater cyanobacterium Anabaena flos-aquae NRC 525-17. *Toxicon* **24**, 425-434 (1986).
37 Cook, W. O. *et al.* Comparison of effects of anatoxin-a(s) and paraoxon, physostigmine and pyridostigmine on mouse brain cholinesterase activity. *Toxicon* **26**, 750-753 (1988).
38 Mahmood, W. A. & Carmichael, W. W. Anatoxin-a(s), an anticholinesterase from the cyanobacterium Anabaena flos-aquae NRC-525-17. *Toxicon* **25**, 1211-1227 (1987).
39 Matsunaga, S., Moore, R. E., Niemczura, W. P. & Carmichael, W. W. Anatoxin-a(s), a potent anticholinesterase from Anabaena flos-aquae. *J Am Chem Soc* **111**, 8021-8023 (1989).
40 Wiese, M., D'Agostino, P. M., Mihali, T. K., Moffitt, M. C. & Neilan, B. A. Neurotoxic alkaloids: saxitoxin and its analogs. *Mar Drugs* **8**, 2185-2211, doi:10.3390/md8072185 (2010).

41 Campbell, D. T. & Hille, B. Kinetic and pharmacological properties of the sodium channel of frog skeletal muscle. *J Gen Physiol* **67**, 309-323 (1976).
42 Lipkind, G. M. & Fozzard, H. A. A structural model of the tetrodotoxin and saxitoxin binding site of the Na+ channel. *Biophys J* **66**, 1-13, doi:10.1016/S0006-3495(94)80746-5 (1994).
43 Su, Z., Sheets, M., Ishida, H., Li, F. & Barry, W. H. Saxitoxin blocks L-type ICa. *J Pharmacol Exp Ther* **308**, 324-329, doi:10.1124/jpet.103.056564 (2004).
44 Wang, J., Salata, J. J. & Bennett, P. B. Saxitoxin is a gating modifier of HERG K+ channels. *J Gen Physiol* **121**, 583-598, doi:10.1085/jgp.200308812 (2003).
45 Mons, M. N., Van Egmond, H. P. & Speijers, G. J. A. Paralytic shellfish poisoning; a review. *RIVM Report 388802 005* (1998).
46 Banack, S. A., Caller, T. A. & Stommel, E. W. The cyanobacteria derived toxin Beta-N-methylamino-L-alanine and amyotrophic lateral sclerosis. *Toxins (Basel)* **2**, 2837-2850, doi:10.3390/toxins2122837 (2010).
47 Karlsson, O., Roman, E. & Brittebo, E. B. Long-term cognitive impairments in adult rats treated neonatally with beta-N-Methylamino-L-Alanine. *Toxicol Sci* **112**, 185-195, doi:10.1093/toxsci/kfp196 (2009).
48 Karlsson, O. *et al.* Neonatal exposure to the cyanobacterial toxin BMAA induces changes in protein expression and neurodegeneration in adult hippocampus. *Toxicol Sci* **130**, 391-404, doi:10.1093/toxsci/kfs241 (2012).
49 Lobner, D., Piana, P. M., Salous, A. K. & Peoples, R. W. Beta-N-methylamino-L-alanine enhances neurotoxicity through multiple mechanisms. *Neurobiol Dis* **25**, 360-366, doi:10.1016/j.nbd.2006.10.002 (2007).
50 Gemma, S., Molteni, M. & Rossetti, C. Lipopolysaccharides in Cyanobacteria: a brief overview. *Advances in Microbiology* **6**, 391-397 (2016).
51 Durai, P., Batool, M. & Choi, S. Structure and Effects of Cyanobacterial Lipopolysaccharides. *Mar Drugs* **13**, 4217-4230, doi:10.3390/md13074217 (2015).
52 Stewart, I., Webb, P. M., Schluter, P. J. & Shaw, G. R. Recreational and occupational field exposure to freshwater cyanobacteria--a review of anecdotal and case reports, epidemiological studies and the challenges for epidemiologic assessment. *Environ Health* **5**, 6, doi:10.1186/1476-069X-5-6 (2006).
53 Jakubowska, N. & Szelag-Wasielewska, E. Toxic picoplanktonic cyanobacteria--review. *Mar Drugs* **13**, 1497-1518, doi:10.3390/md13031497 (2015).
54 Fujiki, H. *et al.* Indole alkaloids: dihydroteleocidin B, teleocidin, and lyngbyatoxin A as members of a new class of tumor promoters. *Proc Natl Acad Sci U S A* **78**, 3872-3876 (1981).
55 Fujiki, H. *et al.* The third class of new tumor promoters, polyacetates (debromoaplysiatoxin and aplysiatoxin), can differentiate biological actions relevant to tumor promoters. *Gan* **73**, 495-497 (1982).
56 Fujiki, H., Sugimura, T. & Moore, R. E. New classes of environmental tumor promoters: indole alkaloids and polyacetates. *Environ Health Perspect* **50**, 85-90 (1983).
57 Jeffrey, A. M. & Liskamp, R. M. Computer-assisted molecular modeling of tumor promoters: rationale for the activity of phorbol esters, teleocidin B, and aplysiatoxin. *Proc Natl Acad Sci U S A* **83**, 241-245 (1986).
58 Wood, R. Acute animal and human poisonings from cyanotoxin exposure - A review of the literature. *Environ Int* **91**, 276-282, doi:10.1016/j.envint.2016.02.026 (2016).
59 Manganelli, M. *et al.* Emerging health issues of cyanobacterial blooms. *Ann Ist Super Sanita* **48**, 415-428, doi:DOI: 10.4415/ANN_12_04_09 (2012).
60 Stewart, I. *et al.* Epidemiology of recreational exposure to freshwater cyanobacteria--an international prospective cohort study. *BMC Public Health* **6**, 93, doi:10.1186/1471-2458-6-93 (2006).

61 Giannuzzi, L., Sedan, D., Echenique, R. & Andrinolo, D. An acute case of intoxication with cyanobacteria and cyanotoxins in recreational water in Salto Grande Dam, Argentina. *Mar Drugs* **9**, 2164-2175, doi:10.3390/md9112164 (2011).
62 Wood, S. A. & Dietrich, D. R. Quantitative assessment of aerosolized cyanobacterial toxins at two New Zealand lakes. *Journal of Environmental Monitoring* **13**, 1617-1624, doi:10.1039/C1EM10102A (2011).
63 Osborne, N. J. T., Webb, P. M. & Shaw, G. R. The toxins of Lyngbya majuscula and their human and ecological health effects. *Environment International* **27**, 381-392, doi:http://dx.doi.org/10.1016/S0160-4120(01)00098-8 (2001).
64 Osborne, N. J., Shaw, G. R. & Webb, P. M. Health effects of recreational exposure to Moreton Bay, Australia waters during a Lyngbya majuscula bloom. *Environment International* **33**, 309-314, doi:http://dx.doi.org/10.1016/j.envint.2006.10.011 (2007).
65 Stewart, I., Schluter, P. J. & Shaw, G. R. Cyanobacterial lipopolysaccharides and human health - a review. *Environ Health* **5**, 7, doi:10.1186/1476-069X-5-7 (2006).
66 Berg, K. A. *et al.* Virulence genes of Aeromonas isolates, bacterial endotoxins and cyanobacterial toxins from recreational water samples associated with human health symptoms. *J Water Health* **9**, 670-679, doi:10.2166/wh.2011.206 (2011).
67 WHO. World Health Organization. Guidelines for safe recreational water environments. Volume 1: Coastal and fresh waters. (2003).
68 Byth, S. Palm Island mystery disease. *Med J Aust* **2**, 40, 42 (1980).
69 Hawkins, P. R. & Griffiths, D. J. Artificial destratification of a small tropical reservoir: effects upon the phytoplankton. *Hydrobiol* **254**, 169-181 (1993).
70 Teixeira Mda, G., Costa Mda, C., de Carvalho, V. L., Pereira Mdos, S. & Hage, E. Gastroenteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil. *Bull Pan Am Health Organ* **27**, 244-253 (1993).
71 Harding, W. R. A research strategy for the detection and management of algal toxins in water sources. (Water Research Commission of South Africa (TT 277/06), 2006).
72 Jochimsen, E. M. *et al.* Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N Engl J Med* **338**, 873-878, doi:10.1056/NEJM199803263381304 (1998).
73 Ueno, Y. *et al.* Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* **17**, 1317-1321 (1996).
74 Fleming, L. E. *et al.* Blue green algal (cyanobacterial) toxins, surface drinking water, and liver cancer in Florida. *Harmful Algae* **1**, 157-168, doi:http://dx.doi.org/10.1016/S1568-9883(02)00026-4 (2002).
75 Svircev, Z. *et al.* Epidemiology of primary liver cancer in Serbia and possible connection with cyanobacterial blooms. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **31**, 181-200, doi:10.1080/10590501.2013.824187 (2013).
76 Dietrich, D. R., Ernst, B. & Day, B. W. 132 (In: 7th International Conference on Toxic Cyanobacteria (ICTC), Brazil, 2007).
77 Hallegraeff, G. M. *Harmful algal blooms: a global overview. In: Hallegraeff G.M., Anderson D.M., Cembella A.D. (Ed.). Manual on Harmful Marine Microalgae*. 1-22 (1995).
78 Faber, S. Saxitoxin and the induction of paralytic shellfish poisoning. *Journal of Young Investigators* (2012).
79 Orr, P. T., Jones, G. J., Hunter, R. A. & Berger, K. Exposure of beef cattle to sub-clinical doses of Microcystis aeruginosa: toxin bioaccumulation, physiological effects and human health risk assessment. *Toxicon* **41**, 613-620 (2003).
80 Feitz, A. J. *et al.* Absence of detectable levels of the cyanobacterial toxin (microcystin-LR) carry-over into milk. *Toxicon* **40**, 1173-1180 (2002).

81 Jarvenpaa, S. *et al.* Effects of microcystins on broccoli and mustard, and analysis of accumulated toxin by liquid chromatography-mass spectrometry. *Toxicon* **49**, 865-874, doi:10.1016/j.toxicon.2006.12.008 (2007).
82 Haas, L. W. Improved epifluorescence microscopy for observing planktonic micro-organisms. *Annales de l'Institut Oceanographique Paris* **58**, 261-266 (1982).
83 Hobbie, J. E., Daley, R. J. & Jasper, S. Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology* **33**, 1225-1228 (1977).
84 Venrick, E. L. Phytoplankton manual - Chapter 7.1.2 - How many cells to count? (1978).
85 Utermöhl, H. Zur Vervollkommnung der quantitativen Phytoplankton. *Methodik - Mitt. Int. Ver. Theor. Angew. Limnol.* **9**, 1-38 (1958).
86 INAG, I. P. Manual para a avaliação da qualidade biológica da água. Protocolo de amostragem e análise para o Fitoplâncton. (2009).
87 Willén, T. Studies on the phytoplankton of some lakes connected with or recently isolated from the Baltic. *Oikos* **13**, 169-199 (1962).
88 Komárek, J. & Anagnostidis, K. Spektrum Akademischer Verlag Cyanoprokaryota, Teil: Chroococcales. **1**, 224-236 (1999).
89 Rutten, T. P., Sandee, B. & Hofman, A. R. Phytoplankton monitoring by high performance flow cytometry: a successful approach? *Cytometry A* **64**, 16-26, doi:10.1002/cyto.a.20106 (2005).
90 Srivastava, A., Singh, S., Ahn, C. Y., Oh, H. M. & Asthana, R. K. Monitoring approaches for a toxic cyanobacterial bloom. *Environ Sci Technol* **47**, 8999-9013, doi:10.1021/es401245k (2013).
91 Fietz, S. & Nicklisch, A. An HPLC analysis of the summer phytoplankton assemblage in Lake Baikal. *Freshwater Biology* **49**, 332-345, doi:10.1111/j.1365-2427.2004.01183.x (2004).
92 Dring, M. J. *The biology of marine plants*. (1992).
93 (HMSO), H. M. S. O. *The Determination of Chlorophyll a in Aquatic Environments 1980. In: Methods for the estimation of waters and associated materials.*, (1980).
94 Welschmeyer, N. A. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. **39**, 1985-1992 (1994).
95 Asai, R. *et al.* Detection of phycobilin pigments and their seasonal change in lake Kasumigaura using a sensitive in situ fluorometric sensor *Analytical letters* **34**, 2521-2533 (2007).
96 Izydorczyk, K., Tarczynska, M., Jurczak, T., Mrowczynski, J. & Zalewski, M. Measurement of phycocyanin fluorescenceas an online early warning system for cyanobacteria in reservoir intake water. *Environmental Toxicology* **20**, 425-430 (2005).
97 Bastien, C. *et al.* Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria. *Journal of Environmental Monitoring* **13**, 110-118.
98 Zamyadi, A., McQuaid, N., Prévosta, M. & Dorner, S. Monitoring of potentially toxic cyanobacteria using an online multi-probe in drinking water sources. *Journal of Environmental Monitoring* **14**, 579-588 (2012).
99 Snortheim, C. A. *et al.* Meteorological drivers of hypolimnetic anoxia in a eutrophic, north temperate lake. *Ecological Modelling* **343**, 39-53, doi:http://dx.doi.org/10.1016/j.ecolmodel.2016.10.014 (2017).
100 Wagner, C. & Adrian, R. Cyanobacteria dominance: Quantifying the effects of climate change. *Limnology and Oceanography* **54**, 2460-2468, doi:10.4319/lo.2009.54.6_part_2.2460 (2009).
101 Kitsiou, D. & Karydis, M. Coastal marine eutrophication assessment: A review on data analysis. *Environment International* **37**, 778-801, doi:http://dx.doi.org/10.1016/j.envint.2011.02.004 (2011).
102 Moreira, C., Matos, A., Mendes, R. & Antunes, A. in *Plant Toxins* (eds P. Gopalakrishnakone, Célia R. Carlini, & Rodrigo Ligabue-Braun) 1-23 (Springer Netherlands, 2015).

103 Moreira, C., Ramos, V., Azevedo, J. & Vasconcelos, V. Methods to detect cyanobacteria and their toxins in the environment. *Applied Microbiology and Biotechnology* **98**, 8073-8082, doi:10.1007/s00253-014-5951-9 (2014).
104 Auckenthaler, R. & Risch, M. [Do Multiplex PCR techniques displace classical cultures in microbiology?]. *Ther Umsch* **72**, 77-85, doi:10.1024/0040-5930/a000647 (2015).
105 Fergusson, K. M. & Saint, C. P. Multiplex PCR assay for Cylindrospermopsis raciborskii and cylindrospermopsin-producing cyanobacteria. *Environ Toxicol* **18**, 120-125, doi:10.1002/tox.10108 (2003).
106 Ouahid, Y., Perez-Silva, G. & del Campo, F. F. Identification of potentially toxic environmental Microcystis by individual and multiple PCR amplification of specific microcystin synthetase gene regions. *Environ Toxicol* **20**, 235-242, doi:10.1002/tox.20103 (2005).
107 Saker, M. L., Vale, M., Kramer, D. & Vasconcelos, V. M. Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. *Applied Microbiology and Biotechnology* **75**, 441-449, doi:10.1007/s00253-006-0813-8 (2007).
108 Furukawa, K. *et al.* Highly sensitive real-time PCR assay for quantification of toxic cyanobacteria based on microcystin synthetase A gene. *J Biosci Bioeng* **102**, 90-96, doi:10.1263/jbb.102.90 (2006).
109 Moreira, C. *et al.* Application of real-time PCR in the assessment of the toxic cyanobacterium Cylindrospermopsis raciborskii abundance and toxicological potential. *Applied Microbiology and Biotechnology* **92**, 189-197, doi:10.1007/s00253-011-3360-x (2011).
110 Rinta-Kanto, J. M. *et al.* Quantification of ToxicMicrocystisspp. during the 2003 and 2004 Blooms in Western Lake Erie using Quantitative Real-Time PCR. *Environmental Science & Technology* **39**, 4198-4205, doi:10.1021/es048249u (2005).
111 Koreivienė, J. & Belous, O. Methods for Cyanotoxins detection. *Botanica Lithuanica* **18**, doi:10.2478/v10279-012-0008-4 (2012).
112 Kaushik, R. & Balasubramanian, R. Methods and Approaches Used for Detection of Cyanotoxins in Environmental Samples: A Review. *Critical Reviews in Environmental Science and Technology* **43**, 1349-1383, doi:10.1080/10643389.2011.644224 (2013).
113 Navarro, E., Serrano-Heras, G., Castano, M. J. & Solera, J. Real-time PCR detection chemistry. *Clin Chim Acta* **439**, 231-250, doi:10.1016/j.cca.2014.10.017 (2015).
114 Lettieri, T. Microbial biodiversity detection and monitoring using molecular biology tools. Report No. EUR 22600 EN, (European Commission, Joint Research Centre (JRC), 2006).
115 Pearson, L. A. & Neilan, B. A. The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. *Curr Opin Biotechnol* **19**, 281-288, doi:10.1016/j.copbio.2008.03.002 (2008).
116 Rudi, K., Skulberg, O. M., Skulberg, R. & Jakobsen, K. S. Application of Sequence-Specific Labeled 16S rRNA Gene Oligonucleotide Probes for Genetic Profiling of Cyanobacterial Abundance and Diversity by Array Hybridization. *Applied and Environmental Microbiology* **66**, 4004-4011 (2000).
117 Rantala, A., Rizzi, E., Castiglioni, B., De Bellis, G. & Sivonen, K. Identification of hepatotoxin-producing cyanobacteria by DNA-chip. *Environmental Microbiology* **10**, 653-664 (2008).
118 Matsunaga, T., Nakayama, H., Okochi, M. & Takeyama, H. Fluorescent detection of cyanobacterial DNA using bacterial magnetic particles on a MAG-microarray. *Biotechnol Bioeng* **73**, 400-405, doi:10.1002/bit.1073 (2001).
119 Zeck, A., Eikenberg, A., Weller, M. G. & Niessner, R. Highly sensitive immunoassay based on a monoclonal antibody specific for [4-arginine]microcystins. *Analytica Chimica Acta* **441**, 1-13, doi:http://dx.doi.org/10.1016/S0003-2670(01)01092-3 (2001).
120 Metcalf, J. S., Bell, S. G. & Codd, G. A. Production of novel polyclonal antibodies against the cyanobacterial toxin microcystin-LR and their application for the detection and quantification of microcystins and nodularin. *Water Research* **34**, 2761-2769, doi:10.1016/s0043-1354(99)00429-7 (2000).

121 Rapala, J., Erkomaa, K., Kukkonen, J., Sivonen, K. & Lahti, K. Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography–UV detection and enzyme-linked immunosorbent assay. *Analytica Chimica Acta* **466**, 213-231, doi:10.1016/s0003-2670(02)00588-3 (2002).

122 Sheng, J.-W., He, M., Shi, H.-C. & Qian, Y. A comprehensive immunoassay for the detection of microcystins in waters based on polyclonal antibodies. *Analytica Chimica Acta* **572**, 309-315, doi:http://dx.doi.org/10.1016/j.aca.2006.05.040 (2006).

123 Triantis, T. *et al.* Development of an integrated laboratory system for the monitoring of cyanotoxins in surface and drinking waters. *Toxicon* **55**, 979-989, doi:http://dx.doi.org/10.1016/j.toxicon.2009.07.012 (2010).

124 Fischer, W. J. *et al.* Congener-independent immunoassay for microcystins and nodularins. *Environ Sci Technol* **35**, 4849-4856 (2001).

125 Spoof, L., Vesterkvist, P., Lindholm, T. & Meriluoto, J. Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography-electrospray ionisation mass spectrometry. *J Chromatogr A* **1020**, 105-119, doi:http://dx.doi.org/10.1016/S0021-9673(03)00428-X (2003).

126 Shen, P. P. *et al.* Analysis of microcystins in cyanobacteria blooms and surface water samples from Meiliang Bay, Taihu Lake, China. *Environ Int* **29**, 641-647, doi:10.1016/S0160-4120(03)00047-3 (2003).

127 Mountfort, D. O., Holland, P. & Sprosen, J. Method for detecting classes of microcystins by combination of protein phosphatase inhibition assay and ELISA: comparison with LC-MS. *Toxicon* **45**, 199-206, doi:10.1016/j.toxicon.2004.10.008 (2005).

128 Lane, J. Q., Roddam, C. M., Langlois, G. W. & Kudela, R. M. Application of Solid Phase Adsorption Toxin Tracking (SPATT) for field detection of the hydrophilic phycotoxins domoic acid and saxitoxin in coastal California. *Limnol Oceanogr-Meth* **8**, 645-660, doi:10.4319/lom.2010.8.645 (2010).

129 Kleinteich, J. *et al.* Potent toxins in Arctic environments – Presence of saxitoxins and an unusual microcystin variant in Arctic freshwater ecosystems. *Chemico-Biological Interactions* **206**, 423-431, doi:http://dx.doi.org/10.1016/j.cbi.2013.04.011 (2013).

130 Tillmanns, A. R., Pick, F. R. & Aranda-Rodriguez, R. Sampling and analysis of microcystins: Implications for the development of standardized methods. *Environ Toxicol* **22**, 132-143, doi:10.1002/tox.20250 (2007).

131 Preece, E. P., Moore, B. C. & Hardy, F. J. Transfer of microcystin from freshwater lakes to Puget Sound, WA and toxin accumulation in marine mussels (Mytilus trossulus). *Ecotoxicol Environ Saf* **122**, 98-105, doi:10.1016/j.ecoenv.2015.07.013 (2015).

132 Gurbuz, F., Uzunmehmetoğlu, O. Y., Diler, Ö., Metcalf, J. S. & Codd, G. A. Occurrence of microcystins in water, bloom, sediment and fish from a public water supply. *Science of The Total Environment* **562**, 860-868, doi:http://dx.doi.org/10.1016/j.scitotenv.2016.04.027 (2016).

133 Sedda, T. *et al.* Determination of microcystin-LR in clams (Tapes decussatus) of two Sardinian coastal ponds (Italy). *Mar Pollut Bull* **108**, 317-320, doi:10.1016/j.marpolbul.2016.04.022 (2016).

134 Dixit, R. B., Patel, A. K., Toppo, K. & Nayaka, S. Emergence of toxic cyanobacterial species in the Ganga River, India, due to excessive nutrient loading. *Ecological Indicators* **72**, 420-427, doi:http://dx.doi.org/10.1016/j.ecolind.2016.08.038 (2017).

135 Almeida, V. P. S., Cogo, K., Tsai, S. M. & Moon, D. H. Colorimetric test for the monitoring of microcystins in cyanobacterial culture and environmental samples from southeast - Brazil. *Brazilian Journal of Microbiology* **37**, 192-198, doi:10.1590/s1517-83822006000200017 (2006).

136 Heresztyn, T. & Nicholson, B. C. Determination of cyanobacterial hepatotoxins directly in water using a protein phosphatase inhibition assay. *Water Research* **35**, 3049-3056, doi:http://dx.doi.org/10.1016/S0043-1354(01)00018-5 (2001).

137 Lambert, T. W., Boland, M. P., Holmes, C. F. & Hrudey, S. E. Quantitation of the microcystin hepatotoxins in water at environmentally relevant concentrations with the protein phosphatase bioassay. *Environ Sci Technol* **28**, 753-755, doi:10.1021/es00053a032 (1994).

138 Ward, C. J., Beattie, K. A., Lee, E. Y. C. & Codd, G. A. Colorimetric protein phosphatase inhibition assay of laboratory strains and natural blooms of cyanobacteria: comparisons with high-performance liquid chromatographic analysis for microcystins. *FEMS Microbiology Letters* **153**, 465-473, doi:10.1111/j.1574-6968.1997.tb12611.x (1997).

139 Kaloudis, T. *et al.* Determination of microcystins and nodularin (cyanobacterial toxins) in water by LC–MS/MS. Monitoring of Lake Marathonas, a water reservoir of Athens, Greece. *Journal of Hazardous Materials* **263, Part 1**, 105-115, doi:http://dx.doi.org/10.1016/j.jhazmat.2013.07.036 (2013).

140 Ortea, P. M. *et al.* Determination of toxic cyclic heptapeptides by liquid chromatography with detection using ultra-violet, protein phosphatase assay and tandem mass spectrometry. *Chemosphere* **55**, 1395-1402, doi:10.1016/j.chemosphere.2003.11.025 (2004).

141 Bouhaddada, R., Nélieu, S., Nasri, H., Delarue, G. & Bouaïcha, N. High diversity of microcystins in a Microcystis bloom from an Algerian lake. *Environmental Pollution* **216**, 836-844, doi:http://dx.doi.org/10.1016/j.envpol.2016.06.055 (2016).

142 Kohoutek, J., Babica, P., Bláha, L. & Maršálek, B. A novel approach for monitoring of cyanobacterial toxins: development and evaluation of the passive sampler for microcystins. *Analytical and Bioanalytical Chemistry* **390**, 1167-1172, doi:10.1007/s00216-007-1785-y (2008).

143 Spoof, L. & Meriluoto, J. Rapid separation of microcystins and nodularin using a monolithic silica C18 column. *Journal of Chromatography* **947** 237-245 (2002).

144 McElhiney, J. & Lawton, L. A. Detection of the cyanobacterial hepatotoxins microcystins. *Toxicology and Applied Pharmacology* **203**, 219-230, doi:http://dx.doi.org/10.1016/j.taap.2004.06.002 (2005).

145 Dell'Aversano, C., Eaglesham, G. K. & Quilliam, M. A. Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography–mass spectrometry. *Journal of Chromatography A* **1028**, 155-164, doi:http://dx.doi.org/10.1016/j.chroma.2003.11.083 (2004).

146 Dell'Aversano, C., Hess, P. & Quilliam, M. A. Hydrophilic interaction liquid chromatography–mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins. *Journal of Chromatography A* **1081**, 190-201, doi:http://dx.doi.org/10.1016/j.chroma.2005.05.056 (2005).

147 Diener, M., Erler, K., Christian, B. & Luckas, B. Application of a new zwitterionic hydrophilic interaction chromatography column for determination of paralytic shellfish poisoning toxins. *J Sep Sci* **30**, 1821-1826, doi:10.1002/jssc.200700025 (2007).

148 Poyer, S. *et al.* Identification and separation of saxitoxins using hydrophilic interaction liquid chromatography coupled to traveling wave ion mobility-mass spectrometry. *J Mass Spectrom* **50**, 175-181, doi:10.1002/jms.3515 (2015).

149 Chen, J. *et al.* Simultaneous screening for lipophilic and hydrophilic toxins in marine harmful algae using a serially coupled reversed-phase and hydrophilic interaction liquid chromatography separation system with high-resolution mass spectrometry. *Analytica Chimica Acta* **914**, 117-126, doi:http://dx.doi.org/10.1016/j.aca.2016.01.062 (2016).

150 Buszewski, B. & Noga, S. Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique. *Analytical and Bioanalytical Chemistry* **402**, 231-247, doi:10.1007/s00216-011-5308-5 (2012).

151 Gugger, M. *et al.* First report in a river in France of the benthic cyanobacterium Phormidium favosum producing anatoxin-a associated with dog neurotoxicosis. *Toxicon* **45**, 919-928, doi:http://dx.doi.org/10.1016/j.toxicon.2005.02.031 (2005).

152 Furey, A., Crowley, J., Hamilton, B., Lehane, M. & James, K. J. Strategies to avoid the mis-identification of anatoxin-a using mass spectrometry in the forensic investigation of acute neurotoxic poisoning. *Journal of Chromatography A* **1082**, 91-97, doi:http://dx.doi.org/10.1016/j.chroma.2005.05.040 (2005).

153 Bogialli, S., Bruno, M., Curini, R., Di Corcia, A. & Laganà, A. Simple and rapid determination of anatoxin-a in lake water and fish muscle tissue by liquid-chromatography–tandem mass spectrometry. *Journal of Chromatography A* **1122**, 180-185, doi:http://dx.doi.org/10.1016/j.chroma.2006.04.064 (2006).

154 Furey, A., Crowley, J., Lehane, M. & James, K. J. Liquid chromatography with electrospray ion-trap mass spectrometry for the determination of anatoxins in cyanobacteria and drinking water. *Rapid Commun Mass Spectrom* **17**, 583-588, doi:10.1002/rcm.932 (2003).

155 Oehrle, S. A., Southwell, B. & Westrick, J. Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectrometry. *Toxicon* **55**, 965-972, doi:10.1016/j.toxicon.2009.10.001 (2010).

156 Kubo, T., Kato, N., Hosoya, K. & Kaya, K. Effective determination method for a cyanobacterial neurotoxin, β-N-methylamino-l-alanine. *Toxicon* **51**, 1264-1268, doi:http://dx.doi.org/10.1016/j.toxicon.2008.02.015 (2008).

157 Li, A. *et al.* Detection of the neurotoxin BMAA within cyanobacteria isolated from freshwater in China. *Toxicon* **55**, 947-953, doi:http://dx.doi.org/10.1016/j.toxicon.2009.09.023 (2010).

158 Krüger, T., Mönch, B., Oppenhäuser, S. & Luckas, B. LC–MS/MS determination of the isomeric neurotoxins BMAA (β-N-methylamino-l-alanine) and DAB (2,4-diaminobutyric acid) in cyanobacteria and seeds of Cycas revoluta and Lathyrus latifolius. *Toxicon* **55**, 547-557, doi:http://dx.doi.org/10.1016/j.toxicon.2009.10.009 (2010).

159 Li, A. *et al.* Elucidation of matrix effects and performance of solid-phase extraction for LC-MS/MS analysis of beta-N-methylamino-L-alanine (BMAA) and 2,4-diaminobutyric acid (DAB) neurotoxins in cyanobacteria. *Analyst* **137**, 1210-1219, doi:10.1039/c2an15887f (2012).

160 Banack, S. A. & Johnson, H. E. Production of the Neurotoxin BMAA by a Marine Cyanobacterium. **5**, 180-196 (2007).

161 Takino, M., Daishima, S. & Yamaguchi, K. Analysis of anatoxin-a in freshwaters by automated on-line derivatization–liquid chromatography–electrospray mass spectrometry. *Journal of Chromatography A* **862**, 191-197, doi:http://dx.doi.org/10.1016/S0021-9673(99)00943-7 (1999).

162 Cameán, A., Moreno, I. M., Ruiz, M. J. & Picó, Y. Determination of microcystins in natural blooms and cyanobacterial strain cultures by matrix solid-phase dispersion and liquid chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry* **380**, 537-544, doi:10.1007/s00216-004-2755-2 (2004).

163 Allis, O. *et al.* Liquid chromatography-tandem mass spectrometry application, for the determination of extracellular hepatotoxins in Irish lake and drinking waters. *Anal Chem* **79**, 3436-3447, doi:10.1021/ac062088q (2007).

164 Cong, L. *et al.* Determination of trace amount of microcystins in water samples using liquid chromatography coupled with triple quadrupole mass spectrometry. *Analytica Chimica Acta* **569**, 157-168, doi:http://dx.doi.org/10.1016/j.aca.2006.03.052 (2006).

165 Fayad, P. B., Roy-Lachapelle, A., Duy, S. V., Prévost, M. & Sauvé, S. On-line solid-phase extraction coupled to liquid chromatography tandem mass spectrometry for the analysis of cyanotoxins in algal blooms. *Toxicon* **108**, 167-175, doi:http://dx.doi.org/10.1016/j.toxicon.2015.10.010 (2015).

166 Perez, S. & Aga, D. S. Recent advances in the sample preparation, liquid chromatography tandem mass spectrometric analysis and environmental fate of microcystins in water. *Trac-Trend Anal Chem* **24**, 658-670, doi:10.1016/j.trac.2005.04.005 (2005).
167 Wang, J., Pang, X., Ge, F. & Ma, Z. An ultra-performance liquid chromatography-tandem mass spectrometry method for determination of microcystins occurrence in surface water in Zhejiang Province. *Toxicon* **49**, 1120-1128 (2007).
168 Zastepa, A., Pick, F. R., Blais, J. M. & Saleem, A. Analysis of intracellular and extracellular microcystin variants in sediments and pore waters by accelerated solvent extraction and high performance liquid chromatography-tandem mass spectrometry. *Anal Chim Acta* **872**, 26-34, doi:10.1016/j.aca.2015.02.056 (2015).
169 Hedman, C. J., Krick, W. R., Karner Perkins, D. A., Harrahy, E. A. & Sonzogni, W. C. New measurements of cyanobacterial toxins in natural waters using high performance liquid chromatography coupled to tandem mass spectrometry. *J Environ Qual* **37**, 1817-1824, doi:10.2134/jeq2007.0368 (2008).
170 Rodriguez, I. *et al.* Toxin profile in samples collected in fresh and brackish water in Germany. *Toxicon* **91** 35-44 (2014).
171 Faassen, E. J., Gillissen, F. & Lürling, M. A Comparative Study on Three Analytical Methods for the Determination of the Neurotoxin BMAA in Cyanobacteria. *PLoS One* **7**, doi:10.1371/journal.pone.0036667 (2012).
172 Banack, S. A. *et al.* Distinguishing the cyanobacterial neurotoxin β-N-methylamino-l-alanine (BMAA) from its structural isomer 2,4-diaminobutyric acid (2,4-DAB). *Toxicon* **56**, 868-879, doi:http://dx.doi.org/10.1016/j.toxicon.2010.06.006 (2010).
173 Banack, S. A. *et al.* Distinguishing the cyanobacterial neurotoxin beta-N-methylamino-L-alanine (BMAA) from other diamino acids. *Toxicon* **57**, 730-738, doi:10.1016/j.toxicon.2011.02.005 (2011).
174 Metcalf, J. S. *et al.* Co-occurrence of beta-N-methylamino-L-alanine, a neurotoxic amino acid with other cyanobacterial toxins in British waterbodies, 1990-2004. *Environ Microbiol* **10**, 702-708, doi:10.1111/j.1462-2920.2007.01492.x (2008).
175 Al-Sammak, M. A., Hoagland, K. D., Snow, D. D. & Cassada, D. Methods for simultaneous detection of the cyanotoxins BMAA, DABA, and anatoxin-a in environmental samples. *Toxicon* **76**, 316-325, doi:http://dx.doi.org/10.1016/j.toxicon.2013.10.015 (2013).
176 Al-Sammak, M., Hoagland, K., Cassada, D. & Snow, D. Co-occurrence of the Cyanotoxins BMAA, DABA and Anatoxin-a in Nebraska Reservoirs, Fish, and Aquatic Plants. *Toxins* **6**, 488 (2014).
177 Meriluoto, J., Karlsson, K. & Spoof, L. High-Throughput Screening of Ten Microcystins and Nodularins, Cyanobacterial Peptide Hepatotoxins, by Reversed-Phase Liquid Chromatography-Electrospray Ionisation Mass Spectrometry. *Chromatographia* **59**, 291-298, doi:10.1365/s10337-003-0163-y (2004).
178 Zhang, L., Ping, X. & Yang, Z. Determination of microcystin-LR in surface water using high-performance liquid chromatography/tandem electrospray ionization mass detector. *Talanta* **62**, 191-198, doi:10.1016/j.talanta.2003.07.001 (2004).
179 Lajeunesse, A. *et al.* Detection and confirmation of saxitoxin analogues in freshwater benthic Lyngbya wollei algae collected in the St. Lawrence River (Canada) by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A* **1219**, 93-103, doi:http://dx.doi.org/10.1016/j.chroma.2011.10.092 (2012).
180 Xu, W. *et al.* Development and application of ultra performance liquid chromatography-electrospray ionization tandem triple quadrupole mass spectrometry for determination of seven microcystins in water samples. *Anal Chim Acta* **626**, 28-36, doi:10.1016/j.aca.2008.07.040 (2008).
181 Messineo, V. *et al.* Cyanobacterial toxins in Italian freshwaters. *Limnologica* **39**, 95-106, doi:10.1016/j.limno.2008.09.001 (2009).

182 Wood, S. A., Holland, P. T. & MacKenzie, L. Development of solid phase adsorption toxin tracking (SPATT) for monitoring anatoxin-a and homoanatoxin-a in river water. *Chemosphere* **82**, 888-894, doi:10.1016/j.chemosphere.2010.10.055 (2011).
183 Di Gregorio, F. N., Bogialli, S., Ferretti, E. & Lucentini, L. First evidence of MC-HtyR associated to a Plankthothrix rubescens blooming in an Italian lake based on a LC-MS method for routinely analysis of twelve microcystins in freshwaters. *Microchemical Journal* **130**, 329-335, doi:10.1016/j.microc.2016.10.012 (2017).
184 Howard, K. L. & Boyer, G. L. Quantitative analysis of cyanobacterial toxins by matrix-assisted laser desorption ionization mass spectrometry. *Anal Chem* **79**, 5980-5986, doi:10.1021/ac0705723 (2007).
185 Meisen, I. *et al.* Direct coupling of high-performance thin-layer chromatography with UV spectroscopy and IR-MALDI orthogonal TOF MS for the analysis of cyanobacterial toxins. *Anal Chem* **81**, 3858-3866, doi:10.1021/ac900217q (2009).
186 Saker, M. L., Fastner, J., Dittmann, E., Christiansen, G. & Vasconcelos, V. M. Variation between strains of the cyanobacterium Microcystis aeruginosa isolated from a Portuguese river. *J Appl Microbiol* **99**, 749-757, doi:10.1111/j.1365-2672.2005.02687.x (2005).
187 Welker, M., Fastner, J., Erhard, M. & von Dohren, H. Applications of MALDI-TOF MS analysis in cyanotoxin research. *Environ Toxicol* **17**, 367-374, doi:10.1002/tox.10073 (2002).
188 Fang, X., Fan, X., Tang, Y., Chen, J. & Lu, J. Liquid chromatography/quadrupole time-of-flight mass spectrometry for determination of saxitoxin and decarbamoylsaxitoxin in shellfish. *J Chromatogr A* **1036**, 233-237 (2004).
189 Maizels, M. & Budde, W. L. A LC/MS method for the determination of cyanobacteria toxins in water. *Analytical Chemistry* **76**, 1342-1351 (2004).
190 James, K. J. *et al.* Anatoxins and degradation products, determined using hybrid quadrupole time-of flight and quadrupole ion-trap mass spectrometry: Forensic investigations of cyanobacterial neurotoxin poisoning. *Rapid Communications in Mass Spectrometry* **19**, 1167-1175 (2005).
191 Ortelli, D., Edder, P., Cognard, E. & Jan, P. Fast screening and quantitation of microcystins in microalgae dietary supplement products and water by liquid chromatography coupled to time of flight mass spectrometry. *Anal Chim Acta* **617**, 230-237, doi:10.1016/j.aca.2008.03.033 (2008).
192 Moura, S., Ultramari Mde, A., de Paula, D. M., Yonamine, M. & Pinto, E. 1H NMR determination of beta-N-methylamino-L-alanine (L-BMAA) in environmental and biological samples. *Toxicon* **53**, 578-583, doi:10.1016/j.toxicon.2008.12.013 (2009).
193 Dagnino, D. & Schripsema, J. 1H NMR quantification in very dilute toxin solutions: application to anatoxin-a analysis. *Toxicon* **46**, 236-240, doi:http://dx.doi.org/10.1016/j.toxicon.2005.04.014 (2005).
194 Isaacs, J. D. *et al.* Microcystins and two new micropeptin cyanopeptides produced by unprecedented Microcystis aeruginosa blooms in North Carolina's Cape Fear River. *Harmful Algae* **31**, 82-86, doi:10.1016/j.hal.2013.09.010 (2014).
195 WHO. *World Health Organization. Cyanobacterial toxins: microcystin-LR. In: Guidelines for Drinking Water Quality (Ed.) Addendum to vol.2. Health Criteria and Other Supporting Information, 2nd ed. World Health Organization, Geneva, pp.95–110* (1998).
196 EFSA. Marine biotoxins in shellfish - Saxitoxin group. Scientific opinion of the panel on contaminants in the food chain. *The EFSA Journal* **1019**, 1-76 (2009).
197 Burrell, S., Crum, S., Foley, B. & Turner, A. D. Proficiency testing of laboratories for paralytic shellfish poisoning toxins in shellfish by QUASIMEME: A review. *TrAC Trends in Analytical Chemistry* **75**, 10-23, doi:http://dx.doi.org/10.1016/j.trac.2015.09.004 (2016).
198 Hess, P. *et al.* Three Rs approaches in marine biotoxin testing. *Alternatives to Laboratory Animals* **34**, 193-224 (2006).

199 EC. *European Commission. Regulation (EC) No. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004. OJ L 338, 22.12* 27-59 (2005).
200 Ledoux, M. & Hall, S. Proficiency testing of eight French laboratories in using the AOAC mouse bioassay for paralytic shellfish poisoning: Interlaboratory collaborative study. *Journal of AOAC International* **83**, 305-310 (2000).
201 Park, D. L., Adams, W. N., Graham, S. L. & Jackson, R. C. Variability of mouse bioassay for determination of paralytic shellfish poisoning toxins. *J Assoc Off Anal Chem* **69**, 547-550 (1986).
202 Turner, A. D. *et al.* Comparison of AOAC 2005.06 LC official method with other methodologies for the quantitation of paralytic shellfish poisoning toxins in UK shellfish species. *Analytical and Bioanalytical Chemistry* **399**, 1257-1270, doi:10.1007/s00216-010-4428-7 (2011).
203 Louzao, M. C., Vieytes, M. R., Baptista de Sousa, J. M., Leira, F. & Botana, L. M. A fluorimetric method based on changes in membrane potential for screening paralytic shellfish toxins in mussels. *Anal Biochem* **289**, 246-250, doi:10.1006/abio.2000.4942 (2001).
204 Louzao, M. C., Rodriguez Vieytes, M., Garcia Cabado, A., Vieites Baptista De Sousa, J. M. & Botana, L. M. A fluorimetric microplate assay for detection and quantitation of toxins causing paralytic shellfish poisoning. *Chem Res Toxicol* **16**, 433-438, doi:10.1021/tx025574r (2003).
205 Jellett, J. F., Doucette, L. I. & Belland, E. R. The MIST™ shippable cell bioassay kits for PSP: An alternative to the mouse bioassay. . *Journal of Shellfish Research* **17**, 1653-1655 (1998).
206 Mackintosh, F. H., Gallacher, S., Shanks, A. M. & Smith, E. A. Assessment of MIST Alert, a commercial qualitative assay for detection of paralytic shellfish poisoning toxins in bivalve molluscs. *J AOAC Int* **85**, 632-641 (2002).
207 Mackintosh, F. H. & Smith, E. A. Evaluation of MIST Alert™ rapid test kits for the detection of paralytic and amnesic shellfish poisoning toxins in shellfish. *Journal of Shellfish Research* **21**, 455-460 (2002).
208 Usleber, E., Dietrich, R., Burk, C., Schneider, E. & Martlbauer, E. Immunoassay methods for paralytic shellfish poisoning toxins. *J AOAC Int* **84**, 1649-1656 (2001).
209 Preece, E. P., Moore, B. C., Swanson, M. E. & Hardy, F. J. Identifying best methods for routine ELISA detection of microcystin in seafood. *Environ Monit Assess* **187**, 12, doi:10.1007/s10661-014-4255-y (2015).
210 Berry, J. P., Lee, E., Walton, K., Wilson, A. E. & Bernal-Brooks, F. Bioaccumulation of microcystins by fish associated with a persistent cyanobacterial bloom in Lago de Patzcuaro (Michoacan, Mexico). *Environ Toxicol Chem* **30**, 1621-1628, doi:10.1002/etc.548 (2011).
211 Ernst, B., Dietz, L., Hoeger, S. J. & Dietrich, D. R. Recovery of MC-LR in fish liver tissue. *Environ Toxicol* **20**, 449-458, doi:10.1002/tox.20131 (2005).
212 Geis-Asteggiante, L. *et al.* Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with ELISA. *Anal Bioanal Chem* **401**, 2617-2630, doi:10.1007/s00216-011-5345-0 (2011).
213 Vieytes, M. R. *et al.* Solid-phase radioreceptor assay for paralytic shellfish toxins. *Anal Biochem* **211**, 87-93, doi:10.1006/abio.1993.1237 (1993).
214 Doucette, G. J., Logan, M. M., Ramsdell, J. S. & Van Dolah, F. M. Development and preliminary validation of a microtiter plate-based receptor binding assay for paralytic shellfish poisoning toxins. *Toxicon* **35**, 625-636 (1997).
215 Ruberu, S. R. *et al.* Receptor binding assay for paralytic shellfish poisoning toxins: optimization and interlaboratory comparison. *J AOAC Int* **86**, 737-745 (2003).
216 Lawrence, J. F., Niedzwiadek, B. & Menard, C. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid

chromatography with fluorescence detection: interlaboratory study. *J AOAC Int* **87**, 83-100 (2004).

217 Lawrence, J. F., Niedzwiadek, B. & Menard, C. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J AOAC Int* **88**, 1714-1732 (2005).

218 CEN. European Committee for Standardization. European Standard DIN EN 14526. Foodstuffs — determination of saxitoxin and dc-saxitoxin in mussels — HPLC method using precolumn derivatization with peroxide or periodate oxidation, 2004-11. .

219 Rourke, W. A. *et al.* Rapid postcolumn methodology for determination of paralytic shellfish toxins in shellfish tissue. *J AOAC Int* **91**, 589-597 (2008).

220 Rey, V., Botana, A. M. & Botana, L. M. Quantification of PSP toxins in toxic shellfish matrices using post-column oxidation liquid chromatography and pre-column oxidation liquid chromatography methods suggests post-column oxidation liquid chromatography as a good monitoring method of choice. *Toxicon* **129**, 28-35, doi:10.1016/j.toxicon.2017.02.003 (2017).

221 Boundy, M. J., Selwood, A. I., Harwood, D. T., McNabb, P. S. & Turner, A. D. Development of a sensitive and selective liquid chromatography-mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. *J Chromatogr A* **1387**, 1-12, doi:10.1016/j.chroma.2015.01.086 (2015).

222 Turner, A. D., McNabb, P. S., Harwood, D. T., Selwood, A. I. & Boundy, M. J. Single-Laboratory Validation of a Multitoxin Ultra-Performance LC-Hydrophilic Interaction LC-MS/MS Method for Quantitation of Paralytic Shellfish Toxins in Bivalve Shellfish. *J AOAC Int* **98**, 609-621, doi:10.5740/jaoacint.14-275 (2015).

223 Sayfritz, S. J., Aasen, J. A. B. & Aune, T. Determination of paralytic shellfish poisoning toxins in Norwegian shellfish by liquid chromatography with fluorescence and tandem mass spectrometry detection. *Toxicon* **52**, 330-340, doi:http://dx.doi.org/10.1016/j.toxicon.2008.06.001 (2008).

224 Zhuo, L. *et al.* Determination of paralytic shellfish poisoning toxins by HILIC-MS/MS coupled with dispersive solid phase extraction. *Food Chem.* **137**, 115-121 (2013).

225 Li, A., Chen, H., Qiu, J., Lin, H. & Gu, H. Determination of multiple toxins in whelk and clam samples collected from the Chukchi and Bering seas. *Toxicon* **109**, 84-93, doi:http://dx.doi.org/10.1016/j.toxicon.2015.11.016 (2016).

226 Ibelings, B. W., Backer, L. C., Kardinaal, W. E. & Chorus, I. Current approaches to cyanotoxin risk assessment and risk management around the globe. *Harmful Algae* **49**, 63-74, doi:10.1016/j.hal.2014.10.002 (2015).

List of abbreviations and definitions

1H-NMR	Proton Nuclear Magnetic Resonance
2,4-DAB	2,4-diaminobutyric acid
ALDH2	Aldehyde Dehydrogenase 2
AOAC	Association of Official Analytical Chemists
APX	Aplysiatoxin
ATX-a	Anatoxin-a
ATX-a(s)	Anatoxin-a(s)
BGAS	Blue Green Algae Supplements
BMAA	β-N-methylamino-L-alanine
BMP	Bacterial Magnetic Particles
cDNA	Complementary Desoxyribonucleic Acid
CRL	Community Reference Laboratory
CYN	Cylindrospermopsin
DNA	Desoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EU	European Union
hATX-a	Homoanatoxin-a
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
i.p.	Intraperitoneal
i.v.	Intravenous injection
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Triple Quadrupole Mass Spectrometry
LC-TOF-MS	Liquid Chromatography –Time–of-Flight Mass Spectrometry

LD_{50}	Lethal Dose 50
LOAEL	Lowest Adverse Effect Level
LOQ	Limit of Quantification
LPS	Lypopolysaccharide
LT	Lyngbyatoxin
LTA	Lyngbyatoxin A
LTB	Lyngbyatoxin B
LTC	Lyngbyatoxin C
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MCs	Microcystins
MC-LR	Microcystin-LR
MC-RR	Microcystin-RR
MRL	Maximum Residue Limit
MS	Mass Spectrometry
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous end Joining
NOAEL	No Observed Adverse Effects Level
NODs	Nodularins
NRPS	Non-Ribosomal Peptide Synthetase
NMR	Nuclear Magnetic Resonance
OATP	Organic Anion Transporting Polypeptides
PCR	Polymerase-Chain Reaction
PDA	Photodiode-Array
PHE	phenylalanine
PKC	Protein Kinase C
PKS	Poliketide Synthase
PMAC	Provisional Maximum Concentration
PMAV	Provisional Maximum Value
PPIA	Protein Phosphatase Inhibitor Assay
PP1	Protein Phosphatases 1
PP2A	Protein Phosphatases 2A
PSP	Paralytic Shellfish Poisons
QIT	Quadrupole Ion-Trap
qPCR	Quantitative Real-Time Polymerase Chain Reaction
QqTOF	Quadrupole Time-Of-Flight
rDNA	Ribosomal Desoxyribonucleic Acid

RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal Ribonucleic Acid
SNP	Single Nucleotide Polymorphisms
SPE	Solid Phase Extraction
STXs	Saxitoxins
TLR4	Toll-like Receptor 4
TNF-α	Tumor Necrosis Factor-α
TPA	Tumor Promoter ^{3}H-12-*O*-Tetradecanoylphorbol-13-Acetate
UBA	Umweltbundesamt
UHPLC	Ultrahigh Pressure Liquid Chromatography
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

List of figures

List of tables

Europe Direct is a service to help you find answers to your questions about the European Union.

Freephone number (*)**:**

00 800 6 7 8 9 10 11

(*) The information given is free, as are most calls (though some operators, phone boxes or hotels may charge you).

More information on the European Union is available on the internet (http://europa.eu).

HOW TO OBTAIN EU PUBLICATIONS

Free publications:

- one copy:
  via EU Bookshop (http://bookshop.europa.eu);
- more than one copy or posters/maps:
  from the European Union's representations (http://ec.europa.eu/represent_en.htm);
  from the delegations in non-EU countries (http://eeas.europa.eu/delegations/index_en.htm);
  by contacting the Europe Direct service (http://europa.eu/europedirect/index_en.htm) or
  calling 00 800 6 7 8 9 10 11 (freephone number from anywhere in the EU) (*).

  (*) The information given is free, as are most calls (though some operators, phone boxes or hotels may charge you).

Priced publications:

- via EU Bookshop (http://bookshop.europa.eu).

KJ-NA-28624-EN-N

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.

EU Science Hub
ec.europa.eu/jrc

@EU_ScienceHub

EU Science Hub - Joint Research Centre

Joint Research Centre

EU Science Hub

Publications Office

doi:10.2760/36186

ISBN 978-92-79-69099-0