Faculties of Pharmacy and Medicine

Department of Chemistry and Drug Technologies

PHD THESIS IN

PHARMACEUTICAL SCIENCE

- XXXIII CYCLE–

"High Resolution Mass Spectrometr: new methods of analysis for risk assessment by cyanotoxins and cyromazine in water for human consumption"

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DEDICATED TO
MY HYSBAND AND MY SONS,
MY PARENTS AND MY BROTHERS

"Non permettere mai a nessuno di dirti che non sai fare qualcosa. Se hai un sogno tu lo devi proteggere. Quando le persone non sanno fare qualcosa lo dicono a te che non la sai fare.
Se vuoi qualcosa, vai e inseguila. Punto."

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ACRONYMS

ADDA	3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-DecaDienoic Acid
ALF	Alert Level Framework
ATP	Adenosine Triphosphate
ANA-a	Anatoxin-a
ANAB	Anabaenopeptins
BMAA	Beta-Methylamino-L-Alanine
BSA	Bovine Serum Albumin
CE	Collision Energy
CIMF	Cyanobacterial Incident Management Framework
CYN	Cylindrospermopsin
CYP	Cyanopeptolin
CV	Coefficient of Variation
i.d.	Inner diameter
DAD	Diode Array Detector
EDCs	Endocrine-disrupting chemical
EP	Emerging pollutant
IS	Internal standard
ISS	*Istituto Superiore di Sanità*
DRP	Dissolved Reactive Phosphorus
SD	Standard Deviation
ELISA	Enzyme-Linked Immunosorbent Assay
FLD	Fluorescence Detector
FRP	Filtred Reactive Phosphorus
GCB	Graphitized Carbon Black
HPLC	High performance liquid chromatography
HRMS	High Resolution Mass Spectrometry
IARC	International Agency for Research on Cancer
LC	Liquid Chromatography
LOD	Limit of Detection
LPS	Lipopolysaccharides
MICRO	Microginins
MC	Microcystins
MS	Mass Spectrometry
NOAEL	No Observed Adverse Effect Level
NOD	Nodularin
NOM	Natural Organic Matter
NRPS	Non-Ribosomal Peptide Synthetase
PAR	Photosynthetically Active Radiation
PCB	Polychlorinated biphenyls
PKS	Polyketide synthase
PPCP	Pharmaceuticals and personal care products
POP	Persistent organic pollutant
Q-TOF	Quadrupole Time Of Flight
RF	Response Factor
RP	Reactive Phosphorus
S/N	Signal/Noise
SPE	Solid Phase Extraction

SRP	Soluble Reactive Phosphorus
TDI	Tolerable Daily Intake
TEF	Toxicity Equivalent Factor
TIC	Total Inorganic Carbon
TP	Total Phosphorus
t_r	Retention time
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet Light
WHO	World Health Organization
WSP	Water Safety Plan

INTRODUCTION

The recent implementation of the EU Directive 1787/2015 introduced in Italy the Water Safety Plan (WSP), a preventive model to guarantee drinking water through control measures extended to the entire water supply chain, from collection, treatment and distribution to the user.

In this context of prevention, protection and monitoring of the quality of water intended for human consumption, it becomes important to develop analytical methods that allow the search for an increasing number of contaminants that could be present in the water, with particular attention to substances considered emerging contaminants (EPs).

EPs encompass a wide range of natural and man-made chemicals which are in use worldwide and which are indispensable for modern society, that currently are not included in routine monitoring programmes but that could have nocive effects on human health.

In particular, among the emerging contaminants, my attention has focused on the secondary metabolites of cyanobacteria and on Cyromazine, a pesticide used as a drug of abuse, for two different reason:

- the proliferation of cyanobacteria in water used for human consumption is an emerging issue in Italy in recent years, involving almost all the Regions, with potential impact on environmental and human health. Changes induced, directly or indirectly, by human activity in surface water bodies preside over, in fact, an abnormal proliferation of constituent bodies of aquatic biota, can cause undesirable or toxic metabolites (cyanotoxins), to affect the quality of water and cause a significant health risk - that requires proper management - water for the supply chain for the production of drinking water.
- the recent issue of a press release by the Directorate General for Hygiene and Safety of Food and Nutrition regarding the revocation of the authorizations of plant protection products containing the active substance cyromazine, following the expiry of its Community approval period pursuant to Regulation (EU) 844/2012.

In the first part of the research work of this PhD, an analytical method of solid phase extraction (SPE) and subsequent determination in ultra-high performance liquid chromatography (UPLC) coupled to a high resolution mass spectrometer (QTOF) for the simultaneous determination of 21 cyanotoxins in water for human consumption was developed.

The method was then validated and applied during a monitoring activity in an Italian volcanic lake in Viterbo (Lazio Region, Italy), due to a severe algal proliferation in January 2018 – January 2020 period.

Obtained results were used for the assessment of cyanobacteria proliferation risk and of cyanotoxin production in drinking water chain.

The development of another analytical method without SPE for the determination of further two cyanotoxins has been started but the method is not complete and needs a further optimization step.

As final step a method for the identification and quantification of cyromazine in drinking water samples used in chicken coops was developed and validated.

CHAPTER 1 : STATE OF ART

1. EMERGING POLLUTANT

Water must comply with the chemical and physical characteristics required by law, (Legislative Decree 31/2001 and smi), to be defined as drinking water.

The law establishes which substances and bacteria can be dissolved in water intended for human consumption or not, and above all in which concentrations. Over the years, however, attention has also shifted to what are defined as "emerging pollutants", ie substances dissolved in water only recently discovered or substances for which scientific studies and assessments have confirmed the danger to human health.

Emerging pollutants (EPs) encompass a wide range of man-made chemicals (such as pesticides, cosmetics, personal and household care products, pharmaceuticals, among others), which are in use worldwide and which are indispensable for modern society[1].

EPs can be defined as pollutants that are currently not included in routine monitoring programmes at the European level and which may be candidates for future regulation, depending on research on their (eco)toxicity, potential health effects and public perception and on monitoring data regarding their occurrence in the various environmental compartments.

These are substances or metals that present in certain concentrations can represent health hazards, such as arsenic, cadmium, lead and mercury, or the more recent Pfas (perfluoroalkyl organic substances), or allergens such as nickel. However, there is no shortage of chemicals such as herbicides, medicines, drugs or secondary metabolite of other organic coumpound.

On the basis of the original application of the substances a more detailed classification for EC is used[2,3] :

- Persistent organic pollutants (POPs) are toxic chemicals that originate from man-made sources associated with the production, use, and disposal of certain organic chemicals. Some of the POPs such as pesticides and polychlorinated biphenyls (PCBs) are intentionally produced, while others such as dioxin and furans are unintentional by-products of industrial processes or result from the combustion of organic chemicals.

- Pharmaceuticals and personal care products (PPCPs), include any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance growth or health of livestock.

PPCPs comprise a diverse collection of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, sunscreens, detergents, and cosmetics.

- Endocrine-disrupting chemicals (EDCs), including synthetic estrogens and androgens, naturally occurring estrogens, and other chemicals that affect hormonal functions.
- Nanomaterials, such as carbon nanotubes, or nano-scale particulate titanium dioxide used in sunscreens, cosmetics, paints and coatings.

In this study two different "class" of emergin pollutants were considered: cyanotoxins and secondary metabolites of cyanobacteria and cyromazine.

2. CYANOBACTERIA

Cyanobacteria are photosynthetic prokaryotes able to synthesize chlorophyll-a and several accessory pigments, such as phycobilins (allophycocyanin, phycocyanin and phycoerythrin) and carotenoids (such as beta-carotene, echinenone, canthaxanthin, myxoxanthofilla, zeaxanthin and oscillaxantina). These accessory pigments absorb light at wavelengths rarely used by other species of phytoplankton so that cyanobacteria have a greater ability to colonize different environments. Ecophysiological properties specific to the different cyanobacteria are very different and allow them to occupy different ecological niches in aquatic ecosystems. The understanding of their response to environmental factors is therefore crucial for the definition of the objectives of management of water bodies.

However, the growth of cyanobacteria is influenced by different environmental factors like' light intensity, nutrients and hydrology of the basin. It is known that cyanobacteria prefer relatively high temperatures of the water and high level of light intensity[4,5].

In addition, there are some species, including major producers of toxins, which are exceptions to this generalization [6,7,8].

For these reasons, any attempt to develop effective management strategies should include knowledge of the taxonomic composition and elements of site-specific ecology of the species concerned.

The light intensity available in quantity and quality varies with depth. It decreases exponentially due to absorption and scattering caused by particles and colored compounds[9]. In particular, the selective removal of some wavelengths causes changes in the spectral distribution of the light . The clear water

absorbs light at the wavelengths of the red light, while the dissolved organic compounds and particles strongly absorb at the wavelengths of blue light.

The phytoplankton changes the spectral distribution of the light: the green algae absorb little in the wavelengths of orange and yellow, which are absorbed by phycobilins. This is a competitive advantage for cyanobacteria which absorb light in a wide range of wavelengths including those used by the chlorophylls.

The ecological effects of temperature and light are essentially inseparable because of the interrelationship between metabolism and light saturation[10]. The light intensity influences the rate of photosynthesis and therefore on the growth of cyanobacteria. The response to light is species-specific and cyanobacteria show a remarkable ability to adapt to changing light intensity. In general, the saturation value of light intensity of photosynthesis increases with the water temperature.

Up to the saturation value, photosynthesis is limited by photochemical reactions that are relatively independent of the temperature, if not at very low temperatures[11]. Reached the saturation value of light, photosynthesis is limited by biochemical enzymatic reactions that are governed by the temperature[12]. Cyanobacteria are known to have a large capacity for adaptation to light and temperature, which allows them to occupy a wide range of environments. For example, *Cylindrospermopsis raciborskii* has proven to be able to grow in a large range of temperatures (20 to 35 °C) and light intensity (30-400 µmol photons m^{-2} s^{-1})[13], even if the growth rates maximum occurring at about 30 °C and 80 µmol photons m^{-2} s^{-1}. Cyanobacteria need very little energy to maintain the function and structure of cells [14,15] and this can be a competitive advantage for cyanobacteria against other algae.

Experimental evidence shows that high temperatures, stratification induced by the temperature and the type of mixing, may affect the growth of the species with gaseous vacuoles and promote algal bloom. The growth of cyanobacteria can also take place at low temperature, even if the growth potential is significantly greater at temperatures above about 15° C, while the maximum growth rates are achieved by most of the cyanobacteria at temperatures above 25° C[2].

It has been shown that these optimum values of temperature are higher than those of green algae and diatoms[7]. However, most of the studies upon which these assumptions have been made in warm water bodies and in conditions of thermal stratification, where it might just be the stratification, the more that the temperature, to represent the determining factor in the regulation of growth of cyanobacteria[6].

Cyanobacteria blooms occur frequently in eutrophic lakes, and therefore it is assumed that cyanobacteria require high concentrations of phosphorus (P) and nitrogen (N). High concentrations

of phosphorus may indirectly support the growth of cyanobacteria, increasing the amount of biomass that the resources of an ecosystem can support. However, cyanobacteria blooms have been identify even at low concentrations of dissolved phosphorus[16].

Furthermore there is a serious difficulty in deciphering what fractions of phosphorus and nitrogen were measured in the different studies in the literature, which makes it difficult to understand the environmental conditions in which blooms occurred.

Normally, the concentration of total P (TP) is measured to characterize the trophic status of a lake. One cause of confusion may arise from the measurement of P reactive instead of P total. The reactive phosphorus can be found abbreviated as FRP (filtered reactive phosphorus), SRP (soluble reactive phosphorus), DRP (dissolved reactive phosphorus) or RP (reactive phosphorus).

Until fifty years ago it was considered that the reactive phosphorus represented the phosphorus in inorganic form, such as orthophosphate. However, it includes, besides that orthophosphate, but also other forms that react with the compounds used for the analysis[17]. There are still open questions about the meaning of the RP, the composition of which probably varies from lake to lake[18], but the consensus is that the forms of phosphorus measured as the RP can be quickly metabolized by the organisms, so that the measured concentrations may be near zero or below the detection limit, even in the presence of a flowering. Therefore, the RP is considered a measure of the phosphorus available immediately, while the TP measures the amount of phosphorus present in a given body of water, either in solution within the plankton.

Several studies have also shown that many organisms can utilize nutrients organic fractions[19], giving further support for the use of the TP to characterize the trophic status of a lake and to determine the conditions prevailing during the algae blooms. However, also as regards the measurement of TP, it should be noted that different values are obtained depending on the analytical technique used.

Most part of studies using an analytical technique that includes an oxidation step, which converts much of the phosphorus present in the sample in RP, and a subsequent spectrophotometric determination of the RP. Therefore different techniques of oxidation, or the application of different instrumental methods, can lead to find different values of TP[20].

Nitrogen, the main component in the construction of gas vesicles, is another important environmental factor that promotes the growth of cyanobacterial species[21]. The algae utilize nitrogen mainly in the form of ammonia (NH_4) which nitrate (NO_3), while the nitrogen gas can be used only by species nitrogen-fixing tools[22]. The fixation of atmospheric nitrogen will only happen if the other forms of

nitrogen are not abundant. It is generally accept that a limitation of nitrogen favors species that produce heterocysts capable of fixing atmospheric nitrogen.

2.1. Cyanobacterial species

The abundant growth of potentially toxic planktonic cyanobacteria (bloom) is a common occurrence in freshwater, brackish and marine areas having a direct impact on environment and health. Of the approximately 150 known genera of cyanobacteria, more than 40 comprise species responsible for the production of cyanotoxins and precisely according to the ability to produce, these compounds are distinguished in producers and non-producers[23]. In the last decades, the ability to synthesize toxins has also been confirmed in type benthic cyanobacteria and subaerial environment[24,25]. The most commonly toxins produced belong to the classes of hepatotoxins (microcystins and nodularins), neurotoxins (anatoxin-a, anatoxin-a (S) and saxitoxin), cytotoxins (cylindrospermopsins) and dermatotoxins (aplysiatoxins and debromoaplyatoxins)[26]. In freshwater environments the microcystins are most commonly produced by species belonging to the *Microcystis*, *Planktothrix* (*Oscillatoria*) and *Dolichospermum* (*Anabaena*) genera[23]. It was observed the production of microcystin by cyanobacteria belonging to the genus Nostoc from aquatic habitats and subaerial[27,28], and *Hapalosiphon*[29] and *Phormidium*[30] genera. In brackish environments such as in the Baltic Sea or salt lakes and estuaries such as in Australia and New Zealand, *Nodularia spumigena* produces the nodularin toxin[24,31]. Neurotoxins are generally produced by *Dolichospermum* (Anabaena) and *Anabaena*, *Aphanizomenon*, less commonly, by *Lyngbya* and *Oscillatoria*[23]. *Cylindrospermopsis, Anabaena, Aphanizomenon, Raphidiopsis* and *Umezakia* produce cylindrospermopsins[32] (10), while several species of *Lyngbya*, *Oscillatoria* and Schizothrix are mainly responsible of the dermatotoxins production (Table 1.1)[23]. The toxic cyanobacteria may be responsible for the production of different types of toxins, and thus, it is possible that the same species may produce more than one type of toxin, as well as it is possible that a particular species can produce different variants of the same class of toxins[33]. This is extensively described for *Microcystis aeruginosa*[34]and for populations of *Planktothrix rubescens* [35,36,37,38,39]. Production of microcystin congeners may be related to the presence of various cyanobacteria populations and the occurence of producers and non-producers strains. In order to discriminate forms of *Planktothrix rubescens* active in the production of microcystins are studies of molecular studies are conducted on genotypes containing myc genes responsible for the biosynthesis of microcystins[40].

Table 1. 1: Classes and general characteristics of cyanotoxins and species responsible for their production (Rapporto Istisan 11/35 Pt. 1)

Toxins	Structures	Generes	Species
Epatotoxins			
Mycrocystins	Cyclic Eptapeptide	*Dolichospermum (Anabaena) Anabaenopsis Aphanizomenon*, *Aphanocapsa Hapalosiphon Limnothrix Microcystis Nostoc Planktothrix Oscillatoria*	*D. circinale D. flos-aquae D. lemmermannii D. viguieri Anab. milleri Aph. ovalisporum Aphanoc.cumulus H. hibernicus L. redekeii M. aeruginosa M. flos-aquae M. viridis M. wesenbergii M. botrys P. agardhii, P. rubescens, O. tenuis*
Nodularins	Cyclic pentapeptide	*Nodularia*	*N. spumigena*
Neurotoxins			
Anatoxin-a	Tropane-related alkaloids	*Dolichospermum (Anabaena) Aphanizomenon Cylindrospermum Oscillatoria Planktothrix Phormidium Raphidiopsis*	*D. circinale D. flos-aquae D. planctonicum D. spiroides P. rubescens P. formosa Pho. formosum R. mediterranea*
Anatoxin-a(s)	Guanidine methyl phosphate ester	*Dolichospermum*	*D. flos-aquae, D. lemmermannii*
Saxitoxins	Alkaloids carbamates	*Dolichospermum Anabaena Aphanizomenon Cylindrospermopsis Lyngbya Planktothrix*	*D. circinale, D. lemmermannii D. spiroides A. perturbata* var. *tumida Aph. isatschenkoi, Aph. flos-aquae, C. raciborskii L. wollei Planktothrix* sp. FP1
Dermatotoxins (irritant) and citotoxins			
Cylindrospermopsins	Guanidine alkaloids	*Anabaena Aphanizomenon Cylindrospermopsis Raphidiopsis*, *Umezakia*	*A. bergii A. lapponica Aph. ovalisporum Aph. flos-aquae, L. wollei C. raciborskii R. curvata U. natans*
Lyngbyatoxin-a	Alkaloid	*Lyngbya Oscillatoria Schizotrix*	*L. majuscula*
Aplysiatoxins and debromoapsyatoxins	Alkaloid	*Lyngbya Oscillatoria Schizotrix*	*O. nigroviridis S. calcicola*
Irritating endotoxins			

2.2. Secondary metabolites

Cyanobacteria are among the most promising microorganisms for the search for new bioactive compounds. These compounds are represented by a group of small linear or cyclic peptides with structural variability using both ribosomal and not-ribosomal biosynthetic pathways[41].

In the last two decades a large number of these secondary metabolites obtained from cyanobacteria in natural samples and in isolated culture have been isolated and characterized. More than 600 peptides or peptide metabolites are know; these compounds are been isolated mostly from species belonging to the *Oscillatoriales* and *Nostocales* orders and, followed by Chroococcales and Stigonematales orders, while they are still little known metabolites produced by *Pleurocapsales* [39].

These numbers are, however, determined by the availability of the strains and by the possibility of biomass analysis from natural environments. For example, the *Lyngbya* (Oscillatoriales) and *Microcystis* (Chroococcales) species are easily obtained and manipulated in terms of growth and abundance so that it's possible get sufficient quantities for the determination of these secondary metabolites, while *Pleurocapsa* requires long times and labor-intensive interventions for the extraction of the same compounds.

The majority of secondary metabolites produced by cyanobacteria are oligopeptides or compound with synthesized structures and they are synthesized through a completely non-ribosomal biosynthetic pathway (NRPS, Non-Ribosomal Peptide Synthetase) or partially non-ribosomal (NRPS / PKS, polyketide synthase).

In Table 1.2 is reported the various classes of secondary metabolites list and their related synonyms and the various genres involved in the production of these compounds. Have been determined more than 200 variants, and these must be added a series of peptides of the new generation of class of cianobactine[42].

Table 1. 2: Classes of secondary metabolites produced by cyanobacteria (Rapporto Istisan 11/35 Pt. 1)

Classes	Synonyms	Origin	Variants
Aeruginosins	microcina, spumigina	*Microcystis*, *Nodularia, Planktothrix*	27
Microginins	cianostatina, oscillaginina, nostoginina	*Microcystis*, *Nostoc, Planktothrix*	38
Anabaenopeptins	oscillamide, acido ferintoico, cheramamide, chonbamide, mozamide, nodulapeptina, plectamide, schizopeptina	*Anabaena*, *Aphanizomenon, Microcystis*, *Nodularia*, *Planktothrix*, *Plectonema*, *Schizothrix*	32
Cyanopeptolins	aeruginopeptina, anabaenopeptilide, dolostatina, hofmannolina, microcistilide, micropeptina, nostociclina, nostopeptina, oscillapeptilide, oscillapeptina, planctopeptina, sciptolina, somamide, simplostatina, tasipeptina	*Anabaena*, *Lyngbya*, *Microcystis*, *Planktothrix*, *Scytonema*, *Symploca*	82
Microviridine		*Microcystis*, *Nostoc, Planktothrix*,	10
Ciclamidi	aaniasciclamide, bistratamide, dendroamide, microciclamide, nostociclamide, obianamide, raociclamide, tenueciclamide, ulongamide, westiellamide	*Lyngbya*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Stigonema*, *Westelliopsis*	21

More than one hundred cianobactine found in cyanobacteria living in a free form or in symbiotic association with some species of ascidians species are described[39]. The biosynthetic pathway of genes involved in the production of cianobactine has been described in species belonging to *Anabaena*, *Lyngbya*, *Microcystis*, *Nostoc*, *Prochloron* and *Trichodesmium* genera[39,43 ,44,45]. In order to know and better understand the biosynthetic pathway of cianobactine has been conducted, recently, a study of molecular type of one of the genes responsible for the formation of cianobactine; this study has involved the use of 132 strains from brackish water and sweet including filamentous forms such as *Planktothrix*, *Anabaena* forms filamentous eterocistiche like, *Aphanizomenon*, *Nodularia* and colonial forms such as *Microcyst*is and *Snowella*[46].

2.3. Geographical distribution of cyanobacteria in Italian lakes

Excessive fertilization of water basins has caused the massive growth of certain organisms, such as cyanobacteria and algae which in the maximum phase of their growth cause *algal bloom*[47]. Cyanobacteria are the algal component that has a bigger impact on the frequency of these blooms in fresh water and can produce cyanotoxins can be dangerous for humans and for animals [48,49]. Since

1970, in different parts of the world, there was a constantly increasing in the frequency of algal blooms also associated with species that produce toxins, increased frequency of episodes of poisoning of animals, including humans have been reported in different areas[45].

In Italy, blooms of toxic cyanobacteria species are causing ecological and health problems; these events have involved both in natural lakes and in reservoirs and have been related to the general increase in the trophic status of the various basins[50,51,52,53].

Episodes due to the presence and development of blooms of toxic cyanobacteria in 61 lakes and reservoirs in Italy are reported in literature. *Planktothrix rubescens* was found in the lakes of northern Italy (Figure 1.1). Extensive studies on the phytoplankton community of the deep subalpine Como, Garda, Iseo, Lugano and Maggiore Lakes have gathered many chemical, physical and biological data showing a state of degradation of water quality due to a gradual process of environments eutrophication. In addition, in most of the lakes of northern Italy have been observed, even species belonging to the *Anabaena, Aphanizomenon* and *Microcystis* genera (Figure 1.2)[54,55].

Algal blooms are also defined as *oligotrophic bloom* because it occurs even in environments with a low trophic level as Maggiore and Garda Lakes[56].

Regarding the small subalpine lakes, a frequent development of cyanobacterial blooms was observed in those lakes compromised in terms of trophic evolution: an emblematic example is the Alserio, Pusiano and Varese Lakes in Lombardy Region.

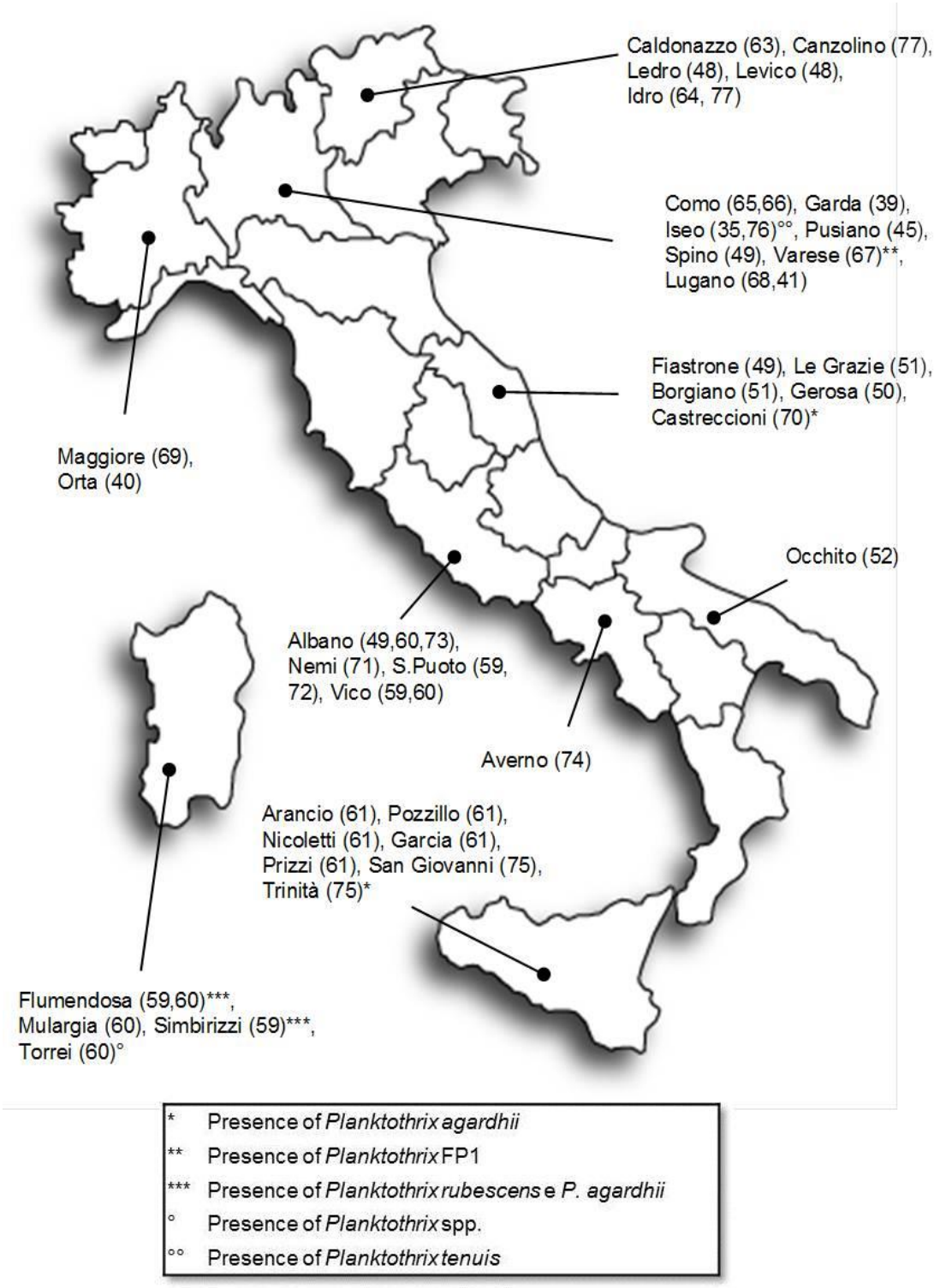


Figure 1. 1:Distribution of Planktothrix rubescens blooms in lakes and reservoirs Italian between 1992 and 2009(Rapporto Istisan 11/35 Pt.1)

Figure 1.2: Cyanobacteria species blooms belonging to the Dolichospermum, Aphanizomenon, Cylindrospermopsis and Microcystis genera in Italian lakes and reservoirs(1992-2010) (Rapporto Istisan 11/35 Pt.1)

In these basins blooms of *Planktothrix rubescens* (Pusiano Lake)[57]; *Microcystis* spp. and *Anabaena* spp. (Varese Lake)[58]; *Aphanizomenon flos-aquae* (Alserio Lake)[59] are frequently reported.

Even for some lakes of Trentin Region the most widespread species is *P. rubescens;* also the of *Aphanizomenon flos-aquae* and *Microcystis aeruginosa* presence was observed[60].

In central and southern Italy species *P. rubescens* is grow abundantly in volcanic lakes both Lazio

(Albano, Nemi and Vico Lake) and Campania Region (Averno Lake) . Other cases of blooms have been detected in Fiastrone, Grazie, Borgia and Gerosa Lakes[61,62,63] and in Occhito Lake in Puglia Region[64, 65].

In central Italy, the only reports on the presence of *Cylindrospermopsis raciborskii* [66] involving the Trasimeno Lake in Umbria Region[67] and Albano Lake in Lazio Region[68].

The abundant presence of cyanobacteria in lakes of Lazio Region has long been known and already in 1953 an episode of exceptional bloom of *Aphanizomenon* ovalisporum in Albano and Nemi Lake was identified[69].

Recently, studies on the trophic conditions and the phytoplankton community of Albano Lake have shown critical conditions of water with a tendency towards a state of meso-eutrophic and biodiversity reduced coupled instead to the development of different species of cyanobacteria such as *Planktothrix* and *Anabaena* spp., which represented up to 47-65% of the total phytoplankton[70,71,72].

The presence of cyanobacteria species responsible for blooms or potential producers of toxins has also been reported for the Nemi Lake[69] and St. Puoto Lake[73]. Moreover, the presence of *Microcystis aeruginosa* is reported in other lakes of central Italy: Massaciuccoli Lake in Tuscany Region, Trasimeno Lake in Umbria Region, Liscione Lake in Molise Region and Polverina Lake in the Marche Region where it has been also possible to detect toxicity for MC-RR[74]. Despite lakes and reservoirs located in the semi-arid parts of the Italian peninsula, represent the most important source of water for various human activities, the presence of toxic cyanobacteria blooms in the southern areas are still poorly reported and are not currently evaluated in their effective dissemination.

However, on the islands have been described blooms of *P. rubescens* in the Italian island: in the Orange, Pozzillo, Nicoletti, Garcia, Prizzi Lakes (Sicily Region)[75] and in lakes Simbrizzi, Flumendosa Mulargia and Torrei Lakes (Sardinia Region).

Furthermore, *M. aeruginosa* and *Dolichospermun flos-aquae*[76] have been reported as recurrent in most of the 27 lakes and reservoirs in Sicily, in which the formation of cyanobacterial blooms since 1979 has been encouraged by the growing phenomenon of eutrophication; while in Sardinia Region 36 basins affected by the presence and / or cyanobacteria blooms are numerous and monitored by time (Table 1.3).

In summary, in Italy data on the presence of cyanobacterial toxic species are avaible only for 61 among the 500 lakes distributed throughout Italy (not counting the minor basis) in 13 out of 20 regions (Table 1.3).

Completely lack data on the rest of the lakes and reservoirs.

Table 1. 3: Cyanobacteria described in the literature from 1992 to 2010 (includes some species of cyanobacteria that were not always present in conjunction with the species considered toxic) (Rapporto Istisan 11/35 Pt.1

Lake	Species
Trentino-Alto Adige	
Idro	*Microcystis* sp.[1]
Caldonazzo	*Anabaena princeps*[2], *Aphanizomenon* sp.[3]
Terlago	*Microcystis* sp.[2],*Oscillatoria* sp.[2]
Lombardy	
Iseo	*Aphanotece clathrata*[4], *Chroococcus limneticus*[4], *Planktolyngbya limnetica*[4], *Gomphosphaeria lacustris*[4], *Aphanocapsa*/*Aphanothece*[5], Leptolyngbyoideae[5*], *Snowella* spp.[5], *Pseudoanabaena limnetica*[6], *Microcystis stagnalis*[7], *Aphanothece clathrata*[7], *Chroococcus minimus*[7], *Chroococcus minutus*[7], *Anabaena catenula*[7]
Garda	*Planktolyngbya limnetica*[8], *Aphanocapsa*/*Aphanothece*[5], Limnotrichoideae[5], Leptolyngbyoideae[5*], *Snowella* cf. *aracnoidea*[9], *Limnothrix* sp.[7]
Como	*Planktolyngbya limnetica*[10], *Chroococcus* sp.[10], *Aphanocapsa*/*Aphanothece*[5], *Pseudoanabaena limnetica*[6], Limnotrichoideae[5], *Limnothrix* sp.[7], *Aphanothece clathrata*[7], *Aphanothece nidulans*[7], *Gomphosphaeria lacustris*[5], Leptolyngbyoideae[9]
Pusiano	*Aphanothece clathrata*[11], *Merismopedia tenuissima*[11], *Pseudoanabaena* sp.[11]
Lugano	*Aphanocapsa/Aphanothece*[5], *Pseudoanabaena limnetica*[6], Limnotrichoideae[5], Leptolyngbyoideae[5*], *Gomphosphaeria lacustris*[5], *Lyngbya limnetica*[7], *Limnothrix* sp.[7]
Piedmont	
Maggiore	*Aphanocapsa*/*Aphanothece*[5], Limnotrichoideae[5], *Limnotrix* sp.[7], Leptolyngbyoideae[5*], *Gomphosphaeria lacustris*[5], *Pseudoanabaena limnetica*[6]
Marche	
Castreccioni	*Aphanocapsa delicatissima*[12], *Aphanocapsa incerta*[12], *Aphanocapsa planctonica*[12], *Chroococcus limneticus*[12], *Merismopedia glauca*[12], *Oscillatoria limosa*[12], *Rhabdogloea smithii*[12], *Spirulina gigantea*[12]
Lazio	
Nemi	*Pseudoanabaena limnetica*[13], *Merismopedia trolleri*[13]
Bolsena	*Snowella*-like[14], *Microcystis* sp.[14]
Albano	*Anabaena* sp.[14]
Molise	
Liscione	*Pseudoanabaena mucicola*[15], *Aphanocapsa* spp.[15], *Anabaena* spp.[15], *Aphanothece* spp.[15]
Siciliy	
Arancio	*Dolichospermum smithii*[16], *Anabaena solitaria* f.*planctonica*[17], *Microcystis panniformis*[18], *Gomphosphaeria nägeliana*[19], *Pseudoanabaena* sp.[19], *Sphaerospermopsis aphanizomenoides*[17], *Dolichospermum crassum*[17], *Anabaena* spp.[17], *Coelosphaerium kuetzingianum*[17], *Raphidiopsis mediterranea*[17], *Woronichinia naegeliana*[17]
Disueri	Oscillatoriales[17], Chroococcales[17]
Pozzillo	*Anabaena nodularioides*[17], *Microcystis* sp.[17], Oscillatoriales[17]
Prizzi	*Anabaenopsis elenkinii* f. *circularis*[17]
Rosamarina	*Aphanizomenon* sp.[17], *Planktothrix* sp.[17], *Merismopedia* spp.[17]
Villarosa	*Microcystis* sp.[17], Chroococcales[17]
Piana degli Albanesi	*Anabaena solitaria* f.*planctonica*[17], *Dolichospermum crassum*[17],
Gammauta	*Dolichospermum smithii*[16], *Dolichospermum crassum*[17], Chroococcales [17]
Rubino	*Planktothrix* sp.[17], *Anabaena* spp.[17], Oscillatoriales[17]
Soprano	*Anabaenopsis elenkinii*[17], *Aphanotece* sp.[17], *Oscillatoria* spp.[17], *Phormidium* sp.[17], Oscillatoriales[17]
Gorgo	*Anabaena* sp.[17], *Anabaenopsis elenkinii* f. *circularis*[17], Oscillatoriales[17]
San Giovanni	*Microcystis* spp.[17], *Anabaena* spp.[17], *Anabaenopsis elenkinii* f. *circularis*[17], Oscillatoriales[17]
Castello	*Planktothrix* sp.[17]
Trinità	*Anabaena* spp.[17], *Coelosphaerium kuetzingianum*[17], Oscillatoriales[17]
Scansano	*Dolichospermum spiroides*[17], Oscillatoriales[17], *Anabaena* spp.[17] *Microcystis* spp.[17]
Guadalami	*Dolichospermum smithii*[17], *Dolichospermum crassum*[17], *Planktothrix* sp.[17], Chroococcales[17], Oscillatoriales[17]

Biviere di Cesarò	*Oscillatoria* spp.[17]
Santa Rosalia	*Anabaena* spp.[17], Oscillatoriales[17]
Olivo	*Anabaena nodularioides*[17]
Cimia	*Merismopedia* spp.[17]
Vasca Ogliastra	*Anabaena* spp.[17], *Microcystis* spp.[17]
Biviere di Gela	*Microcystis* spp.[17], *Lyngbya* spp.[17]
Ogliastro	*Oscillatoria* spp.[17]
Pergusa	*Oscillatoria* spp.[17], *Spirulina* sp.[17], Chroococcales[17]
Comunelli	*Lyngbya* spp.[17], *Phormidium* sp.[17]
Sardinia	
Flumendosa	*Oscillatoria mougetii*[20], *Oscillatoria* spp.[21]; *Gomphospaeria aponina*[21]; *Aphanothece* spp.[21]
Simbirizzi	*Anabaena* sp.[15]
Mulargia	*Anabaena* spp.[15], *Oscillatoria mougetii*[20]; *Oscillatoria* spp.[21]
Gusana	*Aphanocapsa* spp.[15], *Lyngbya* sp.[15]
Liscia	*Gomphospaeria aponina*[21]
Monteleone	*Anabaena* sp.[15], *Microcystis* sp.[15], *Aphanocapsa* spp.[15], *Aphanizomenon* spp.[15]
Cucchinadorza	*Lyngbya* sp.[15], *Anabaena* sp.[21], *Aphanocapsa* sp.[15]
Torrei	*Aphanizomenon* spp.[15], *Lyngbya* sp.[15]
Bidighinzu	*Aphanocapsa* sp.[21]
Posada	*Anabaena* spp.[15], *Aphanocapsa* sp.[15], *Pseudoanabaena mucicola*[15]; *Lyngbya* sp.[15], *Microcystis* spp.[15], *Gomphospaeria aponina*[21]; *Oscillatoria* spp.[21]
Govassai	*Merismopedia* sp.[15]; *Aphanocapsa* sp.[15], *Aphanothece* spp.[21]
Cedrino	*Microcystis* spp.[15]
Benzone	*Lyngbya* sp.[15], *Aphanocapsa* sp.[15], *Oscillatoria* spp.[21]
Pattada	*Aphanizomenon* spp.[15], *Woronichinia* spp.[15], *Anabaena* spp.[15], *Gomphospaeria* spp.[21]; *Aphanocapsa* sp.[21], *Oscillatoria* spp.[21]
Cuga	*Pseudoanabaena mucicola*[21]
Omodeo	*Merismopedia punctata*[21], *Aphanothece* spp.[21]
Monteleone Roccadoria	*Pseudoanabaena mucicola*[21], *Aphanocapsa* sp.[21], *Gomphospaeria aponina*[21]
Bunnari alto	*Merismopedia punctata*[21], *Aphanocapsa* sp.[21]
Casteldoria	*Anabaena* spp.[21]
Santa Lucia	*Aphanothece* spp.[21], *Oscillatoria* spp.[21], *Gomphospaeria aponina*[21]
Monte Pranu	*Oscillatoria* spp.[21]
Coghinas	*Aphanocapsa* sp.[21]
Cixerri	*Oscillatoria* spp.[21], *Pseudoanabaena mucicola*[21]
Is Barroccus	*Aphanothece* spp.[21], *Aphanocapsa* sp.[21]
Surigheddu	*Oscillatoria* spp.[21],
Monteponi	*Aphanocapsa* sp.[21]
Medau Zirimilis	*Oscillatoria* spp.[21]
Sos Canales	*Anabaena* spp.[21]
Bau Pressiu	*Aphanothece* spp.[21]
Barzolu	*Anabaena* spp.[21]
Corongiu	*Aphanocapsa* sp.[21]
Leni	*Aphanocapsa* sp.[21]

2.4. Cyanobacterial toxins

The cyanotoxins are a group formed by natural toxins different from both chemical and toxicological point of view; they are responsible for both acute and chronic poisoning in animals and humans.
The main classes include: the hepatotoxins (microcystins and nodularins), neurotoxins (anatoxin-a, homoanatoxin-a, anatoxin-a (s), saxitoxin, BMAA), the cytotoxins such as cylindrospermopsin, gastrointestinal toxins and compounds with acute skin effects such as aplysiatoxin,

debromoaplysiatoxin and lingbiatoxin produced by marine cyanobacteria and lipopolysaccharide endotoxin (LPS), potentially irritating[77] [78].

In general, the MC and Nod are frequently toxins found.

2.5. Microcystins

2.5.1. Chemic structures and properties

The microcystins (MCs) are monocyclic heptapeptides with low molecular weight, consisting of a carbohydrate locking, seven amino acid residues and one methylamine. This class of compounds are two L-amino acid variables (L-R1 and L-R2) (Figure 1.3).

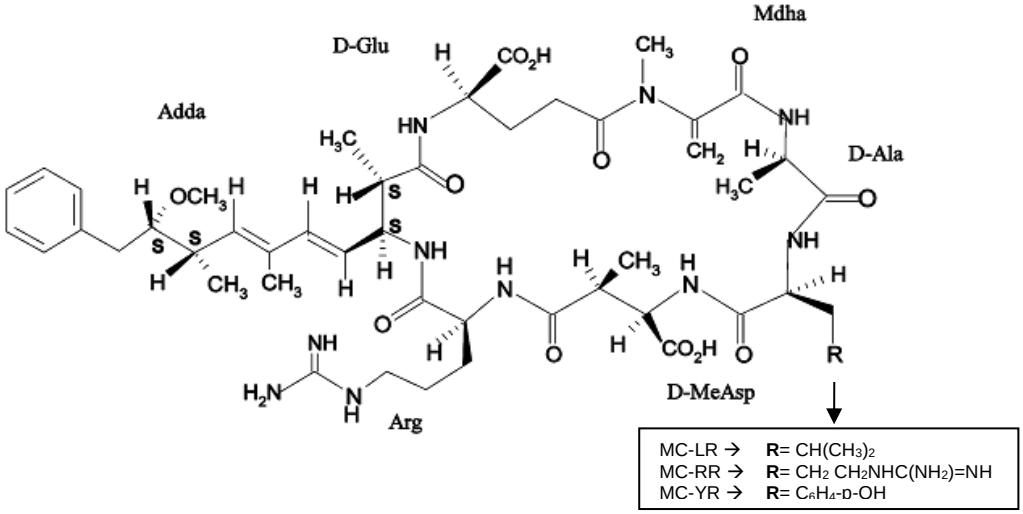


Figure 1. 3:Chemical structure of microcystins most common

In the world more than 80 different variants from the first toxin identified, the MC-LR, have been isolated [75].

MCs are soluble in water, methanol and ethanol, insoluble in acetone, ether, chloroform and benzene; they are resistant to hydrolysis and chemical oxidation at neutral pH values. A rapid chemical hydrolysis can occur only in controlled laboratory conditions such as in the presence of 6M HCl at high temperatures; they are instead oxidized by ozone and other strong oxidizing agents.

The MCs are very stable to sunlight, while the UV light to the values of maximum absorption of the MC-LR and MC-RR degrades rapidly[79].

2.5.2. Toxicity

- *Mechanism of action*

The MC-LR and most of its congeners are highly water soluble and generally not able to cross cell membranes of vertebrates and need, therefore, to a carrier protein dependent adenosine-triphosphate (ATP).

Through the ileum and the system of organic anion transporting, the MC-LR reaches the liver[76]; here, into hepatocytes, carries out its activity as a potent inhibitor of phosphatases 1 and 2A.

This inhibition, at high doses, leads to hyperphosphorylation of the cytoskeletal proteins and final rupture of the ultrastructure of the liver. The liver swells to double its volume due to a large hemorrhage intrahepatic lobular center, preceded by swelling of hepatocytes and the rupture of the liver sinusoids. At lower doses there is induction of cell proliferation and hypertrophy of the liver.

Certain chemicals have been used experimentally in laboratory animals to prevent hepatotoxicity of MC. These include cyclosporine A, rifampin and silymarin. Their effectiveness is greatest when given before or simultaneously with toxin[80]. The intestines and kidneys are other organs that can accumulate significant amounts of the toxin.

Some Japanese authors[81] have determined the toxicity of 21 variants of MC and NOD on the basis of their ability to inhibit the phosphatase 2A (IC 50: Concentration Inibitory = 50%).

The results indicate that the MC-LR is the most potent inhibitor of phosphatase 2A.

On the basis of these inhibition values the authors have calculated a conversion factor to calculate the concentrations of MC and NOD as equivalents of MC-LR, as reported in Table 1, using the following formula:

Conversion factor = IC 50 MC-LR/IC50 MC considered.

Some published studies suggest that MC could act as tumor promoters, agents that do not cause cancer, but they stimulate the proliferation of cancer cells. In June 2006, the IARC (International Agency for Research on Cancer) has assembled a group of experts to assess the toxicity of MC-LR and NOD[82,83]. The committee concluded that there was adequate evidence in experimental animals for the carcinogenicity of MC-LR.

Table 1. 4: Conversion factors and IC50 values for 21 variants of microcystins and nodularin

Toxin	IC_{50} (nM)	Conversion factor
MC-LR	0,032±0,004	1,000
MC-RR	0,056±0,002	0,571
MC-FR	0,069±0,003	0,464
MC-LF	0,096±0,0019	0,333
[D-Asp^3]MC-HtyR	0,098±0,006	0.327
[D-Asp^3, (Z)-Dhb^7]MC-HtyR	0,110±0,008	0,291
MC-LW	0,114±0,003	0,291
[D-Asp^3, (E)-Dhb^7]MC-HtyR	0,122±0,005	0,262
MC-YR	0,125±0,005	0,256
MC-LA	0,161±0,002	0,199
[D-Asp^3, (Z)-Dhb^7]MC-LR	0,164±0,010	0,195
[Dha7]MC-LR	0,167±0,003	0,192
MC-WR	0,179±0,011	0,179
[D-Asp^3, (E)-Dhb^7]MC-LR	0,201±0,003	0,159
[D-Asp^3, Dha^7]MC-RR	0,220±0,012	0,145
[D-Asp^3, Dha^7]MC-LR	0,254±0,004	0,126
[Dha^7]MC-RR	0,293±0,012	0,109
[D-Asp^3]MC-RR	0,300±0,009	0,107
[Dha^7]MC-YR	0,379±0,003	0,084
NOD	0,540±0,063	0,059
[6-(Z)-ADDA^5]MC-RR	0,126±0,314	0,003

- *Pharmacokinetics*

The liver appears to be the main target organ both as regards the accumulation that the excretion of the MC. In tissue distribution studies on laboratory animals following intravenous and intraperitoneal administration of MC-LR, 50-70% was recovered in the liver, another 7-10% in the intestine and the remaining amount distributed throughout the body. It is likely that the transport can also occur in the kidney, since this organ also has a transport system of bile, similar to that of the intestinal cells of the rats. The MC are resistant to enzymatic hydrolysis and thus the degradation in tissues[84], and their excretion in the bile occurs as toxins as such or as a result of their conjugation[85]. The liver has a crucial role on the detoxification of these toxins[86]. The detoxification products were detected in the urine and feces. Have identified three metabolic products derived from conjugation reactions respectively with glutathione, with cysteine and with the diene ADDA oxidized[87]. Following studies in mice has resulted in a biexponential plasma elimination of MC-LR, with half-lives of 0.8 and 6.9 minutes[88]. The MC-LR is excreted rapidly, 75% of the total excretion occurs within 12 hours. The remaining 24% is excreted after 6 days, of which 9% in the urine and 15% more slowly with the feces[89].

- *Human Exposure*

Humans can be exposed to toxins orally or through the consumption of water through the intake of supplements based on algae or dermal through contact with contaminated water from lakes and rivers during sports activities[77]. A minor source of exposure is inhalation through the showers and during water sports (inhalation of spray and droplets)[75] .

- *Short-term effects*

Several incidents of acute poisoning by consumption of contaminated water from MC with implications for human health from gastroenteritis to death are reported in literature[90]. The consumption of fish living in water presenting blooms of cyanobacteria, especially of its liver, can cause the Haff syndrome, vomit, production of dark brown urine, muscle pain, death from respiratory failure[77]. Humans can also be exposed through the consumption of food supplements based on algae, potentially hazardous if they contain some toxic species of cyanobacteria. Many of these products contain *Aphanizomenon flos-aquae,* blue green algae which coexists with *Mycrocistis aeruginosa*, which can thus enter into the composition of these products for human use. The Departments of Health and Agriculture in Oregon (USA) have established a legal limit of 1 µg/g for the presence of MC in the products based on blue-green algae and the obligation of tests to detect the presence of algal toxins[91]. Dermal exposure, however, may take place during the course of recreational activities, or during the use of showers fed with water contaminated.
This exposure can cause production of blisters on the lips and allergic reactions such as contact dermatitis, asthma, hay fever and conjunctivitis[77].

- *Long-term effects*

Health effects resulting from chronic exposure to low doses of MC are not known[74]. In China, studies in order to determine the importance of the MC as a risk factor in the development of hepatocellular carcinoma in humans have been conducted. The incidence of this disease in China is very high, with a variable geographic distribution. The cyanobacteria blooms, for example, are very abundant in the surface waters in the south-east China, where the incidence of this tumor is the highest in the country[83].

- *NOAEL and TDI estimation*

In 1998, the *World Health Organization* has drawn up a provisional guideline value for the presence of the only MC-LR in water intended for human consumption[92]. In the conclusions of WHO guidelines on drinking water was highlighted that guideline values for other MCs could not be fixed. This impossibility is still valid. The limit for the MC-LR in waters for human consumption derived from a NOAEL *(No Observed Adverse Effect Level),* for liver damage, of 40 μg/kg of body weight obtained from 13 weeks long study in mice treated with water watering containing MC-LR. Considering this value has been derived a TDI *(Tolerable Daily Intake)* of 0.04 μg/kg body weight/ day, using a safety factor of 1000 (100 for the differences between species and intra-species and 10 for the low level of available data). From TDI has been obtained a guidance value *(Guidance Value,* GV) of μg/L for the concentration of MC-LR, having considered that the intake through drinking water represents 80% of the total intake *(Allocation Facto,* AF 0.80) and a consumption of 2 L of water / day for a person weighing 60 kg (10).This value is supported by a 44 days long study on pigs watered with water containing an extract of *M. aeruginosa* producing MC-LR.

If other MCs are present, the use of the *Toxicity Equivalent Factor* (TEF) may be necessary; this factor express the toxicity of the mixture containing different MCs in MC-LR equivalents. Wolf and Frank[93] have calculated the values of TEFS for other MCs on the basis of the value of LD_{50} *(Lethal Dose* 50%) from acute toxicity studies in mice intaperitoneal. The bibliography toxin can be considered the MC-LR, so that its TEF is = 1. The TEF individual of a toxin X can be calculated from the ratio between the value of LD_{50} of MC-LR toxin and X, according to the equation:

$$TEF_X = LD_{50}\ \text{MC-LR} / LD_{50}\ (X)$$

the same value of MC-LR was adopted for the MC-LA,-YR and-YM, for the MC (D-ASP^3 (E)-Dhb^7)-RR and RR values were 0.2 and 0.1 respectively.

2.6. Nodularins

2.6.1. Chemical structures and properties

The nodularins (NODs) (Figure 1.4) are monocyclic pentapeptide with a structure similar to the MC, containing the amino acid ADDA[94]: to date few congeners are known, identified for the variability of the only L-amino acid present at position 2, in addition to small structural changes such as

demetilation. The various congeners may have very different toxicity; a no-toxic variant contains the 6Z-stereoisomer of Adda has been also identified. In the sponge of marine origin *Thenella swinhoei* was found an analogue of NOD called motuporina, which has in place of the hydrophobic L-valine instead the polar L-arginine. The motuporina could be of cyanobacterial origin since the sponge that produces welcomes cyanobacterial symbionts. The NOD is only produced by *Nodularia spumigena*, cyanobacterium living in brackish waters. Saito *et al.*[95] have isolated and identified a new NOD, called NOD-Har, which presents the homoarginine instead of arginine.

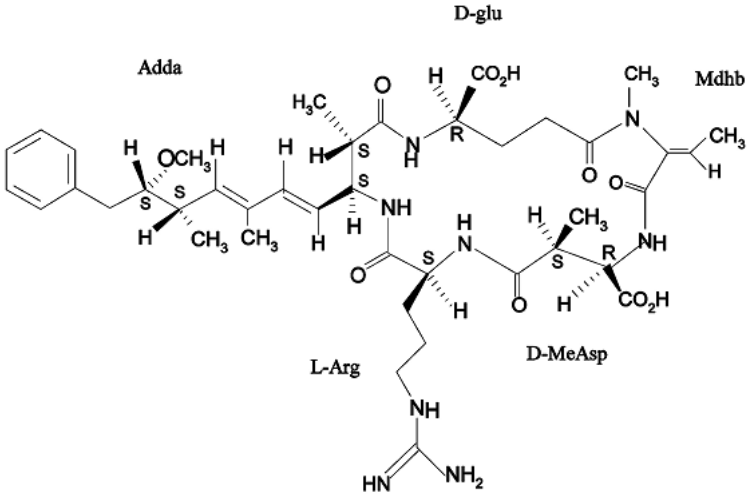


Figure 1. 4:Chemical structure of Nodularin

2.6.2. Toxicity

The toxicity mechanism of the NOD is very similar to that of MC; they are potent hepatotoxic. The toxins enter immediately into the bloodstream through the ilium transported by bile acid transporters that convey toxins through the mucosa. Subsequently, the toxins are transported preferentially in hepatocytes via the bile and finally toxins induce changes in actin microfilaments, in the elements of the cytoskeleton of the cells with the result of a dense aggregation of microfilaments in the vicinity of the center of the cell. The loss of the cellular support cause cell swelling and rupture of the cells endothelial sinusoids. In some cases, the destruction of the parenchymal cells of the liver sinusoids and can cause lethal intrahepatic hemorrhage in a matter of a few hours or liver failure within a few days[88]. The hepatotoxic and carcinogenic activity, as in the case of MC, is associated with inhibition

of phosphatase 1 and 2 [96,97]. The NOD induces bleeding liver in mice, with an LD_{50} of 50 µg/kg (intraperitoneal). At lower doses may act as a tumor promoter by favoring the division of liver cells[98].

- *Effects on humans*

There are no data on the toxic effects on humans of *N. spumigena* (3). In 1991 in the Alexandrina Lake (Australia), some people showed eczema skin after contact with water containing toxins mainly from *Microcystis* and *Nodularia*[99].

- *NOAEL and TDI estimation*

It was developed a NOAEL for NOD due to lack of suitable toxicological data. Since the mechanism of toxicity of MC-LR and NOD is very similar, the guide value for the MC-LR may also be used for the NOD.

2.7. Anabaenopeptin

2.7.1. Chemical structures and properties

The anabaenopeptins (in Figure 1.5 are reported Anabaenopeptin A and Anabaenopeptin B) are cyclic heptapeptides consisting of five amino acid residues that consist of a ring and a further exocyclic residue linked to the ring via an amide bond.

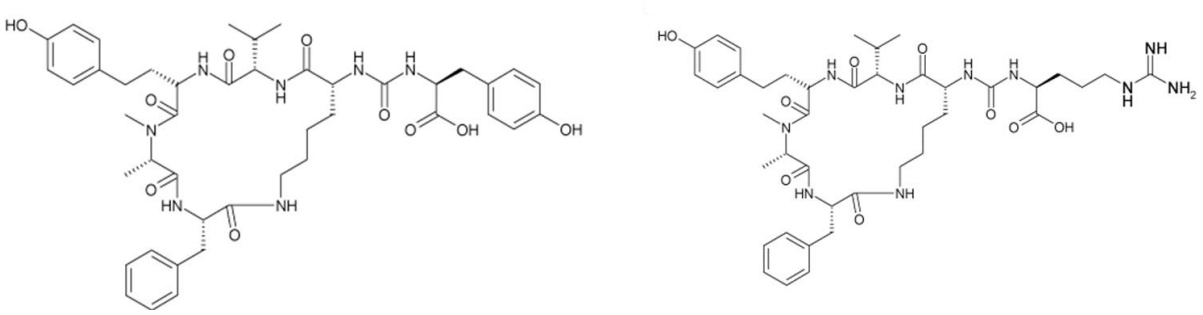


Figure 1. 5 : Anabaenopeptin A (left) and Anabaenopeptin B (right) structures

In the generic structure of anabaenopeptins, the D-lysine residue in position 2 and the amide bond are conserved, while in the remaining positions different amino acids can be found, thus obtaining a great

variety of genera. The first found were anabaenopeptin A and B, produced by Anabaena flos aquae, up to the determination of a considerable number of congeners[100].

2.7.2. Toxicity

The anabaenopeptins show a significant diversity as regards the bioactivity, which arises from the structural variety; all congeners inhibit carboxypeptidase A, some protein phosphatase 1 and 2A, still others the serine protease[101].

2.8. Microginins

2.8.1. Chemical structures and properties

Microginins (in Figure 1.6 one of the analites structure is reported) constitute a class of about 30 linear oligopeptides, mainly produced by Microcystis Aeruginosa, with a number of amino acid residues ranging from four to six. The generic structure consists of a derivative of decanoic acid (Ahda, 3-amino-2-hydroxydecanoic acid)[102] and an N-terminal component, the presence of two tyrosine units and a terminal C is frequently found[103].

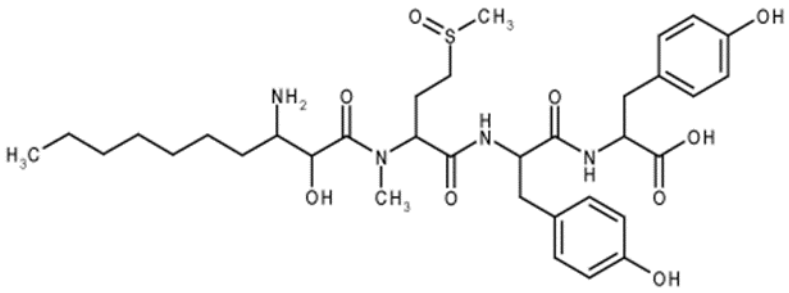


Figure 1. 6: Structure of microginin-690

2.8.2. Toxicity

Microginins are inhibitors of the angiotensin converting enzyme (ACE), which intervenes in the cardiovascular system, and of leucine aminopeptidase (LAP), due to the 2S configuration of the AhDA group. They show no activity against trypsin, chymotrypsin or protein phosphatase 1 A[100].

2.9. Cyanopeptoline

2.9.1. Chemical structures and properties

Cyanopeptolines (Figure 1.7) are oligopeptides whose general structure includes seven amino acid residues, of which six form a ring. This class of cyanotoxins consists of a 3-amino-6-hydroxy-2-piperidone residue and an ester bond between the hydroxyl group of L-threonine and the C-terminal amino acid[104].

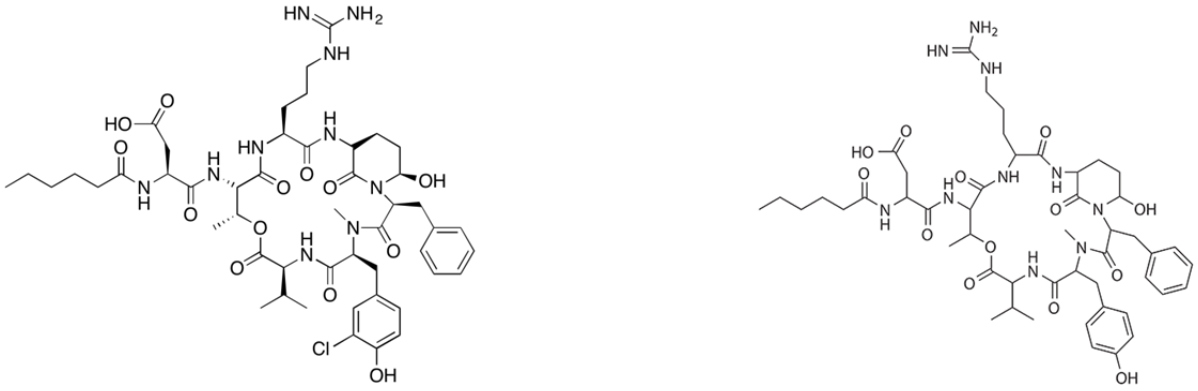


Figure 1. 7: Cyanopeptolin 1041 (left, containig Cl atoms) and Cyanopeptolin 1007 (right, without Cl atoms)

Cyanopeptolines are produced by Anabaena, Microcystis and Planktothrix agardhii, the same cyanobacteria from which microcystins originate. There are countless known varieties, about 70, some of which have chlorine atoms, which regulate biological activity.

1.9.2. Toxicity

Cyanopeptolines inhibit the serine protease by binding to the amino acid residue tyrosine or chlorinated tyrosine via two active sites of the enzyme. The presence or absence of chlorine bound to the tyrosine ring influences the interaction with the active sites, modulating the inhibition activity. The existence of halogenated and non-cyanopeptoline forms makes the determination of a unique biological function difficult, as the latter is strongly influenced by structural variability[105].

1.10. Cylindrospermopsins

1.10.1. Chemical structures and properties

The cylindrospermopsin (CYN) belongs to the class of guanidine alkaloids. The molecule consists of a guanidico tricyclic group combined with hydroxymethyl-uracil (Figure 1.8). It is considered as a cytotoxin, since it produces both cytotoxic nephrotoxic effects nephrotoxic, although other organs (thymus and lung) may be damaged by exposure to the toxin is also considered a potential carcinogen[106]. The orally administered can cause gastroenteritis due to injury to the walls of the intestine, hepatitis to liver cell damage, dysfunction in the functioning of kidneys for renal cell damage and hemorrhage to damage to the blood vessels.

Eight species of cyanobacteria producers of CYN have been identified: *Cylindrospermopsis raciborskii*, *Aphanizomenon ovalisporum* and *Aphanizomenon flos-aquae*, *Umezakia natans*, *Rhaphidiopsis curved* and *Anabaena bergii*, *Anabaena lapponica*, and *Lygnbya wollei*[107].

Among these *Cylindrospermopsis raciborskii* is the species that is the major problem on a global scale[108]. The CYN is highly hydrophilic, and its intestinal absorption requires active transport systems as well as entry into hepatocytes, using as the bile transport system. Since the small size of the molecule, a passive diffusion can occur, even if limited, through the cell membrane, as shown by *in vitro* studies demonstrate that the cytotoxic effects on a cell line without the presence of bile as the transport system[109].

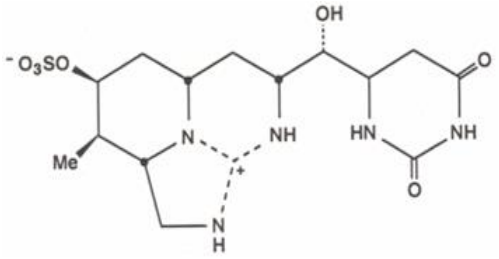


Figure 1. 8:Chemical structure of cylindrospermopsin

1.10.2. Toxicity

At low doses the CYN suppresses the glutathione-conjugated protein synthesis, probably by inhibiting ribosomal translation by binding to a protein associated with eukaryotic translation system, but at higher concentrations dominates the process as quickly as toxic, metabolism-dependent[110, 111] and its acute toxicity appears to be mediated by cytochrome P 450 metabolites - generated[112]. It has an acute and progressive delayed. The acute hepatic injury is located in the center lobular areas with vacuolization of hepatocytes and increased pigmentation of the nuclei and the cytoplasm. The main actions toxic to the kidney occur with necrosis and increased cross section of the proximal tubules

and alteration of the glomeruli. The CYN could also act as endocrine disruptor as a study showed that the toxin might alter the relation progesterone/estrogen in women[113].

Studies on laboratory animals are in favor of a possible genotoxic[114] and carcinogenic effects[115].

- *Pharmacokinetics*

Studies in mice treated intraperitoneally with 0.2 mg / kg of ^{14}C CYN have shown that most of the radioactivity was excreted in the first 12 hours (70.9%), mainly in the urine (59.6% in animals that showed toxic effects and 70.5% in animals without toxic effects). The accumulation occurred mainly in the liver with a peak of 20.6% after 6 hours, and to a lesser extent in the kidneys[116].

- *Effects on humans*

The oral exposure with contaminated water can cause gastrointestinal disturbances such as bloody diarrhea, severe dehydration with loss of protein, electrolytes, glucose and ketones in the urine. All cases of people exposed to CYN needed of hospitalization, where they received intensive treatment with intravenous therapy.

- *NOAEL and TDI estimation*

Two studies have been used for the calculation of a NOAEL / TDI. The first 90-day long study in mice given water with contaminated water produced a NOAEL of 150 µg/kg body weight, and based on this value has been calculated a TDI of 0.3 g / kg bw / day using a safety factor 500 (10 for intraspecies variability, 10 for that interspecies and 5 for the duration of exposure less than the duration of the life of the animal). TDI was obtained from a GV of 9 g / L having considered that the intake through drinking water represents 100% of the total intake and consumption of 2 L of water / day for a person weighing 60 kg.

The second study was conducted on mice treated by gavage for 11 weeks, with a NOAEL of 30 µg/kg body weight and a TDI of 0.06 g / kg bw/day using a safety factor of 500 (10 for the intraspecies variability, 10 for that interspecies and 5 for the duration of exposure less than the duration of the life of the animal). TDI was obtained from a GV of 1.8 g / L having considered that the intake through drinking water represents 100% of the total intake and consumption of 2 L of water / day for a 60 kg

person weight[117]. Some authors recommend an additional safety factor of 10 for potential genotoxic effects.

1.11. Anatoxins

1.11.1. Chemical structure and properties

The anatoxin-a (ANA-a) and the in-homoanatoxin are low molecular weight alkaloids characterized by neurotoxic action. In particular, the ANA-a is a bicyclic amine alkaloid with a molecular weight of 165 Da, a secondary amine 2-acetyl-9-azabicyclo (4-2-1) non-2-ene)[118]. It is produced by *Anabaena flos-aquae, Anabaena* spp. *(Flos-aquae-lemmermannii group), planktonica Anabaena, Oscillatoria,* and *Aphanizomenon Cylindrospermum,* is synthesized in the cell from the amino acid ornithine via putrescine with the participation of the enzyme ornithine decarboxylase. The ANA-in is not susceptible to enzymatic hydrolysis by cholinesterase since it is not an ester. The homoanatoxin-a (179 Da) is an analogue of the ANA-in and is isolated from a strain of *Oscillatoria formosa (Phormidium formosum).* It has a propionyl group in position C-2 instead of the acetyl group present in ANA-a. The ANA-a (s) has a different chemical structure being a phosphoric ester of N-hydroxy guanidine(Figure 1.9).

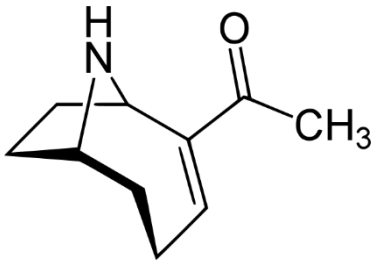


Figure 1. 9: Chemical structure of anatoxin-a

1.11.2. Toxicity

- *Mechanism of action*

The ANA-a is a potent pre- and postsynaptic depolarizing agent. It binds to acetylcholine receptors in the central nervous system and peripheral neuromuscular junctions, causing block the transmission of nerve impulses following by death from muscle paralysis and asphyxiation. The acute effects seem to be the main risk to human health. The ANA-a (s) inhibits the acetylcholinesterase activity only in the peripheral nervous system. The blockades of hydrolysis causes acetylcholine accumulate resulting

in nerve hyperexcitability. The type of action is similar to that of many organophosphates, commonly used as pesticides[119].

- *Effects on humans*

There are no data available on humans, although a recent episode of accidental death of a boy occurred in the United States has been attributed to the ingestion of contaminated water with ANA-a during recreational activities.

- *NOAEL and TDI estimation*

There are insufficient data to obtain a NOAEL or LOAEL and calculate a TDI.

1.12. Available methods for the analysis of cyanotoxins

1.12.1. Sampling for the detection of algal toxins

Cyanotoxins in a water body are mainly contained within the toxin-producer-cells (intracellular toxins), although high concentrations of toxins can be released into the water primarily as a result of senescence and cell lysis (extracellular toxins or free).
The risk associated with the presence of cyanotoxins in freshwater and drinking water can be significantly reduced by removal or filtration of algal biomass in the water[120]. However, treatments used for the removal of the cells as well oxidation processes for water treatment, responsible for cell lysis, may increase the release of extracellular toxins within the water body.
The choice of the determination of total, intracellular or extracellular toxin concentration is primarily relate to the specific needs of the risk assessment, for exposure assessment or the efficiency of water treatments. In adopting precautionary principles for the protection of human health, especially during an intense algal bloom, it is advisable to determine the total concentration of toxins that may be present both in freshwater and drinking water.
For screening and / or confirmatory analyses, storage of samples must be carried out in line with the requirements for determining the total (intracellular + extracellular) and / or free (extracellular) toxin concentration.

As containers for water sampling are indicated polyethylene or glass dark bottles washed with ultrapure water without traces of the analytes[111]. The samples must be stored in the dark and at temperatures in the range 1-10 °C to prevent degradation of the analytes due to the action of light and microbiological agents. In these conditions, storage is limited to 24h as maximum time; on the other hand, freezing of the samples will be necessary for longer periods of storage.

- ***Analysis of the total level of toxins***

  Store samples in polyethylene or glass bottles and proceed at least one cycle of freezing-thawing to promote cell lysis. In case of necessity of filtration use filters black band.

- ***Content analysis of extracellular toxins***

  Store samples in polyethylene or glass bottles in the dark and at temperatures in the range 1-10 °C for 24h as maximum time. In case of necessity of filtration, the porosity of the filters must not be above 0.45 mM in order to retain the algal cells.

1.12.2. Methods of screening

In risk management, it is useful to have screening methods for early detection both of the presence of cyanotoxins and the type of the class produced. The screening methods are biological methods, immunological and biochemical qualitative and / or semi-quantitative those do not require external analytical standards. Screening methods must ensure adequate sensitivity to the level of toxicological interest, simplicity of execution and the possibility to quickly and inexpensively analyze a large number of samples. Generally, they are able to identify the class of toxins, but not specifically the single compound.

The same considerations previously described for risk assessment associated with the total content or extracellular toxins are valuable for screening methods. Water sample must be stored and pretreated in agreement with the requirements reported for the analysis of total toxins or free.

In Italian guideline for cyanobacteria in water for human consumption, the authors reported a selection of screening methods[111].

- *Biological assays in vivo*

The mouse assay *(Mouse BioAssay,* MBA) has been in the past the *in vivo* test most commonly used to determine the toxicity of samples containing cyanotoxins.

The MBA is an economic test and it can provide information on the overall toxicity of the sample within a few hours, including the toxicological class to which the toxin belongs to epatotoxin and neurotoxin[121,122]. The MBA disadvantages are the lack of sensitivity and selectivity, and last but not least, ethical issues related to the use of laboratory animals. In recent years other methods have been developed based on the use of shellfish, traditionally used in ecotoxicological assays, such as *Daphnia* spp., *Moina* spp. And *Thamnocephalus platyurus* between those freshwater[123,124,125] and *Artemia salina* among those marine.These assays have a fast response (24 hours) and are easy to perform; however they have the same limitations of the MBA and are also not able to discriminate between the classes of toxins, because the toxicity is expressed only in terms of EC_{50}.

- *Immunological methods*

The enzyme immunoassay ELISA method *(Enzyme Linked Immunosorbent Assay)* allows cyanotoxins determination in freshwater and drinking water, including spring waters, swimming pool water and those used for the production of water for dialysis, according the definitions listed in the regulations. On the market are available ELISA kit for the analysis of microcystins (MCs) and cylindrospermopsin (CYN).

For MCs kits are available that can determine concentrations ranging from 0.1 to 5.0 μg/L of MC-LR, while for CYN the concentration range is from 0.04 to 2.0 μg/L. Higher concentrations of analytes can be measured after dilution of the sample; many analytical protocols have been validated on the analysis of real water sample. The reliability and sensitivity of an ELISA essentially depend on the type of antibody used and its ability to bind to target compounds. The choice of the most suitable ELISA test is dependent on the need to determine a specific compound (monoclonal antibodies) or to be effective as screening for a class of substances (polyclonal antibodies).

The most widespread polyclonal test for MCs determination employs specific antibodies able to recognize and bind to the Adda, the amino acid representative of the class of MC and nodularins (NOD) and is therefore not able to discriminate between the different congeners. The results are expressed as equivalents of MC-LR, and the total amount of MC in the sample is determined by interpolation on the calibration curve. The qualitative and quantitative analysis is based on a colorimetric reaction between a reagent and peroxidase bound to MC-LR. These tests meet all the ADDA-containing analytes, including possible degradation or conjugation compounds. For this reason, together with the impossibility of knowing the specific reactivity of the different MC-LR

congeners, these tests are considered semi-quantitative and not useful for a risk assessment of the toxicological potential of the different MC variants.

Similar considerations can be made on the polyclonal ELISA for CYN, for which it has also been found an overestimation of one order of magnitude compared to chemical methods[126,127] in the presence of *Aphanizomenon sp.* In this case, *cross-reaction* with isomers or congeners of CYN was assumed.

The tests for MC using monoclonal antibodies are based on the indirect competition between a protein complex of the MC (eg. a conjugate with bovine serum albumin - *Bovine Serum Albumin:* MC-LR-BSA) that functions as an antigen, and that contained in the sample.

Both types of ELISA are based on spectrophotometric detection, then the test sample, must be free of any endogenous compound or reagent capable of interfering with the colorimetric response.

In the most popular ELISA kit adopted for MC determination to levels close to their detection limits, false positive in varying degrees 6-17% was estimated[128].

Recently, methods of analysis based on the realization of synthetic receptors capable of reacting with the MC-LR were described. These *Molecularly Imprinted Polymers* (MIPs) are very sensitive (detection limit of 0.1 µg/L) but show little reactivity towards other MC congeners[129]. These methods have also been used as materials for extraction.

- *Biochemical methods*

MCs and NOD are potent natural inhibitors of protein phosphatases (serine / threonine) PP1 and PP2A[130]. Inhibition test enzymatic activity are available with a good sensitivity for the determination of cyanotoxins; thus, it's possible to use both the PP1 is the PP2A, with different performance in terms of sensitivity[131, 132](12, 13) but still adequate to WHO limits without the need for pre-treatment of the sample.

The quantification of the inhibition can be made with different spectrophotometric (range of response from 0.1 to 2.5 µg/L) or radiometric techniques[133]. This latter are more sensitive than spectrophotometric techniques.

Biochemical methods are not selective enzyme inhibition against several congeners of MC, as the immunological tests; however, the response is proportional to the total toxicity of the sample and can then be used to assess the potential toxicological risk associated with these compounds.

1.12.3. Confirmatory methods for the determination of cyanotoxins

Confirmatory methods are based on the determination of physico-chemical properties such as molecular weight, presence of chromophores or functional groups able to give specific reactions. The physico-chemical methods of confirmation, if sufficiently selective, may allow the simultaneous analysis of MCs, CYN and anatoxin-a (ANA-a) and compounds of degradation and/or structurally similar, such as the homo-anatoxin, the dihydro- and epoxy- anatoxin[134] and deoxy-cylindrospermopsin.

For an accurate analysis of cyanotoxins in freshwater and drinking water and for a proper management of water treatments, it is advisable to estimate both the amount of intracellular toxins and the dissolved fraction in the water.

If instrumentation with high sensitivity and selectivity is available, the direct injection of the sample in the detection system are preferred because they minimize the possibility of alteration of the sample and error propagation[135,136]. However, in these cases, it is necessary to take into account the influence of the aqueous sample may have on the accuracy and reliability of the method, with particular bibliography to "matrix effects", reproducibility and robustness of the method.

In the analyses of cyanotoxins with chemical methods, however, the pretreatment, the extraction and pre-concentration of the sample are often necessary to achieve both adequate sensitivity and to perform a simultaneous purification from organic and inorganic compounds present in the water.

It is advisable to make use of analytical protocols involving the use of a process standard or internal standard, in order to ensure the reliability of the analysis and compensate for any errors in the preparation step of the sample. The standard process should be virtually absent in the sample to be analyzed and structurally similar to the analytes to be determined. It is recommended, when available, the use of isotopes or compounds similar. For the analysis of MCs, the NOD can be used as a standard process, after confirmation of its absence in the samples to be analyzed.

The commercial availability of certified analytical standards remains a weak link in the chemical determination of cyanotoxins. Currently, there are standards of 12 different MCs of about 80 known congeners, 8 of saxitoxin, 2 of cyanopeptolins (CYP), 2 of anabaenopeptins, 5 of microginins, 2 of anatoxin and 2 of CYN.

1.12.4. Sample preparation for cyanotoxins determination

- *Storage and pre-treatment*

The water sample must be stored according to the analytical method adopted for the determination of extracellular or total toxin concentration.
In the case of water samples subjected to purification process, the residual oxidant compound, typically free chlorine, can alter the result of the analysis. The removal of the residual chlorine can be obtained by treatment with a solution of sodium thiosulfate.
If the analytical method involves the use of a standard process, this must be added after the treatment with the antioxidant.
Often the procedures provide that the pH of the water sample is modified depending on the type of interactions between these analytes anBVJHUJd the stationary phase used in the preparation of the sample.
If a filtration step is required, different types of filters must be used for the determination of the concentration of extracellular or total cyanotoxins. In the first case, filters of porosity of not more than 0.45 μm should be used for retaining the algal cells. In the case of the analysis of the total content, if necessary, the sample can be filtered with a black band filters.

- *Extraction and purification*

The most common techniques of extraction and pre-concentration are based on solid phase extraction *(Solid Phase Extraction,* SPE), through the use of a cartridge filled with several stationary phases[137]. The most used materials are C-18[138], polymeric materials in a divinylbenzene-polystyrene, also functionalized with polar groups (HLB)[131,139] and Graphitized Carbon Black (GCB)[140,141] . It was also proposed the simultaneous extraction of MCs and ANA-a by using ion pair chromatography[142]. Cartridge with immunosorbent anti-MC-LR[143] and from MIP[144] have also been used.
In contrast, for the extraction of polar compounds such as ANA and CYN-in, including similar compounds (eg. homo-anatoxin) and degradation products, polar stationary phases, such as ion exchangers have been used[145,146]. A faster alternative to the cartridges for SPE provides the use of discs for extraction consisting of similar stationary phases; moreover, these procedures have been effectively used for the extraction of contemporary MCs and ANA-a[147].

By adopting this technique, the pretreated and the filtered sample are transferred directly onto the cartridge. The volume of the sample depends on the limits of detection to be achieved and the detection system used. In general, the range of volumes is between 0.1 L and 1 L.

Analytes are extracted from the cartridge generally after a washing step to remove potentially interfering compounds in the matrix. The most common organic phases used for the extraction of MCs are constituted by methanol, acetonitrile, dichloromethane-methanol solutions spiked with different acid or basic modifiers[148].

For the re-elution of CYN and ANA-a, the solvent most frequently used is water, generally acidified, in conjunction with mixtures of water/methanol[149,150].

For aqueous matrices, extraction by SPE is generally adopted as effective steps of purification. However, the possible presence of endogenous compounds is capable of interfering with the final determination. In the case of detectors with a low selectivity, it is necessary a second passage on SPE cartridges that should be made of different materials than those used in the extraction step. In general, the scheme applicable to the analysis of MCs, involves the use of hydrophobic materials during extraction step and hydrophilic materials for purification step, such as silica.

The extracted solution from the SPE cartridge is usually subjected to further concentration by evaporation in water baths at temperature ≤ 50 °C. The residue is reconstituted, filtered in the case where the turbidity makes it necessary, and an aliquot is injected into the detection system.

In literature are available methods for the analysis of the intracellular, extracellular or total (sum of intracellular and extracellular) toxin level. The methods available for the determination of the content of extracellular cyanotoxins generally involve the filtration of the sample with filters not greater than 0.45 μm. The analysis of the intracellular content should be carried out by extracting the target compounds from the residue of the filtration step. These procedures, however, are generally less reliable with a low reproducibility. Alternatively, it is possible to determine the total content of toxins and, for difference, the intracellular toxins level after cell lysis obtained by means of sonication and/or cycles of freeze-thawing. This is preferable to the first approach because the performance of the methods for the determination of extracellular and total content of toxins are comparable, as it is possible to use the same protocol analysis except for the pretreatment step of the sample.

- *Detection systems*

High performance liquid chromatography (HPLC) - coupled to spectrophotometric, amperometric or mass spectrometric detectors - is the system of choice for the chemical determination of cyanotoxins. Methods of analysis based on Ultra Performance Liquid Chromatography (UPLC) have recently been developed; this technique reduces significantly the time of determination by increasing the resolution and the number of theoretical plates[151]. Analytical standards of individual toxins are required for the qualitative and quantitative analysis, based on a comparison with the retention times and detector signals.

The use of C-18 as stationary phase and water, methanol and acetonitrile as mobile phases are generally adopted as chromatographic conditions. For the separation of cyanotoxins hydrophilic, as ANA-a, it is also possible to use polar columns[152], type HILIC (Hydrophilic Interaction Liquid Chromatography). Acidic agents are commonly used to facilitate the chromatographic separation and the increase in the signal when using the mass spectrometer as a detector in positive ionization mode. In this case can be used formic acid in concentration ranging from 1 to 20 mM[153,154]while for the UV detection is preferred to use trifluoroacetic acid (TFA)[155] because it ensures a background noise lowest in the region of wavelengths typical of MCs (220-240 nm), ANA-a (227 nm) and CYN (260 nm).

UV detection has found many applications even if it is necessary the availability of a photodiode array detector (DAD) to ensure the necessary selectivity[156], especially for the identification of individual variants of MCs[141]. The use of fluorescence detection (FLD) with a process of derivatization (30) allows achieving high sensitivity and selectivity, with detection limits of the order of ng/L.

However, for an unambiguous identification of the individual variants of cyanotoxins, it is necessary the use of mass spectrometry (MS). Three-dimensional and linear ion traps (LIT), single spectrometers and especially triple quadrupole (LC-MS/MS)[157,158,159], are the most suitable detectors for reliable, sensitive and specific analysis of cyanotoxins. Thus, the high selectivity ensures a very low probability of the presence of signals due to interfering compounds; however in some cases it is still necessary to pay attention to chromatographic and mass- spectrometric problem, as in the case of possible erroneous identification of ANA-a in place of amino acid phenylalanine[160].

Useful information about the presence of MCs can be obtained from the presence of a fragment ion at m/z 135, characteristic of the MCs and arising from the break of the amino acid fragment ADDA.

The presence of multiple basic sites may give rise to multi-charged molecular ions, as in the case of the MC-RR group, which contains two arginine residues. It is therefore important to consider as scan range, even during the optimization phase of the instrumental conditions, both the single charged ions, corresponding to molecular weights, and the double charged ions, with m/z in the range 400-700 (20, 21).

Recently, the high-resolution mass spectrometer, using as detectors the time of flight- quadrupole (Q-TOF) (31), Matrix Assisted Laser Desorption Ionization - ToF (MALDI - ToF)[161,162] or Orbitrap ™ [163,164] has provided much useful information on the identification of new variants of MCs and / or degradation products. The qualitative analysis is very fast because the detector is so specific that it is often possible to eliminate the sample preparation step and can then be used as a screening for the presence of advanced cyanotoxins. For quantitative analysis, however, it is necessary the availability of analytical standards.

1.13. Cyromazine

1.13.1. Chemical structure and properties

Cyromazine is a triamino-1,3,5-triazine (Figure 1.10) and it is a growth regulating insecticide with inhibitory effect on the development of diptera larvae and with a prolonged action. It has systemic properties: applied to the vegetation, it quickly penetrates the leaf tissues (translaminar effect) where it acts on the miner larvae, inhibiting their growth; when applied to the soil, it is absorbed by the roots and translocated inside the plant protecting the aerial part.

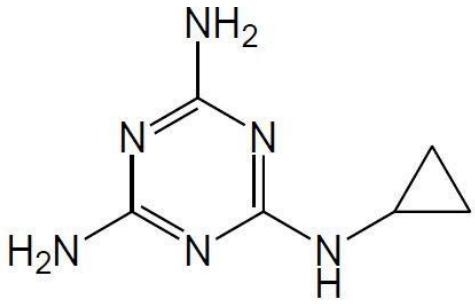


Figure 1. 10:Chemical structure of Cyromazine

The use is not allowed in the poultry sector on bedding placed in contact direct with animals because cyromazine is toxic if ingested, inhaled or absorbed through the skin.

1.13.2. Toxicity

- *Effects on humans*

No adverse health effects have been reported in connection with the handling of cyromazine in the synthesis or formulation. Based on animal testing, poisoning symptoms may constitute body weight loss.
Exposure of persons entering/working in animal housing after application might not be completely excluded. However, actual inhalation exposure is considered negligible due to the low vapour pressure of cyromazine. Dermal exposure would be limited to direct contact with the treated surfaces, i.e. manure. The first is considered to be negligible as shoes (boots) are expected to be worn into animal housing. For the latter at least a small amount of exposure of (mostly) hands might not be excluded while cleaning animal housing. However, due to dilution of the product within the manure and potential degradation of cyromazine, this exposure is considered negligible as well.

- *Bioaccumulation*

The potential for aquatic and terrestrial bioaccumulation has been investigated via testing and open literature data, respectively. In addition, the aquatic and terrestrial bioconcentration factors have been estimated theoretically indicating that cyromazine has a negligible potential of bioaccumulation via the aquatic and terrestrial food chain[165].

- *NOAEL and TDI estimation*

There are insufficient data to obtain a NOAEL or LOAEL and calculate a TDI.

1.14. International strategies for controlling risks in drinking water supply chain: Water Safety Plan (WSP) and Alert Level Framework (ALF)

During the last years, the criteria and strategies of quality control systems related to water for human consumption, until now characterized by a surveillance over more or less defined segments of the

catchment → treatments → distribution → users cycle and/or by a random monitoring on distributed waters, have been substantially redefined. The development of risk analysis knowledge has, indeed, definitely moved the interest towards the creation of a global risk management system involving the entire water supply chain, from catchment to the final user point.

This is the approach included in the WSPs recently introduced by the WHO during the review of the guidelines concerning the drinking water quality[166] and strengthened in the following editions until the most recent of 2011[167]. This approach has been implemented at regulatory level in several Countries of the European area and it has been proposed for a possible introduction in the review of Directive 98/83/EC concerning the quality of water for human consumption.

The WSPs model, extremely straightforward in its general aspects, is aimed to drastically reduce waters contamination chances at the catchment, to diminish or eliminate chemical and microbiological risk factors through properly designed water treatments, carried out and controlled and, eventually, to prevent possible recontaminations during water storage stage and distribution to the final user point.

The strategy presents a high flexibility and it can be applied to any production and distribution system regardless of its nature, legal form, policy, size and complexity.

The principles contained in the WSPs, and synthetically reported in Table 1.5, can be considered as a reassessment and reorganization of several criteria and management procedures that, until now, have led to the production and distribution of waters with an adequate quality for human consumption, especially when based on quality assurance systems equivalent to ISO 9001:2001; among them, it is found the multi barrier control system based on an integrated process to prevent microbiological contamination of water. At the same time, there have appeared crucial elements of risk analysis and management borrowed from other productive sectors and, mainly, from the HACCP system (*Hazard Analysis and Critical Control Points*), compulsory for the food industry and standardized at regulatory level[168].

Plan stage	**Purpose**
Creation of a multidisciplinary team with identification of roles and responsibilities	▪ To establish the risks related to each single component/stage of the water system. ▪ To evaluate the system effectiveness in granting appropriate hygiene and sanitary quality standards.
Water system description	▪ To represent the system and all its components/stages in detail (flow chart): catchment area, catchment, treatments, storage and distribution network, internal distribution systems. ▪ To identify users segments and uses of distributed waters.

Risks analysis and identification of risk priorities	▪ To identify potential factors of biological, physical and chemical risk related to different elements of the system and the possible events that can cause a health risk for final users. ▪ To establish a risk priority scale based on potential effects and likelihood of occurrence, as basis of each decision-making process.
Definition and validation of adequate measures for monitoring risks	▪ To identify and verify actions so as to monitor each significant risk, through physical barriers or appropriate activities to prevent, eliminate or reduce the likelihood of occurrence or mitigate consequences.
Control and monitoring measures	▪ To carry out, on a systematic basis, a series of process and products controls so as to ensure the effectiveness of the system in taking the risk under control: each control measure must be planned in terms of implementation procedures, safety limits and corrective actions to be taken in case of significant deviations from those limits.
Plan testing	▪ To evaluate the overall effectiveness of plan in granting water compliance - at user point – to hygiene and sanitary quality standards.
Papers and review	▪ To ensure and document, over time, plan functioning effectiveness, based on the results obtained or following to the occurrence of accidents or emergencies.

Table 1. 5: Synthetic representation of WSPs principles

A WSP should, due to its nature, be developed for each specific water system. Several difficulties in plan designing and implementation stage, can be especially observed in small size water supply systems (*Small Water Supplies*, SWS) which represent a significant part of the Italian aqueduct system and that also find a specific place in WSPs application manuals[169].

1.14.1. WSP for cyanotoxins monitoring

Cyanobacteria blooms can occur in undamaged natural environments and, more frequently, in water bodies affected by human interferences which directly or indirectly favour algal development. In many cases, this is encouraged by the introduction of nutrients with consequent eutrophication due to agricultural, livestock activities or wastewater, or, in other circumstances, by the change in river course due to the creation of reservoirs for water catchment which increase the retention time and the exposure of the water body to the sunlight[170]. Even climate change plays its role in the expansion of algal bloom phenomena related to cyanobacteria. It not only influences temperatures but it can also entail drastic changes in the hydrodynamic regime of reservoirs, as in the case of shallows rapidly followed by flood flows freeing the nutrients tied up in the sediments[171,172].

The massive development of toxic cyanobacteria frequently occurs in reservoirs previously not affected by proliferation phenomena; on the other hand, the reoccurrence of blooming phenomena in already affected reservoirs, has to be considered as normal. In fact, in this last case, populations of cyanobacteria, once installed, persist in the aquatic environment and tend to proliferate in favourable

environmental conditions. The likelihood to interrupt the occurrence of these phenomena, over time, is related to complex long-term ecological recovery measures such as the monitoring of nutrients introduction, the limitation of sedimentation activity or sediments removal. The scope of these interventions, the discussion of which goes beyond the purpose of these guidelines, entails the involvement of many functions within the global management of internal water, environmental policy, and resources development and allocation strategies.

Figure 1.11 represents a series of preventive interventions and monitoring measures that can be realized in the water body and in the drinking water supply chain so as to eliminate or reduce the possible occurrence of cyanotoxins in water for human consumption; the scheme gives the idea of how the measures have been divided – seen as multiple barrier control in the different stages of the chain –, of the nature of different measures, also in terms of interventions space and time extension and, at the same time, of the need to merge the different actions in a sole integrated and global prevention and control strategy based on WSPs and WHO principles[173].

The structuring of a cyanotoxins monitoring based on WSPs approach, whose main elements have been previously recalled, offers some crucial advantages that can be summarized as follows:

– The preventive approach allows reducing the exposure related to possible overcoming of toxins levels in the distributed water that, in the event of monitoring of distributed water, could be backwardly noticed.

– The preventive measures adopted for cyanotoxins risk are effective for water protection compared to several other risk factors, for example the monitoring of livestock waste prevent other problems related to the eutrophication as well as the spreading of oro-fecal transmission disease agents and protozoa (ex.: *Giardia, Cryptosporidium*).

– Similarly, treatment measures adopted to mitigate risks due to cyanotoxins (ex.: activated charcoal filtration) contribute to the monitoring of other critical parameters such as disinfection by-products and trihalomethans which tend to gather due to the higher concentration of organic substance in the water caused by the algal mass.

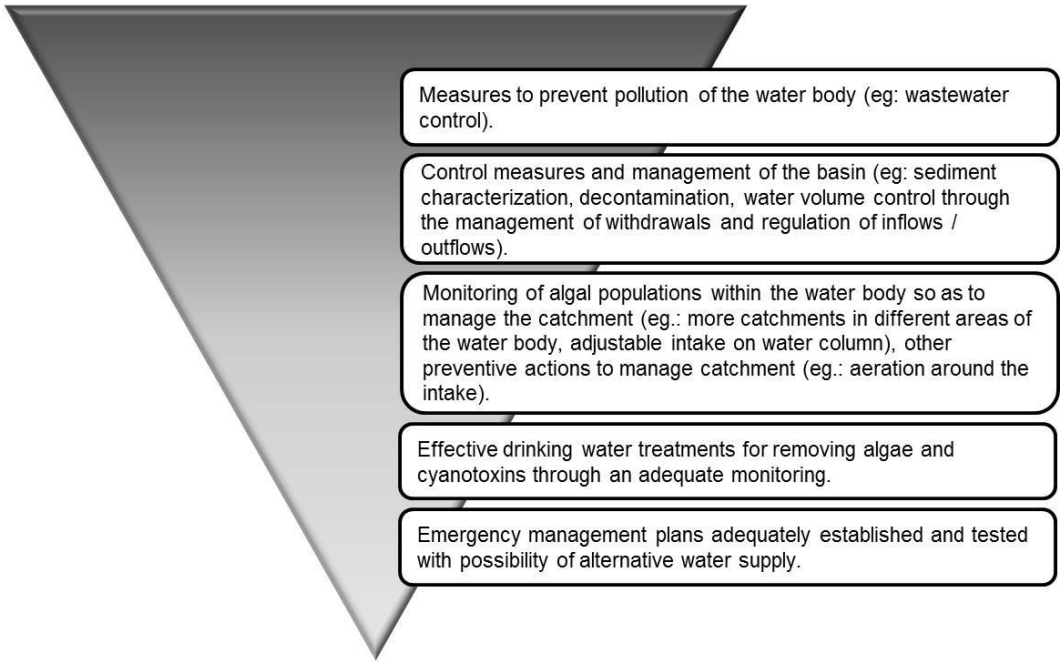


Figure 1. 11: Preventive and control measures in the water body and in the supply chain hydro-drinking to eliminate or reduce the risks of the presence of cyanotoxinsin the water distributed for human consumption (Rapporto Istisan 11/35 Pt.2)

1.14.2 ALF approach for cyanotoxins monitoring

From the healthcare point of view, the main consequence of the events related to cyanobacteria proliferations, is associated to drinking water use. The risk is determined by the possible presence in the water of toxic metabolites produced by phytoplanktonic organisms. Cyanotoxins, in fact, which are found in significant concentrations under intra-cellular and/or dissolved form, in water for human consumption, if not efficiently removed from the drinking water treatments chain, could persist reaching the final consumer and, if in concentrations exceeding the safety levels, could represent a risk factor for water consumption.

From the surveillance point of view, cyanotoxins represent, in each regard, chemical risk factors. However, the systematic determination of these lasts in the water, contrary to what happen with other parameters which are normally subject to monitoring, is not regularly carried out. Cyanotoxins are not expressly included among the parameters to be monitored in compliance with Directive 98/83/EC and with its implementation at national level, Legislative Decree 31/2001 and amendments. On the other hand, based on specific risk assessments, some Countries have judged useful to establish a cyanotoxins monitoring obligation within the regulation on drinking water quality[174].

In general terms, being the cyanobacteria proliferation generally confined to limited time intervals, the presence of toxins in potentially significant concentrations for human health persists just for short-term periods and, therefore, a periodical control during the entire year could be inappropriate from the point of view of resources allocation. To this, it can be added that within the blooming periods, cyanotoxins levels can significantly change in few days, with the consequent need of a close surveillance whereas a standard continuous control over the year should necessarily envisage prolonged time intervals between samplings and could not be able to identify health risk conditions. Toxins monitoring methods for confirmation purposes are, at present, not available for the overall territory and demand costly instrumental and human resources.

According to this, there have been implemented several monitoring and risk management strategies based on an integrated surveillance of the water body and the drinking water chain, adjusted on existing risk levels within the raw waters and on treatment systems implemented. A consolidated approach at international level and on which the system suggested in these guidelines is based, is the monitoring-actions sequence called ALF and hereunder described.

- *ALF systems*

The ALF approach establishes a multistage-type model, organized through a series of measures envisaging several water controls through differentiated risk management measures that are functional to the contamination risk level assessed on surface water (detection and alert levels) and through possible mitigation actions carried out within the water treatment chain. ALF general criteria are also used for reservoirs with an intended use other than human consumption, such as recreational or irrigation waters, according to a different risk assessment, that is functional to the specific water use. Consequently, in these kinds of contexts, the decision basis, the safety limitations adopted and the actions taken, can also be significantly different from those presented in this document.

Taking into account the water for human consumption, it is useful to have a look to the approaches appeared in the last two decades and gradually move to the risk management model recommended at national level by the guidelines.

– *Burch system*

  The system suggested by Burch in 1993, uses, as elements for the implementation of differentiated measures, the number of cells detected in raw waters, and it defines three different alert levels[175]:

- *Alert level 1:* low cell levels: 500 – 2,000 cells/mL;
- *Alert level 2*: moderate cell levels: 2,000 – 15,000 cells/mL;
- *Alert level 3*: high, persistent cell levels, more than 15,000 cells/mL.

In levels 1 and 2, waters are considered as appropriate for human consumption while, in the absence of specific risk mitigation measures, level 3 states the non-suitability for human consumption. Different actions are suggested based on alert stages, both from the surveillance (species-specific identification of algal population, cyanotoxins analysis) and solution (changes in the intake work depth, water treatments) point of view, together with recommendations on the decision-making process.

– *WHO system*

Some years later, the WHO reconsidered the ALF approach based on the assessment of cyanobacteria concentration detected in the source waters defining the three following stages:

- *Surveillance level*: related to cyanobacteria detection which demands the enhancement of algal monitoring;
- *Alert level 1*: activated for cyanobacteria concentrations greater than 2,000 cells/mL (chlorophyll-a greater than 1 µg/mL), in which there is the possibility of cyanotoxins occurrence equivalent to the guide value (1,0 µg/L for MC-LR) and it is needed the activation of analytical measurement regarding cyanotoxins levels and the implementation of appropriate treatment measures for the removal of algal cells and toxins, followed by a report to the health authorities;
- *Alert level 2*: equivalent to concentrations greater than 100,000 cells/mL (chlorophyll-a greater than 50 µg/L) for toxic cyanobacteria, in correspondence of which, beyond monitoring enhancement and treatment systems enhancement/optimization, the emergency alternative water supply identification is carried out, followed by an adequate communication between health authorities and media.

– *CIMF system*

ALF principles have been integrated within more general management plans defined as *Cyanobacterial Incident Management Framework* (CIMF)[176] that envisages a more coordinated system based on regular monitoring, surveillance level and three levels of alert, where the passage from an alert stage to the next one is determined by the positivity of different indicators

among which, beyond those aimed at the identification of algal cells and cyanotoxins, the bioassay is also suggested.

– *Australian system*

The Burch model has been redefined and integrated in the Australian national protocol for the monitoring of cyanobacteria and cyanotoxins in the surface waters establishing:

- *Detection level*: concentration of cyanobacteria greater than 500 cells/mL;
- *Alert level 1*: concentration of cyanobacteria greater than 2,000 cells/mL;
- *Alert level 2*: concentration of cyanobacteria greater than 5,000 cells/mL;
- *Alert level 3*: concentration of cyanobacteria greater than 50,000 cells/mL.

This system also uses the cyanobacteria biovolume measurement as an alternative to algal counts and it takes into consideration the identification of cyanotoxins in the last stages of alert as a criterion of risk assessment for water consumption.

– *Newcombe system*

A more recent evolution of the system based on WHO principles[177] and developed according to knowledge progress even in respect to toxins species-specific production potential, has been suggested by Newcombe[178] and it identifies a detection level and three different levels of alert. The definition of the different levels and the actions associated to each level of alert are hereunder briefly described:

- *Detection level*: concentrations of cyanobacteria roughly included in the range 500 – 2,000 cells/mL.

  This is useful to identify an early stage of algal bloom. Whereas in the water management system there is not an operative and appropriate monitoring of cyanobacteria, it is recommended to implement it on a weekly basis, thus integrating the information with frequent visual inspections of the water body so as to detect the presence of foams or water stains.

- *Alert level 1*: concentrations of cyanobacteria *Microcystis aeruginosa* in the range 2,000 – 6,500 cells/mL, in waters collected at the catchment.

  This is defined based on conservative criteria so as to ensure a time interval of 4-6 days, before the population development reach levels presenting cyanotoxins concentrations equivalent to the guide value (alert level 2). At alert level 1, it is recommended to notify the

situation to local health authorities and, if possible, to activate analytical determinations of cyanotoxins. Other decisions must be taken on a case by case basis according to the information available on species toxicity, the pre-existing scenario, with a particular attention to cyanobacteria proliferations episodes already occurred, the immediate availability of possible alternative supplies, the kind and effectiveness of water treatment plant.

- *Alert level 2*: it reports, in the absence of specific data on toxin levels, the possibility that the water entering the drinking water supply chain presents microcystins concentrations near to the guide value; the appraisal is conservative taking for granted that the algal population is highly toxic and that the entire toxin produced is released into the water and cannot be removed by treatments. The concentration of *M. aeruginosa* which defines level 2, is in fact calculated assuming, at worst, a part of toxin per cell (*toxin quota)* equal to 0.2 pg, that, taking into account a concentration equal to 6,500 cells/mL, would result in a toxin concentration equal to 1.3 μg/L, the guide value considered in the Australian guidelines[179]. The appraisal clearly presents a high degree of approximation in that the *toxin quota* in natural populations of cyanobacteria is considerably variable and difficult to be defined and, furthermore, it is different from one species to another. Based on this, assuming in the appraisal the same criteria mentioned above and the *toxin quota* values referred to each species, in Australia there has been suggested a more specific evaluation for alert level 2 for the most spread algal species, according to the following values[175]:
  - *Microcystis aeruginosa:* 6,500 cells/mL;
  - *Anabaena circinalis:* 20,000 cells/mL;
  - *Cylindrospermopsis raciborskii:* 15,000 cells/mL;
  - *Nodularia spumigena*: 40,000 cells/mL.

  This level of alert entails a decision on the notification to the health authorities and on possible use restrictions where there are no water treatment systems and it is not possible to regularly determine toxins concentrations. At operative level, a constant monitoring of cyanotoxins and cyanobacteria composition is suggested, at least on a weekly basis.

- *Alert level 3*: active for concentrations greater than 6,500 cells/mL; it is referred to *Microcystis aeruginosa* toxic cells and represents a potential toxin production in water for human consumption with concentration near or ten times greater than the guide value.

A notification must be sent to the health authority, if not previously sent, and an accurate risk assessment must be established, taking first of all into account the treatment measures taken and their suitability – both for technologies used, existing systems effectiveness and maintenance status - also considering the existence of sensitive users' categories. If risks mitigation measures cannot be considered as appropriate, use restrictions provisions and emergency response plans implementation are needed. In any case, a constant monitoring is demanded (frequency recommended 3-7 days), so as to highlight population decline and the reduction of toxin levels within a safety threshold. Specific measures, especially in case of water use restrictions, must be adopted so as to ensure an adequate communication with media and population, from the side of health authorities.

The passage from a high level of alert to a lower one is determined by cyanobacteria population decline and/or by the adoption of risk prevention and/or mitigation measures that the health authority considers as appropriate.

References

[1] Gavrilescu, M. et al., Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation, New Biotechnol. (2014), http://dx.doi.org/10.1016/j.nbt.2014.01.001

[2] https://clu-in.org

[3] http://www.eugris.info

[4]Paerl HW, Bland PT, Bowles ND, Haibach ME. Adaption to high intensity, low wavelength light among surface blooms of the cyanobacterium Microcystis aeruginosa. Applied and Environmental Microbiology 1985;49(5):1046-1052.

[5] Robarts RD, Zohary T. Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. New Zealand Journal of Marine and Freshwater Research 1987;21(3):391-399.

[6] Reynolds CS. Phytoplankton periodicity: interactions of form, function and environmental variability. Freshwater Biology 1984; 14(2):111-142.

[7] Walsby AE, Ng G, Dunn C, Davis PA. Comparison of the depth where Planktothrix rubescens stratifies and the depth where the daily insolation supports its neutral buoyancy. New Phytologist 2003;162(1):133-145.

[8] Ellwood NTW, Albertano PA, Galvez R, Mosello R, Funiciello R. Water chemistry and trophic evaluation of Lake Albano (Central Italy): a 4 year water monitoring study. Journal of Limnology 2009;68(2):288-303.

[9] Oliver RL, Ganf GG. Freshwater Blooms. In: Whitton BA, Potts M (Ed.). The Ecology of Cyanobacteria. Dordrecht: Kluwer Academic Publishers; 2001. p 149-194.

[10] Wetzel RG. Limnology. Lake and River Ecosystems. San Diego: Academic Press; 2001.

[11] Wilhelm C. The biochemistry and physiology of light harvesting processes in chlorophyll b- and c- containing algae. Plant Physiology and Biochemistry 1990;28:293-306.

[12] Davison IR. Environmental effect of algal photosynthesis: Temperature. Journal of Phycology 1991;27(1):2-8.

[13] Briand JF, Leboulanger C, Humbert JF, Bernard C, Dufour P. Cylindrospermopsis raciborskii (Cyanobacteria) invasion at mid-latitudes: selection, wide physiological tolerance, or global warming? Journal of Phycology 2004;40(2):231–238.

[14] Mur LR, Gons HJ, van Liere L. Competition of the green alga Scenedesmus and the blue-green alga Oscillatoria in light limited environments. FEMS Microbiology Letters 1977;1(6):335-338.

[15] . Van Liere L, Mur LR, Gibson CE, Herdman M. Growth and physiology of Oscillatoria agardhii and some related species, a survey. Dev. Hydrobiology 1979;2:67-77.

[16] Mur LR, Skulberg OM, Utkilen H. Cyanobacteria in the environment. In: Chorus I, Bartram J (Ed.). Toxic cyanobacteria in water: a guide to their public health consequences, monitoring, and management. London and New York: E&FN Spon; 1999. p. 15-40.

[17] . Rigler FH. The phosphorus fractions and the turnover time of inorganic phosphorus in different types of lakes. Limnology and Oceanography 1964; 9(4):511-8.

[18] Nürnberg G, Peters RH. Biological availability of soluble reactive phosphorus in anoxic and oxic freshwaters. Canadian Journal of Fisheries and Aquatic Sciences 1984;41(5):757-65.

[19] Whitton BA, Al-Shehri AM, Ellwood NTW, Turner BL. Ecological aspects of phosphatase activity in cyanobacteria, eukaryotic algae and bryophytes. In: Turner BL, Frossard E, Baldwin DS (Ed.). Organic Phosphorus in the Environment. Wallingford, UK: CAB International; 2005. p. 205-41.

[20] Griesbach SJ, Peters RH. The effects of analytical variations on estimates of phosphorus concentration in surface waters. Lake and Reservoir Management. 1991;7(1):97-106.

[21] Oliver RL, Ganf GG. Freshwater blooms. In: Whitton BA, Potts M (Ed.). The ecology of cyanobacteria. Dordrecht: Kluwer Academic Publishers; 2000. pp. 149-194.

[22] Tandeau de Marsac N, Houmard J. Adaption of cyanobacteria to environmental stimuli: new step towards molecular mechanisms. FEMS Microbiology Reviews. 1993;104(1-2):119-90.

[23] Van Apeldoorn ME, van Egmond HP, Speijers GJA, Bakker GJI. Toxins of cyanobacteria. Molecular Nutritional Food Research 2007;51(1):7-60.

[24] Bláha L, Babica P, Maršálek B. Toxins produced in cyanobacterial water blooms – toxicity and risks. Interdisciplinary Toxicology 2009;2(2):36-41.

[25] Surakka A, Sihvonen LM, Lehtimaki JM, Wahlsten M, Vuorela P, Sivonen K. Benthic cyanobacteria from the Baltic Sea contain cytotoxic Anabaena, Nodularia, and Nostoc strains and an apoptosis-inducing Phormidium strain. Environmental Toxicology 2005;20(3):285-92.

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

[27] Sivonen K. Emerging high throughput analyses of cyanobacterial toxins and toxic cyanobacteria. In: Hudnell HK (Ed.). Cyanobacterial harmful algal blooms: state of the science and research needs. New York: Springer; 2008. p. 539-57

[28] Oksanen I, Jokela J, Fewer DP, Wahlsten M, Rikkinen J, Sivonen K. Discovery of rare and highly toxic microcystins from lichen-associated cyanobacterium Nostoc sp. strain IO-102-I. Applied and Environmental Microbiology 2004;70(10):5756-63.

[29] Michèle RP. Further pterocellins from the New Zealand marine bryozoans Pterocella vesiculosa. Journal of Natural Product 2008;71(1):134-6.

[30] . Izaguirre G, Neilan BA. Benthic Phormidium species that produces microcystin–LR, isolated from three reservoirs in Southern California. Sixth International Conference on Toxic Cyanobacteria Abstract book, 2004.

[31] Heresztyn T, Nicholson BC. Nodularin concentrations in Lakes Alexandrina and Albert, South Australia, during a bloom of the cyanobacterium (Blue-Green Alga) Nodularia spumigena and degradation of the toxin. Environmental Toxicology Water Quality 1997;12:273-82.

[32] Fergusson KM, Saint CP. Multiplex PCR assay for Cylindrospermopsis raciborskii and cylindrospermopsin-producing cyanobacteria. Environmental Toxicology 2003;18(2):120-5.

[33] Mbedi S, Welker M, Fastner J, Wiedner C. Variability of the microcystin synthetase gene cluster in the genus Planktothrix (Oscillatoriales, Cyanobacteria). FEMS Microbiology Letters 2005;245(2): 299-306.

[34] . Kützing FT. *Tabulae phycologicae; oder, Abbildungen der Tange*. Nordhausen: Gedruckt auf kosten des Verfassers; 1846. Vol. 1, fasc. 1, pls 1-10. p. 1-8.

[35] Gomont M. Monographie des Oscillariées (Nostocacées homocystées). Annales des Sciences Naturelles, Botanique, Series 7 1892;16:91-264.

[36] Anagnostidis K, Komárek J. Modern approach to the classification system of cyanophytes. 3. Oscillatoriales. Archiv für Hydrobiologie 1988;80:327-472.

[37] Kurmayer R, Christiansen G, Chorus I. The abundance of microcystin-producing genotypes correlates positively with colony size in Microcystis sp. and determines its microcystin net production in Lake Wannsee. Applied and Environmental Microbiology 2003;69(2):787-95.

[38] Kurmayer R, Christiansen G, Fastner J, Börner T. Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium Planktothrix spp. Environmental Microbiology 2004;6(8):831-41.

[39] . Briand JF, Jacquet S, Flinois C, Avois-Jacquet C, Maisonnette C, Leberre B, Humbert JF. Variations in the microcystin production of *Planktothrix rubescens* (Cyanobacteria) assessed from a four-year survey of Lac du Bourget (France) and from laboratory experiments. *Microbial Ecology* 2005;50: 418-28.

[40] Christiansen G, Rainer Kurmayer R, Liu Q, Börner T. Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring Planktothrix spp. Applied and Environmental Microbiology 2006;72(1):117-23.

[41] Sivonen K, Leikoski N, Fewer DP, Jokela J. Cyanobactins—ribosomal cyclic peptides produced by cyanobacteria. Applied Microbiology and Biotechnology 2010;86(5):1213-25.

[42] Welker M, von Döhren H. Cyanobacterial peptides – Nature's own combinatorial biosynthesis. FEMS Microbiology Reviews 2006;30(4):530-63.

[43] Sudek S, Haygood MG, Youssef DTA, Schmidt EW. Structure of trichamide, a cyclic peptide from the bloom-forming cyanobacterium *Trichodesmium erythraeum*, predicted from the genome sequence. *Applied and Environmental Microbiology* 2006;72(6):4382-7.

[44] Ziemert N, Ishida K, Quillardet P, Bouchier C, Hertweck C, Tandeau de Marsac N, Dittmann E. Microcyclamide biosynthesis in two strains of *Microcystis aeruginosa*: from structure to genes and vice versa. *Applied and Environmental Microbiology* 2008;74(6):1791-7.

[45] . Leikoski N, Fewer DP, Jokela J, Wahlsten M, Rouhiainen L, Sivonen K. Highly diverse cyanobactins in strains of the genus *Anabaena*. *Applied and Environmental Microbiology* 2010;76(3):701-9.

[46] Leikoski N, Fewer DP, Sivonen K. Widespread occurrence and lateral transfer of the cyanobactin biosynthesis gene cluster in cyanobacteria. *Applied and Environmental Microbiology* 2009;75(3):853-7.

[47] Istvánovics V, Somlyódy L. Factors influencing lake recovery from eutrophication – the case of basin 1 of lake Balaton. *Water Research* 2001;35(3):729-35.

[48] Chorus I, Bartram J (Ed.). Toxic cyanobacteria in water: a guide to their public health consequences, monitoring, and management. London and New York: E & F.N. Spon; 1999.

[49] Van den Hoek C, Mann DG, Jahns HM. Algae an introduction to phycology. U.K.: Cambridge University Press; 1995.

[50] Garibaldi L, Anzani A, Marieni A, Leoni B, Mosello R. Studies on the phytoplankton of the deep subalpine Lake Iseo. Journal of Limnology 2003;62(2):177-89.

[51] Garibaldi L, Brizzio MC, Galanti G, Varallo A, Mosello R. Idrochimica e fitoplancton del Lago d'Idro. In: Mosello R, Giussani G. (Ed.). Documenta Istituto Italiano Idrobiologia Volume 61, 1997. p.153-72.

[52] Carollo A, Libera V. Geographical and characteristics of the main Italian lakes. Memorie Istituto Italiano Idrobiologia 1992;50:29-35.

[53] Cordella P, Salmaso N. Studies on some reservoirs and lakes in North-East Italy. Memorie Istituto Italiano Idrobiologia 1992;50:259-71

[54] Salmaso N. Factors affecting the seasonality and distribution of cyanobacteria and chlorophytes: a case study from the large lakes south of the Alps, with special bibliography to Lake Garda. *Hydrobiologia* 2000;438(1-3):43-63.

[55] Morabito G. Six years' (1992-1997) evolution of phytoplankton communities after recovery by liming in Lake Orta, northern Italy. *Lakes and Reservoirs: Research and Management* 2001;6(4):305-12.

[56] Salmaso N, Morabito G, Buzzi F, Garibaldi L, Simona M, Mosello R Phytoplankton as an indicator of the water quality of the deep lakes south of the Alps. *Hydrobiologia* 2006;563(1):167-87.

[57] Legnani E, Copetti D, Oggioni A, Tartari G, Palombo MT, Morabito G. Planktothrix rubescens' seasonal dynamics and vertical distribution in Lake Pusiano (North Italy). Journal of Limnology 2005;64(1):61-73.

[58] Premazzi G, Cardoso AC, Rodari E, Austoni M, Chiaudani G. Hypolimnetic withdrawal coupled with oxygenation as lake restoration measures: the successful case of Lake Varese (Italy). Limnetica 2005;24(1-2):123-32.

[59] Leoni B, Morabito G, Rogora M, Pollastro D, Mosello R, Arisci S, Forasacco E, Garibaldi L. Response of planktonic communities to calcium hydroxide addition in a hardwater eutrophic lake: results from a mesocosm experiment. Limnology 2007;8(2):121-30.

[60] Flaim G, Pinamonti V. I cianobatteri nei laghi trentini. In: Funari E (Ed.). Aspetti sanitari della problematica dei cianobatteri nelle acque superficiali italiane. Workshop. Atti. Roma, 16-17 dicembre 1999. Roma: 2000 (ISTISAN 00/30). p. 111-6.

[61] Viaggiu E, Calvanella S, Melchiorre S, Bruno M, Albertano P. Toxic blooms of Planktothrix rubescens (Cyanobacteria/Phormidiaceae) in three water bodies in Italy. Archiv für Hydrobiologie, Algological Studies 2003;109:569-77.

[62] Manganelli M, Scardala S, Stefanelli M, Vichi S, Mattei D, Bogialli S, Ceccarelli P, Corradetti E, Petrucci I, Gemma S, Testai E, Funari E. Health risk evaluation associated to *Planktothrix rubescens*: An integrated approach to design tailored monitoring programs for human exposure to cyanotoxins. *Water Research* 2010;44(5):1297-306.

[63] Bruno M, Serenelli F, Germozzi R, Scagnetti V, Leoni T, Antonelli B, Melchiorre S, Messineo V. *L'eutrofizzazione tossica in un complesso artificiale multilacuale: i laghi del Fiastrone (1998-2007)*. Roma: Istituto Superiore di Sanità; 2009. (Rapporti ISTISAN09/21).

[64] Ferretti E, Lucentini L, Ottaviani M, Bogialli S, Veschetti E, Giovanna R, Ledalardo C, Cannarozzi De Grazia M, Ungaro N, Rosaria Petruzzelli R, Tartari G, Guzzella L, Mingazzini M, Copetti D, Abis P, Fiori G, De Leo A. Exceptional bloom of cyanobacterium *Planktothrix rubescens* and microcystin production in an Italian drinking water basin. *The 8th International Conference on Toxic Cyanobacteria (ICTC8)*. Istanbul, 29 agosto-4 settembre 2010. p. 117.

[65] Bogialli S, Nigro di Gregorio F, Lucentini L, Ferretti E, Ottaviani M, Ungaro N, et al., Management of a Toxic Cyanobacterium Bloom (Planktothrix rubescens) affecting an Italian Drinking Water Basin: A Case Study., Environ. Sci. Technol. 47 (2013) 574–83.

[66] Seenayya G, Subba Raju N. On the ecology and systematic of the alga known as Anabaenopsis raciborskii. In: Diskachary TV (Ed.). First International Symposium and Taxonomy and Biology of blu-green algae. Madras, India; 1972. p. 52-7.

[67] Manti G, Mattei D, Messineo V, Bogialli S, Sechi N, Casiddu P, Luglié A, Di Brizio M, Bruno M. First report of Cylindrospermopsis raciborskii in Italy. Harmful Algal News 2005;28:8-9.

[68] Bogialli S, Bruno M, Curini R, Di Corcia A, Fanali C, Laganà A. Monitoring algal toxins in lake water by liquid chromatography tandem mass spectrometry. Environmental Science Technology 2006 40(9):2917-23.

[69] Bazzichelli PG, Abdelahad N. Morphometric and statistic characterization of two Aphanizomenon populations of the group Aphanizomenon ovalisporum Forti from the lakes of Nemi and Albano (Italy). Archiv für Hydrobiologie Algological Studies 1994;73:1-21.

[70] Chondrogianni C, Ariztegui D, Guilizzoni P, Lami A. Lakes Albano and Nemi (central Italy): an overview. Memorie Istituto Italiano Idrobiologia 1996;55:17-22.

[71] Lai A, Giordano P, Barbini R, Fantoni R, Ribrezzo S, Palucci A. Bio-optical investigation on the Albano lake. In: Proceedings of EARSeL-Sig-Workshop LIDAR, Dresden (Germany), June 16-17, 2000. p. 185-95.

[72] Margaritora FG, Bazzanti M, Ferrara O, Mastrantuono L, Seminara M., Vagaggini D. Classification of the ecological status of volcanic lakes in Central Italy. Journal of Limnology 2003;62(1):49-59

[73] Bruno M, Gucci P M B, Pierdominici E, Sestili P, Ioppolo A, Sechi N, Volterra L. Microcystin-like toxins in different freshwater species of Oscillatoria. Toxicon 1992;30(10):1307-11.

[74] Messineo V, Bogialli S, Melchiorre S, Sechi N, Luglié A, Casiddu P, Mariani MA, Padedda BM, Di Corcia A, Mazza R, Carloni E, Bruno M. Cyanobacterial toxins in Italian freshwaters. Limnologica 2009;39(2):95-106.

[75] Naselli Flores L, Barone R, Chorus I, Kurmayer R. Toxic cyanobacterial blooms in reservoirs under a semiarid mediterranean climate: the magnification of a problem. Environmental Toxicology 2007;22(4):399-404.

[76] Waklin P, Hoffman L, Komárek J. Nomenclatural validation of the genetically revised cyanobacterial genus Dolichospermum (Ralfs ex Bornet et Flahault) comb. nova. Fottea 2009;9(1):59-64.

[77] Chorus I, Bartram J (Ed.). Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. London and New York: E & F.N. Spon; 1999.

[78] Carmichael WW. Health effects of toxin-producing cyanobacteria:"The CyanoHabs". Human and Ecological Risk Assessment: An International Journal 2001;7(5):1393-407.

[79] Hitzfeld BC, Höger SJ, Dietrich DR. Cyanobacterial toxins, removal during drinking water treatment, and human risk assessment, Environment Health Perspect 2000;108(Suppl 1):113-22.

[80] Rao PV L, Gupta N, Bhaskar ASB, Jayaraj R. Toxins and bioactive compounds from cyanobacteria and their implications on human health. *Journal of Environmental Biology* 2002;23(3):215-24.

[81] Ikehara T, Imamura S, Sano T, Nakashima J, Kuniyoshi K, Oshiro N, Yoshimoto M, Yasumoto T. The effect of structural variation in 21 microcystins on their inhibition of PP2A and the effect of replacing cys269 with glycine. *Toxicon* 2009;54(4):539-44.

[82] IARC. *Ingested nitrates and nitrites, and cyanobacterial peptide toxins.* Lyon: International Agency for Research on Cancer; 2006.

[83] Grosse Y, Baan R, Straif K, Secretan B, El Ghissassi F, Cogliano V. Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. *The Lancet Oncology* 2006;7(8):628-9.

[84] Runnegar MTC, Gerdes RG, Falconer IR. The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. *Toxicon* 1991;29(1):43-51.

[85] Lambert TW, Holmes CFB, Hrudney SE. Microcystin class of toxins: health effects and safety of drinking water supplies. *Environmental Review* 1994;2(2):167-86.

[86] Runnegar MTC, Falconer IR. Isolation, charachterization, and pathology of the toxinsfrom the blue-green alga *Microcystis aeruginosa*. In: Carmichael WW (Ed.). *The water environment: algal toxins and health*. New York: Plenum Press; 1981. p. 325-42.

[87] Falconer IR, Buckley TH, Runnegar MTC. Biological half-life, organ distribution and excretion of 125I-labelled toxic peptide from blue-green alga *Microcystis aeruginosa*. *Australian Journal of Biological Sciences* 1986;39(1):17-21.

[88] Brooks WP, Codd GA. Distribution of *Microcystis aeruginosa* peptide toxin and interactions with hepatic microsomes in mice. *Pharmacology and Toxicology Journal* 1987;60(3):187-191.

[89] Kondo F, Matsumoto H, Yamada S, Ishikawa N, Ito E, Nagata S, Ueno Y, Suzuki M, Harada KI. Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. *Chemical Research Toxicology* 1996;9(8):1355-9.

[90] DeVries SE, Galey FD, Namikoshi M, Woo JC. Clinical and pathologic findings of blue-green algae (*Microcystis aeruginosa*) intoxication in a dog. *Journal of Veterinary Diagnostic Investigation*, 1993;5(3):403-8.

[91] Gilroy DJ, Kauffman KW, Hall RA, Huang X, Chu FS. Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements. *Environmental Health Perspect* 2000;108(5):435-9.

[92] World Health Organization. *Guidelines for drinking-water quality 1998, 2nd Ed. Addendum to Volume 2 Health criteria and other supporting information*. Geneva: WHO; 1998. p. 94–110.

[93] Wolf HU, Frank C, Toxicity assessment of cyanobacterial toxin mixtures. *Environmental Toxicology* 2002;17(4):395-9.

[94] Rinehart KL, Harada K, Namikoshi M, Chen C, Harvis CA. Nodularin, microcystin and the configuration of Adda. *Journal of the American Chemical Society* 1988;110(25):8557-8.

[95] Saito K, Konno A, Ishii H, Saito H, Nishida F, Abe T, Chen C. Nodularin- Har, a new nodularin from *Nodularia*. *Journal of Natural Products* 2001;64(1):139-41.

[96] Eriksson JE, Meriluoto JA, Kujari HP, Osterlund K, Fagerlund K, Hallbom L. Preliminary characterization of a toxin isolated from the cyanobacterium *Nodularia spumigena*. *Toxicon* 1988;26(2):161-6.

[97] Honkanen RE, Dukelow M, Zwiller J, Moore RE, Khatra BS, Boynton AL. Cyanobacterial nodularin is a potent inhibitor of type 1 and type 2A protein phosphatases. *Molecular Pharmacology* 1991;40(4):577-83.

[98] Ohta T, Sueoka E, Iida N, Komori A, Suganuma M, Nishiwaki R, Tatematsu M, Kim SJ, Carmichael WW, Fujiki H. Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Research* 1994;54:6402-6.

[99] Duy TN, Lam PKS, Shaw G, Connell DW. Toxicology and risk assessment of freshwater cyanobacterial (bluegreen algal) toxins in water. *Reviews of Environmental Contamination & Toxicology* 2000;163:113-86.

[100] E. Entfellner, M. Frei, G. Christiansen, L. Deng, J Blom, R. Kurmayer, *"Evolution of anabaenopeptin peptide structural variability in the cyanobacterium Planktothrix", Frontier Microbiology, 2017, Volume 8

[101] L. Spoof, A. Błaszczyk, J. Meriluoto, M. Cegłowska, H. Mazur-Marzec, "Structures and activity of new anabaenopeptins produced by baltic sea cyanobacteria", Marine Drugs, 2016, Issue 1, Volume 14, page 8

[102] R. L. Carneiro, F. A. Dörr, F. Dörr, S. Bortoli, N. Delherbe, M. Vásquez, E. Pinto, "Co-occurrence of microcystin and microginin congeners in brazilian strains of Microcystis sp.", FEMS Microbiology Ecology, 2012, Issue 3, Volume 82, pages 692-702

[103] B. Bober, J. Bialczyk, "Determination of the toxicity of the freshwater cyanobacterium Woronichinia naegeliana (Unger) Elenkin", Journal of Applied Phycology, 2017, Issue 3, Volume 29, pages 1355-1362

[104] T. B. Rounge, T. Rohrlack, A. Tooming-Klunderud, T. Kristensen, K. S. Jakobsen, "Comparison of Cyanopeptolin Genes in Planktothrix, Microcystis, and Anabaena Strains: Evidence for Independent Evolution within Each Genus", Applied and Environmental Microbiology, 2007, Issue 22, Volume 73, pages 7322-7330

[105] A. Tooming-Klunderud, T. Rohrlack, K. Shalchian-Tabrizi, T. Kristensen, K. S. Jakobsen, "Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from Microcystis: implications for evolution of cyanopeptolins", Microbiology, 2007, Issue 5, Volume 153, pages 1382-1393

[106] Humpage AR, Fenech M, Thomas P, Falconer IR. Micronucleus induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. *Mutation Research* 2000;472(1-2):155-61.

[107] Pearson L, Mihali T, Moffitt MK, Kellmann R Neilan B. On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Marine Drugs* 2010;8(5):1650-80.

[108] Neilan BA, Saker ML, Fastner J, Torokne A, Burns BP. Phylogeography of the invasive cyanobacterium *Cylindrospermopsis raciborskii*. *Molecular Ecology* 2003;12(1):133-40.

[109] Chong MWK, Wong BSF, Lam PKS, Shaw GR, Seawright AA. Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. *Toxicon* 2002;40(2):205-11.

[110] Froscio SM, Humpage AR, Burcham PC, Falconer IR. Cell-free protein synthesis inhibition assay for the cyanobacterial toxin cylindrospermopsin. *Environmental Toxicology* 2001;16(5):408-12.

[111] Froscio SM, Humpage AR, Wickramasinghe W, Shaw G, Falconer IR. Interaction of the cyanobacterial toxin cylindrospermopsin with the eukaryotic protein synthesis system. *Toxicon* 2008;51(2):191-8.

[112] Humpage AR, Fontaine F, Froscio S, Burcham P, Falconer IR. Cylindrospermopsin genotoxicity and cytotoxicity : role of cytochrome P-450 and oxidative stress. *Journal of Toxicology and Environmental Health. Part A*, 2005;68(9):739-53.

[113] Young FM, Micklem J, Humpage AR. Effects of blue-green algal toxin cylindrospermopsin (CYN) on human granulosa cells *in vitro*. *Reproductive Toxicology* 2008;25(3):374-380.

[114] Rogers EH, Zehr RD, Gage MI, Humpage AR, Falconer IR, Marr M, Chernoff N. The cyanobacterial toxin, cylindrospermopsin, induces fetal toxicity in the mouse after exposure late in gestation. *Toxicon* 2007;49(6):855-64.

[115] Falconer IR, Humpage AR. Preliminary evidence for *in vivo* tumour initiation by oral administration of extracts of the blue-green alga *Cylindrospermopsis raciborskii* containing the toxin cylindrospermopsin. *Environmental Toxicology* 2001;16(2):192–195.

[116] Briand JF, Jacquet S, Bernard C, Humbert JF. Health hazards for terrestrial vertebrates from toxic cyanobacteria in surface water ecosystems. *Veterinary Research* 2003;34(4):361-77.

[117] Griffiths DJ, Saker ML. The palm island mystery disease 20 years on, a review of research on the cyanotoxin cylindrospermopsin. *Environmental Toxicology* 2002;18(2):78-93.

[118] USEPA. *Creating a cyanotoxin target list for the unregulated contaminant monitoring rule*. USEPA Technical Service Center, Cincinnati, Ohio, USA, Meeting Summary May 17–18, 2001.

[119] Devlin JP, Edwards OE, Gorham PR, Hunter NR, Pike RK, Stavric B. Anatoxin-a, a toxic alkaloid from *Anabaena flos-aquae* NRC-44h. *Canadian Journal of Chemistry* 1977;55(8):1367-71.

[120] Lucentini l, Ottaviani M per il "Gruppo nazionale pe la gestione del rischio cianobatteri in acque destuinate al consumo umano" (Ed). Cianobatteri in acque destinate a consumo umano. Stato delle conoscenze per la valutazione del rischio. Volume 1. Roma:Istituto Superiore di Sanità; 2011. (Rapporto Istisan 11/35 Pt1.)

[121] Falconer IR, Bartram J, Chorus I, Kuiper-Goodman T, Utkilen H, Burch M, Codd GA. Safe levels and safe practices. In: Chorus I, Bartram J (Ed.). *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring, and management.* London: E & FN Spon; 1999. p. 155-78.

[122] Seawright AA, Nolan CC, Shaw GR, Chiswell RK, Norris RL, Moore MR, Smith MJ. The oral toxicity for mice of the tropical cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska). *Environmental Toxicology* 1999;14:135-42.

[123] Haney JF, Sasner JJ, Ikawa M. Effects of products released by *Aphanizomenon flos-aquae* and purified saxitoxin on the movements of *Daphnia carinata* feeding appendages. *Limnology and Oceanography* 1995;40:263–72.

[124] Törökné A, Vasdinnyei R, Asztalos BM. A rapid microbiotest for the detection of cyanobacterial toxins. *Environmental Toxicology* 2007;22:64-8.

[125] . Ferrão-Filho AS, Soares MC, de Magalhães VF, Azevedo SM. A rapid bioassay for detecting saxitoxins using a *Daphnia* acute toxicity test. *Environmental Pollution* 2010;158:2084-93.

[126] Yilmaz M, Phlips, EJ, Szabo NJ, Badylak S. A comparative study of Florida strains of *Cylindrospermopsis* and *Aphanizomenon* for cylindrospermopsin production. *Toxicon* 2008;51:130-9.

[127] Bláhová L, Oravec M, Maršálek B, Šejnohová L, Šimek Z, Bláha L. The first occurrence of the cyanobacterial alkaloid toxin cylindrospermopsin in the Czech Republic as determined by immunochemical and LC/MS methods. *Toxicon* 2009;53:519-24.

[128] Triantis T, Tsimeli K, Kaloudis T, Thanassoulias N, Lytras E, Hiskia A. Development of an integrated laboratory system for the monitoring of cyanotoxins in surface and drinking waters. *Toxicon* 2010;55:979-89.

[129] Chianella I, Lotierzo M, Piletsky SA, Tothill IE, Chen B, Karim K, Turner APF. Rational Design of a polymer specific for microcystin- LR using a computational approach. *Anaytical Chemistry* 2002;74:1288-93.

[130] Matsushima R, Yoshizawa S, Watanabe M, Harada KF, Furusawa M, Carmichael WW, Fujiki H. Invitro and invivo effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin and fibroblasts. *Biochemical and Biophysical Research Communications* 1990;171:867-74.

[131] Honkanan RE., Codispoti BA., Tse K., Boynton AL. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. *Toxicon* 1994;32:339-50.

[132] Heresztyn T, Nicholson BC. Determination of cyanobacterial hepatotoxins directly in water using a protein phosphatase inhibition assay. *Water Reserch* 2001;35:3049-56.

[133] McElhiney J, Lawton LA. Detection of the cyanobacterial hepatotoxins microcystins. *Toxicology and Applied Pharmacology* 2005;203:219-30.

[134] KJ, Furey A, Sherlock IR, Stack MA, Twohig M, Caudwell FB, Skulberg OM. Sensitive determination of anatoxin-a, homoanatoxin-a and their degradation products by liquid chromatography with fluorimetric detection. *Journal of Chromatography A* 1998;798:147-57

[135] 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* 2006;1122:180-85.

[136] Bogialli S, Bruno M, Curini R, Di Corcia A, Fanali C, Laganà A. Monitoring algal toxins in lake water by liquid chromatography tandem mass spectrometry. *Environmental Science & Technology* 2006;40:2917-23.

[137] Pérez S, Aga DS. Recent advances in the sample preparation, liquid chromatography tandem mass spectrometric analysis and environmental fate of microcystins in water. *TRAC- Trends in Analytical Chemistry. 2005*;24:658-70.

[138] Meriluoto J, Lawton L, Harada KI. Isolation and detection of microcystins and nodularins, cyanobacterial peptide hepatotoxins. In: Holster O (Ed.). *Methods in molecular biology.* New Jersey: Humana Press Inc; 2000. p. 65-87.

[139] Rapala J, Erkomaa K, Kukkonen J, Lahti K. Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography–UV detection and enzyme-linked immunosorbent assay, comparison of methods. *Analytica Chimica Acta* 2002;466:213-31.

[140] ISO 20179. *Water quality – Determination of Microcystins – Method using Solid Phase Extraction (SPE) and High Performance Liquid Chromatography (HPLC) with Ultraviolet (UV) detection*. Geneva: International Organization for Standardization; 2005.

[141] Dimitrakopoulos IK, Kaloudis TS, Hiskia AE, Thomaidis NS, Koupparis MA. Development of a fast and selective method for the sensitive determination of anatoxin-a in lake waters using liquid chromatography–tandem mass spectrometry and phenylalanine-d5 as internal standard. *Analytical and Bioanalytical Chemistry* 2010;397:2245-52.

[142] Pietsch J, Fichtner S, Imhof L, Schmidt W, Brauch HJ. Simultaneous determination of cyanobacterial hepato- and neurotoxins in water samples by ion-pair supported enrichment and HPLC-ESI-MS-MS. *Chromatographia* 2001;5:339-44.

[143] Aranda-Rodriguez R, Kubwabo C, Frank MB. Extraction of 15 microcystins and nodularin using immunoaffinity columns. *Toxicon* 2003; 42: 587-99.

[144] Chianella I, Piletsky SA, Tothill IE, Chen B, Turner APF. MIP-based solid phase extraction cartridges combined with MIP-based sensors for the detection of microcystin-LR. *Biosens Bioelectron* 2003;18:119-27.

[145] Furey A, Crowley J, Lehane M, James KJ. Liquid chromatography with electrospray ion-trap mass spectrometry for the determination of anatoxins in cyanobacteria and drinking water. *Rapid Communication in Mass Spectrometry* 2003;17:583-8.

[146] James KJ, Furey A, Sherlock IR, Stack MA, Twohig M, Caudwell FB, Skulberg OM. Sensitive determination of anatoxin-a, homoanatoxin-a and their degradation products by liquid chromatography with fluorimetric detection. *Journal of Chromatography A* 1998;798:147-57.

[147] Maizels M, Budde ML. A LC/MS Method for the determination of cyanobacteria toxins in water. *Analitycal Chemistry* 2004;76:1342-51.

[148] Ortea PM, Allis O, Healy BM, Lehane M, Ni Shuilleabhain A, Furey A, James KJ. Determination of toxic cyclic heptapeptides by liquid chromatography with detection using ultra-violet, protein phosphatase assay and tandem mass spectrometry. *Chemosphere* 2004;55:1395-1402.

[149] . Osswald J, Rellán S, Gago A, Vasconcelos V. Toxicology and detection methods of the alkaloid neurotoxinproduced by cyanobacteria, anatoxin-a. *Environment International* 2007;33:1070-89.

[150] Namikoshi M, Murakami T,Watanabe MF, Oda T, Yamada J, Tsujimura S, Nagai H, Oishi S. Simultaneous production of homoanatoxin-a, anatoxin-a, and a new nontoxic 4-hydroxyhomoanatoxin-a by the cyanobacterium *Raphidiopsis mediterranea* Skuja. *Toxicon* 2003;42(5):533-8.

[151] Oehrle SA, Southwell B, Westrick J. Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectrometry. *Toxicon* 2010;55:965-72.

[152] Dell'Aversano C, Eaglesham GK, Quilliam MA. Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography-mass spectrometry. *Journal of Chromatography A* 2004;1028:155-64.

[153] 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. *Journal of Chromatography A* 2003;1020:101-15.

[154] Sjovall O, Meriluoto J. SOP: analysis of microcystins by liquid chromatography–mass spectrometry. In: Meriluoto J, Codd, GA (Ed.). *TOXIC: Cyanobacterial monitoring and cyanotoxin analysis.* Turku, Finland: Abo Akademi University Press; 2005. p. 105-110.

[155] Barco M, Rivera J, Caixach J. Analysis of cyanobacterial hepatotoxins in water samples by microbore reversed-phase liquid chromatography-electrospray ionisation mass spectrometry. *Journal of Chromatography A* 2002;959:103-11.

[156] Metcalf JS, Lindsay J, Beattie KA, Birmingham S, Saker ML, Törökné AK, Codd GA. Toxicity of cylindrospermopsin to the brine shrimp *Artemia salina*: comparisons with protein synthesis inhibitors and microcystins. *Toxicon* 2002;40:1115-20.

[157] Harada KI, Nakano T, Fujii K, Shirai M. Comprehensive analysis system using liquid chromatography-mass spectrometry for the biosynthetic study of peptides produced by cyanobacteria. *Journal of Chromatography A* 2004;1033:107-13.

[158] Eaglesham GK, Norris RL, Shaw GR, Smith MJ, Chiswell RK, Davis BC, Neville GR, Seawright AA, Moore MR. Use of HPLC-MS/MS to monitor cylindrospermopsin, a blue-green algal toxin, for public health purposes. *Environmental Toxicology* 1999;14:151-4.

[159] Spoof L, Berg KA, Rapala J, Lahti K. First observation of cylindrospermopsin in *Anabaena lapponica* isolated from the boreal environment (Finland). *Environmental Toxicology* 2006;21:552-60.

[160] Furey A, Crowley J, Hamilton B, Lehane M, James KJ. Strategies to avoid the mis-identification of anatoxin-a using mass spectrometry in the forensic investigation of acute neurotoxic poisoning. *Journal of Chromatography A* 2005;1082:91–7.

[161] Fastner J, Erhard M, Döhren H. Determination of oligopeptide diversity within a natural population of *Microcystis* spp. (cyanobacteria) by typing single colonies by matrix assisted laser desorption ionization-time of flight mass spectrometry. *Applied and Environmental Microbiology* 2001;67:5069-76.

[162] Welker M, Fastner J, Erhard M, Von Dohren H. Applications of MALDI-TOF MS analysis in cyanotoxin research. *Environmental Toxicology* 2002;17:367-74.

[163] Diehnelt CW, Dugan NR, Peterman SM, Budde WL. Identification of microcystin toxins from a strainof *Microcystis Aeruginosa* by liquid chromatography introduction into a hybrid linear ion trap-fourier transform ion cyclotron resonance mass spectrometer. *Analytical Chemistry* 2006;78:501-12.

[164] Merel S, Clement M, Mourot A, Fessard V, Thomas O. Characterization of cylindrospermopsin chlorination. Science of the Total Environment- 2010;408:3433-42.

[165] https://davar.gouv.nc/sites/default/files/atoms/files/2016_ue_assessment_report_cyromazine.pdf

[166] World Health Organization. Guidelines for drinking-water quality. Volume 1. Recommendations. 3rd Edition. Geneva: WHO; 2004.

[167] World Health Organization. Guidelines for drinking-water quality. 4th Edition. Geneva: WHO; 2011

[168] UNI EN ISO 22000. Sistemi di gestione per la sicurezza alimentare. Requisiti per qualsiasi organizzazione nella filiera alimentare. Milano: Ente Nazionale Italiano di Unificazione; 2005

[169] World Health Organization. Water safety plan manual: Step-by-step risk management for drinking-water suppliers. 3rd edition .Vol. 1. Geneva: WHO; 2008.

[170] Chorus I, Bartram J (Ed.). Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. London: E & FN Spon; 1999.

[171] Lucentini L, et al. Case study 7: unprecedented cyanobacterial bloom and MC production in a drinking-water reservoir in the South of Italy. In: Sinisi L, Aertgeerts R (Ed.). Guidance on water supply and sanitation in extreme weather events. Geneva: World Health Organization; 2010.

[172] Assennato G, Blonda M, Cudillo B, Gifuni S, Petruzzelli R, Pastorelli AM, Ungaro N. Cyanobacteria bloom in the Occhito artificial lake (Southern Italy): relationship between Planktothrix rubescens density and microcystin concentration. Fresenius Environmental Bulletin, 2010;9:1795-1801.

[173] World Health Organization. *Water safety plans managing drinking-water quality from catchment to consumer*. Geneva: WHO; 2005.

[174] Lucentini l, Ottaviani M per il "Gruppo nazionale pe la gestione del rischio cianobatteri in acque destuinate al consumo umano" (Ed). Cianobatteri in acque destinate a consumo umano. Stato delle conoscenze per la valutazione del rischio. Volume 1. Roma:Istituto Superiore di Sanità; 2011. (Rapporto Istisan 11/35 Pt1.)

[175] Burch MD. The development of an alert levels and response framework for the management of blue - green algal blooms. In: *Blue-green algal blooms – new developments in research and management.* A symposium convened by the Australian Centre for Water Quality Research and the University of Adelaide. 17th February 1993, Adelaide, S. Australia; 1993

[176] Van Baalen L, Du Preez HH. Incident management framework for blue-green algal toxins, Final Report April 2001. Rand Water, South Africa; 2001.

[177] World Health Organization. *Guidelines for safe recreational water environment*. *Volume 1. Coastal and fresh waters*. Geneva: WHO; 2003

[178] Newcombe G, House J, Ho L, Baker P, Burch M. *Management strategies for cyanobacteria (blue-green algae) and their toxins: a guide for water utilities*. Adelaide: Water Quality Research Australia Limited; 2010. (Research Report 74).

[179] National Water Quality Management Strategy. *Australian drinking water guidelines 6.* Australian Government, National Health and Medical Research Council; 2004. Disponibile all'indirizzo http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/eh34_adwg_11_06.pdf; ultima consultazione 18/02/2011.

CHAPTER 2 : OBJECTIVES

In accordance with the recent introduction of the concept of Water Safety Plans (WSP) through the transposition of Directive (EU) 1787/2015 introduced in Italy, this doctoral work aims to develop analytical methods for the research of a large number of emerging contaminants in water for human consumption.

In particular, the attention was focused on:

- 23 different secondary metabolites of cyanobacteria that could be potentially toxic for human health (12 Microcystins, 2 Cyanopeptolins, 2 Anabaenopeptins and 5 Microginins, Anatoxin-a and Cylindrospermopsin)
- Cyromazine, an herbicide and insecticide regulator of growth effective against the larvae blown on sheep and lambs and other diptera such as flies and mosquitoes

The following specific objectives were established:

1. Development and validation of an analytical method based on ultra performance liquid chromatography coupled to high resolution mass spectrometry (UPLC/HRMS) for the simultaneous determination of the wide range of cyanotoxins potentially affecting water intended for human consumption in Italy.
2. Application of the developed method as analytical approach to the management of an emergency related to the proliferation in Vico Lake of Planktothrix rubescens, a cyanobacterium responsible of cyanotoxins production, by a monitoring analyses conducted in the period January 2018- January 2020 in Vico Lake and the whole drinking water chain of the municipality of Caprarola (Viterbo Province).
3. Development and validation of an analytical method based on ultra performance liquid chromatography coupled to high resolution mass spectrometry (UPLC/HRMS) for the identification and the quantitation of Cyromazine in drinking water, used even for chicken coops.

CHAPTER 3 : EXPERIMENTAL SECTION

3.1 Reagents and Chemicals.

MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF, MC-LY, [D-Asp3]-MC-RR, [D-Asp3]-MC-LR, MC-HtyR, MC–HilR, MC-WR, Anabaenopeptin A, Anabaenopeptin B, Microginin 690, Microginin 690 methyl ester, Microginin 704, Microginin 527 , Microginin 527 methyl ester, Cyanopeptolins 1007, Cyanopeptolins 1041, Anatoxin-a, Cilindrospermopsin and Nodularin (NOD), used as internal standard (IS) for all cyanotoxins, were purchased by Alexis® Biochemicals, La Jolla, CA, USA and the stock solutions of all analytes, were prepared by dissolving each compound with 4 ml of methanol RS (Carlo Erba, Milan, Italy).

The mobile phases were prepared using acetonitrile RS grade (Carlo Erba, Milan, Italy) and water obtained from a Milli-Q system (Millipore Bedford, MA, USA).

Carbograph 4 cartridges, used for the extraction of selected analytes from water samples, were supplied by LARA, Rome, Italy while 125 mm diameter Black Ribbon 589 paper filter was purchased by Schleicher & Schuell, Legnano, Italy. Every chemical reagent used in this study were of analytical grade.

Cyromazine standard was purchased by Merck.

Leucine Enkephalin and Sodium formiate used to calibrate the instrument were purchased by Waters Corp., Milford, MA, USA

3.2 Instrumental analysis

The liquid chromatographic method consisted of an Acquity UPLC system (Waters Corp., Milford, MA, USA) equipped with Acquity UPLC column (Waters Corp., Milford, MA, USA) thermostated at 40 °C and interfaced with a XEVO G2S Q-TOF mass spectrometer (Waters Corp., Milford, MA, USA). Daily, the detector check and the lock mass check were performed with a solution of Leucine Enkephalin 1 ng/µl 50:50 H_2O:ACN 0.1% formic acid and QTOF calibration check were performed with a solution of Sodium formiate 0.5 mM 90:10 isopropanol:H_2O.

3.3 Blank sample

The "blank sample" is a sample of the same matrix with characteristics similar to the sample under

investigation.

The white sample must be free of analytes and it may be used:

- To verify the specificity of the method;
- For quality control;
- For preparation of samples artificially contaminated

3.4 Cyanotoxins analysis

3.4.1 Blank samples

Three different kind of blank samples were tested for the cyanotoxins analysis:

- Tap water after removal of residues of disinfectant agents, in particular chlorine, which act as oxidants against cyanotoxins and the standard process, knocking down the content; the removal of residual chlorine can be obtained by treatment with a solution of sodium thiosulfate (prepared by dissolving 1 g of sodium thiosulfate in 100 mL of deionized water), adding 100 ml of this solution of sodium thiosulfate per liter of water to be treated;
- Surface water;
- Deionized water.

All the blank samples were prepared spiking 250 ml of water with Nodularin at the final concentration of 1 μg, subjecting to the same SPE procedure provided for the preparation of samples and they were analysed in advance to make sure of the absence of the analytes selected. All the matrices analyzed didn't show any interference during the analysis and it was decided to use deionized water produced by a MilliQ system for the preparation of all blank samples.

3.4.2 Standard solution and sample preparation

A standard solution of 21 cyanotoxins, containing 0.1 μg/l of all the analytes and 1 μg/l of Nodularin as internal standard (IS), was prepared in 70:30 H_2O:ACN 10 mM formic acid without any extraction.

Samples for calibration curves were produced by spiking blank samples with analytes at four different concentration (0.1 μg/l, 0.5 μg/l, 1 μg/l, 2 μg/l); the same SPE proce4dure provided for the preparation of real samples was applied to all samples.

3.4.3 Sampling site Description

Vico Lake (Figure 3.1) is a volcanic lake in central Italy located in Lazio Region in the province of Viterbo (Figure 3.2). It has the distinction of altitude among the great Italian lakes, with its 507 m above sea level. Due to its unique natural features, the area of Vico Lake is included among the areas of special natural value of Lazio and between habitats of great natural interest in Italy. It is surrounded by the mountain range of the Cimini mountains, in particular, is surrounded by the Monte Fogliano (965 m) and Mount Venus (851 m), is part of the Natural Reserve of Lake Vico. Today, the lake covers an area of about 12 km^2, of which 8.2 falling in the municipality of Caprarola, and are part of the Nature Reserve. It has a perimeter of 18 km, an average depth of 22.2 meters and reaches 49.5 meters in depth.

Figure 3. 1: Vico Lake

Figure 3. 2: The Vico basin and selected sampling sites of distributed waters

3.4.4 Sample collection

Water samples were taken monthly in Vico Lake, used as a source of drinking water, at the Water Treatment Plant (WTP) and over the piped distribution system in Caprarola municipality (Lazio Region), at the following stations (Figure 3.3):

1) raw water from Vico Lake at the WTP inlet;

2) treated water at the head of the distribution system;

3) drinking waters sampled in two different sites of the distribution system, in Caprarola municipality.

Samples were taken in 500 ml polyethylene bottles and temperature, pH, conductivity and free chlorine content were measured for each sample (data not shown).

In order to analyse the total content of toxins samples must be submitted to at least one cycle of freezing and thawing (-18 ± 3 °C) to facilitate cellular lysis.

After thawing, water samples were spiked with IS and filtered.

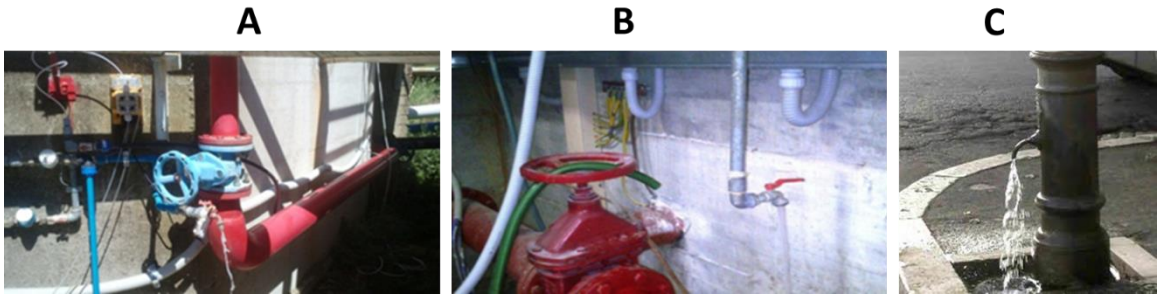


Figure 3. 3: sampling points A) raw water; B treated water; C) drinking water

3.4.5 Solid phase extraction (SPE)

For the sample preparation a previous method was updated adding Cyanopeptolins, Anabeanenopeptins, and Microginins as analytes and optimizing the extraction efficiency for all the cyanotoxins[1].

The extraction of selected analytes was performed by solid phase extraction (SPE), using a device developed in the laboratory and shown schematically in Figure 3.4.

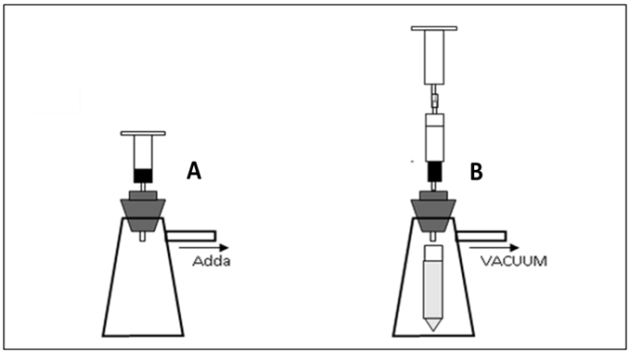


Figure 3. 4: (A) Extraction apparatus SPE; (B) Elution

A tube of propylene (inner diameter of 1 cm, 6 mL capacity), containing a frit on the bottom of propylene, was filled with 0.5 g of Carbograph4 (Lara, Rome, Italy) and a second frit was lying on adsorbent bed to prevent the escape of particles of carbon. Carbograph 4 is an example of Carbon Black graphitized, surface area equal to 200 m^2 / g .

For the elution front mode, it is used a cylindrical hollow piston in teflon and tip " Luer ", all obtained from LARA, Rome, Italy .

Before the passage of the samples through the cartridge, this was washed with 12 mL of the same phase eluent (dichloromethane / methanol 80/20 v / v, 10mM in TFA) and 6 mL of methanol, and then activated with 20 mL of water acidified with HCl (pH 2), followed by 6 mL of distilled water MilliQ (see Figure 3.5) .

SPE procedure was used for the extraction of Microcystins, Cyanopeptolins, Anabeanenopeptins, and Microginins while Anatoxin-a and Cylindrospermopsin were analized without any extraction.

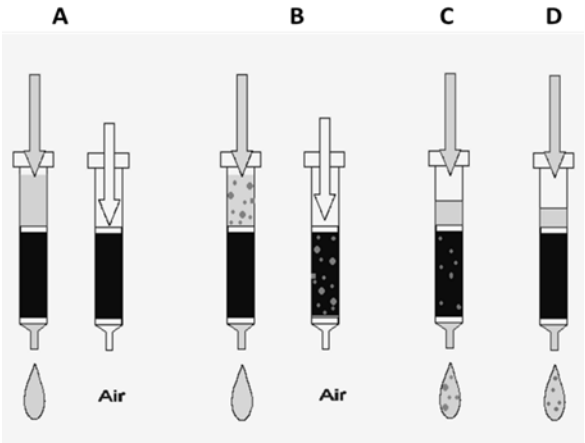


Figure 3. 5: A) Washing / activation of the cartridge; (B) Sample passage and analytes extraction (C) Wash the cartridge and remove of compounds not interfering in detection; (D) Analytes elution.

250 mL of the water samples, filtered, were spiked with 250 µg/L of a Nodularin solution (I.S.). 18 mg/L of sodium thiosulfate was added only to the treated water samples, after acidification, to prevent the I.S. oxidation. The spiked samples were filtered with black ribbon filters for the analysis of the total content of toxins and, after the addition of I.S., samples were passed through a Carbograph 4 SPE cartridge, previously washed and conditioned with dichloromethane/methanol solution as described previously.

The flow rate at which the aqueous samples passed through the cartridges was maintained at about 10 mL /min by vacuum. The SPE cartridge was washed with 6 ml distilled water and the following 0.5 mL of methanol. Analytes were eluted with 6 mL of dichlotromethane/ methanol mixture (80:20, v/v) acidified with 10Mm TFA solution. The eluate was evaporated under a nitrogen stream in a sample concentrator equipped with a dry-block heater set at temperature ≤ 50 °C. Samples were evaporated removing all of liquid phase. The residue was reconstituted with 1 mL of water/acetonitrile mixture 70:30 (v/v) and 10 µL of this extract were injected into the UPLC column.

3.4.6 Extracted Cyanotoxin method

The liquid chromatographic method for extracted cyanotoxins, consisted of an Acquity UPLC system (Waters Corp., Milford, MA, USA) equipped with Acquity UPLC BEH C18 column (2,1 mm i.d. x 100 mm, 1,7 µm, Waters Corp., Milford, MA, USA) thermostated at 40 °C and interfaced with a XEVO G2S Q-TOF mass spectrometer (Waters Corp., Milford, MA, USA).

The experiments were acquired in polarity ES+, Sensitivity Mode with a source temperature of

130°C, a desolvation temperature of 500 °C and a desolvation gas flow of 1000.0 l/Hr.
Two different acquisition modes were available:

- MS mode: a full scan 50-1200 without collision energy to observe the molecular ion signal as MH^+ or MH^{n+}
- MSMS mode: to observe the fragmentation signals applying specific collision energies.

Automatic correction of the mass was applied using lock mass signal during all the experiments. The monocharged and the bicharged ion masses (Table 3.1) were calculated for all the analytes to extrapolate the signals of single ion from the total ion chromatogram.

TOXIN	MONOCHARGED ION	BICHARGED ION
MC-RR	1038.5731	**519.7902**
[D-Asp3]-MC-RR	1024.5574	**512.7824**
MC-YR	**1045.5353**	523.2713
[D-Asp3]-MC-LR	**981.5404**	491.2738
MC-LR	**995.556**	498.2817
MC-LA	**910.492**	455.7497
MC-LY	**1002.5183**	501.7628
MC-LW	**1025.5342**	513.2708
MC-LF	**986.5233**	493.7653
MC-WR	**1068.5513**	534.7793
MC-Htyr	**1059.551**	530.2791
MC-HilR	**1009.5717**	505.2895
CYP-1041	**1041.4807**	521.244
CYP-1007	**1007.5197**	504.2635
Microginin-690	**691.3371**	346.1722
Microginin-690 me	**705.3528**	353.18
Microginin 527	**528.2738**	264.6405
Microginin 527 me	**542.2894**	271.6484
Anab B	**837.4618**	419.2345
Anab A	**844.424**	422.7156
Microginin-704	**705.3528**	353.18
Nodularin (I.S.)	**825.4505**	413.2289

Table 3. 1: List of all extracted cyanotoxins. Monocharged and bicharged ion mass observed for each toxin during the experiment are shown in bold.

3.5 Cyromazine analysis

3.5.1 Blank samples

Tap water, surface water and deionized water was used to prepare three different blank sample.

3.5.2 Standard solution

A standard solution containing 50 μg/l of Cyromazina was prepared in 70:30 H_2O:ACN 10 mM formic acid without any extraction.

Samples for calibration curves were produced by spiking blank samples with analytes at five different concentration (2.5 μg/l, 5 μg/l, 10 μg/l, 25 μg/l, 50 μg/l) without any SPE procedure.

References

[1] F. Nigro Di Gregorio, S. Bogialli, E. Ferretti, L. Lucentini, 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, Microchem. J. 130 (2017) 329–335. doi:10.1016/j.microc.2016.10.012.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Extracted Cyanotoxin

4.1.1 Optimization of experimental conditions

In order to optimize the chromatographic separation of the 21 extracted analytes, 10 µl of a standard solution were injected in the UPLC/QTOF system; a full scan 50-1200 was acquired in MS mode and different chromatographic gradients were employed using water (component A) and acetonitrile (component B) as mobile phases, both containing 10 mM formic acid, with a flow of 450 µl/min.

The purpose of this experiment was to separate the analytes as much as possible and to create different acquisition windows to be used in MSMS experiments.

As an example, three of the adopted experimental conditions and the related obtained chromatograms are reported in figure 4.1. In particular, in figure 4.1 (a) the first attempt is reported indicating the overlapping of retention times for most of the toxins considered; on the other hand, in figure 4.1 (b) and 4.1(c), the optimization of the experimental condition is evidenced by the enhancement of separation. The gradient reported in figure 4.2 was then adopted in all the further experiments leading to the separation of all toxins considered as reported in Figure 4.3.

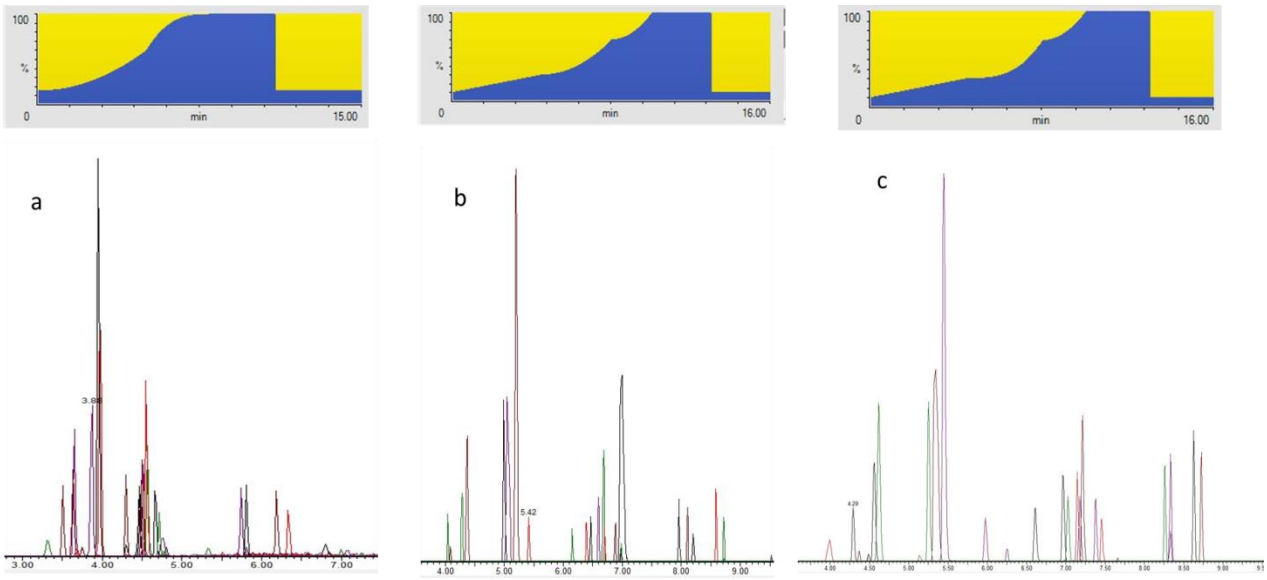
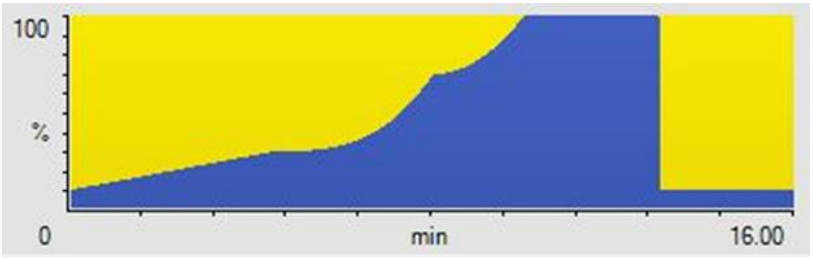


Figure 4. 1:Chromatograms obtained from the analysis of a standard solution containing all analytes at 0.1 µg/l with a full scan (50-1200) in MS mode, applying different chromatographic gradients. Component A (yellow): water 10 mM formic acid; component B (blue)



Time (min)	Flux (ml/min)	%A	%B	Curve
0	0,450	90	10	
4	0,450	70	30	6
8	0,450	30	70	8
10	0,450	0	100	7
12	0,450	0	100	6
13	0,450	90	10	11
16	0,450	90	10	11

Figure 4. 2: Gradient for which the best separation of all analytes was obtained. Component A (yellow): water 10 mM formic acid; component B (blue): acetonitrile 10 mM formic acid with a flow of 0.450 ml/min.

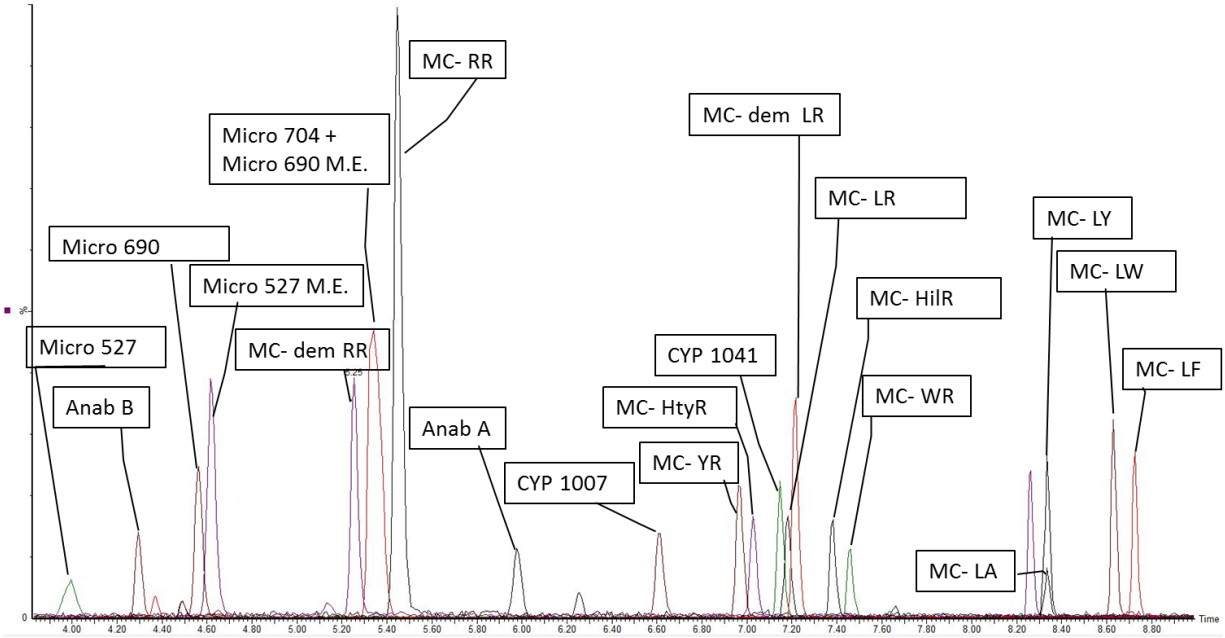


Figure 4. 3: Chromatogram obtained from the analysis of a standard solution containing all analytes at 0.1 µg/l with a full scan (50-1200) in MS mode, applying the gradient reported in Fig 3. IS signal is out of scale and it is not reported.

In addition, the collision energy (CE) for all the analytes has been optimized.

Microcystins, Anabaenopeptin A and Anabaenopeptin B energy collision was achieved through direct infusions of a solution containing single analyte and observing the signals of the fragments with mass 135.0806 (ADDA), 84.0860 and 201.0987 respectively.

For the other selected toxins it was necessary to inject 10 µl of a standard solution in the UPLC/QTOF system, to acquire in MSMS mode with different collision energy ramps and to select the one for which the obtained fragment ion showed higher intensity.

In figure 4.4 some examples of extrapolated chromatograms for Microginins and Cyanopeptolins, are showed while in Table 4.1 the experimental condition for all the toxins considered are reported.

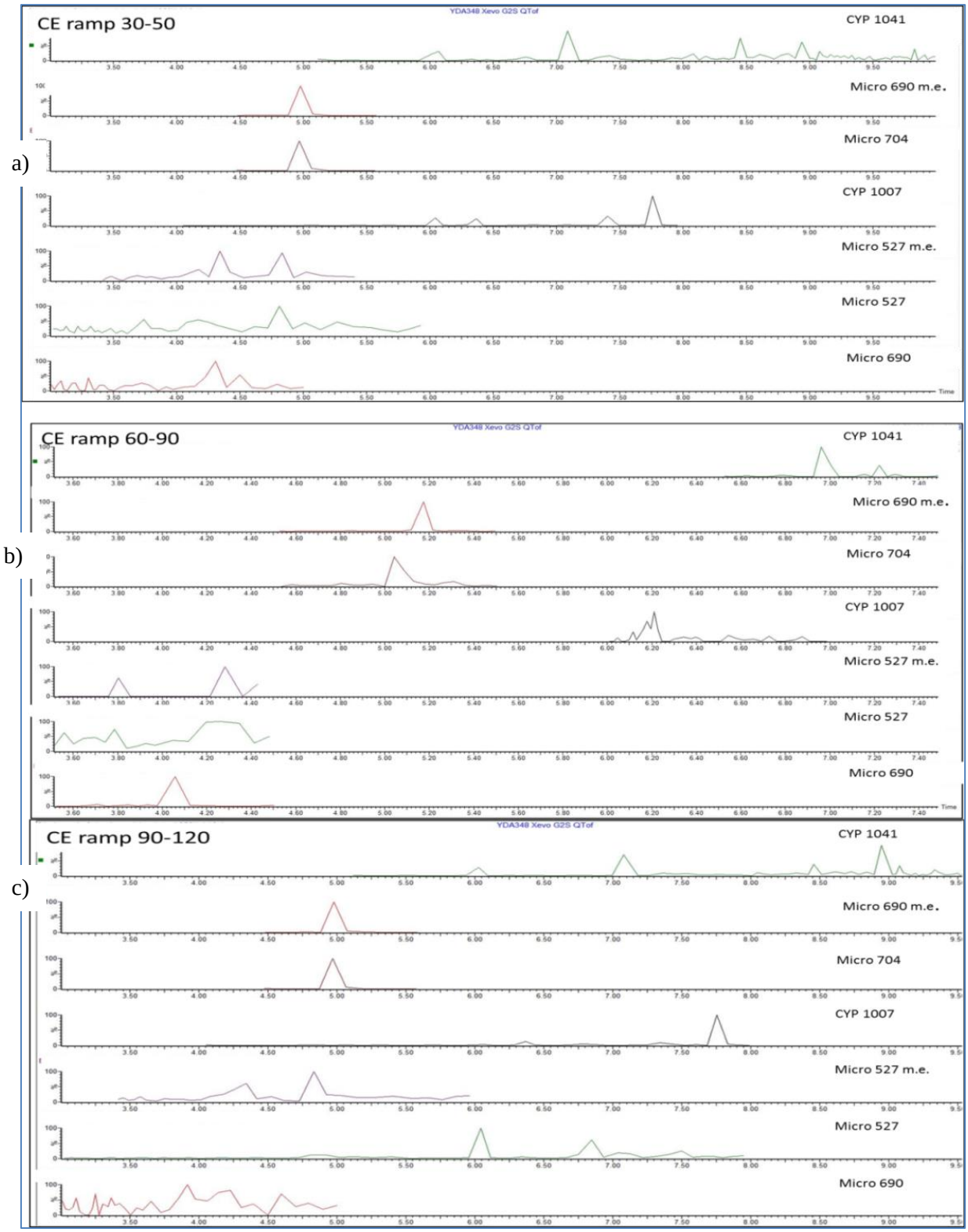


Figure 4. 4: chromatograms for Microginins and Cyanopeptolins acquired in MSMS mode applying different collision energy (CE) ramp a) CE ramp 30-50; b) CE ramp 60-90; c) CE ramp 90-120

ANALYTE	CE (eV)	FRAGMENT
[D-Asp3]-MC-RR	30	135.0806
MC-RR	50	135.0806
MC-YR	60	135.0806
[D-Asp3]-MC-LR	80	135.0806
MC-LR	80	135.0806
MC-HtyR	60	135.0806
MC-HilR	100	135.0806
MC-WR	70	135.0806
MC-LF	60	135.0806
MC-LW	60	135.0806
MC-LA	70	135.0806
MC-LY	60	135.0806
CYP-1041	60-90	150.0918
CYP 1007	50-90	150.0918
Microargin 527 methyl estere	50-90	128.1436
Microargin 690 methyl estere	60-90	128.1436
Microargin 704	60-90	128.1436
Microargin 527	30-50	128.1436
Microargin 690	50-80	128.1436
Anab A	60	84.0860
Anab B	90	201.0987
NOD (i.S.)	60	135.0806

Table 4. 1: Experimental conditions for all extracted cyanotoxins

4.1.2 Validation method

The analytical method has been validated in terms of repeatability, reproducibility, linearity and detection limit.

The presence of significant interferences in blank water samples at the retention times of the analytes and IS, was evaluated by the analysis of 3 blank samples; no matrix effect has been detected for any of the considered samples.

On the other hand, 9 water samples, spiked with all the analytes at 0.1 µg/l and IS at 1 µg/l, were prepared and analysed over three days. The results were used for the assessment of accuracy, as

sum of recoveries and intra-laboratory reproducibility and internal standard accuracy was used as quality control for all measurements during the monitoring.

The method has proven to be robust, precise and accurate with recovery percentages above 85% and with relative standard deviations ≤16%, fitting for the intended purposes at the concentrations of interest.

A calibration curve for each analyte was obtained analyzing water samples spiked at four different concentration levels (0.1, 0.5, 1.0 and 2.0 µg/l) and ~~a~~ good linearity was achieved, with correlation coefficients in the range $0.9902 \leq R^2 \leq 0.9999$ (see Figure 4.5, for example)

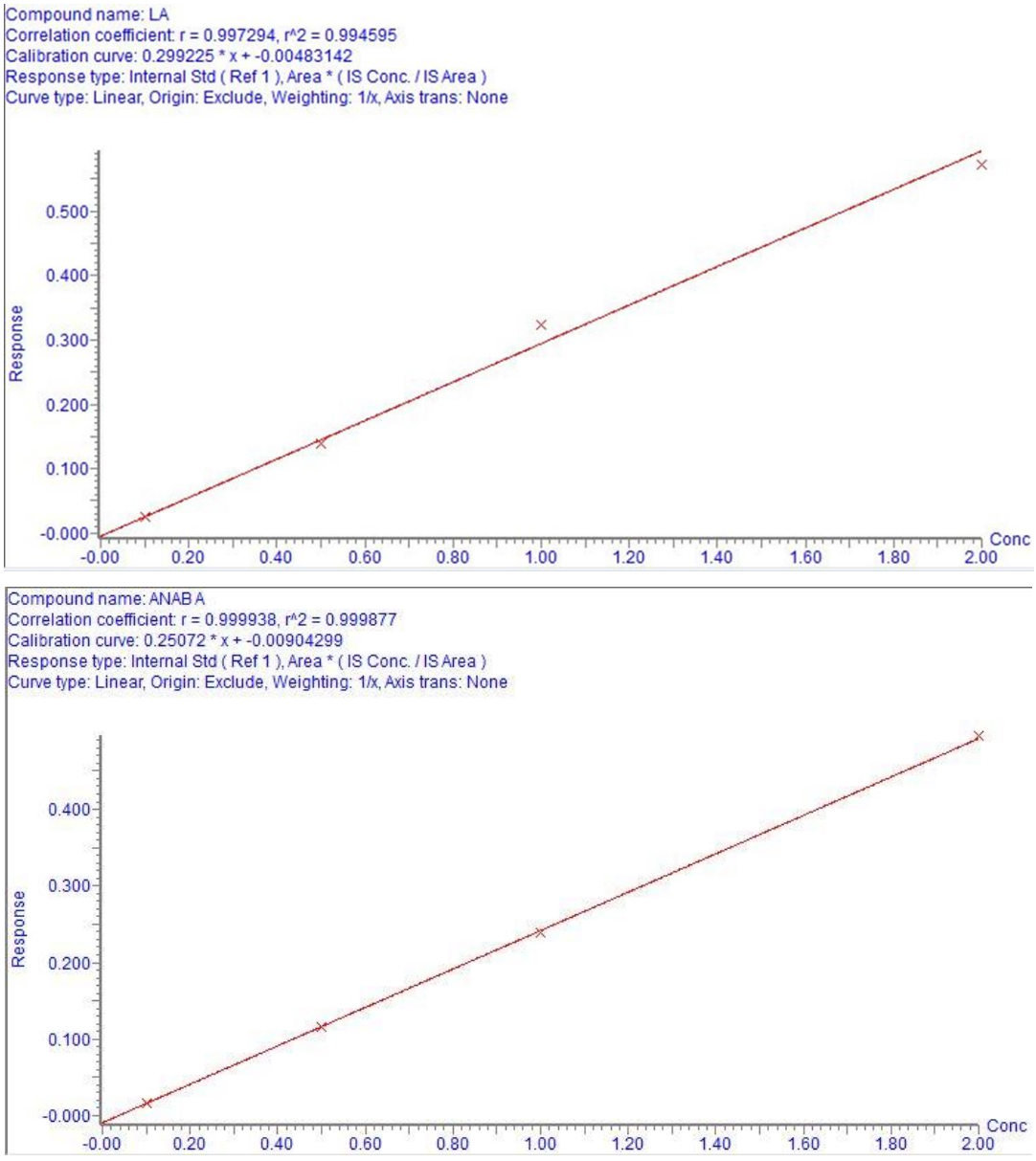


Figure 4. 5: Linearity obtained for MC-LA and Anab A

The limit of detection (LOD) has been determined on the basis of the S / N ratio by calculating the ratio between the height of the peak and the average of the background noise and estimating the concentration of analytes for which an S / N was equal to 3.

The LODs were in the range 0.002 to 0.047 μg/l: comparing the LODs obtained for the protocol described in this study with those of the updated method taken as reference[1] (see Table 4.2), it is possible to note that the obtained values are in the same order of magnitude while for 50% of considered toxins the obtained LODs are much lower with respect to reference method. Nevertheless, we still obtain values at least 20-fold lower than the guideline value proposed by WHO for drinking water (1.0 μg/l for microcystin-LR).

ANALYTE	LOD (SPE-UPLC-HRMS/MS (μg/l))	LOD (SPE-LC-MS/MS(μg/l))
[D-Asp3]-MC-RR	0.002	0.003
MC-RR	0.002	0.003
MC-YR	0.002	0.008
[D-Asp3]-MC-LR	0.020	0.004
MC-LR	0.002	0.004
MC-HtyR	0.004	0.007
MC-HilR	0.002	0.006
MC-WR	0.022	0.030
MC-LF	0.031	0.003
MC-LW	0.022	0.030
MC-LA	0.017	0.003
MC-LY	0.004	0.005
CYP-1041	0.012	0.032
CYP 1007	0.012	0.020
Microginin 527 methyl estere	0.009	0.004
Microginin 690 methyl estere	0.034	0.010
Microginin 704	0.047	0.008
Microginin 527	0.023	0.004
Microginin 690	0.008	0.010
Anab A	0.003	0.020
Anab B	0.004	0.008

Table 4. 2:Comparison between LOD obtained with SPE-UPLC-HRMS/MS method and SPE-LC-MS/MS updated method

4.1.3 Monitoring activity

The analytical protocol has been applied during a monitoring activity in Vico Lake and in drinking water chain.

An example of the chromatograms obtained analysing a raw water sample in MS and MSMS mode was reported in figure 4.6 and 4.7, respectively: it is necessary to observe the presence of the molecular ion and the related fragment signals at the same retention time to detect a specific analyte (see figure 4.8).

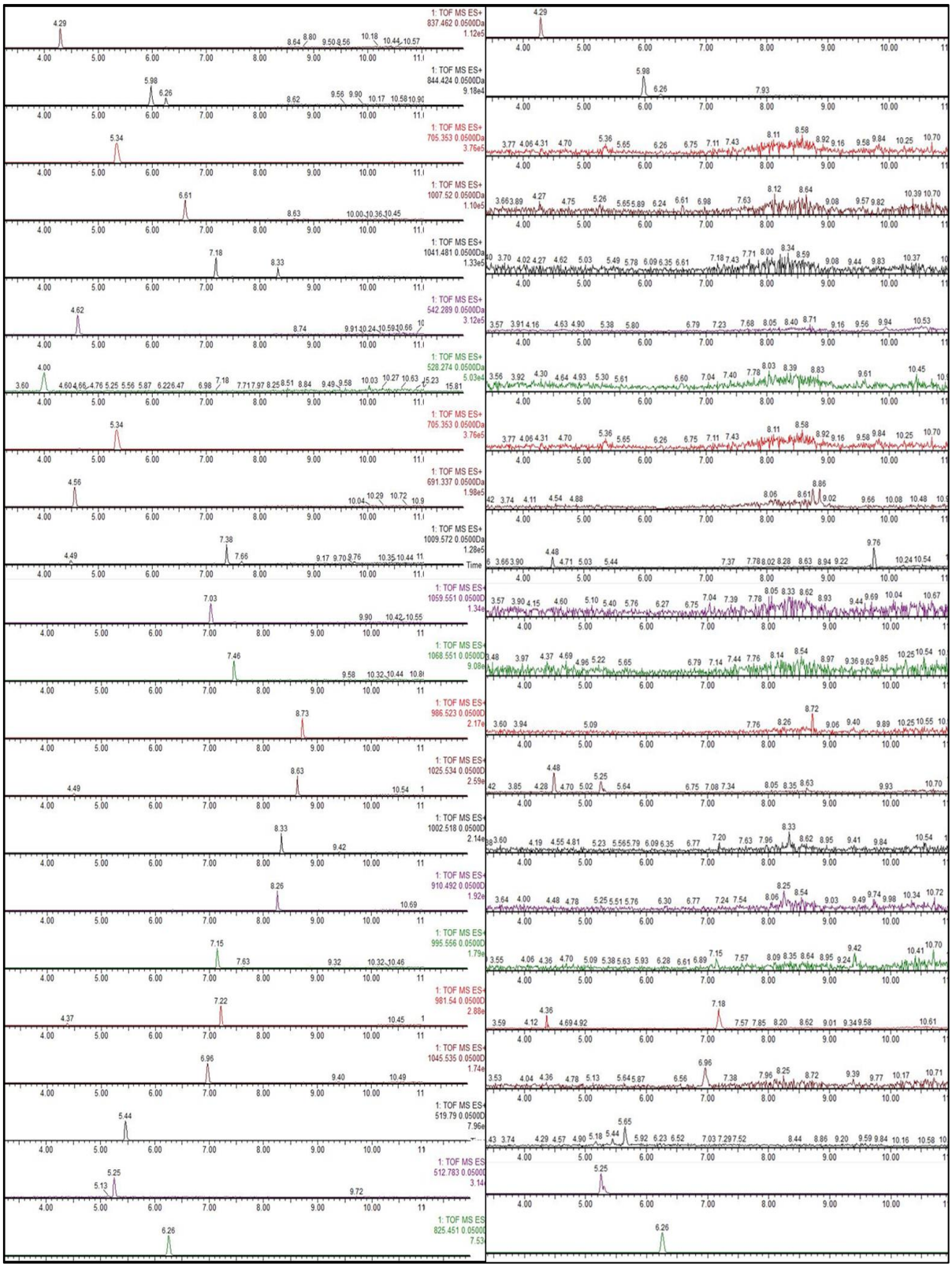


Figure 4. 6: Chromatogram acquired from the analysis in MS mode with a full scan 50-1200, of a standard solution containing 0.1µg/l of all the analytes and 1 µg/l of Nodularin (chromatogram left) and raw water sample spiked with 1 µg/L of Nodularin (chromatogram right)

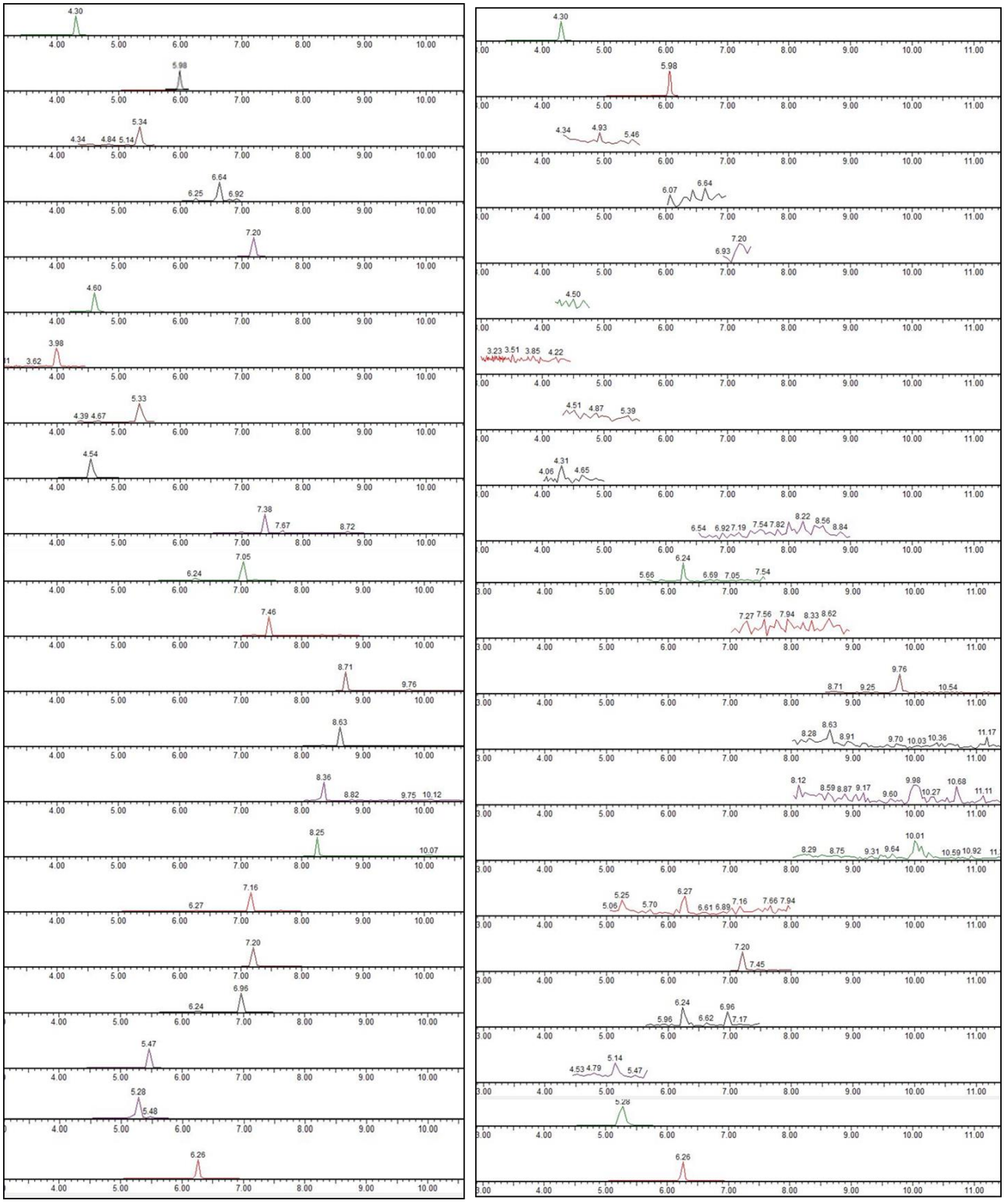


Figure 4. 7: Chromatogram acquired from analysis in MSMS mode of standard solution containing 0.1µg/l of all the analytes and 1 µg/l of Nodularin (chromatogram left) and raw water sample spiked with 1 µg/L of Nodularin (chromatogram right)

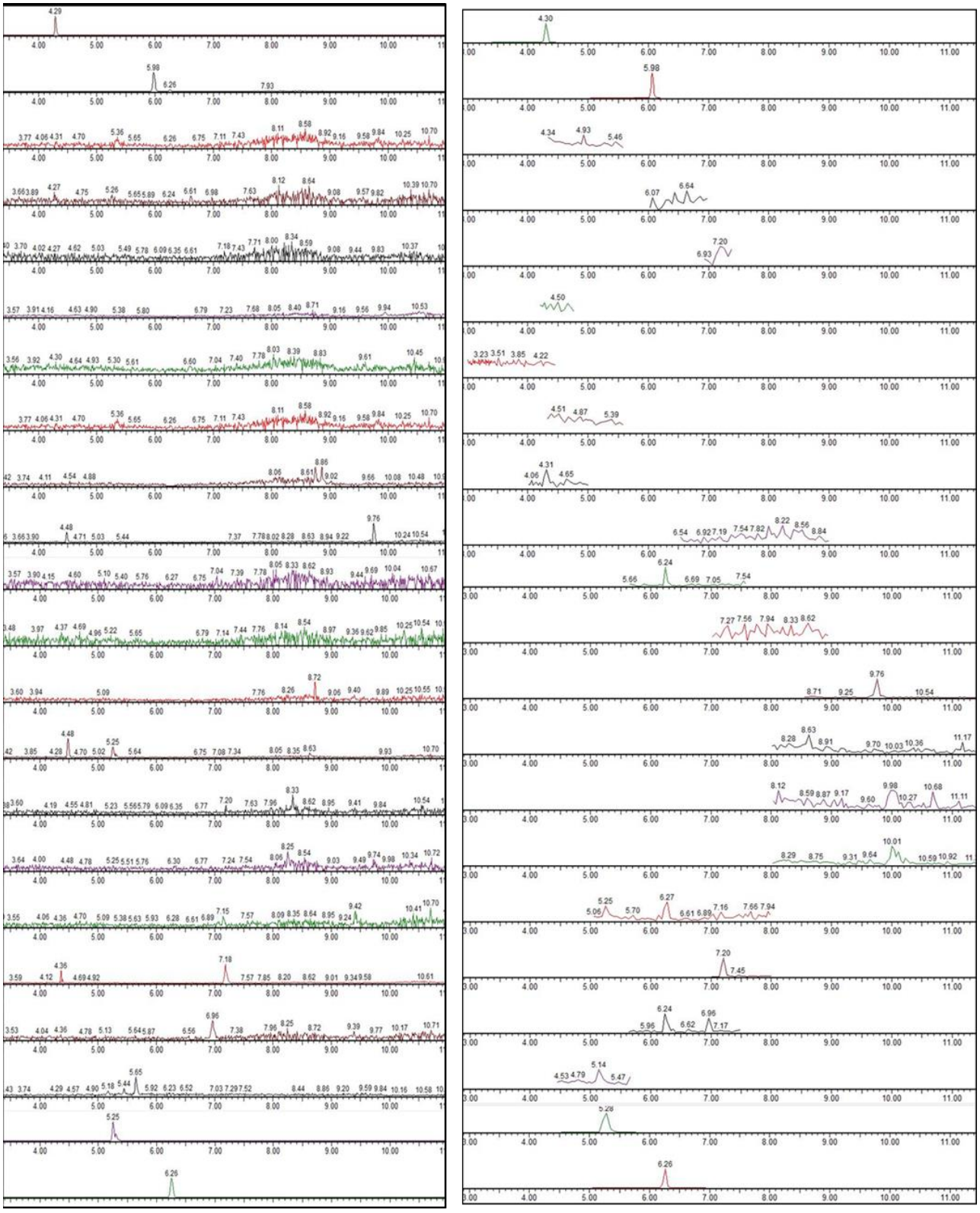


Figure 4. 8: Chromatogram acquired from analysis of a raw water sample spiked with 1 µg/L of Nodularin in MS mode (chromatogram left) and MSMS mode (chromatogram right)

The real samples were analysed by UPLC-HRMS/MS method described in this study and LC-MS/MS updated method, obtaining comparable results.

The results obtained from analysis of water samples evidenced the presence of [D-Asp3]-MC-RR, MC-RR, MC-YR, [D-Asp3]-MC-LR, Anabaenopeptin A and Anabaenopeptin B (see Figure 4.9) with maximum concentrations respectively of 2.29, 0.068, 0.774, 0.338, 1.81 and 2.39 μg/L in raw water. No toxins were detected in raw water samples during the warmest months of the year 2018 and 2019 (July-August).

The most detected algal species during the entire monitoring period were *P. rubescens* and *L. redekei* with maximum cell density levels respectively of $1.23 \cdot 10^8$ and $3.92 \cdot 10^8$ cells/l in raw water.

Toxin production of cyanobacteria appears highly variable between blooms and during all sampling period. *L. redekei* showed high level of cell abundance during the warmest months of 2018 (June-July-August) with $4.9 \cdot 10^7$ cell/l but the toxins were absent in the water samples. From November 2018 to April 2019 an increase of both toxic cyanobacteria species and total cyanotoxins concentration was observed. It has been frequently reported that seasonal changes in toxin production by cyanobacteria are due to changes in the proportion of toxic/nontoxic

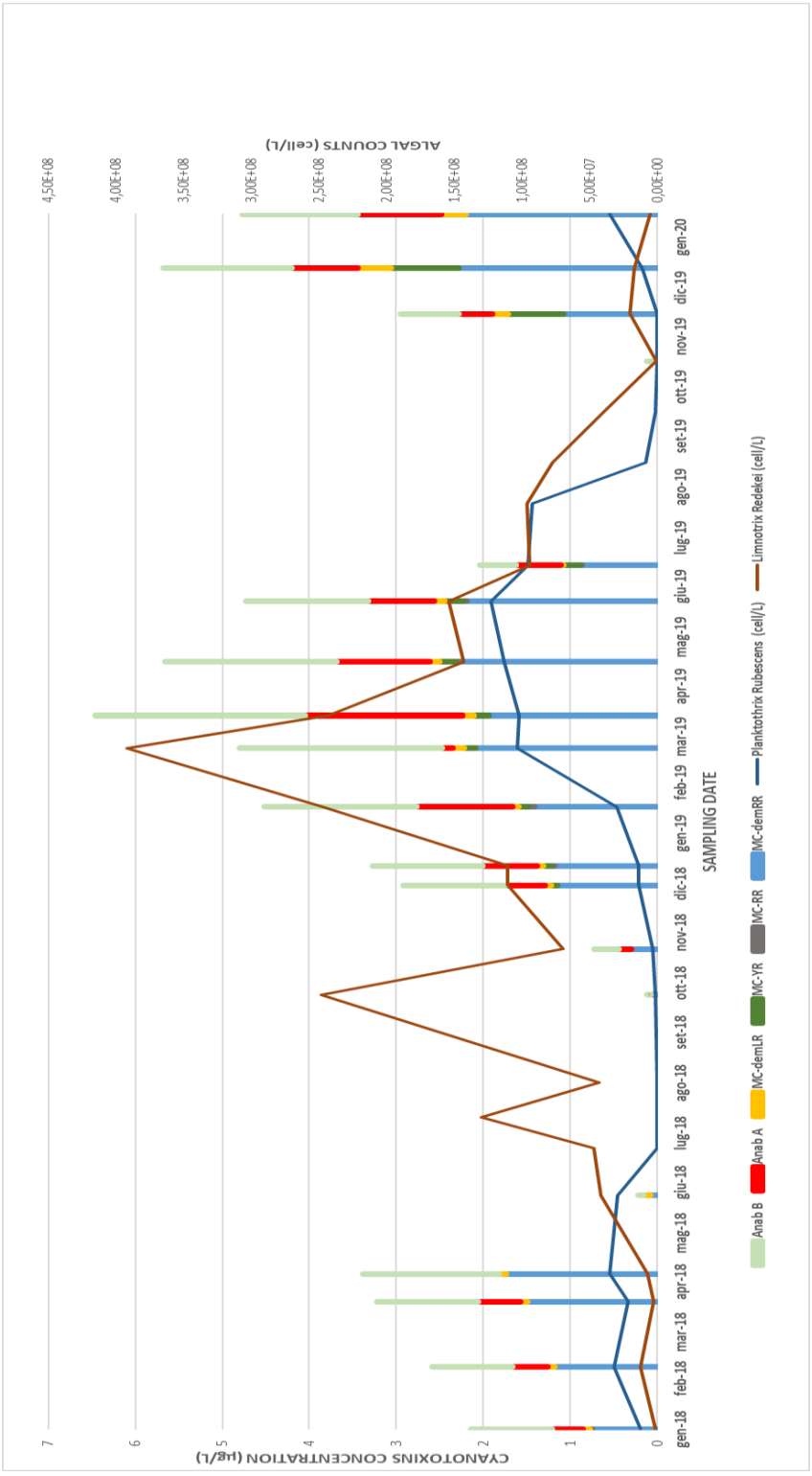


Figure 4. 9:Temporal trend of cell densities [cells/l] of cyanobacteria identified in the Vico basin and cyanotoxins level, as determined with QTOF analysis and expressed as total concentration [µg/l] of the sum of selected compounds, both recorded in raw waters of the Caprarola Water Treatment Plant.

The results obtained from the analysis of raw, treated and distributed water samples (see figure 4.10) were used for the risk assessment related to the proliferation of cyanobacteria and the production of cyanotoxins in the surface water chain allowing to evaluate the effectiveness of treatments and possible risks to human health.

The values related to treated and distributed water, limited to the samples analysed, did not show health risks correlated to phenomena of acute or chronic toxicity in the water, considering the World Health Organization (WHO) provisional guideline value for MC-LR .

However, the treatment processes were not always efficient, considering the algal concentration levels found in raw water samples and drinking water, referring to some threshold values reported in the "National Guidelines for the Management of Cyanobacteria Risk for Water for Human Consumption"[2].

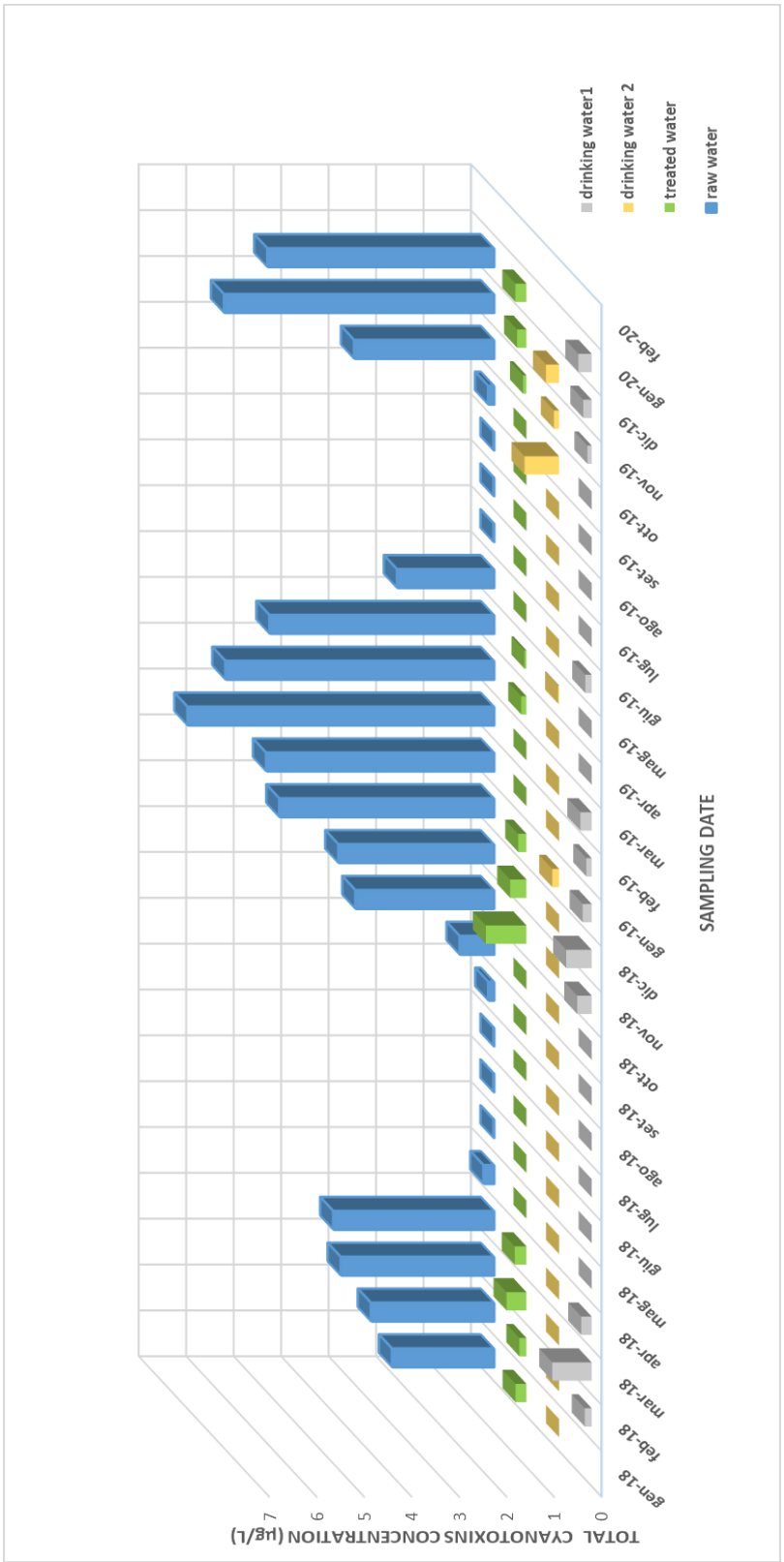


Figure 4. 10: Monitoring of raw treated and distributed water

4.2 Anatoxin-a and Cylindrospermopsin

4.2.1 Optimization of experimental conditions

All the experiments were acquired in polarity ES+, Sensitivity Mode with a source temperature of 130°C, a desolvation temperature of 500 °C and a desolvation gas flow of 1000.0 l/Hr.
In MS mode the molecular ion signals were observed as MH^+ or MH^{n+} with a full scan 50-500 without CE while a specific CE was applied to observe the fragmentation signals.
Automatic correction of the mass was applied using lock mass signal during all the experiments.
The monocharged and the bicharged ion masses (Table 4. 2) were calculated for all the analytes to extrapolate the signals of single ion from the total ion chromatogram.

TOXIN	MONOCHARGED ION
ANA-a	166.1224
CYN	416.1235

Table 4. 3: calculated monocharged ion for ANA-a and CYN

A standard solution containing 50 µg/l of ANA and CYN was used to optimized the esperimental condition while three different chromatographic columns were tested to obtain the separation of ANA and CYN in water samples to be destined for human consumption: two reverse phase C18 columns (BEH and HSS-T3), and a HILIC column (BEH AMIDE).
Acquity UPLC BEH C18 column (2,1 mm ID x 100 mm, 1,7 µm, Waters Corp., Milford, MA, USA) was the firtst to be tested, using H_2O and acetonitrile as mobile phases, both acidified with 10 mM formic acid.
Different gradients were tested, with a flow of 450 µL / min and a column temperature of 45 °: in all the tests carried out, the mass peaks of both analytes were detected, but they were never retained by the column. The best chromatographic gradient and the separation obtained were reported in Table 4.4 and Figure 4.11, respectively.
The percentage of formic acid was also varied, increasing it from 10 to 100 mM but no result was obtained.

Time (min)	% A	%B	Curve
0	90	10	
4.5	70	30	6
8	30	70	8
10	0	100	7
12	0	100	6
13	90	10	11
16	90	10	11

Table 4. 4: Gradient for which the best separation of ANA and CYN was obtained. Component A:water 10 mM formic acid; component B: acetonitrile 10 mM formic acid with a flow of 0.450 ml/min.

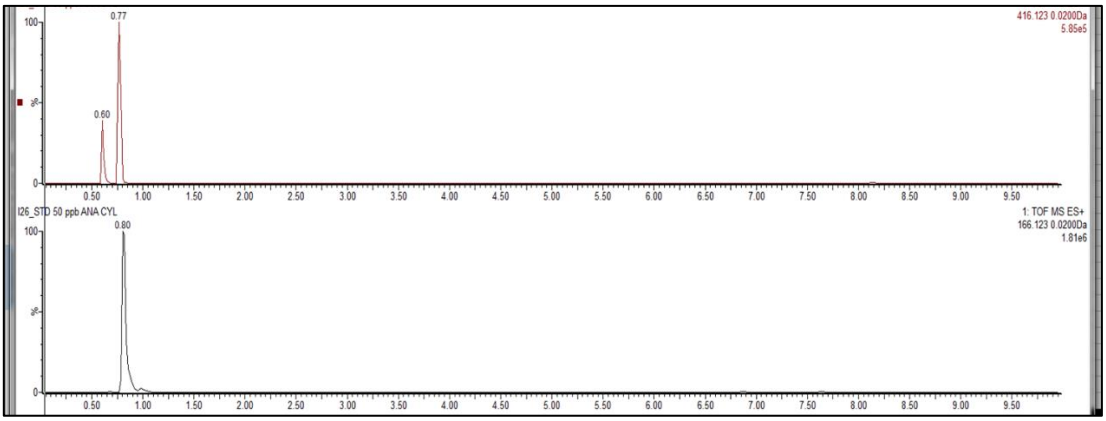


Figure 4. 11: Chromatogram acquired from analysis in MS mode of standard solution containing 50 µg/l of ANA(lower chromatogram) and CYN(upper chromatogram)

Acquity UPLC HSS T3 column (2,1 mm ID x 100 mm, 1,7 µm, Waters Corp., Milford, MA, USA) was tested: this column allows the use of higher percentages of aqueous phase without damaging the column.

Different gradients were tested using water and acetonitrile both with 10 mM formic acid, with a flow of 450 µL / min and a column temperature of 45 °.

The mass peaks of both analytes were detected and all the analytes were eluited after the dead time; unfortunately, due to the high percentage of water used, the peak associated to the anatoxin-a fragments could not be observed.

The best chromatographic gradient was reported in Table 4.5 while the best chromatographic separation in MS was reported in Figure 4.12.

Time (min)	% A	%B	Curve
0	98	2	
3	98	2	1
5	5	95	6
6	5	95	6
7	98	2	4
1O	98	2	11

Table 4. 5: Gradient for which the best separation of ANA and CYN was obtained. Component A:water 10 mM formic acid; component B: acetonitrile 10 mM formic acid with a flow of 0.450 ml/min

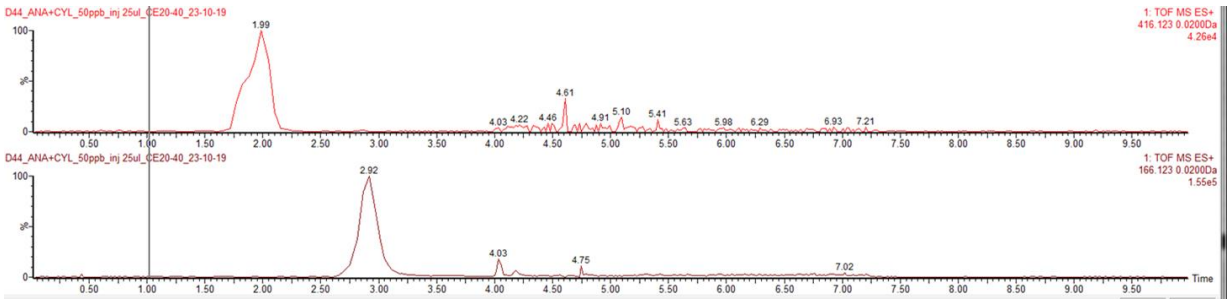


Figure 4. 12: Chromatogram acquired from analysis in MS mode of standard solution containing 50 µg/l of ANA(lower chromatogram) and CYN(upper chromatogram)

Two different CE ramps were applyed in MSMS mode and the obtained results were reported in Figure 4.13

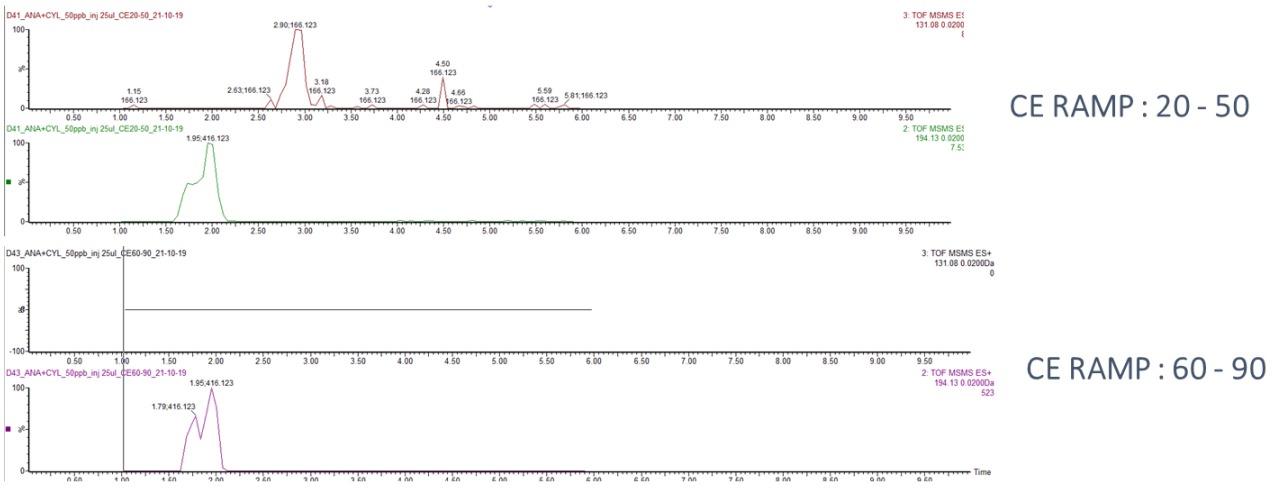


Figure 4. 13:Chromatogram acquired from analysis in MS mode of standard solution containing 50 µg/l of ANA(upper chromatogram) and CYN(lower chromatogram)

The best results were obtained with CE ramp 20-50 but not good resolution was obtained.
The last column to be tested was the BEH AMIDE(2.1 mm ID X 150 mm, 1.7 µm, Waters Corp., Milford, MA, USA), a HILIC type column using H_2O and acetonitrile as mobile phases, both containing a concentration of 10 mM of formic acid, but no good resolution was obtained for the analytes, as reported in figure 4.14: applying the chromatogram reported in Table 4.6 Ana-a was eluited in the dead time.

Time (min)	% A	%B	Curve
0	5	95	
1	5	95	1
2	45	55	9
3	50	50	8
4	50	50	6
4.5	5	95	4
8	5	95	11

Table 4.6: Gradient for which the best separation of ANA and CYN was obtained. Component A:water 10 mM formic acid; component B: acetonitrile 10 mM formic acid with a flow of 0.450 ml/min

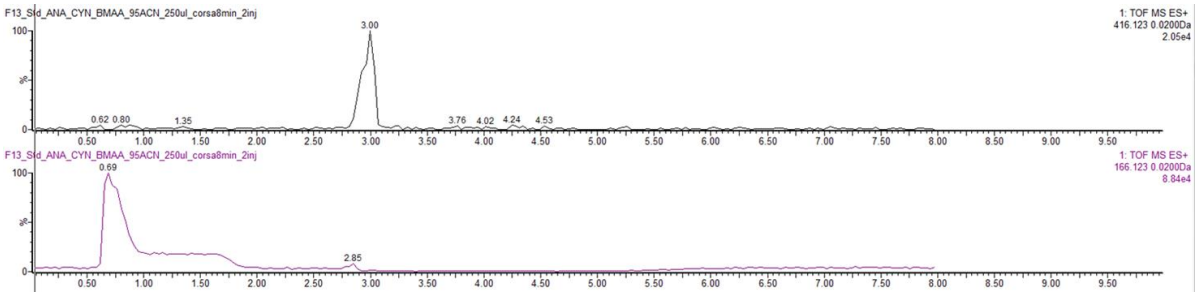


Figure 4. 14: Chromatogram acquired from analysis in MS mode of standard solution containing 50 µg/l of ANA(lower chromatogram) and CYN(upper chromatogram)

As last test, I tried to use H_2O and acetonitrile as mobile phases, both containing a concentration of 10 mM of ammonium acetate, with a flow of 0.450 ml/min and a colum temperature of 45°C. In these conditions, applying the gradient reported in Table 4.7 both the toxins were retained by the column but only for anatoxin-a a good resolution of both the mass peak and the respective fragment was obtained, while the cylindrospermopsin peak was not resolved optimally (Figure 4.15).

Time (min)	% A	%B	Curve
0	5	95	
3	20	80	10
4	30	70	4
4.5	50	50	2
5.5	50	50	6
6	5	95	2
10	5	95	11

Table 4. 7: Gradient for which the best separation of ANA and CYN was obtained. Component A:water 10 mM ammonium acetate; component B: acetonitrile 10 mM ammonium acetate

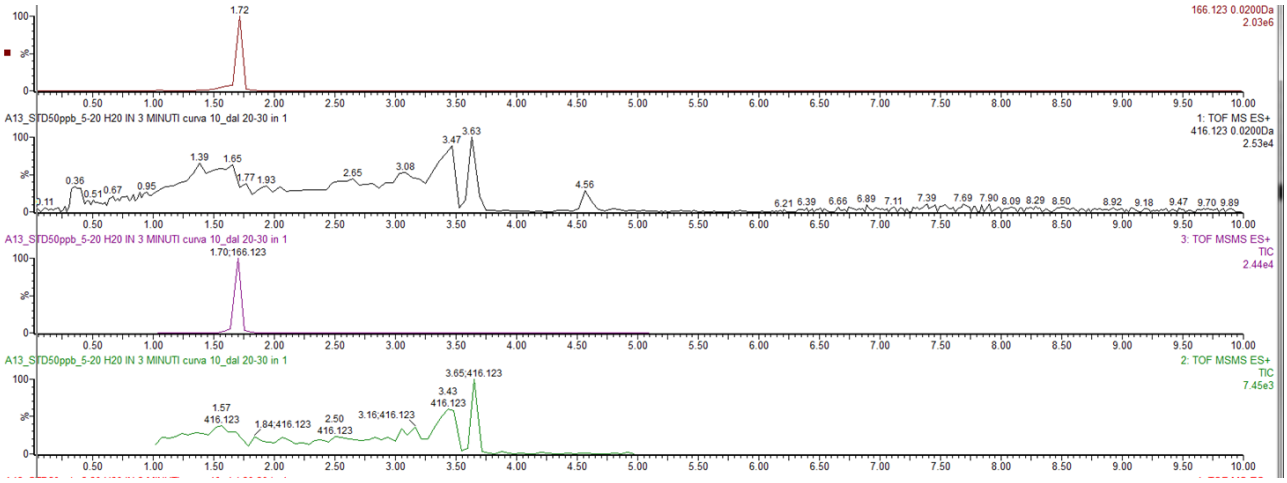


Figure 4. 15: Chromatogram acquired from analysis in MS and MSMS mode of standard solution containing 50 µg/l of ANA(1,70 min) and CYN(3.65 min)

4.3 Cyromazine

4.3.1 Optimization of experimental conditions

In order to optimize the chromatographic separation of Cyromazine, 10 µl of a standard solution cointaing 50 µg/L of the analyte were injected in the UPLC/QTOF system.

The experiment was carried out in polarity ES+, Sensitivity Mode with a source temperature of 130°C, a desolvation temperature of 500 °C and a desolvation gas flow of 1000.0 l/Hr.

The UPLC system was equipped with a BEH AMIDE column (2.1 mm ID X 150 mm, 1.7 µm, Waters Corp., Milford, MA, USA) termosted to 50°C.

The calculated value for MH^+ of cyromazine is 166.1045 so a full scan 50-300 was selected in MS and MSMS mode and different chromatographic gradients were employed using water

(component A) and acetonitrile (component B) as mobile phases, both containing 10 Mm formic acid, with a flow of 550 µl/min.

The gradient reported in Table 4.8 was adopted to obtain the best chromatographic separation (Figure 4.16).

Time (min)	% A	%B	Curve
0	5	95	
0.5	5	95	6
2	20	80	2
3	30	70	6
3.5	40	50	6
6	40	60	6
6.10	5	95	6
10	5	95	11

Table 4. 8: Gradient for which the best separation of Cyromazine was obtained. Component A:water 10 mM formic acid; component B: acetonitrile 10 mM formic acid

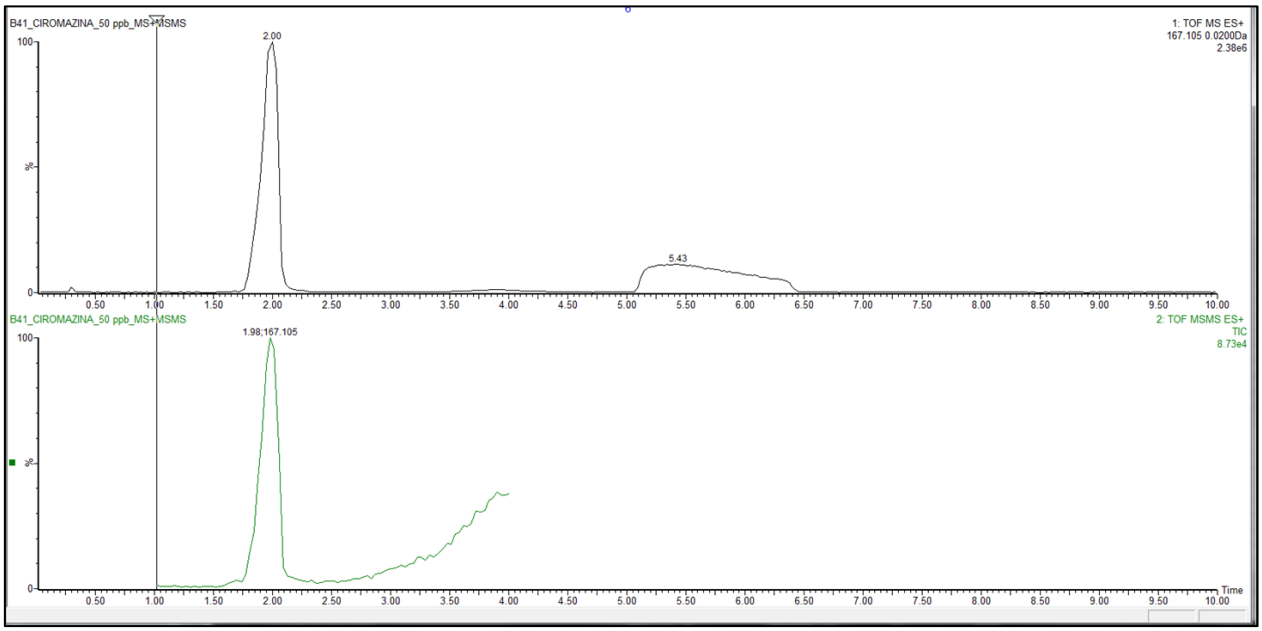


Figure 4. 16: Chromatogram acquired from analysis in MS (upper chromatogram) and MSMS mode (lower chromatogram) of standard solution containing 50 µg/l of Cyromazine

4.3.2 Validation method

The analytical method has been validated in terms of matrix effect, repeatability, reproducibility, linearity and detection limit.

In order to evaluate the matrix effect three different blank samples were prepared and analyzed withou any spike, applyng the condition described in the paragraph 4.3.1.

The results obtained were reported in Figure 4.17: none of the analyzed blank samples showed signals that could interfere with the analysis.

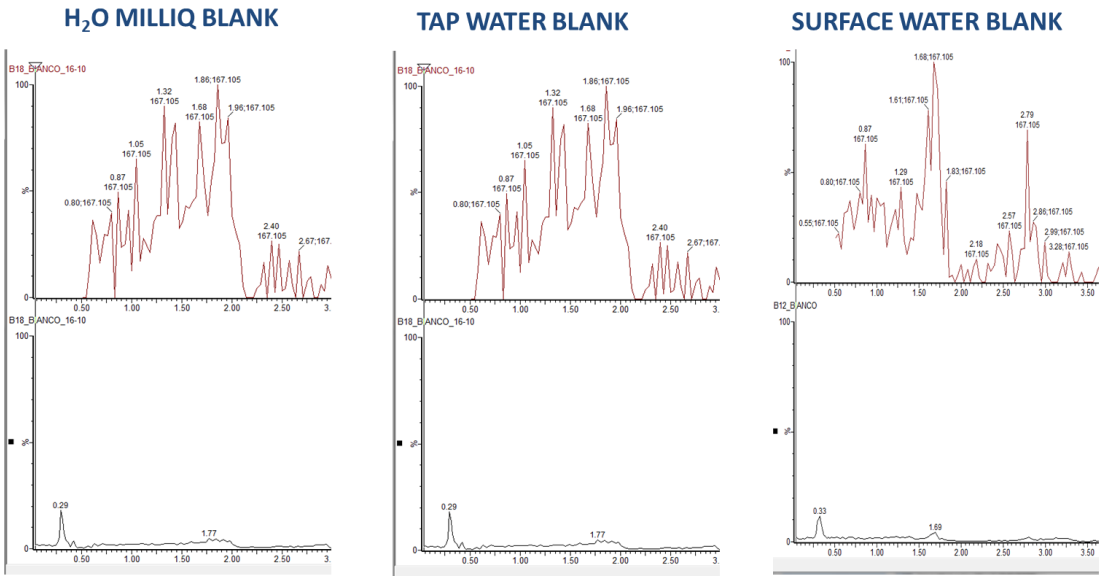


Figure 4. 17: Chromatogram acquired from analysis in MS (upper chromatogram) and MSMS mode (lower chromatogram) of blank sample

Then three different standard were prepared, spiking the blank sample with a concentration of 10 µg/l of Cyromazine and the obtained results were reported in Figure 4.18.

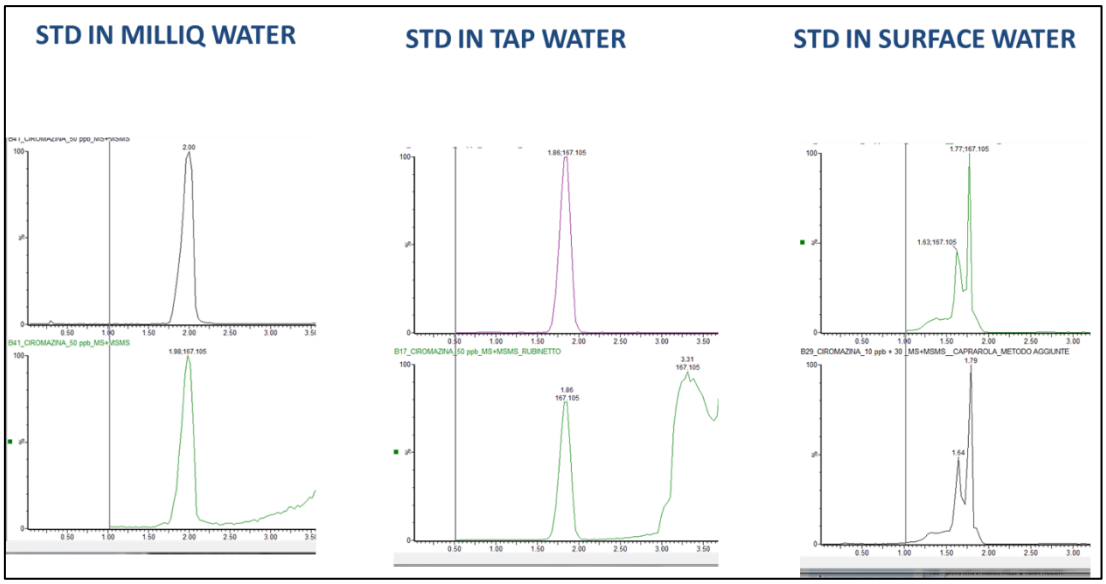


Figure 4. 18:Chromatogram acquired from analysis in MS (upper chromatogram) and MSMS mode (lower chromatogram) of standard solution prepared in three different matrix

Observing the first two standards chromatogram (milli-Q and tap water) it is possibile to note that there is no matrix effect; on the other hand, in the case of the standard prepared in suferce water, a worsening in the shape and resolution of the peak is noted.

It was therefore decided to carry out tests according to the method of standard addtion to verify whether this effect would affect not only the identification phase but also the quantification phase. So four standard solution cointanint 10-20-30-40 µg/l of Cyromazine was prepared in surface water and analized; a good linearity was obtained as reported in Figure 4.19

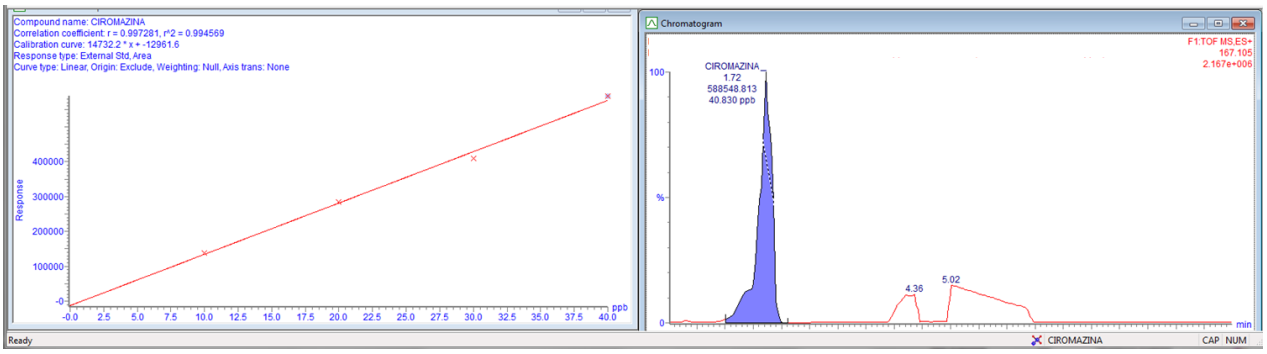


Figure 4. 19: linearity obtained with method of standard addition

Considering the obtained results Milli Q water was selected for the prepation of the blank sample and standard solution.

9 water samples were spiked with cyromazine at 10 µg/l analysed over three days: the results were used for the assessment of accuracy, as sum of recoveries and intra-laboratory reproducibility and proving thet the method was robust, precise and accurate with a relative standard deviations ≤10%.

A calibration curve for each analytes was obtained analyzing water samples spiked at five different concentration levels ((2.5 µg/l, 5 µg/l, 10 µg/l, 25 µg/l, 50 µg/l) and ~~a~~ good linearity was achieved, with correlation coefficients $R^2 = 0.9873$ (see Figure 4.20)

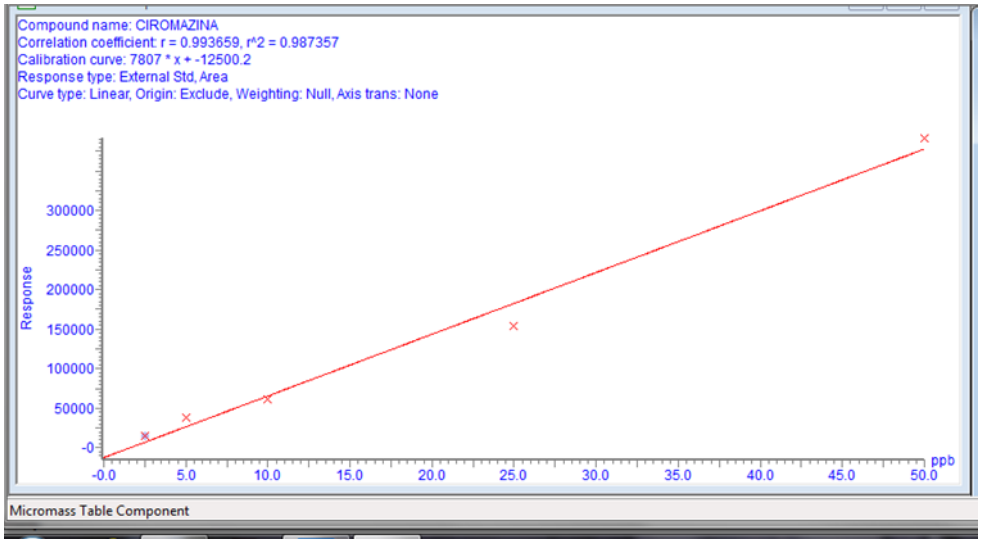


Figure 4. 20: Linearity for Cyromazine

As final step LOD has been calculated on the basis of the S / N ratio, by calculating the ratio between the height of the peak and the average of the background noise and estimating the concentration of analytes for which an S / N was equal to 3.

The LOD was equal to 0.5 µg/l.

References

[1] F. Nigro Di Gregorio, S. Bogialli, E. Ferretti, L. Lucentini, First evidence of MC-HtyR associated to a Planktothrix rubescens blooming in an Italian lake based on a LC-MS method for routinely analysis of twelve microcystins in freshwaters, Microchem. J. 130 (2017) 329–335. doi:10.1016/j.microc.2016.10.012.

[2] Lucentini L., Ferretti E., Cyanobacteria in water for human consumption. Guidelines for risk management. English translation (Rapporti Istisan 13/35), 2013

CONCLUSIONS

To protect human health from the adverse effects of any contamination of water intended for human consumption, the EU legislation established individual parametric values for a number of substances which are important throughout the Community relying upon the guideline values provided by World Health Organisation's 'Guidelines for drinking water quality. However, Member States must set values for other additional parameters not expressively mentioned in the dir 98/83/CE where that is necessary to protect human health within their territories.

Moreover, European Parliament is considering to add the 1.0 µg/l value of MC-LR in the next Revision of the Drinking Water Directive, as reported in the "Proposal for a Directive of and of the Council on the quality of water intended for human consumption".

This study was focused on the development of new criteria and methods for the risk assessment and the risk management of cyanobacteria development and cyanotoxin production in water intended for human consumption, relying upon a prevention integrated strategy covering the entire water chain; from the protection and surveillance of the water resource to be destined to human consumption to the water treatment and distribution, in order to assure the quality of water at the consumer's tap.

In this study a method for simultaneous determination of 21 cyanotoxins in drinking water, based on Ultra Performance Liquid Chromatography coupled with a Q-TOF mass spectrometer, has been developed and applied to a monitoring activity, for the risk assessment in presence of algal bloom.

The method resulted robust, in terms of repeatability, reproducibility and linearity, with recovery percentages above 85% and with relative standard deviations ≤16%, fitting for the intended purposes at the concentrations of interest.

The LODs were in the range 0.002 to 0.047 µg/l for all the analites, at least 20-fold lower than the guideline value proposed by WHO for drinking water for MC-LR.

This method allows the simultaneous identification of target compounds, in a short time of analysis (16 minutes) and low injection volume (10 µl).

The analytical method was then applied for a monitoring plan during a drinking water emergency occurred in Vico Lake in the period January 2018- January 2020.

The results obtained from analysis of water samples evidenced the presence of [D-Asp3]-MC-RR, MC-RR, MC-YR, [D-Asp3]-MC-LR, Anabaenopeptin A and Anabaenopeptin B with maximum concentrations respectively of 2.29, 0.068, 0.774, 0.338, 1.81 and 2.39 μg/L in raw water.while no toxins were detected in raw water samples during the warmest months of the year 2018 and 2019 (July-August).

Other toxins (LW, LY, LF, HilR, WR, HtyR, Cyanopeptolin, Anabaenopeptins and Microginins) have never been detected during the twenty-four sampling months.

It was also sporadically detected the presence of toxins in treated and drinking water, although at levels below the guideline value set by the WHO for MC -LR.

The data obtained on the treated water showed that the treatments conventionally carried out on the raw water generally prove effective in reducing almost all of the contents of the analytes in the influent water to WTP.

One of the objectives pursued by the research activity is to ensure that water supply companies meet the quality standards of cyanotoxin in drinking water through appropriate water protection measures in the aquatic environment and / or through appropriate water treatment measures to be applied before supply, providing the greatest number of data and information useful for the implementation of a new model of intervention criteria aimed at the level of risk estimated in the drinking water reservoir and at the treatment of water before human consumption.

The activity carried out made it possible to define practical, technical-scientific tools for risk analysis along the entire water production and distribution chain and the critical elements in the management of emergency scenarios were finely tuned, with respect to communication risk.

Based on the extensive applicative experience acquired in the course of the thesis, the method results to be fully functional to the more advanced preventive approach to control cyanotoxins within the drinking water supply chain according to the Water Safety Plan (WSP) model, as it is applied to raw and tap water, by allowing an early detection of (simultaneous) algal toxin production, the actual effectiveness of mitigation measures,

the adoption of the UPLC-HRMS approach as validation/confirmatory approach for on-line monitoring of algal and toxins in water bodies and water distribution systems.

The development of another analitical method for the direct analysis of Anatoxin-a and Cylindrospermopsin has been started but the tests carried out didn't lead to satisfactory results and, consequently, the method needs further optimization.

Finally a method for the identification and quantification of Cyromazine has been developed and validated.

A relevant matrix effect was observed in the chromatographic separation using surface water as blank sample while no matrix effect was observed using Milli Q or tap water.

The method resulted robust, precise and accurate with a relative standard deviation $\leq 10\%$, a good linearity was achieved in the range 2.5 - 50 μg/l and a LOD was equal to 0.5 μg/l.

Since the use of pesticides containing cyromazine will be allowed until June 2021, the developed method may be useful to verify the absence of this compound in water.

PUBLICATIONS

1. **Di Pofi G**, Favero G, Nigro Di Gregorio F, Ferretti E, Viaggiu E, Lucentini L "An ultra performance liquid chromatography coupled with high resolution mass spectrometry method for the screening of cianotoxins content in drinking water samples";. MethodsX Volume 7, 2020
2. **Di Pofi G**, Favero G, Nigro Di Gregorio F, Ferretti E, Viaggiu E, Lucentini L. . Articolo dal titolo "Multi-residue Ultra Performance Liquid Chromatography-High resolution mass spectrometric method for the analysis of 21 cyanotoxins in surface water for human consumption."; Talanta Volume 211, 1 May 2020
3. Sottogruppo di lavoro "Cromatografia liquida associata alla spettrometria di massa" (Cianotossine, interferenti endocrini, contaminanti emergenti)." nel Rapporti ISTISAN: 19/7 Metodi analitici per il controllo delle acque da destinare e destinate al consumo umano ai sensi del DL.vo 31/2001 e s.m.i.- Metodi chimici;
4. "Advantages of ultra performance liquid chromatography –high resolution mass spectrometer for the analysis of cyanotoxins in water for human consumption"; Abstract book of Workshop "Validation of targeted and non-targeted methods of analysis, Tartu 20-21Maggio 2019;
5. "First determination of cylindrospermopsin in drinking water chain of vico lake with SPE-LC-MS/MS"; Abstract book of XXVII Congresso Divisione di Chimica Analitica della Società Chimica Italiana, Bologna 16-20 Settembre 2018;
6. "Multi-residue Ultra Liquid Chromatography-high Resolution Mass Spectrometric Method for the Analysis of 21 Cyanotoxins in Surface Water for Human Consumption"; Abstract book of 47th International Symposium on High performance liquid phase separation and related technique 29 Luglio-2 Agosto 2018;
7. "Solid Phase Extraction-Liquid Chromatography Mass Spectrometric Protocol for determination of Cylindrospermopsin in Surface Water Sample: First Identification in Italian Lake". Abstract book of 47th International Symposium on High performance liquid phase separation and related technique, Washington 29 Luglio-2 Agosto 2018

ACKNOWLEDGEMENTS

I am grateful to Professor Favero and to Dr. Lucentini for giving me the opportunity to do this PhD: in these three years I have grown a lot not only in terms of scientific knowledge but also on a personal level.

You allowed me to participate in congresses and conferences, interface with experts, and have experiences that I could not have done in other contexts.

I thank all the team in the Water Quality and Health department who welcomed me and made me feel at home.

I thank Emanuele and Valentina for the friendly talks and for encouraging me in some difficult moments but a special thanks goes to Federica: you were an excellent colleague and a true friend ..... you supported me in the most difficult moments, when everything seemed go wrong and you celebrated with me when the good news came.

In some situations, I felt like I was reliving the years 2010-2011: the tools did not help us but in any case we did not lose heart and we found a way to laugh!

I thank my parents and brothers for supporting my choices, encouraging me and helping me with my children when I was not at home for work reasons, allowing me to fully enjoy the opportunities that were offered and offered to me.

The final, biggest and special thanks go to Daniele, Viola and Luca: you have always supported me and even when I was away for one or more days, you never wanted to weigh me down. You are fantastic!!!!! I LOVE YOU ☺