# New criteria and methods for cyanobacteria risk assessment and risk management in water for human consumption

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*Dedicated to Emanuele and my Parents*

Faculties of Pharmacy and Medicine
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# PHD THESIS IN

# PHARMACEUTICAL SCIENCE

# - XXVI CYCLE –

## *New criteria and methods for cyanobacteria risk assessment and risk management in water for human consumption*

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# ACRONYMS

| | |
|---|---|
| **ADDA** | 3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-DecaDienoic Acid |
| **ALF** | Alert Level Framework |
| **API** | Atmospheric Pressure Ionization |
| **ATP** | Adenosine Triphosphate |
| **ANA-a** | Anatoxin-a |
| **BMAA** | Beta-Methylamino-L-Alanine |
| **CID** | Collision Induced Dissociation |
| **CIMF** | Cyanobacterial Incident Management Framework |
| **MAC** | Maximum Acceptable Concentrations |
| **CV** | Coefficient of Variation |
| **CYN** | Cylindrospermopsin |
| **CYP** | Cyanopeptolin |
| **i.d.** | Inner diameter |
| **DAD** | Diode Array Detector |
| **DOC** | Dissolved Organic Carbon |
| **DP** | Declustering Potential |
| **IPD** | Individual Protection Devices |
| **ISS** | *Istituto Superiore di Sanità* |
| **DRP** | Dissolved Reactive Phosphorus |
| **SD** | Standard Deviation |
| **EDTA** | EthyleneDiamineTetraacetic Acid |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **EP** | Entrance Potential |
| **FEP** | Fluorinated Ethylene Propylene |
| **FLD** | Fluorescence Detector |
| **FP** | Focusing Potential |
| **FRP** | Filtred Reactive Phosphorus |
| **GAC** | Granular Activated Carbon |
| **GC** | Gas Cromatography |
| **GPS** | Global Positioning System |
| **HPLC** | High Performance Liquid Chromatography |
| **HRMS** | High Resolution Mass Spectrometry |
| **IARC** | International Agency for Research on Cancer |
| **LC** | Liquid Chromatography |
| **LLE** | Liquid/Liquid Extraction |
| **LOD** | Limit of Detection |
| **LPS** | Lipopolysaccharides |
| **MC** | Microcystins |
| **MRM** | Multiple Reaction Monitoring |
| **MS** | Mass Spectrometry |
| **NOAEL** | No Observed Adverse Effect Level |
| **NOD** | Nodularin |
| **NOM** | Natural Organic Matter |
| **NRPS** | Non-Ribosomal Peptide Synthetase |
| **NTU** | Nephelometric Turbidity Unit |
| **PAC** | Powdered Activated Carbon |

| | |
|---|---|
| **PAR** | Photosynthetically Active Radiation |
| **PKS** | Polyketide Synthase |
| **POC** | Particolate Organic Carbon |
| **PTFE** | Polytetrafluoroethylene |
| **Q-TOF** | Quadrupole Time Of Flight |
| **RF** | Response Factor |
| **RP** | Reactive Phosphorus |
| **SPE** | Solid Phase Extraction |
| **SRP** | Soluble Reactive Phosphorus |
| **TC** | Total Carbon |
| **TDI** | Tolerable Daily Intake |
| **TEF** | Toxicity Equivalent Factor |
| **TIC** | Total Inorganic Carbon |
| **TIS** | Turbo Ion Spray |
| **TLC** | Thin Layer Chromatography |
| **TOC** | Total Organic Carbon |
| **TP** | Total Phosphorus |
| **$t_r$** | Retention time |
| **UPLC** | Ultra Performance Liquid Chromatography |
| **UV** | Ultraviolet Light |
| **VOC** | Volatile Organic Compound |
| **WHO** | World Health Organization |
| **WSP** | Water Safety Plan |

# INTRODUCTION

The availability of water resources for a long time determines the possibility of development of each civilization and quality of water for human consumption presides over the state of health of the population. The progress in knowledge on the interrelationships between water and human health and, in parallel, cultural evolution and ethics in the field of environmental protection and balance of biosystems, framed in an objective economic and social scenario, has been included on the regulatory side, in the current, consistent, *acquis communitaire* - faithfully transposed into national legislation – in where the health issues related to the uses of water resources are addressed same to the more strictly environmental, pertaining to the full protection of the waters, to regardless of their origin and nature. The two objectives of the joint European legislation matter, as articulated and complex this may appear, concern, in fact, on the one hand the achieve levels of water quality that do not result in unacceptable impacts or risk human health and the environment and, second, the guarantee of a water use that is sustainable in the long run . In this context, the protection and surveillance of the quality of water intended for human consumption is an essential measure for primary prevention against diseases with a high-pitched, mainly caused by microbiological contaminants, and chronic degenerative diseases, generally referable to chemicals. Just think, in proof that a simple practice such as disinfection of water has essentially eradicated many epidemic diseases that have plagued humanity for millennia, resulting in beneficial effects on the health of the population of similar importance, if not superior to that obtained with antibiotic therapy. 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 activities undertaken in this PhD research have been mainly on risk from cyanobacteria in water with different uses.

In this context, the research program has been aimed to assessment and management of risk from cyanobacteria in water to be used for consumption developing good practices, methods and procedures for the prevention, control and mitigation of the toxic in systems water.

Particularly, the initial step of project focused on the elaboration of tools necessary for the prevention and control of complex phenomena related to the presence of cyanobacteria in a water body. The *first section* of this thesis concerns the analysis of the state of art of cyanobacteria presenting the key elements for risk assessment and analysis of the potential vulnerability of water bodies and water supply, taking into account environmental factors that govern the development and production of toxins. Specific treatment is still controversial given to the definition of toxic species and of biological activity and toxicity of different toxins. A further pivotal phase within the project involved the development, validation and application of a LC-/MS/MS method for simultaneous determination of different cyanotoxins in water intended for human consumption. In the *experimental section* the performance of a liquid chromatography-tandem mass spectrometric method for analysis of 23 algal toxins in raw, treated and distributed water are reported. Data for a monitoring campaign conducted in the period 2011-2013 in Vico Lake and drinking water chain following a recent water emergency associated with the presence of cyanobacteria in the basin are also shown.

 As final activity of the research project, a new comprehensive approach for risk management throughout the supply chain and based on Water Safety Plan and Alert Level criteria. This approach is described in detail in the last section.

# Chapter 1

## State of Art

## 1.1. Environmental factors presiding the proliferation of 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 (1, 2). In addition, there are some species, including major producers of toxins, which are exceptions to this generalization (3-5).

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. In particular, the selective removal of some wavelengths causes changes in the spectral distribution of the light (6). 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 (7). 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 (8). Reached the saturation value of light, photosynthesis is limited by biochemical enzymatic reactions that are governed by the temperature (9). 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 $\mu$mol photons m$^{-2}$ s$^{-1}$) (10), even if the growth rates maximum occurring at about 30 °C and 80 $\mu$mol photons m$^{-2}$ s$^{-1}$. Cyanobacteria need very little energy to maintain the function and structure of cells (11, 12) 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 (13).

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 (14). There are still open questions about the meaning of the RP, the composition of which probably varies from lake to lake (15), 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 (16), 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 (17).

Nitrogen, the main component in the construction of gas vesicles, is another important environmental factor that promotes the growth of cyanobacterial species (18). 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 (19). 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.

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## 1.2. 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 (1). In the last decades, the ability to synthesize toxins has also been confirmed in type benthic cyanobacteria and subaerial environment (2, 3). 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) (4). In freshwater environments the microcystins are most commonly produced by species belonging to the *Microcystis*, *Planktothrix* (*Oscillatoria*) and *Dolichospermum* (*Anabaena*) genera (4). It was observed the production of microcystin by cyanobacteria belonging to the genus Nostoc from aquatic habitats and subaerial (5,6), and *Hapalosiphon* (7) and *Phormidium* (8) 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 (5, 9). Neurotoxins are generally produced by *Dolichospermum* (Anabaena) and *Anabaena*, *Aphanizomenon*, less commonly, by *Lyngbya* and *Oscillatoria* (4). *Cylindrospermopsis, Anabaena, Aphanizomenon, Raphidiopsis* and *Umezakia* produce cylindrospermopsins (10), while several species of *Lyngbya*, *Oscillatoria* and Schizothrix are mainly responsible of the dermatotoxins production (Table 1.1) (4). 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 (11). This is extensively described for *Microcystis aeruginosa* (12) and for populations of *Planktothrix rubescens* (13-17). 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 (18).

**Table1.1: 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** | | | |
| Lipopolysaccharide toxins | Lipopolysaccharides | Part of cyanobacteria | |

### 1.1.1. 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 (19).

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* (20).

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 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 (20).

**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 (19). 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 (19-23). 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* (24).

## 1.1.2. 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* (25). 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 (26,27). 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 (26).

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 (28-31).

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) (32, 33).

Algal blooms are also defined as *oligotrophic bloom* because it occurs even in environments with a low trophic level as Maggiore and Garda Lakes (34).

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.
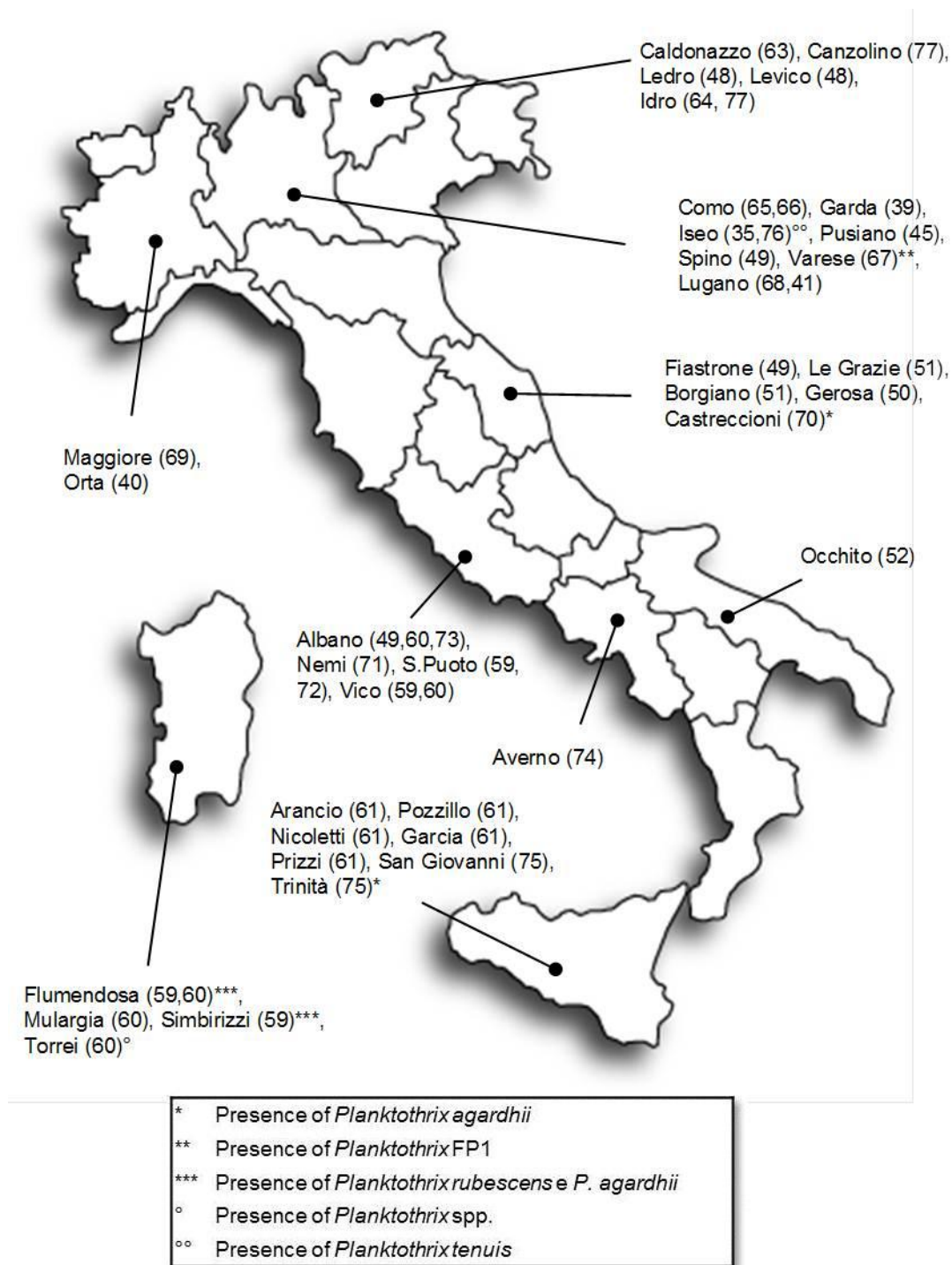
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Caldonazzo (63), Canzolino (77), Ledro (48), Levico (48), Idro (64, 77)

Como (65,66), Garda (39), Iseo (35,76)°°, Pusiano (45), Spino (49), Varese (67)**, Lugano (68,41)

Fiastrone (49), Le Grazie (51), Borgiano (51), Gerosa (50), Castreccioni (70)*

Maggiore (69), Orta (40)

Occhito (52)

Albano (49,60,73), Nemi (71), S.Puoto (59, 72), Vico (59,60)

Averno (74)

Arancio (61), Pozzillo (61), Nicoletti (61), Garcia (61), Prizzi (61), San Giovanni (75), Trinità (75)*

Flumendosa (59,60)***, Mulargia (60), Simbirizzi (59)***, Torrei (60)°

| | |
|---|---|
| * | Presence of *Planktothrix agardhii* |
| ** | Presence of *Planktothrix* FP1 |
| *** | Presence of *Planktothrix rubescens* e *P. agardhii* |
| ° | Presence of *Planktothrix* spp. |
| °° | Presence of *Planktothrix tenuis* |

**Figure 1.1: Distribution of *Planktothrix rubescens* blooms in lakes and reservoirs Italian between 1992 and 2009 (Rapporto Istisan 11/35 Pt.1)**

**Maggiore:** *D.lemmermannii* (80)
*Aph.flos-aquae* (68), *M.aeruginosa* (76)

**Caldonazzo:** *Aph.flos-aquae* (48), *M.aeruginosa* (48)
**Idro:** *D.spiroides* (36)
**Levico:** *Aph.flos-aquae* (48)
**Canzolino:** *M.aeruginosa* (48)
**Serraia:** *M.aeruginosa* (48), *Aph.flos-aquae* (48), *D.spiroides* (36)

**Como:** *D.flos-aquae* (65,66), *M.aeruginosa* (65,79), *M.flos-aquae* (65)
**Garda:** *D.lemmermannii* (80), *M.aeruginosa* (78)
**Iseo:** *D.flos-aquae* (76), *D.lemmermannii* (79), *M.aeruginosa* (35), *O.tenuis* (76), *L.redekei* (76)
**Lugano:** *Aph.flos-aquae* (39,68), *M.flos-aquae* (76), *L.redekei* (76)
**Monate:** *M.aeruginosa* (81), *D.spiroides* (81)
**Pusiano:** *M.aeruginosa* (45), *Aph.flos-aquae* (45), *D.viguieri* (45)

**Massaciuccoli:**
*M.aeruginosa* (60)

**Albano:** *Aph.ovalisporum* (73), *C.raciborskii* (53,54),
**Nemi:** *Aph.flos-aquae v.klebahnii* (71), *L.redekei* (71)
**Vico:** *Aph.ovalisporum* (85), *L.redekei* (85)

**Castreccioni:** *M.aeruginosa* (45)
**Polverina:** *M.aeruginosa* (60)

**Trasimeno:** *M.aeruginosa* (60),
*C.raciborskii* (53)

**Liscione:** *M.aeruginosa* (60)

**Bidighinzu:** *D.flos aquae* (87),
*M.flos-aquae* (87)
**Casteldoria:** *D.flos aquae* (87),
*M.aeruginosa* (87), *M.flos-aquae* (87)
**Cedrino:** *D.planctonicum* (87), *C.raciborskii* (53),
*M.aeruginosa* (60,87), *Aph.flos-aquae* (87)
**Cixerri:** *D.planctonicum* (87), *M.aeruginosa* (87)
**Coghinas:** *D.flos aquae* (87), *M.aeruginosa* (87),
*M.flos-aquae* (87), *Aph.flos-aquae* (87)
**Cuga:** *D.planctonicum* (87), *D.flos aquae* (87), *Aph.flos-aquae* (87), *M.aeruginosa* (87)
**Flumendosa:** *O.tenuis* (59)
**Flumineddu:** *D.planctonicum* (86)
**Gusana:** *M.aeruginosa* (87)
**Is Barroccus:** *Aph.flos-aquae* (87)
**Liscia:** *D.planctonicum* (86), *M.aeruginosa* (87)
**Monte Pranu:** *D.planctonicum* (87), *M.aeruginosa* (87)
**Monteleone Roccadoria:** *D.spiroides* (87), *M.aeruginosa* (87)
**Mulargia:** *D.planctonicum* (86), *D.flos-aquae* (86), *M.aeruginosa* (87)
**Nuraghe Pranu Antoni:** *M.aeruginosa* (87), *D.spiroides* (87)
**Omodeo:** *D.spiroides* (87), *M.aeruginosa* (87), *M.flos-aquae* (87),
**Pattada:** *M.aeruginosa* (87)
**Posada:** *M.aeruginosa* (87), *D.flos-aquae* (87)
**Punta Gennarta:** *M.aeruginosa* (87)
**Santa Lucia:** *M.aeruginosa* (87)
**Simbirizzi:** *O.tenuis* (59)
**Surigheddu:** *Aph.flos-aquae* (87)

**Averno:**
*Aph.ovalisporum* o
*C.raciborskii* (82)

**Cecita:**
*M.aeruginosa* (60)

**Arancio:** *M.aeruginosa* (83),
*M.wesenbergii* (75)
**Rosamarina:** *M.aeruginosa* (84)
**Piano degli Albanesi:**
*M.aeruginosa* (75)
**Rubino:** *D.flos-aquae* (75)
**Soprano:** *D.flos-aquae* (75),
*M.flos-aquae* (75)

**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) (35); *Microcystis* spp. and *Anabaena* spp. (Varese Lake) (36); *Aphanizomenon flos-aquae* (Alserio Lake) (37) 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 (12,38).

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 (39-41) and in Occhito Lake in Puglia Region (42-43).

In central Italy, the only reports on the presence of *Cylindrospermopsis raciborskii* (44) involving the Trasimeno Lake in Umbria Region (45) and Albano Lake in Lazio Region (46).

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 (47).

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 (48-50).

The presence of cyanobacteria species responsible for blooms or potential producers of toxins has also been reported for the Nemi Lake (50) and St. Puoto Lake (51). 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 (52). 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) (53) and in lakes Simbrizzi, Flumendosa Mulargia and Torrei Lakes (Sardinia Region) (59,60).

Furthermore, *M. aeruginosa* and *Dolichospermun flos-aquae* (54) 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 (56); 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] |

*follows*

15

| Lake | Species |
|------|---------|
| **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] |

| Lake | Species |
|------|---------|
| **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] |

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# 1.3. 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 (1, 2).

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

## 1.3.1. Microcystins

### Chemical 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).
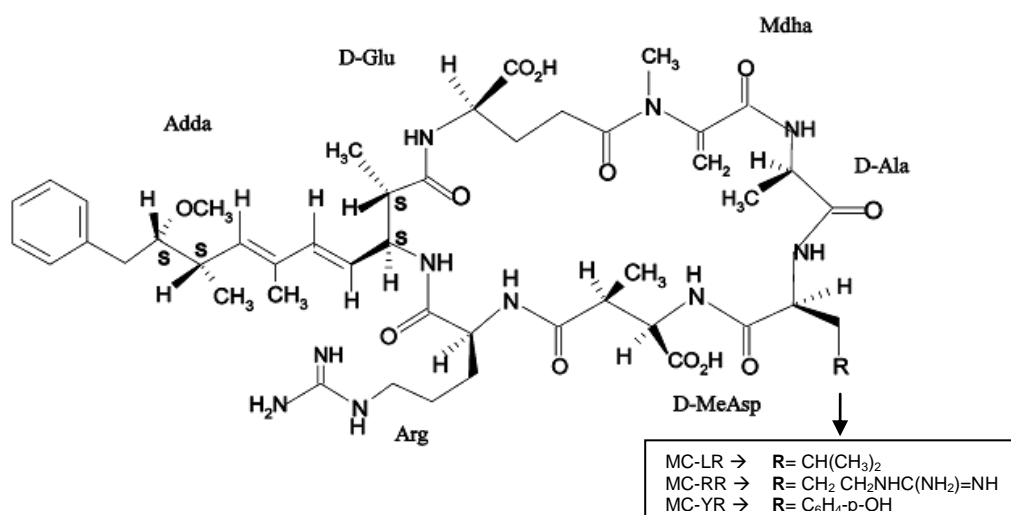


Figure 1.3: Chemical structure of microcystins most common

In the world more than 80 different variants from the first toxin identified, the MC-LR, have been isolated (2).

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 (3).

## 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 (3); 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 (4). The intestines and kidneys are other organs that can accumulate significant amounts of the toxin.

Some Japanese authors (5) 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 (6, 7). 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 (Rapporto Istisan11/35 Pt.1)**

| 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 (8), and their excretion in the bile occurs as toxins as such or as a result of their conjugation (9). The liver has a crucial role on the detoxification of these toxins (10). 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 (11). Following studies in mice has resulted in a biexponential plasma elimination of MC-LR, with half-lives of 0.8 and 6.9 minutes (12). 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 (13).

*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 (4). A minor source of exposure is inhalation through the showers and during water sports (inhalation of spray and droplets) (2).

*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 (14). 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 (4). 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 (15). 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 (4).

## Long-term effects

Health effects resulting from chronic exposure to low doses of MC are not known (1). 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 (10).

## 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 (16). 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 (17) 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:

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

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.

## 1.3.2. Nodularins

### *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 (18): 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.* (19)

27

have isolated and identified a new NOD, called NOD-Har, which presents the homoarginine instead of arginine.
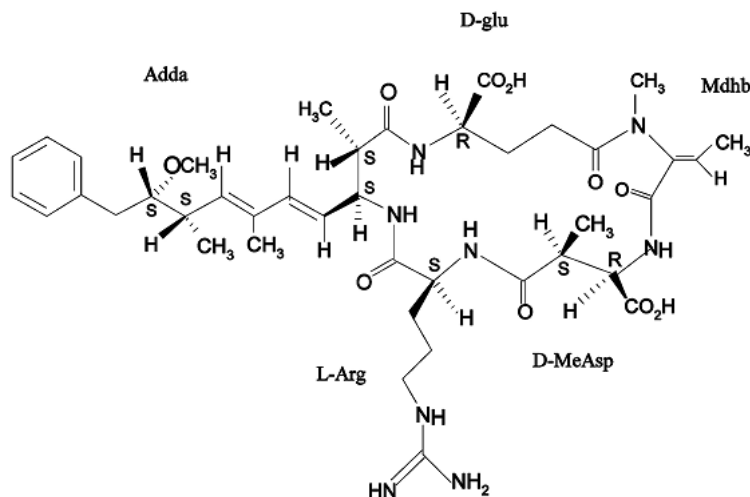


**Figure 1.4: Chemical structure of nodularin**

## 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 (15). The hepatotoxic and carcinogenic activity, as in the case of MC, is associated with inhibition of phosphatase 1 and 2 (20, 21). 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 (22).

*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* (23).

*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.

## 1.3.3. Cylindrospermopsins

### 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 1.5). It is consider 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 (1, 2) is also considered a potential carcinogen (24). 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* (25).

Among these *Cylindrospermopsis raciborskii* is the species that is the major problem on a global scale (26). 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 (27).
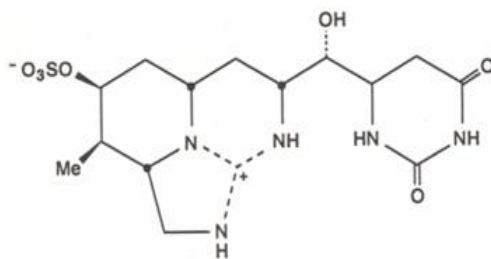


**Figure 1.5. Chemical structure of cylindrospermopsin**

## 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 (28, 29) and its acute toxicity appears to be mediated by cytochrome P 450 metabolites - generated (30). 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 (31).

Studies on laboratory animals are in favor of a possible genotoxic (32) and carcinogenic effects (33).

*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 (34).

## 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 (33).

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 (35). Some authors recommend an additional safety factor of 10 for potential genotoxic effects.

## 1.3.4. Anatoxins

**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) (36). 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 (1, 23). The ANA-a (s) has a different chemical structure being a phosphoric ester of N-hydroxy guanidine.

**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 (2, 3, 37).

*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.

## 1.3.5. Aplysiatoxin, Debromoaplysiatoxin and Lingbiatoxin

The lingbiatoxin-a, found in a strain of *Lyngbya majuscola* present in surface water, has been linked to the onset of dermatitis and severe oral and gastrointestinal inflammation in humans. It can also act as a promoter of skin tumors (39).

The characteristic symptoms resulting from poisoning by aplysiatoxin and debromoaplysiatoxin consist of intestinal disorders and severe irritation of the mouth and throat (39) .

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# 1.4.  Available methods for the analysis of cyanotoxins

## 1.4.1.    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 (1). 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 (1). 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.

## 1.4.2.   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 (1).

### 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 (2, 3). 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 (4-6) 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 (7, 8) 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 (9).

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 (10). 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 (11). 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 (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 (14). 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.

## 1.4.3. 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 (15) 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 (16, 17). 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 recommend, 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.

## 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 and 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 (18). The most used materials are C-18 (19), polymeric materials in a divinylbenzene-polystyrene, also functionalized with polar groups (HLB) (20, 21) and Graphitized Carbon Black (GCB) (20,22). It was also proposed the simultaneous extraction of MCs and ANA-a by using ion pair chromatography (23). Cartridge with immunosorbent anti-MC-LR (24) and from MIP (25) 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 (26, 27). 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 (28).

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 (20, 29).

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 (30, 31).

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 $\leq 50$ °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 (22, 32). 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 (33), 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 (14, 15, 34, 35), while for the UV detection is preferred to use trifluoroacetic acid (TFA) (36) 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 (17, 18,24, 31, 37), especially for the identification of individual variants of MCs (17, 24, 31). 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) (17, 19, 20, 38-40), 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 (19, 25, 41).

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 ) (42, 43) or Orbitrap ™ (44, 45) 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.

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# 1.5 International strategies for controlling cyanobacteria-related risks: Water Safety Plan (WSP) and Alert Level Framework (ALF)

### 1.5.1. WSP for risk assessment and management in the drinking water supply chain

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 (1) and strengthened in the following editions until the most recent of 2011 (2). 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 (3).

**Table 1.5: Synthetic representation of WSPs principles**

| 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.<br>▪ 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.<br>▪ 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.<br>▪ 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. |

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 (2, 4).

## 1.5.1. 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 (5). 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 (6-9).

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 (10). 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 1.6 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 (1, 2, 11).

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.
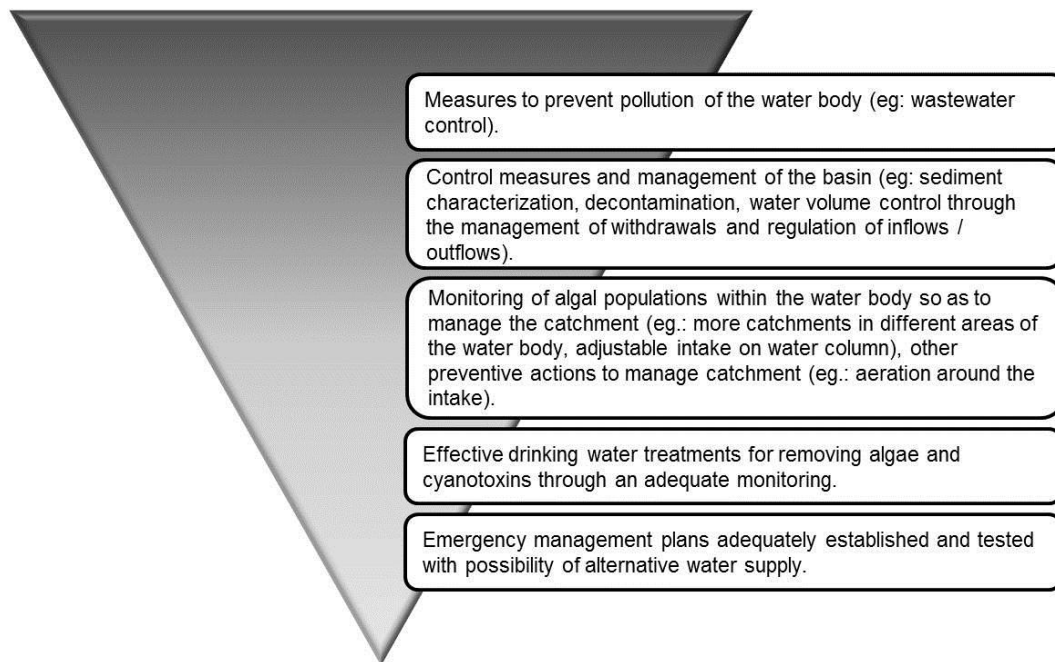
Figure1.6: Preventive and control measures in the water body and in the supply chain hydro-drinking to eliminate or reduce the risks of the presence of cyanotoxinsin the water distributed for human consumption (Rapporto Istisan 11/35 Pt.2)

## 1.5.2. 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 (10).

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 (12):

- *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 (5) 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) (13) 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 (12) has been redefined and integrated in the Australian national protocol (14) 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 (15) and developed according to knowledge progress even in respect to toxins species-specific production potential, has been suggested by Newcombe (16) 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 (14). 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 (16):

- *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.

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# Chapter 2

## Objectives

## 2.2. Objectives

The deep analysis of the knowledge on cyanobacteria development and toxin-production mechanisms, the accurate definition of the scenario of contamination by cyanobacteria and toxic metabolites (cyanotoxins) in the water resources and supply chain of water intended for human consumption in Italy, as presented in the previous section of this document, call for the need of adequate prevention and response measures towards health risk emergencies related to cyanobacteria blooms and water contamination by cyanotoxins.

Accordingly, the activities of the research program reported in this thesis were devoted to develop and validate a methodological model for cyanobacteria risk prevention and control, with possible regulatory value, in line with the recent evidence at the international level, the data on toxic phenomena occurring in the Italian territory the framework of water resources and water treatment technologies currently practiced in water supply systems in Italy.

**GENERAL OBJECTIVE:**

The objective of the thesis was to structure and validate - on the basis of experience gained in the Italian territory – an integrated national methodological model with regulatory valence to be adopted in the asset of water quality surveillance by Local Health Authorities and water management systems for the effective surveillance, prevention, control and emergency management of contamination by cyanobacteria toxins in water intended for human consumption.

In doing this, a novel multi-stage strategy has been developed, involving routine actions for surveillance and alert - relying upon automated techniques for early warning and a new LC-MS/MS confirmatory method - and specific measures of risk management targeted to the different levels of estimated risk of contamination in water (alert levels) and the specific risk mitigation existing within the drinking water supply system.

## SPECIFIC OBJECTIVES

In order to carry out the general objective, the following specific objectives were established:

1. Development and validation of an analytical methods based on liquid chromatography coupled to tandem mass spectrometry ( LC-MS/MS ) 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 a recent 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 2011-2013 in Vico Lake and the whole drinking water chain of the municipality of Caprarola (Viterbo Province).

3. Definition of monitoring plans supporting risk assessment, involving the evaluation of the probability of occurrence of cyanobacterial blooms and estimation of the potential health impact of the toxic phenomena in the reservoir of water intended for human consumption.

4. Definition of operational tools for managing risks along the whole water chain, depending by the mitigation measures existing within the drinking water system;

5. Establishment of a strategy for the management of possible emergencies associated with algal blooms.

# Chapter 3

## Experimental section

## 3.1. Chemicals and Reagents

MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF, MC-LY, [D-Asp3]-MC-RR, [D-Asp3]-MC-LR, ANA-a, CYN, Anabaenopeptin A, Anabaenopeptin B, CYP 1007, CYP 1041, Microginin 527, Microginin 690, Microginin 704, Microginin 527 methyl estere, Microginin 690 methyl estere and nodularin, used as internal standard (IS), were purchased from Alexis® Biochemicals, La Jolla, CA, USA. ANA-a was obtained from ICN Biomedical, Aurora, OH, USA.

Stock solutions of the nine MCs, IS, five Microginins, two Anabaenopeptins, two CYP, CYN and ANA-a were prepared by dissolving each compound with at least 4 mL of methanol. These solutions were stored at -18 °C in the dark to minimize analyte degradation. Working standard solutions of analytes and IS were obtained by suitable diluting stock solutions with mobile phases, at a final concentration of 1 µg/mL and 5 µg/mL respectively. When unused, all working standard solutions were stored at 4 °C in the dark, and renewed after two working weeks.

All solvents and chemicals were of analytical grade (Carlo Erba, Milan, Italy). Extraction cartridges filled with 0.5 g of Carbograph 4 were supplied by LARA, Rome, Italy. A 125 mm diameter Black Ribbon 589 paper filters were purchased from Schleicher &Schuell, Legnano, Italy.

## 3.2. Instrumentation

The sample analyses were carried out with an API 3000 (Applied Biosystems, Darmstadt, Germany) triple-quadrupole mass-spectrometer, equipped with a Turbo Ion Spray (TIS) ion source, coupled with Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA, USA). Chromatographic separation was achieved with a reversed phase Alltima C18 column (2.1 x 150 mm, 5 µm) (Alltech, Sedriano, Italy) (Park Bellefonte, PA, USA) thermostated at 40 °C .

## 3.3. Blank samples

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 can be obtained using:

- 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.

The white sample may be used:

- To verify the specificity of the method;

- For quality control;

- For preparation of samples artificially contaminated.

Blank sample was used as a verification of the specificity the analytical method, for the preparation of samples artificially prepared to be used for the calibration line and the quality controls.

For the preparation of the white sample was used deionized water, analyzed in advance to make sure of the absence of the analytes selected, and subjected to the same SPE procedure provided for the preparation of samples.

## 3.4. 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.
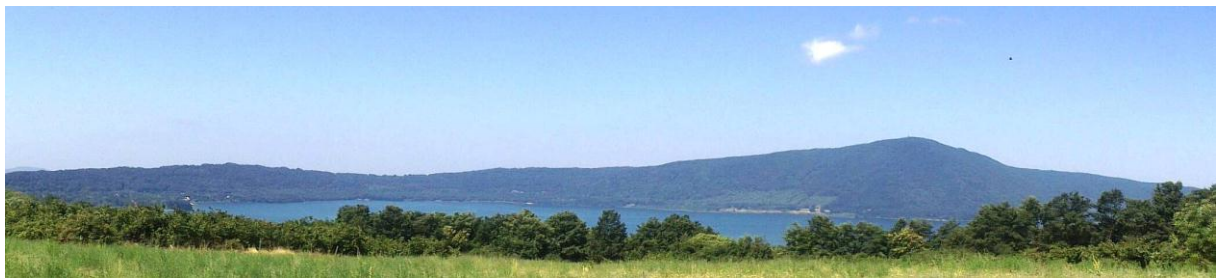


**Figure 3.1: Vico Lake**



**Figure 3.2: The Vico basin and selected sampling sites of distributed waters with their relative distance.**

## 3.5. Sample collection

Over twenty-four months of monitoring (October 2011 -October 2013), water samples were taken at least every fifteen days at the WTP and over the piped distribution system in Caprarola municipality (Lazio Region), at the following three stations (Figures 3.3 – 3.5):

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

2) drinking water, exiting the WTP at the head of the distribution system;

3) sampling point on the distribution system, sited in the centre of Caprarola municipality.

Samples were taken in 500 mL polyethylene bottles as containers or dark glass washed with ultrapure water without traces of the analyte.

Temperature and free chlorine content were measured for each sample (data not shown).

In order to analyze the total content of toxins samples must be stored in polyethylene bottles or glass and submitted to at least one cycle of freezing and thawing (-18 ± 3 °C) to facilitate lysis.

Otherwise to determine the content of extracellular toxins, water samples must be stored in polyethylene bottles or glass in the dark and at a temperature of 1-10 ° C to prevent degradation of the analytes due to the action of light and microbiological agents. In these conditions, the retention time is limited to a maximum of 24 h. After thawing, water samples were spiked with IS and filtered.



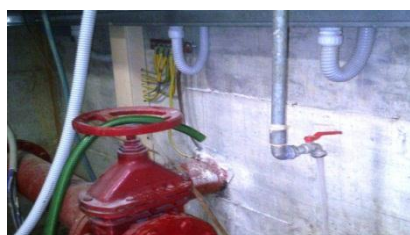**Figure 3.3: Sampling point of raw water**

**Figure 3.4: Sampling point of treated water**

**Figure 3.5: Sampling point of drinking water**

# 3.6. Procedures

## 3.6.1. Solid phase extraction (SPE)

**Apparatus Extraction**

The extraction of selected analytes was performed by solid phase extraction (SPE), using a device developed in the laboratory and shown schematically in Figure 3.6. 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 4 Carbograph (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 3.7) .
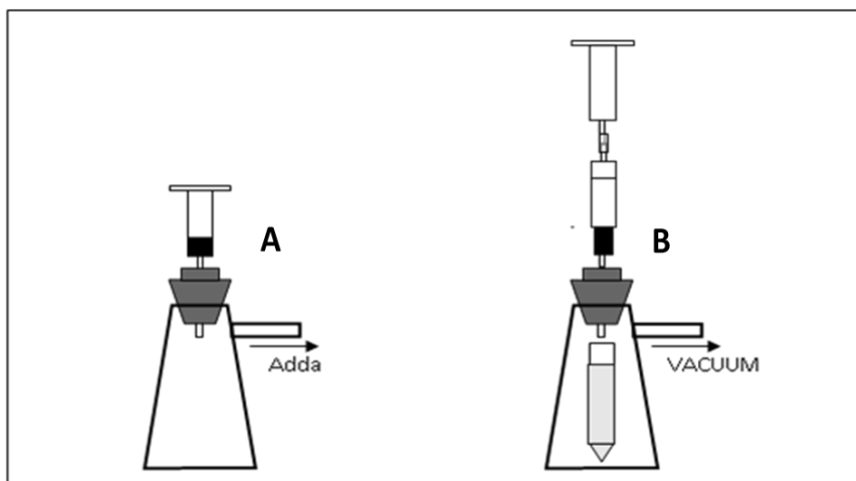
**Figure 3.6: (A) Extraction apparatus SPE; (B) Elution**
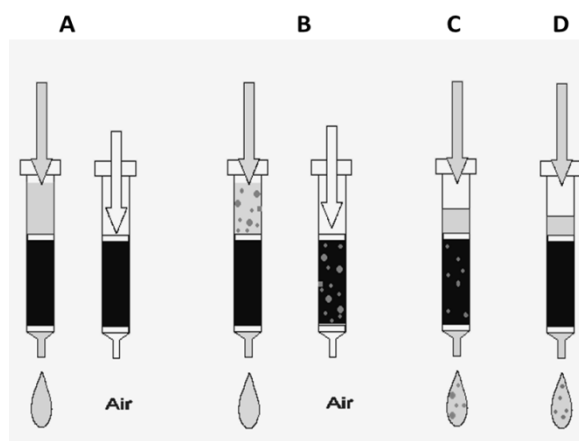


**Figure 3.7: A) Washing / activation of the cartridge; (B) Sample passage and analytes extraction (C) Wash the cartridge and remove of compounds not interfering in detection; (D) Analytes elution.**

## Sample preparation

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. During filtration has been used a system of vacuum filtration with black ribbon filters for the analysis of the total content of toxins and glass fiber filters with a pore diameter ≤ 0.45 µm for the analysis of the content of extracellular toxins. After the addition of I.S., samples were passed through a Carbograph 4 SPE cartridge, previously washed and conditioned with dichloromethane/methanol solution. 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 (80:40, 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 50 µL of this extract were injected into the LC column.

The other hand to analyse CYN and ANA-a toxins, 100 µL of the filtered water sample spiked with IS were directly injected into the LC/MS apparatus according to experimental conditions reported in (1) for CYN and ANA-a, except for using nodularin as IS and a calibration range of 0.5-50 µg/L.

## 3.6.2. Liquid chromatography – mass spectrometry

Different chromatographic gradients were employed for determining MCs, Microginins, CYP, Anabaenopeptins, CYN and ANA-a, using acetonitrile (component A) and water (component B) as mobile phases, both containing 10 mM formic acid, at 0.2 mL/min.

For MCs, Microginins, CYP, Anabaenopeptins the mobile phase gradient profile was as follows (t in min): $t_0$, A= 15%; $t_{10}$, A= 65%; $t_{11}$, A= 80%; $t_{16}$, A= 100%; $t_{19}$, A= 100%; $t_{20}$, A= 20%; $t_{29}$, A= 20%, while for CYN and ANA-a, the mobile phase gradient profile was as follows: $t_0$, A= 15%; $t_4$, A= 15%; $t_5$, A= 70%; $t_{10}$, A= 100%; $t_{11}$, A= 15%, $t_{20}$, A= 15%. High-purity nitrogen was used as curtain and collision gas with flow set at 10 a.u. (arbitrary units) and 6 a.u. respectively. As nebulizer gases was used air set at 12 a.u., while the turbo gas flow was 7 a.u. and the ion source temperature was maintained at 350 °C. Capillary voltage was 5500 V. The select values of declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), collision exit potential (CXP) potential and dwell time were optimized for each analyte (data are reported in Table 3.1).

Full scan spectra were obtained in MS scan mode over range of m/z, depending on the analyte molecular mass, at a cycle time of 500 ms every 1 s and with an interscan of 100ms. The electrospray ionization sourse was operated in positive mode. The multi reaction-monitoring (MRM) mode was used for quantitation by selecting two precursor ion>daughter ion transitions for each analyte, although only one was used for quantification (Table 3.1).

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**Table 3.1. Experimental Conditions for detecting selected cyanotoxins in water**

| Compound | MRM transitions (*m/z*)* | | | | |
|---|---|---|---|---|---|
| Mass-Spectrometric conditions** | | DP[1] | FP[2] | EP[3] CXP[4] | CE[5] |
| MC-RR | 520>135 | 90 | 400 | 10 | 8 | 45 |
| | 520>127 | 90 | 400 | 10 | 8 | 60 |
| MC-LR | 995>135 | 70 | 400 | 11 | 11 | 100 |
| | 995>70 | 70 | 400 | 11 | 10 | 125 |
| MC-LA | 910>135 | 90 | 400 | 10 | 10 | 80 |
| | 910>776 | 55 | 400 | 10 | 21 | 25 |
| MC-YR | 1045>135 | 60 | 400 | 12 | 11 | 100 |
| | 1045>70 | 60 | 400 | 10 | 11 | 125 |
| MC-LF | 986>478 | 60 | 400 | 10 | 13 | 30 |
| | 986>852 | 60 | 400 | 10 | 13 | 30 |
| MC-LW | 1025>446 | 30 | 400 | 10 | 13 | 50 |
| | 1025>891 | 30 | 400 | 10 | 13 | 30 |
| MC-LY | 1002>135 | 90 | 400 | 10 | 13 | 90 |
| | 1002>868 | 40 | 400 | 10 | 13 | 30 |
| [D-Asp[3]]-MC-RR | 513>135 | 70 | 400 | 12 | 11 | 40 |
| | 135>70 | 70 | 400 | 10 | 9 | 100 |
| [D-Asp[3]]-MC-LR | 981>135 | 70 | 400 | 10 | 11 | 100 |
| | 981>70 | 70 | 400 | 10 | 11 | 125 |
| Nodularin (internal standard) | 825>135 | 90 | 400 | 10 | 10 | 80 |
| CYP-1041 | 1041>70 | 70 | 400 | 10 | 11 | 130 |
| | 1041>184 | 70 | 400 | 10 | 19 | 100 |
| CYP-1007 | 1007>150 | 80 | 400 | 10 | 11 | 100 |
| | 1007>70 | 80 | 400 | 10 | 11 | 125 |
| MC-HtyR | 1059>135 | 70 | 400 | 10 | 8 | 90 |
| | 1059>107 | 200 | 400 | 10 | 13 | 125 |
| MC-WR | 1068>135 | 200 | 400 | 10 | 13 | 100 |
| | 1068>159 | 200 | 400 | 10 | 10 | 100 |
| MC-HilR | 1009>135 | 90 | 400 | 14 | 10 | 100 |
| | 1009>213 | 90 | 380 | 15 | 10 | 70 |
| Microargin527 | 528>180 | 55 | 310 | 7 | 10 | 30 |
| | 528>128 | 55 | 320 | 7 | 10 | 50 |
| Microargin690 | 691>70 | 50 | 300 | 7 | 6 | 70 |
| | 691>510 | 50 | 300 | 7 | 13 | 30 |

**follows**

**continues**

| Compound | MRM transitions (*m/z*)* | DP[1] | FP[2] | EP[3] | CXP[4] | CE[5] |
|---|---|---|---|---|---|---|
| **Mass-Spectrometric conditions*** | | | | | | |
| Anabaena B | 837>201 | 70 | 400 | 9 | 13 | 70 |
| | 837>70 | 70 | 400 | 9 | 13 | 130 |
| Anabaena A | 844>58 | 100 | 400 | 9 | 11 | 130 |
| | 844>84 | 90 | 400 | 9 | 11 | 130 |
| Microginin527methyl estere | 542>70 | 70 | 350 | 10 | 13 | 70 |
| | 542>128 | 60 | 350 | 10 | 7 | 55 |
| Microginin704 | 705>128 | 70 | 360 | 12 | 13 | 35 |
| | 705>136 | 65 | 400 | 10 | 13 | 70 |
| Microginin690methyl estere | 705>180 | 52 | 350 | 10 | 11 | 35 |
| | 705>70 | 52 | 350 | 10 | 12 | 100 |
| Anatoxin-a | 166>149 | 30 | 300 | 10 | 8 | 20 |
| | 166>131 | 30 | 300 | 10 | 8 | 20 |
| | 166>107 | 30 | 300 | 10 | 8 | 25 |
| | 166>91 | 30 | 300 | 10 | 8 | 25 |
| Cylindrospermopsin | 416>336 | 100 | 300 | 10 | 13 | 20 |
| | 416>194 | 70 | 300 | 10 | 13 | 50 |
| | 416>176 | 70 | 300 | 10 | 8 | 25 |

* Parent ion > daughter ion transition
**Turbo Ion Spray (TIS): 5500 V; Courtain gas flow 10 a.u.; Nebulizer gas flow 12 a.u.; Turbo-gas flow 30 a.u.; CAD flow 6 a.u.
[1]DP: Declustering Potential, [2]FP: Focusing Potential, [3]EP: Entrance Potential, [4]CXP:Collision Exit Potential, [5]CE: Collision Energy

**Quantitative analysis of cyanotoxins in water**

For LC/MS/MS analysis, the peak area of each analyte transition with the more intense signal to noise ratio, S/N (quantifying transition), related to the area of IS was chosen for the quantification of each analyte. The resulting area ratio (analyte area/IS area) was used to construct an external calibration curve by spiking and extracting blank of deionized water samples with the working solutions of analytes at 0.1, 1.0, 2.5 and 5 µg/L and the IS at 1 µg/L, using the average of noise obtained by the analysis of blank samples as intercept. The Limits of Detection (LODs) were assessed for each analyte from the lowest calibration level, considering the transition with the worst S/N (qualifier/diagnostic), as reported elsewhere (1).When amounts of cyanotoxins injected from extracts of water samples exceeded the upper limit of the linear dynamic range of the detector response, extracts were suitably diluted and re-injected.

## 3.6.3. Validation protocol

The method has been validate in compliance with the Italian implementation of the Drinking Water Directive 98/83/EC (2), including selectivity, matrix effect, linearity, recovery, precision, accuracy and stability.
The validation study was built using spiked deionized water samples.

### *Selectivity and specificity*

The specificity and selectivity of the method was evaluated by analyzing water samples from at least three different origin to investigate the potential interferences at the LC peak region for analytes and IS.

### *Matrix effect*

The matrix effect was investigated to ensure precision, selectivity and specificity that were not compromised by the matrix screened and it was studied by evaluating the ion suppression enhancement effects
Deionized water, surface water from a cyanotoxins-free lake (Bracciano, Italy) and tap water were chosen as matrices for the evaluation of the matrix effect.

## Recovery

Analysis of extracts of deionized water samples spiked with analytes at 0.1 μg/L and IS after evaporation were used as reference for the evaluation of the extraction efficiency. The mean overall recoveries of the analytes and IS were determined analyzing the extracts of deionized water samples spiked with analytes at 0.1 μg/L and IS after evaporation.

## Calibration curves

Calibration curves for quantification water samples were produced by extracting blank samples spiked with analytes at four different concentration (0.1 μg / L, 0.5 μg / L, 1 μg / L, 2.5 μg / L) and a constant concentration of IS equal to 1 μg / L.

## References

1. 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.

2. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal L 330, 32-54, 1998; http://www.bsmi.gov.tw/wSite/public/Attachment/f1224039638719.pdf.

# Chapter 4

Result and Discussion

# 4.1. Analytical issues: determination of cyanotoxins in in water sample

## 4.1.1. Method development

Simultaneous determinations of total cyanotoxins content were performed with LC/MS/MS method. Several new cyanotoxins classes are recently available as certified standards, so a previous method (1) was upgraded, optimizing it in terms of instrumental response, field of application and extraction efficiency for these analytes. In particular, the number of target cyanotoxins was enlarged, including Anabaenopeptin A, Anabaenopeptine B, CYP 1007, CYP 1041, Microginin 527, Microginin 690, Microginin 704, Microginin 527 methyl ester, Microginin 690 methyl ester, MC-HtyR, MC-HilR and MC-WR. Moreover the correct quantification of [D-Asp3]-MC-RR and [D-Asp3]-MC-LR variants is important when a bloom of *P. rubescens* occurs, these MCs being the most abundant ones produced by this cyanobacterium (2, 3). Performance, reliability and feasibility of the method were improved in order to optimize resources of the Health Authority's laboratories for cyanotoxins sampling and analysis in raw, treated and drinking waters.

## Optimization of LC-MS/MS condition

The analytes were separated by reverse phase chromatography, and analyzed in MS / MS with ESI source . The chromatograms were acquired in " Multi reaction monitoring " (MRM) : the advantage of this mode of acquisition is the highest sensitivity because it is possible to significantly decrease the background noise, thus allowing to obtain increased S / N. MRM is selected in the first quadrupole ion, called the "parent ion", which is fragmented in the second and collects in the third quadrupole ion one said "daughter ion". For each analyte infusions in positive mode with a standard solution at a concentration of 5 ng / L has been carried out by varying the experimental parameters in order to obtain two precursor ion>daughter ion transitions with the best S / N ratio.

Figures 4.1-4.3 show the full-scan precursor ion spectrum of three of selected cyanotoxins and tandem mass spectra of precursor ion fragmentation at difference collision energy.
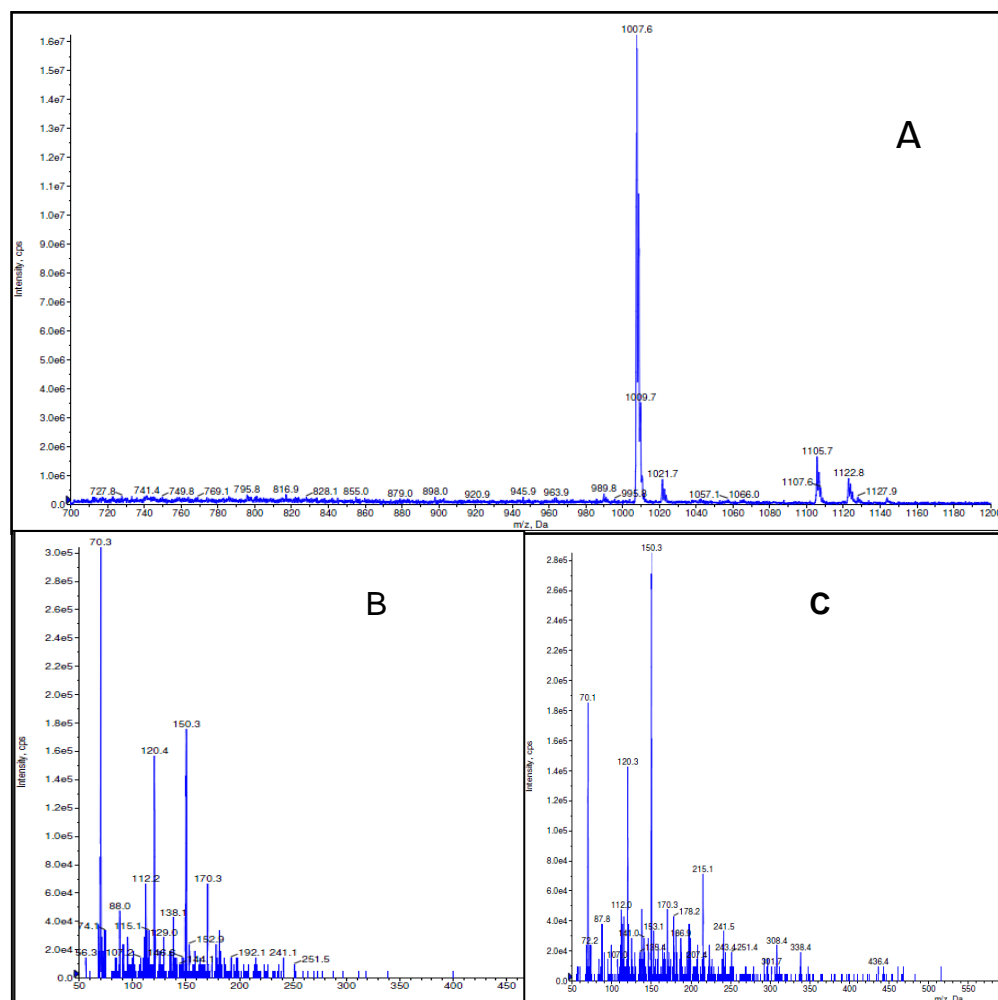


**Fig. 4.1: A) Full-scan MS spectrum of Cyanopeptolin 1007; B) Full-scan MS/MS product ion spectrum of Cyanopeptolin 1007 at collision energy= 70 eV; B) Full-scan MS/MS product ion spectrum of Cyanopeptolin 1007 at collision energy= 150 eV;**
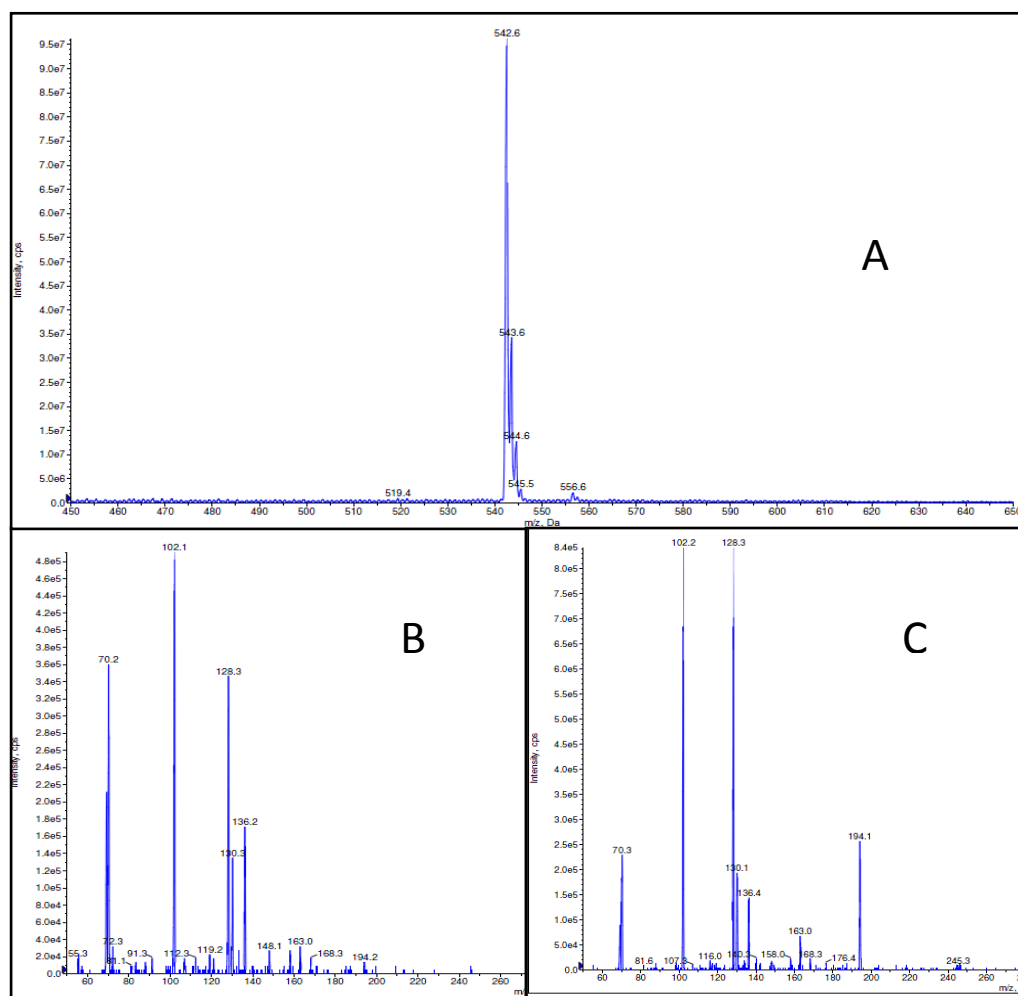
**Fig. 4.2: A) Full-scan MS spectrum of Microginin 527 methyl ester; B) Full-scan MS/MS product ion spectrum of Microginin 527 methyl ester at collision energy= 70 eV; B) Full-scan MS/MS product ion spectrum of Microginin 527 methyl ester at collision energy= 128 eV;**
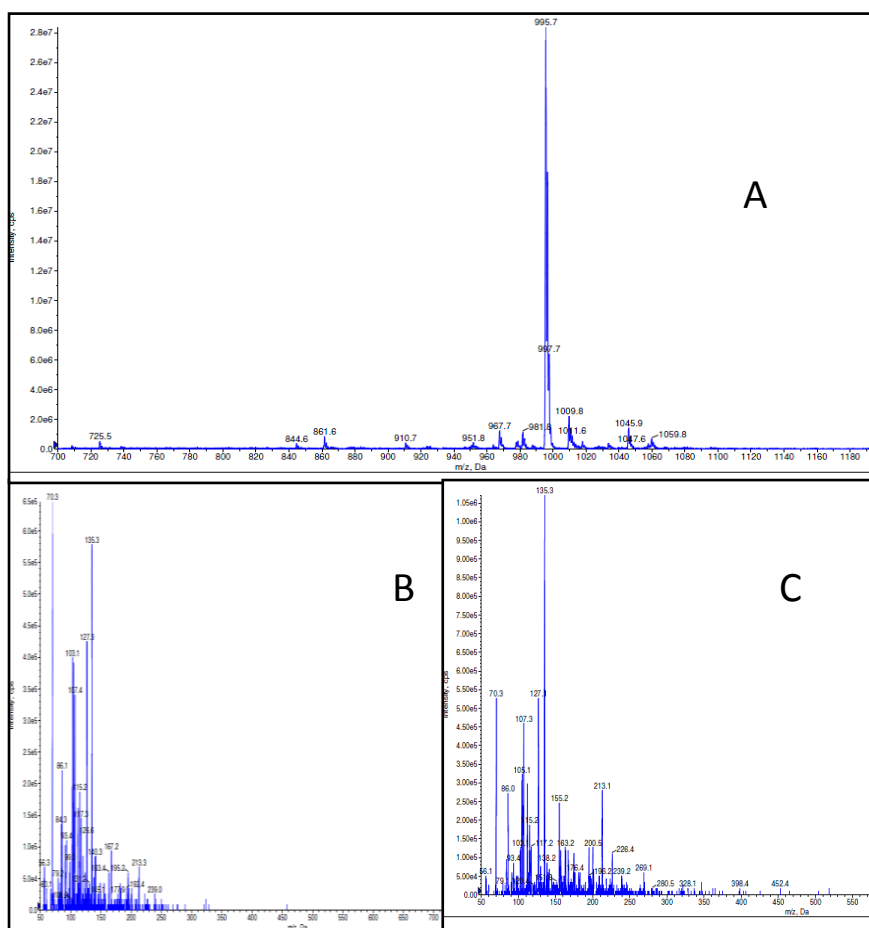
**Fig. 4.3: A) Full-scan MS spectrum of MC-LR; B) Full-scan MS/MS product ion spectrum of MC-LR; B) Full-scan MS/MS product ion spectrum of MC-LR at collision energy= 135 eV;**

The fragment that characterizes the class of MCs and even the common Nod, is that one having a ratio m / z = 135 which comes from the rupture of the side chain of the residue Adda which is always present in this class of compounds. In Figure 4.4 the diagram of the generic fragmentation of an ion double charged of a MC is shown as an example.
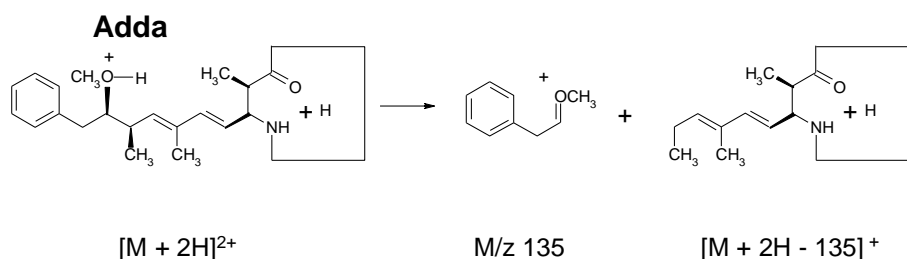
**Figure 4.4: Fragmentation of a generic microcystin with characteristic formation of the ion with m / z value = 135.**

MCs and Nod have the possibility of forming single or double charge ions depending on the basicity of the amino acid component variable which characterizes the structure. (4) The double charge ion formation is mainly for those MCs that hold in the chemical structure two arginine residues, but also depends significantly on the geometry of the ESI source.

Unlike what is reported in the literature, significant variations of the fragmentation of the molecular ion of the analytes have been observed.

The identified pseudomolecular ions were single or double charged and characterized by fragmentation due to rupture generally of fragment ion m/z=135, also found in the literature (5).

Also for the MC- RR (m / z = 127 ), dem - MC- RR (m / z = 127 ), MC -LR (m / z = 127 ), dem - MC -LR (m / z = 70 ), we obtained a more extensive fragmentation characterized by very small fragments whose formation presumably derived from events of fragmentation of the second level of the fragment having m / z 135.

In some monitoring studies, reported in the literature, on water lake during cyanobacterial blooms, the presence of demethylate variants of MC- RR and MC- LR was found (6).

In previous study, the quantitation of demethylate forms and their isomers respective isomers has been obtained giving same instrumental response of the corresponding methylated forms (7), using the transition corresponding to the double charged molecular ion molecular >135 that characterizes this class of compounds, as they were not available as commercial standards. For this research study were

purchased the analitycal standards, today commercially available, of the two demethylate MCs were purchased.On the other hand, the results obtained from full scan MS analyses of other cyanotoxins (Microginins, CYP, Anabaenopeptolins) shown the production of single charged fragment ions of each analytes.

As it concern chromatography conditions, the mobile phases used were water and acetonitrile acidified with formic acid, since the use of this acid as well as to influence the protonation of the silanol groups, promotes positive ionization in the electrospray process.

The binary gradient for the chromatographic separation used in the previous protocol (1) was modified considering the increase in the number of analytes investigated having different chemical structure and polarity with respect to the MCs. Representative chromatograms of a standard solution are depicted in Figures 4.5.
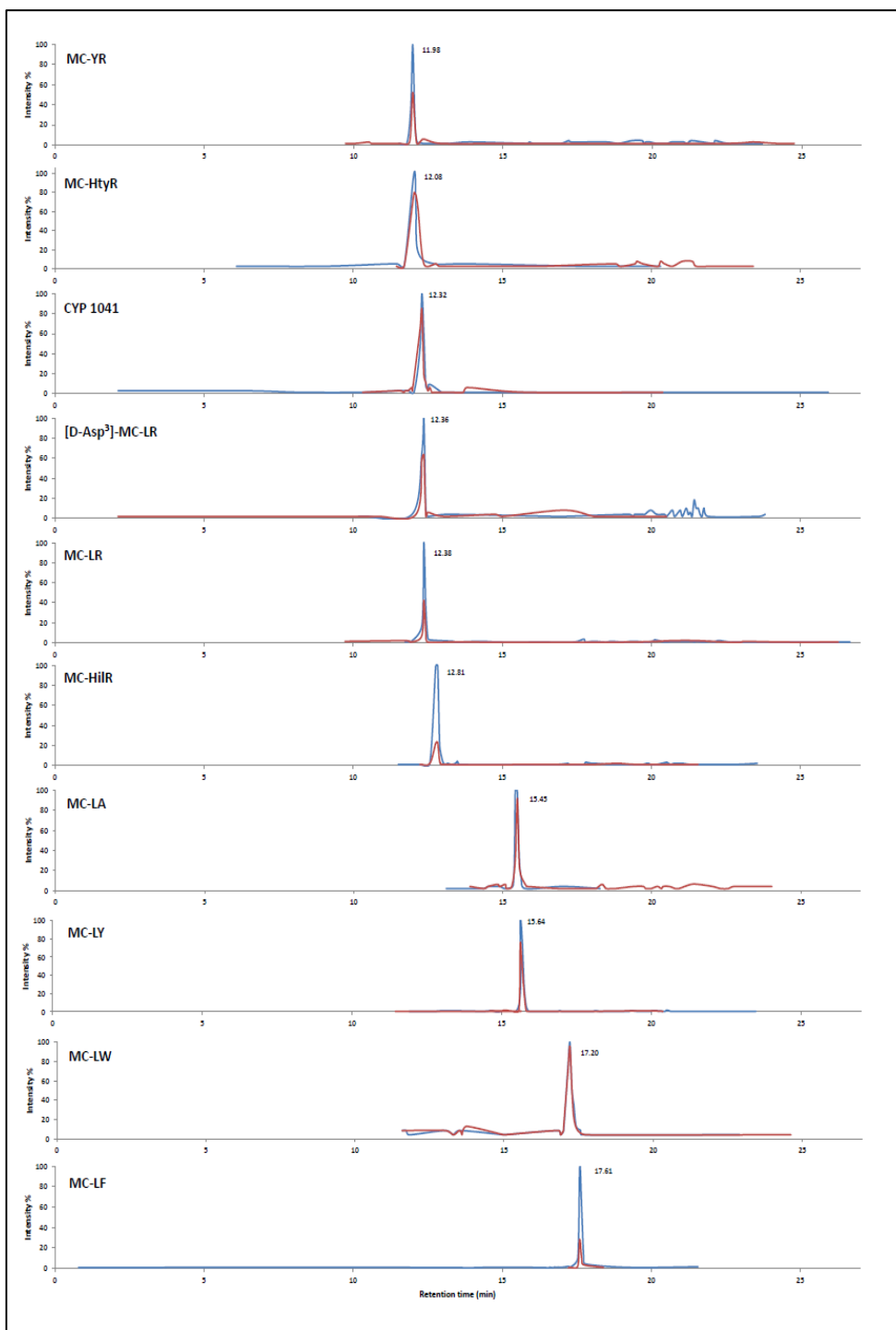


follows

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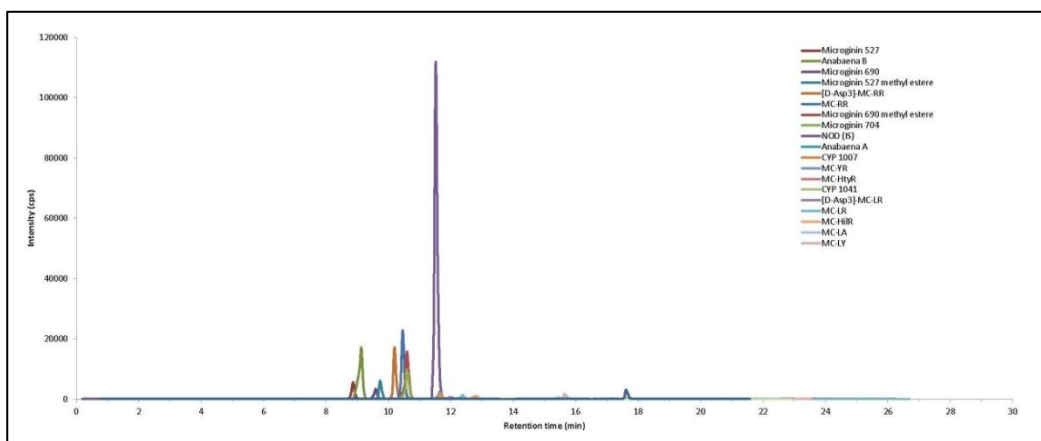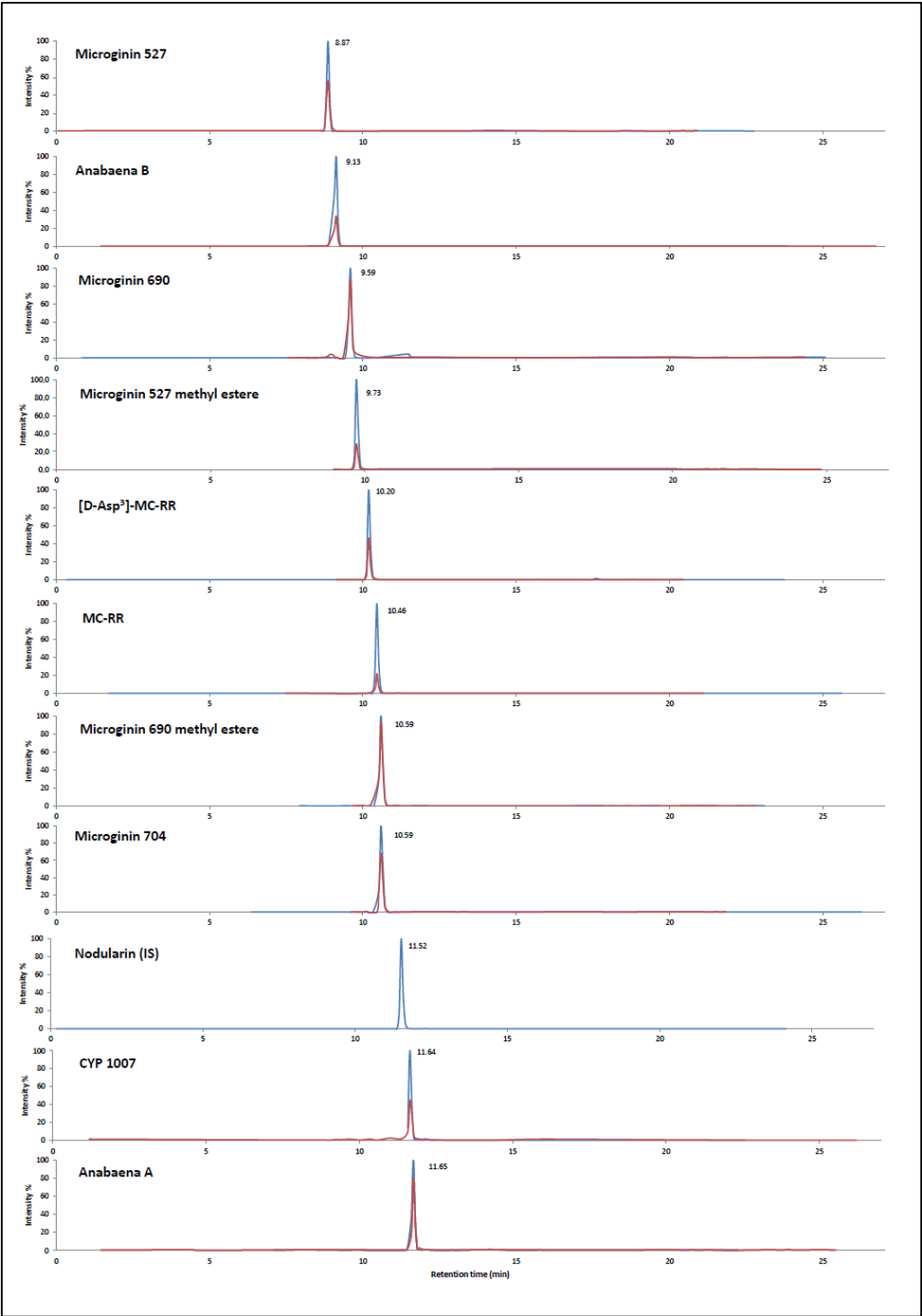
continues

**Figure 4.5: MRM LC/MS/MS chromatogram resulting from analysis of blank water sample spiked with selected cyanotoxinsat 0.1µg/L level and 1 µg/L of nodularin. It is reported in Blue colour the quantitation transition and in red the qualification transition for each analytes**

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## __Optimization of the SPE procedure__

Regarding the extraction procedure, in some methods reported in literature (8) analytes elution was performed in the *back- flushing* mode achieving high extraction efficiency for all the toxins monitored, in particular for the more hydrophobic as in the case of MC -LW . In addition, using this method of extraction, evaporation times are reduced, making the application of the method very fast.

In this study, in contrast, the elution of the analytes through the cartridge was performed in a *frontal mode* to be able to make the application of the method more practical and thus making the method easily transferable to the control agencies have been performed tests volume of re − elution have been performed being able to determine the volume needed to elute all analytes considered including those characterized by a higher hydrophobicity ; compared to 4 mL of eluent phase used for fashion back- flushing the volume of re -elution was increased to 7 mL .

## 4.1.2. Method Validation

The entire method has been validated in terms of sensitivity, selectivity, repeatability, reproducibility, robustness and detection limit, in compliance with the Italian DL 31/2001 (9) transposing the Drinking Water Directive, 98/83/EC (10) considering also criteria of UNI ENV ISO 13530: 2001, so that it can be proposed as a method for the determination of MCs in water intended for human consumption.

It should be noted that the performance characteristics required of an analytical method in the field of water intended for human consumption established by Italian DL 31/2001 are:

1) accuracy

2) precision

3) LOD

The method was validated in terms of linearity, sensitivity, accuracy and precision, by means of tests of repeatability and within-laboratory reproducibility conducted at the laboratories of the ISS.

## *Matrix effect*

Deionised water, surface water from a cyanotoxins-free lake (Bracciano, Italy) and tap water were chosen as real matrices for the evaluation of the matrix effect. Results, reported in Table 4.1 indicated that a weak matrix effect was present for several compounds in all types of water considered. Anyway, the matrix effect is not significantly dependent on the specific matrix selected, as resulted from one-way ANOVA test at the $P = 0.05$ significance level.

Thus, in order to propose an friendly-to-use method for routine analysis, deionised water was chosen as a representative matrix.

**Table 4.1**. **Evaluation of the Matrix Effect in deionized, tap and lake waters spiked with selected microcystins at 0.1 µg/L and the IS at 1 µg/L after the evaporation step.**

| Compound | MQ water | Tap water | Lake water |
|---|---|---|---|
| | R,%[1]±RSD,% | R,%[1]±RSD,% | R,%[1]±RSD,% |
| [D-Asp³]-MC-RR | 101±1 | 106±6 | 114±6 |
| MC-RR | 109±19 | 104±14 | 88±4 |
| MC-YR | 90±12 | 88±9 | 87±17 |
| [D-Asp³]-MC-LR | 85±5 | 87±6 | 84±10 |
| MC-LR | 91±2 | 99±12 | 110±15 |
| MC-LA | 98±8 | 94±8 | 98±12 |
| MC-LF | 100±11 | 98±8 | 94±11 |
| MC-LW | 102±10 | 101±4 | 82±14 |
| MC-LY | 98±5 | 95±5 | 92±12 |
| MC-HtyR | 100±13 | 98±7 | 95±7 |
| MC-HilR | 91±2 | 99±10 | 98±12 |
| MC-WR | 87±6 | 85±2 | 88±4 |
| CYP 1007 | 78±2 | 75±6 | 74±5 |
| CYP 1007 | 71±7 | 74±7 | 71±5 |
| Anabaenopeptin A | 96±11 | 97±2 | 96±7 |
| Anabaenopeptin B | 98±8 | 94±2 | 94±2 |
| Microginin 527 | 85±2 | 88±6 | 84±2 |
| Microginin 690 | 80±5 | 87±2 | 81±7 |
| Microginin 704 | 70±2 | 75±8 | 74±2 |
| Microginin 527 Methyl ester | 81±5 | 79±7 | 82±2 |
| Microginin 690 Methyl ester | 79±2 | 77±7 | 75±8 |

[1] the recoveries (R) are expressed as per cent ratio average (n=3) of the absolute area related to water samples spiked at 0.1 µg/L after evaporation respect to a standard solution at the same concentration.

## Precision and accuracy

Analysis of N=9 water samples spiked at 0.1 µg/L, assayed in triplicate over three days by different operators, for the assessment of accuracy, as sum of trueness (recoveries) and intra-laboratory reproducibility, was performed.

Results of these studies are reported in Table 4.2 .Internal standard accuracy has been used as quality control for all measurements during the monitoring, showing that this protocol is robust and not significantly dependent by different matrices processed (79%±21% on N=20 samples). The extraction efficiency of not previously tested MC congeners proved to be satisfactory, with relative recoveries not lower than 85%, and an intra-lab reproducibility not higher than 16%.

**Table 4.2: Accuracy obtained for determining selected cyanotoxins in water by tandem MS. Accuracy, expressed as sum of trueness and within-laboratory reproducibility, was obtained analyzing on three different days and by different operators N=9 water samples spiked with 0.1 µg/L of each microcystin and IS at 1 µg/L**

| Compound | Trueness[1],% | RSD,% |
|---|---|---|
| [D-Asp³]-MC-RR | 95% | 11% |
| MC-RR | 107% | 10% |
| MC-YR | **121%** | **15%** |
| [D-Asp³]-MC-LR | 118% | 11% |
| MC-LR | 109% | 11% |
| MC-LA | 108% | 16% |
| MC-LF | 119% | 13% |
| MC-LW | 117% | 15% |
| MC-LY | 109% | 11% |
| MC-HtyR | 109% | 11% |
| MC-HilR | 109% | 11% |
| MC-HilR | 109% | 14% |
| MC-WR | 109% | 15% |
| CYP 1007 | 85% | 11% |
| CYP 1007 | 87% | 11% |
| Anabaenopeptin A | 94% | 11% |
| Anabaenopeptin B | 98% | 11% |
| Microginin 527 | 88% | 15% |
| Microginin 690 | 90% | 12% |
| Microginin 527 Methyl ester | 85% | 11% |
| Microginin 690 Methyl ester | 90% | 13% |

## *Selectivity, Linearity and Limit of detection (LOD)*

Analysis of N=3 blank samples for evaluating method selectivity, was carried out.

The interference by water constituents with analytes and IS was assessed by inspection of chromatograms that were derived from processed blank water sample. No significant interferences were found in blank water samples at the retention times of the analytes and IS.

A calibration curve, spiking N=8 (n=2 replicates for each level) water samples at 0.1, 0.5, 1.0 and 2.5 µg/L concentration levels, covering the range of the WHO guideline value (1 µg/L as MC-LR equivalents) for the study of sensitivity and linearity of the method, was obtained (Figure 4.6).

A good linearity was achieved, with correlation coefficients in the range $0.9925 \leq R^2 \leq 0.9998$.

Finally, adding the antioxidant to the treated waters seems to be effective in the analytes and IS protection, enlarging the feasibility of the proposed method to the analysis of drinking water.

The LODs of the method were extrapolated from the LC- MS/MS chromatograms MRM mode register, obtained from the analysis of a sample of deionized water (250 mL) contaminated with 0.1 µg / L of the 21 cyanotoxins and 1.0 µg / L of the internal standard.

After extracting the ion current profiles relative to the transitions for each analyte, the resulting tracks were subjected to two successive " smoothing " processes of, using the Analyst software (Applied Biosystem) so as required by Italian Legislative Decree 31/2001 (9).

The LODs have 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.

The LODs were estimated as the concentration of analytes in a position to give a S / N equal to 3 and are calculated on the worst of the two transitions *precursor ion > daughter ion* . It was dected a limit of detection in the range of 0.002 to 0.200 µg / L for all selected analytes.
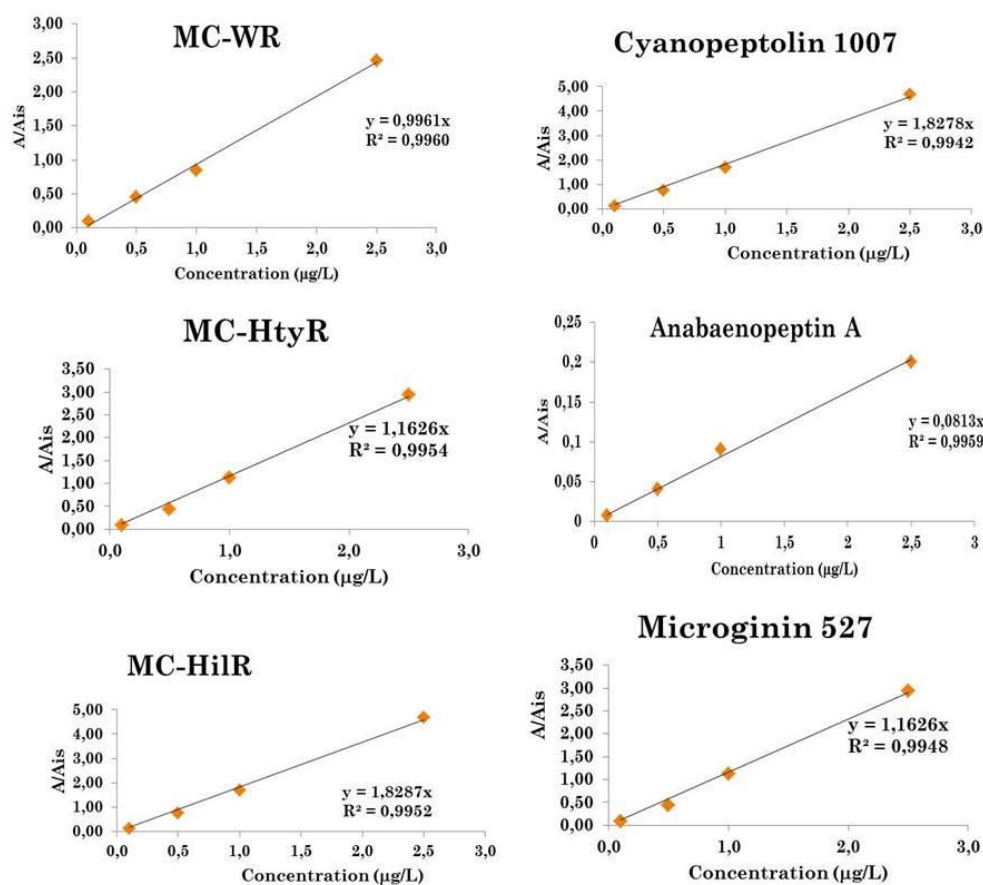
**Figure 4.6: Calibration curves were constructed for each analyte plotting the area of the transition with the more intense S/N related to the area of IS, using the average of noise obtained by the analysis of blank samples as intercept. Spiking levels were 0.1, 0.5, 1 and 2.5 μg/L and the IS at 1 μg/L**

**Table 4.3: Multi-Reaction Monitoring (MRM) transitions, Limit of Detection (LODs)**

| Compound | MRM transition, *m/z* | LOD, µg/L |
|---|---|---|
| Anatoxin-a | 166>149<br>**166>131[1]** | 0.200 |
| Cylindrospermopsin | 416>336<br>**416>194** | 0.100 |
| [D-Asp$^3$]-MC-RR | 513$^2$>135<br>**513$^2$>127** | 0.004 |
| MC-RR | 520$^2$>135<br>**520$^2$>127** | 0.004 |
| MC-YR | 1045>135<br>**1045>70** | 0.013 |
| [D-Asp$^3$]-MC-LR | 981>135<br>**981>70** | 0.004 |
| MC-LR | 995>135<br>**995>213** | 0.006 |
| MC-LA | 910>135<br>**910>776** | 0.002 |
| MC-LF | 986>852<br>**986>478** | 0.002 |
| MC-LW | 1025>891<br>**1025>446** | 0.002 |
| MC-LY | 1002>135<br>**1002>868** | 0.002 |
| MC-HtyR | 1059>135<br>**1059>107** | 0.009 |
| MC-HilR | 1009>135<br>**1009>213** | 0.012 |
| MC-WR | 1068>135<br>**1068>159** | 0.006 |
| CYP 1007 | 1007>150<br>**1007>70** | 0.020 |
| CYP 1007 | 1041>184<br>**1041>70** | 0.032 |
| Anabaenopeptin A | 844>84<br>**844>58** | 0.020 |
| Anabaenopeptin B | 837>201<br>**837>70** | 0.008 |
| Microginin 527 | 528>180<br>**528>128** | 0.004 |
| Microginin 690 | 691>70<br>**691>510** | 0.010 |
| Microginin 527 Methyl ester | 542>128<br>**542>70** | 0.004 |
| Microginin 690 Methyl ester | 705>70<br>**705>180** | 0.010 |
| Microginin 704 | 705>120<br>**705>136** | 0.008 |

[1] transitions with the worst S/N are reported in bold; [2] double charged ion

## 4.1.3. Monitoring of cyanotoxins in the drinking water chain.

During this research project an intense two-years monitoring plan has been set and implemented in which sanitary inspections and sampling at the WTP were carried out in collaboration with the the Italian National Health Institute (ISS) and Local Health Authority (ASL) following the detection of the cyanobacteria bloom that in the winter of 2011-2012 identified occurred in the Vico basin as *P. rubescens* (Figure 4.7).

In this thesis was studied the concentration of toxins in the water following the change of algal bloom in a period of about twenty-four months, the toxins have been identified and quantified by mass spectrometry according to the protocol described in the Experimental section consisting of extraction and SPE LC-MS/MS injection apparatus.

The employment of methods involving the use of a detection system LC / MS tandem, is correlated mainly to the ability to identify and quantify the different variants of cyanotoxins. In this way, it is possible to accurately assess the toxic potential of the sample, provided that reliable data are available for toxicological evaluation.



**Figure 4.7: Vico Lake, red algae bloom**

The most abundant types of cyanotoxins detected in this study were the MC-RR and its demethylated form ([D-ASP3]-MC-RR) and ([D-ASP3]--MC-LR, with the maximum concentration values of respectively 1.52, 0.100 and 0.159 μg / L. During this monitoring was never detected the presence of MC-LA, LF, LY, HtyR, WR, HilR,

ANA-a, CYP 1041, CYP 1007, Anabaenopeptins, Microginins and the CYN sporadically has been found.

Previously, in other Italian lakes affected by bloom of *Planktothrix rubescens*, has been reported the presence of two isomers of [DAsp3]-MC-RR, present as separated chromatographic peaks (8). In this monitoring the presence of a single peak on the [DAsp3]-MC-RR was detected and it was possible to attribute with certainty to one of the two possible structures, the [DAsp3]-MC-RR, since the standards of the variants demethylated have recently been characterized. In this work it was possible to have the standard commercial and carryout identification with a high degree of certainty and accurate quantification.

Particular limnological conditions and nutrients of the lake, as well as genetic, can alter the profiles of productions traditionally reported. Higher levels of algal bloom recorded and expressed as number of cells / L confirm the trend of the species to proliferate at low temperatures.

## Cyanotoxins in raw waters

Figure 4.8 shows data of the cells density and of cyanotoxins concentration obtained from LC/MS analysis, expressed as sum of selected compounds. Two species of cyanobacteria were identified, namely *P. rubescens,* that was always the predominant strain and *Aphanizomenon ovalisporum*. The maximum cells density has been registered in the two winter seasonal particularly in February 2012 and February 2013 when temperatures were certainly higher.

When higher than LODs values, cyanotoxins total levels measured with the LC/MS/MS system in the raw water ranged from 0.004 μg/L (June 2012) to 1.66 μg/L (January 2013). However, the concentration of cyanotoxins was not constant nor predictable on the basis of the cells density, that is generally, not necessarily, associated to high algal toxins levels. This fact was maybe due to changes in the genes expression involved in the toxin production (11,12), so that a toxin quota was not simply inferable. Interestingly, in the second phase of emergency, the drift of cyanotoxins production, as evidenced by LC/MS analysis, seems to be shifted of *ca*

10-15 days respect to the temporal tendency of the *P. rubescens* and *Aphanizomenon ovalisporum* cells density. This observation was consistent with an another study (11) conducted in a Italian basin affected by *P. rubescens* blooms, that has suggested as cause of this temporal shift the release of the intracellular contribute of toxin in water after cells apoptosis. Environmental parameters, such as temperature, light and content of nutrients may had affect the behaviour observed in the toxinogenesis (12).
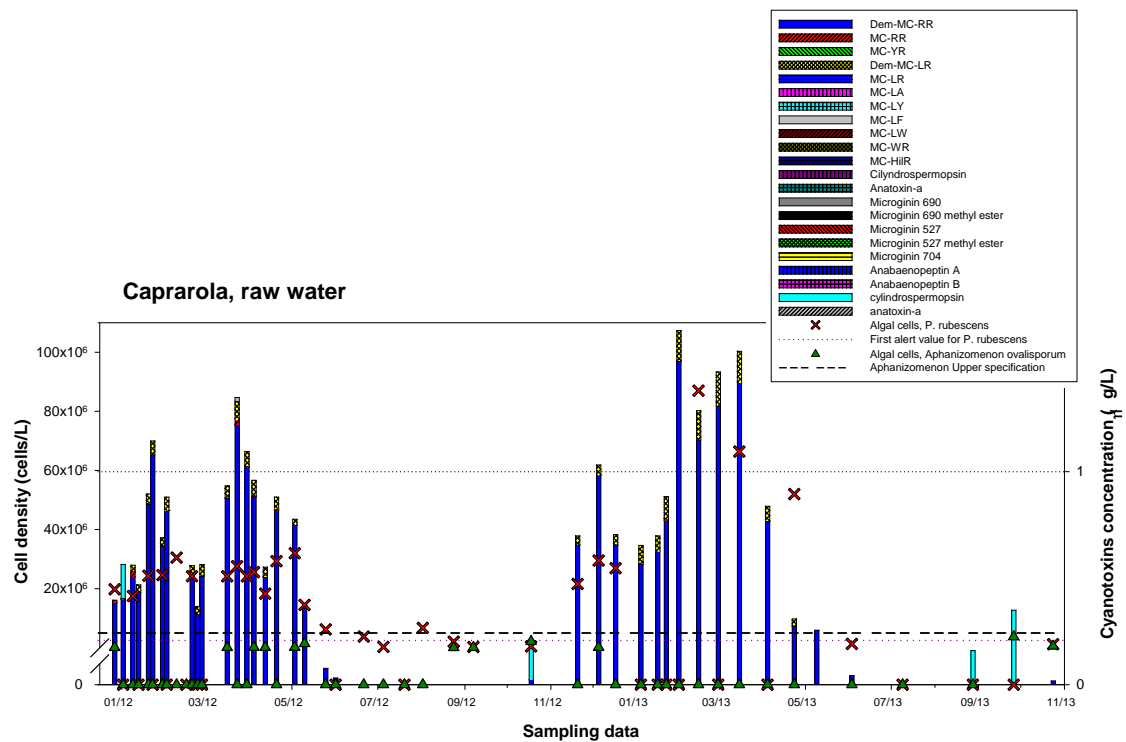
**Figure 4.8: Temporal trend of cell densities [cells/L] of cyanobacteria identified in the Vico basin and cyanotoxins level, as determined with LC/MS analysis and expressed as total concentration [μg/L] of the sum of selected compounds, both recorded in raw waters of the Caprarola Water Treatment Plant**

## Drinking water monitoring and drinking water treatments evaluation

In this project were analyzed influent (raw water) and effluent water from the water treatment plan and in water distribution network in the town.

To a first general reading of the effectiveness of processes for drinking water carried out, it appears that the water distributed in Caprarola municipality (figure 4.9) showed the presence of toxins in concentrations marginal and not significant, reaching maximum values of 0.004 μg / L.

This situation poses no risk to human health, as the guideline value for cyanotoxins in samples of water intended for human consumption proposed by the WHO is 1 μg / L.
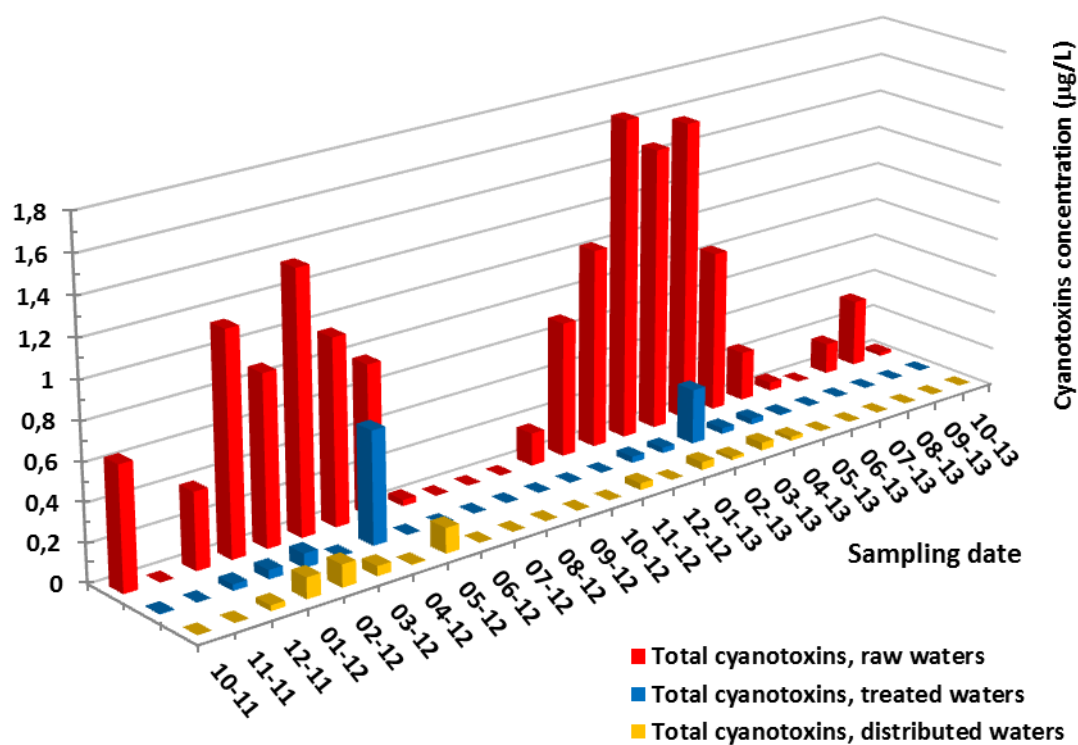


**Figure 4.9: Monitoring of raw, treated and distributed water**

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## 4.2. Surveillance, alert and risk management national system for cyanotoxins monitoring in water for human consumption

According to the results presented, applied to the case study "Emergency Planktothrix Rubescens in Vico Lake" and the general objective of the thesis, this chapter is aimed at providing the operative tools to support the risk management related to cyanobacteria presence, especially potentially toxic species, in water bodies used for drinking water production.

The criteria described in the present section, could be addressed to local health authorities, responsible for risk assessment and compliance determination on water for human consumption according to Legislative Decree 31/2001 and for water systems operators producing water for human consumption.

The model suggested is based on monitoring results of algal count carried out according to regulations by the environmental or health authority, even according to the indications concerning potential system susceptibility and vulnerability to cyanobacteria populations establishment and development; the monitoring must be intensified in reservoirs historically affected by blooms and it is crucial to take into consideration the seasonal blooming timing.

The criteria here proposed, based on *Alert Level Framework* (ALF) principles consolidated at international level, constitute a decision tree in which monitoring and management actions over the water system are implemented in a progressive manner, so as to give an answer to the algal development steps in waters entering the treatment chain and taking into account risk prevention and mitigation measures related to the water supply system.

Alert levels are defined through parameters related to the risk of cyanotoxins presence in the waters. For each level of algal concentration detected in the water body, around the catchment point, there can be established a risk level for the possible presence of cyanotoxins in the water at the user point; on this basis, a series of modular measures should be implemented, including the enhancement of cyanobacteria and cyanotoxins monitoring frequency, the implementation and/or optimisation of water treatments, the

notification to the health authority and, as a last measure, the adoption of restrictions of uses of water and the activation of emergency plans.

Particularly, the approach is based on the model represented in Figure 4.10, and on the summary scheme of risk levels reported in Table A1 (attached to the chapter).

The first one is applied to the drinking water treatment chain and describes the weight of the system protection level, in terms of prevention and mitigation of cyanotoxins presence risk in water delivery/user point. The second one is aimed at risk management in the entire intake water system until the distribution to water supply points.
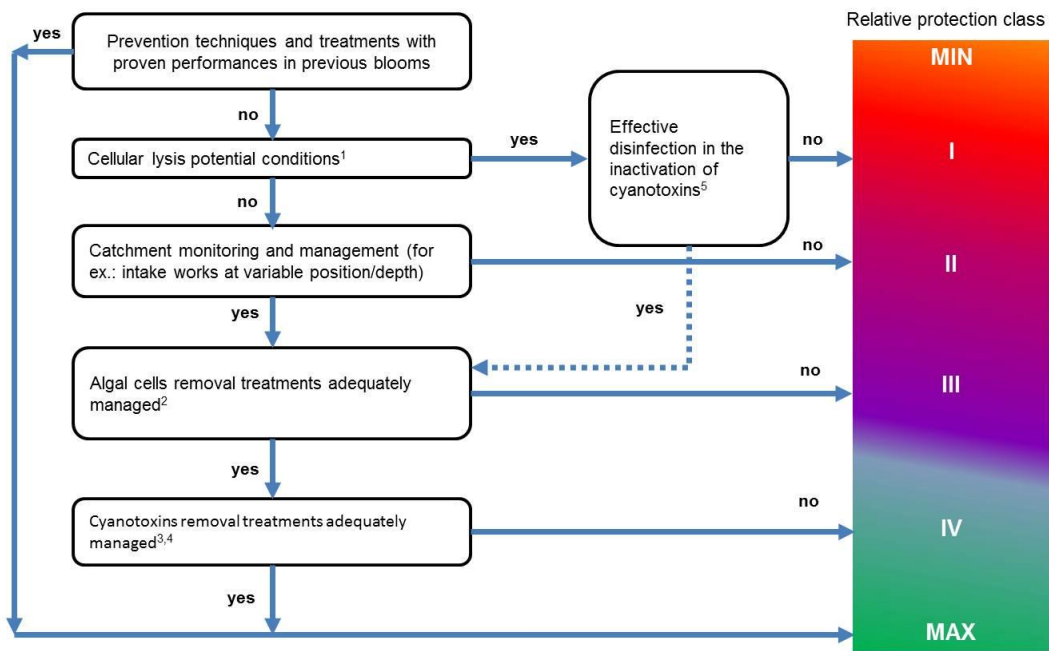


[1] Some circumstances that can induce cellular lysis: algaecide treatments, pre-oxidation, algal population aging process, high pressure pumping
[2] Some effective methods for the removal of cyanobacteria cells: coagulation and sedimentation or flotation, sand filtration, ultrafiltration
[3] Some effective methods for the removal of cyanotoxins: filtration on activated carbon, ultrafiltration, ozone
[4] The removal/inactivation system effectiveness depends on the technologies applied, plant size, water characteristics, entering algae and toxins levels, maintenance, etc.
[5] The disinfection system effectiveness, depends from the technologies applied, plant size, water characteristics, entering algae and toxins, maintenance, etc.

**Figure 4.10: Appraisal of water management system protection level**

The criteria used for the definition of alert levels, risk level assessment, specific actions and protection and mitigation measures to be implemented, are described as follows:

– *Detection level 0*

This level is aimed at highlighting, during a systematic monitoring, the possible occurrence of a bloom at a preliminary stage. The algal count levels, referred to toxic species, are approximately included in the range 500 – 2,500 cells/mL. At this stage, there is no immediate health risk evidence, even where organoleptic changes of the water are visible. It is recommended to intensify algal species monitoring within the water entering the plant, integrating the data with frequent reservoir inspections so as to detect possible presence of foams or colour changes at different depths. To give an example, algal population development can reach concentrations associated to the alert level in a week or less (assuming a time period of almost 4 days for population doubling), based on environmental conditions.

As for the other system stages, it is assumed that the sample is collected near the catchment so as to directly refer to the risk for human consumption; other samples can anyway be collected from the reservoir and can be useful to study the possible distribution of cyanobacteria in the water column or in other water body areas or, in case of collection of foams with considerably high algal concentrations, to highlight the population toxicological profile in terms of composition and toxin levels produced, through confirmatory methods. For this purpose early-warning systems are very useful.

– *Detection level 1*

This level indicates a stable cyanobacteria population and development within the water body able to reveal, in a highly precautionary scenario, a toxin production potential in water for human consumption with concentrations near to the guide value. The algal concentration values appraisal associated to the alert level, also defined as "risk surrogate" (1), is obtained through conservative criteria, considering the entire algal population as toxin producer, assessing a high value of toxin produced

for each single cell and assuming that all the toxins produced are free in the water, represent the most toxic species and are not removed during treatments.

For the assessment, there have been used the criteria established by the *World Health Organization* (WHO) (2) and reconsidered by other authors (1,3,4) assuming a conservative appraisal of toxin value associated to each cell and establishing, from this last, the number of cells needed to obtain a concentration of toxins in the waters near to the bibliography value. As far as the *toxin quota* is concerned, an average value of 0,2 pg/cell (1, 3) is considered for the different toxic algal species. Therefore, in case of *P. rubescens* occurrence, based on the maximum precautionary principle, on the information spread in literature (1, 3, 5) which consider the category associated to a higher cyanotoxins production – indications confirmed by the various national monitoring data assessed during this research project – it is assumed the presence of a *toxin quota* equal to the double of the one established for the other toxic cyanobacteria species, taking as bibliography a cells threshold value consequently reduced.

The actions to be implemented in correspondence of alert level 1 include:

- notification to the local health authority, if the monitoring has been carried out by the operator (internal monitoring), by environmental agencies or by research groups;

- activation of a constant monitoring on a fortnightly or better weekly basis, depending on plant protection status and on resources availability, through algal count;

- risk assessment that can be associated to toxins potential presence in the waters going out from the drinking water treatment plant and for distribution, based on plant level protection system; in this case, if available, the historical data concerning the efficiency of plant during bloom, are crucial.

Protection assessment can be based, in general, on criteria reported in Figure 1. In this model, at alert level 1, a class I-II protection degree is considered to be inadequate, that can occur in small management systems. The assessment must anyway take into account the appropriate sizing of the plant and operation and

maintenance general conditions (it can be useful to inspect the plants, monitor the internal and external – ordinary and extraordinary - maintenance status, time intervals of plants backwashing, internal data related to the operative monitoring, compared, for example, to the operating pressures of filtration plants, etc.); at last, take into account the waters treatment obligation before their distribution for human consumption, in compliance with Legislative Decree 152/2006, concerning pre-treatments based on water body classification.

Whereas the plant protection level is in any case considered as inappropriate, the first action should be addressed to implement cyanotoxin determination on a fortnightly or weekly basis; cyanotoxin determinations must refer to the overall content (intra- and extra- cellular) and they are generally performed on incoming and outgoing waters and, where applicable, during distribution – in this case taking at least into consideration a proximal and distal point of the network where the water is not mixed; optimise, as far as possible, mitigation measures within the drinking water treatment chain thus ensuring an appropriate chlorination; this should be performed by increasing the contact time and, in the absence of other protection measures, by maintaining the residual chlorine levels in distribution at least at concentrations of 0.1 – 0.2 mg/L or even greater, if the health authority judges it to be necessary, at alert stage 1-2, taking also into consideration the monitoring results related to disinfection by-products.

At application purposes, based on accrued national experiences, it is crucial to consider that in a reservoir characterized by a fixed establishment of cyanobacteria, algal concentrations established for the level of alert are extensively exceeded during more or less prolonged time intervals within the year and, usually, they do not entail any use restrictions of water resource because adequately managed in the drinking water treatment chain. In fact, if the water system "coexists" with the more or less periodical occurrence of cyanobacteria, the above said measure, expressed in a general and conservative form, should be adapted to the context and optimised, also in terms of resources, based on the experience gained. In these cases, it is crucial that the water supply operator keeps the internal system documents highlighting the effectiveness of measures taken

according to the historical data obtained in bloom conditions, including internal operative tests data such as for example the *jar test* for removing algal cells.

The monitoring frequency must be maintained until 2 consecutive results state a reduced risk level, after that the frequency can be gradually decreased.

- *High alert level 2*

 Based on the values determined for the alert level, a *high alert level 2* is established to represent the development of cyanobacteria population in the water body able to reveal a potential toxin production in water for human consumption, with concentrations almost ten times greater than the guide value. In this case, for risk management it is crucial to envisage adequate treatment measures within the water system so as to mitigate the risks; in the absence of these measures use restrictions and emergency response plans are needed. So as to integrate the measures described above for lower risk levels, at this stage it is recommended to:

 - inform the local health authority about the phenomenon development;

 - implement a constant monitoring on a fortnightly basis through algal count;

 - determine, on a weekly or (better) fortnightly basis, the presence of cyanotoxins in waters entering and going out from the purifier and in distribution (at least a proximal and distal point of the network where the water is not mixed);

 - optimize mitigation measures in the drinking water treatment chain, thus ensuring an adequate chlorination;

 - be prepared for a possible emergency response plans implementation.

 As far as application purposes are concerned, based on accrued national experiences, it is crucial to consider that in a reservoir characterized by a fixed establishment of cyanobacteria, algal concentrations established for the high level of alert can be overcome in mainly short periods during the year and, if correctly managed, can avoid use restrictions of water resource. Even in these circumstances, if the water system is repeatedly affected by cyanobacteria, the above said measures must be adapted to the context and optimized, even in terms of resources, based on the experience gained.

The described plan, necessarily offers a scheme of three representative risk levels. Intermediate situations (ex.: for algal concentrations between 5,000 and 50,000 cells/L) will be managed through appropriate decisions proportioned to risk level, to be assessed on a case by case basis according to the general scheme.

The monitoring frequency must be maintained until two consecutive results state a reduced risk level, after that the frequency can be gradually decreased.

## 4.2.1. Measures and use restrictions

The current state of knowledge on risk assessment related to the presence of cyanobacteria massive growth in water for human consumption, points out that the health risk is only associated to the production of water for human consumption, contaminated with cyanotoxins levels exceeding the guide values. It is also useful to remember that the bibliography values definition concerns a chronic exposure, that is a prolonged consumption, officially, "during the entire life cycle", of water contaminated with cyanotoxins levels exceeding the bibliography values.

Based on this, use restrictions of drinking water is recommended after having observed a concentration of toxins exceeding the maximum acceptable values in water for distribution.

The maximum temporary acceptable value for the MC-LR in water for human consumption is equal to 1.0 μg/L referred to the total toxin content (intra- and extra-cellular). According to an extensively conservative approach for human health protection, with an overestimation in the toxicity assessment, in the worst appraisal approach, the 1.0 μg/L value must be referred to the sum of different MCs congener concentrations in the sample, considered as equivalent of the MC-LR. For this purpose, there have to be found MC congeners elements through confirmation methods using the best analytical potential available and, as minimum criterion, the congeneric MC congeners for which analytical standards are now commercially available, [D-Asp3]-MC-RR, MC-RR, MC-YR, [D-Asp3]-MC-LR, MC-LR, MC-LA, MC-LY, MC-LF, MC-LW. Cyanotoxins research must be extended to other compounds categories, such as for example CYL, anabaenopeptolins and ANA-a, in the presence of blooming (for

an alert greater than 1) of species producing these toxins; the maximum acceptable value of cyanotoxins other than microcystins could be fixed in compliance with the provisions of the Italian Legislative Decree 31/2001, art. 11(1)(b).

In case of a contamination exceeding the maximum acceptable value, the use restrictions concern, in general, just the drinking water use and the preparation of foods where the water is the main ingredient, with particular respect to risk categories. In case of use restrictions related to the exceeding of bibliography limits it is necessary to activate emergency response plans, to implement alternative water supply and to arrange an epidemiological observatory and communication methods.

However, as far as the adoption of use restrictions is concerned, art. 10(1) of the Italian Legislative Decree 31/2001 has to be considered for the need to take into account the exceeding level and potential risks for human health as well as the risks that could derive from an interruption in the supply or from a use restriction of the delivered water; the possible use restrictions must first of all envisage the recourse to alternative supplies and the urgent implementation of adequate treatments to restore the compliance of the distributed waters.

## 4.2.2. Specific measures recommended based on ALF system

The criteria and strategies have been previously presented in chapter 1.5 according to which it is possible to assess the cyanotoxins risk degree in the production and distribution chain of water for human consumption and establish possible solutions aimed at avoiding exposure of the consumer.

It is nevertheless useful, in this section, to recall the key elements and the actions recommended for the different strategic aspects driving the ALF implementation. For this purpose, Figure 4.11 indicates the main aspects of ALF system structuring and implementation concerning, in particular, risk assessment for the specific water system, surveillance and monitoring methods implemented, emergency response and communication measures.
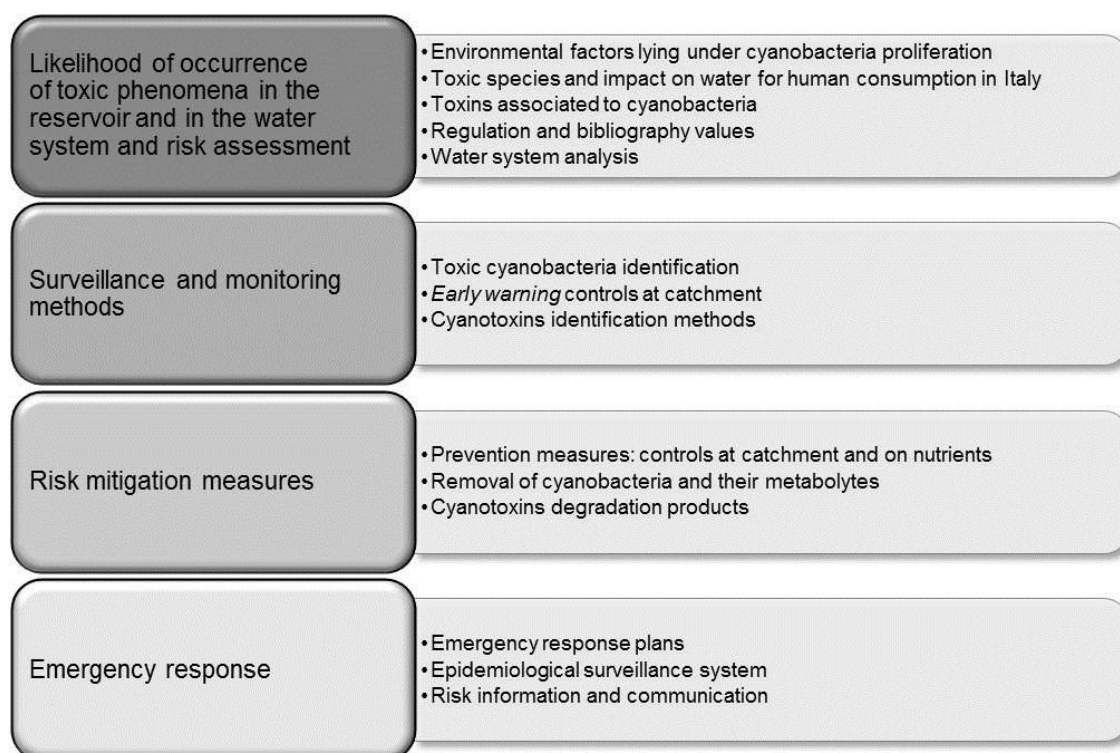
**Figure 4.11 Key aspects of ALF system**

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# Annex A

**Table A1. Summary scheme concerning risk levels adopted in the surveillance system**

| Decision basis | Threshold definition and expected risk[1] | Recommended actions | Measures and possible use restrictions[2] |
|---|---|---|---|
| **Detection level 0** | | | |
| Detection of potentially toxic cyanobacteria presence during monitoring[3,4] | Potentially toxic species [4]:<br><br>500-2.500 cell/mL<br><br>*or*<br><br>Cyanobacteria chlorophyll: 1-2,5 µg/L<br><br>*Detection of reduced concentrations of cyanobacteria. No immediate health risks.* | To deepen reservoir visual inspection.<br><br>To implement a continuous monitoring, at least on a fortnightly basis, of algal or cyanobacteria chlorophyll count[10,11] | - |
| **Alert level 1** | | | |
| Alert for possible health risk: algal count associated to a potential presence of cyanotoxins in waters for human consumption (before drinking water treatment chain) to a level equal to the maximum acceptable value[6,7] | *P. rubescens:* 2.500 cell/mL [4]<br><br>*or*<br><br>Other toxic species: 5.000 cell/mL [4]<br><br>*Settlement and development of cyanobacteria population in the water body so as to envisage, in the worst scenario[6] possible, a potential production of toxin in waters for human consumption with concentrations near to the maximum acceptable value (1,0 µg/L/L MC-LR[7])* | Notification to local health authority in case of recurring/systematic and adequately managed phenomena[5]. To implement a continuous monitoring on a biweekly basis or better weekly basis, through algal count [10,11] at least on system ingoing and outgoing waters.<br>If system protection level is considered as inadequate[8], there is the need to implement the analysis, on a weekly basis[8,9,10,11], of cyanotoxins in system ingoing waters, and if necessary, in outgoing waters and/or in distribution[12]. To optimize, as fare as possible, mitigation measures in the drinking water treatment chain[8,13]<br>To ensure an adequate chlorination[14] | Use restrictions[2] following the detection of toxins concentrations exceeding the maximum acceptable values in distributed waters[7,12] |
| **High alert level 2** | | | |
| High alert level for a possible health risk: algal count associated to a potential presence of cyanotoxins in waters for human consumption (before drinking water treatment chain) to a level equal to 10 times the maximum acceptable[6,7] value in waters for human consumption | P. rubescens: 25.000 cell/mL[4]<br><br>*or*<br><br>Other toxic species: 50.000cell/mL[4]<br><br>*Settlement and development of cyanobacteria population in the water body so as to envisage, in the worst scenario[6] possible, a potential production of toxin in waters for human consumption with concentrations near to 10 times the maximum reference value (1,0 µg/L MC-LR[7]). Adequate prevention and treatment measures must be implemented so as to mitigate risks, otherwise use restrictions and emergency response plans together with an adequate training and communication are demanded* | Notification to local health authority[5]<br>Continuous monitoring on a weekly basis, or better biweekly basis, through algal count[10,11]<br>Cyanotoxins identification on a weekly or better biweekly basis[10] on inwards and outwards waters from the water treatment system and in distribution[7,9,10,11,12]<br>To optimize and/or enhance mitigation measures in the drinking water treatment chain[8,13]<br>To ensure an adequate chlorination[14]<br>Organization of emergency, information and communication plans | Use restrictions[2] following the detection of toxins concentrations exceeding the maximum acceptable values in distributed waters[7,12] |

1. Threshold is the maximum acceptable level of toxins concentration in the reservoir water entering the water management system (as preventive measure it is equal to the maximum acceptable value of cyanotoxin in the water at consumer site), assessed from cyanobacteria level in waters, according to a conservative assessment of potential cellular production level (*toxin quota*); the expected risk is considered as the potential occurrence in the water distributed for human consumption of cyanotoxins contamination level near or exceeding the maximum acceptable value.

2. In use restrictions adoptions must be however taken into consideration art. 10(1) of Legislative Decree 31/2001 and amendments, concerning the need of considering the excess amount and potential risks for human health, as well as those risks that could derive from a supply interruption or from a use restriction of distributed waters; the possible use restrictions must first of all envisage the recourse to alternative supply and the urgent implementation of adequate treatments.

3. The routine monitoring on the reservoir must be envisaged according to the regulation in force or to risk plans if envisaged by the water operator based on a risk assessment according to the WSP approach.

4. Values established on samples collected near the intake work or in inwards waters (raw waters), where the sampling in the reservoir is not possible to carry out; other assessments can be performed according to the count of samples collected in other areas and/or water body deepness, and which highlight a possible concentration equal to the threshold values established for the area near the intake work (taking for example into consideration the likelihood of vertical shifting on the water column of algal mass).

5. If the risk related to cyanobacteria occurs in the water system on a regular or systematic and/or prolonged basis (in general occurring in one ore more months during which the alert level 1-2 persists), data of internal monitoring concerning the risk due to cyanobacteria and toxins, including monitoring data, can be provided by the operator through methods and timings to be agreed with the health authority on a case by case basis, according to contamination level, prevention and mitigation measures adopted by the plant (according to plant size), technologies used for water treatment, level of systems maintenance and monitoring) and to the experience gained by the water system; as a general rule, for water systems with multiannual experience in risk management using adequate and tested monitoring systems, a communication of data on a six-month or annual basis can be envisaged (summary of cyanobacteria concentration data and, if necessary, of toxins levels in inwards, outwards and distribution waters) together with the realized interventions, except for extraordinary contamination events.

6. Maximum precautionary criteria by considering the entire algal population as a toxins producer, assessing a high level of toxin produced for each single cell and assuming that all the toxins produced are free in water, represent the most toxic varieties and are not removed during treatments.

7. The maximum acceptable value for cyanotoxins, in compliance with Legislative Decree 31/2001 (art. 6), is actually fixed for MC-LR at 1,0 µg/L, as sum of different microcystins congeneric concentrations, expressed as MC-LR equivalent. The microcystins congeneric identifiable at the best of analytical potentiality available, must be found through confirmation methods, and as minimum criterion congeneric elements for which analytical standards are commercially available at the current state: dem-MC-RR, MC-RR, MC-YR, dem-MC-LR, MC-LR, MC-LA, MC-LY, MC-LF, MC-LW. Cyanotoxins research must be extended to other compounds, such as for example, cylindrospermopsin and anatoxin in bloom presence (alert greater than 1) of potential producers of these toxins; the maximum acceptable value of cyanotoxins other than microcystins should be fixed in compliance with Legislative Decree 31/2001, art. 11, clause 1, b.

8. As reference, a class I-II protection level indicated in Figure 1, can be considered as inadequate, even if system size and functioning and maintenance conditions are appropriate; moreover waters treatment is also required before they can be delivered for human consumption as stated in the Legislative Decree 152/2006 concerning pre-treatments based on water body classification.

9. Cyanotoxins determinations must be referred to the overall content (intra- and extra-cellular); the analytical method must be adequate for the purpose.

10. Monitoring frequency must be established according to the assessed risk level (first of all algal concentration and toxin levels in inwards waters and where necessary in outgoing and distributed waters, assessment of the reduction through the system during the monitoring and other operative indicators such as for example turbidity, TOC, etc.).

11. To keep monitoring frequency until two consecutive results state a reduced risk level, after that the frequency can be gradually decreased.

12. As far as distributed waters monitoring is concerned, it is required to take into consideration a proximal and a distal point of the network where the water is not mixed with water having other origins;

13. Protection measures III-VI (Figure 1) must be implemented for concentrations exceeding alert level 1.

14. About 0,1-0,2 mg/L of residual chlorine, increasing, where possible, the contact time during disinfection; higher chlorine concentrations (ex.: 0,5 mg/L) can be recommended for short periods of time during an emergency, taking however into consideration that the monitoring of disinfection by-products can be critical due to the increase of organic substance charge caused by algal mass in inwards waters; it is therefore required to monitor the levels of disinfection by-products such as trihalomethanes and/or chlorite according to the techniques adopted for the possible pre-oxidation and post-disinfection.

## 4.3. Emergency response plans

The state of emergency is represented through the evidence of a health risk for the consumer due to the presence in the water of cyanotoxins levels exceeding the maximum acceptable values. It has to be highlighted that, in many cases, the occurrence of a state of emergency is very rapid, even few days, and the time frame in which the emergency occurs usually involves a few weeks.

It is therefore clear that a safety management of the emergency, the health, the economic and social impact of these phenomena over consumers, is related to interventions promptness and actions suitability. Those aspects therefore need:

– a previous preparation of the emergency plan;
– a coordinated and planned engagement of all the parties involved during the emergency stage as far as suitable procedures are concerned;
– preparation of human resources, through an adequate training.

It has to be noticed that emergency plans have already been established by every operator of water systems for human consumption, and different scenarios having effect on the water utility, such as for example, extreme climatic events or potential hostile actions against the system, have already been considered. Plans define, in general, each duty and responsibility and envisage the creation of a "crisis unit" within companies and identify emergency equipments, such as power units, movable tanks and tankers, water provisions and baggers, means of transport, operative room equipment, etc., also through coordination among Companies of the same Province/Region, possibly coordinated by Prefectures.

The emergency response plans described in the present section can usefully include crisis management measures already established by water supply systems

## 4.3.1. Technical roundtable

The preventive organisation of a technical roundtable may grant, during an emergency stage, the presence of a multidisciplinary and coordinated expert team able to manage the different crisis stages at its best.

Ideally, the technical roundtable team must ensure:

− the involvement of local key functions, at health and environmental level, water supply operators, basin authority and other interested parties;

− the presence of experts of different disciplines such as Biology, Chemistry, Toxicology, Medicine, Hydraulic engineering, Agricultural science;

− a support, if needed, by the side of national bodies, such as for example members of the cyanobacteria national group, so as to quickly provide crucial information, scientific and technical tools;

− the availability of complete data in real time on which the decision-making process must be based;

− information, communication and transparency of decisions.

Table 4.4 shows, as example, the task force created to deal with a drinking water emergency due to cyanobacteria which involved, in 2009, the Occhito Lake (1,2) used for several purposes among which the catchment of water for human consumption in a large area of the Puglia Region in Italy.

**Table 4.4.Task force created to deal with the drinking water emergency due to cyanobacteria which involved, in 2009, the reservoir of Occhito**

| Body | Functions, role, contribution |
|---|---|
| Regional Authority | Coordination |
| Province | Information on sensitive consumers, risk perception, economic activities and other specific issues |
| Mayors of municipalities involved | Information on sensitive consumers, risk perception, economic activities and other specific issues<br>Beneficiaries of local measures |
| Civil Defence | Definition of possible water supply measures in state of emergency |
| Local health authority | Responsible, at institutional level, for the decisions on risk assessment and suitability of water for human consumption |
| ISS | Technical and scientific information support, opinion on solutions suggested<br>Determination of toxins levels in waters |
| Regional Authority Environment Protection | Data on the qualitative-quantitative composition of algal species in the basin, incoming and outgoing raw waters from the purifier and in distribution |
| Development Consortium Basin Authority | Data on the hydrodynamic regime of the basin and water use<br>Implementation of established basin management measures (ex.: basin levels, sediments analysis) |
| Water supply operator | Data on internal controls<br>Prevention and mitigation measures for the risk in drinking water supply chain implementing body |
| Epidemiological monitoring centre and first aid service | Arrangement of observations over possible syndromic frameworks due to consumptions of contaminated water |
| Regional communication office | Informing the population |

## 4.3.2. Decision-making information

The availability of consolidated information during the emergency stage, is useful to grant the correct response time for definite needs and decisions. For this purpose, the following information is basically needed:

– list of key roles (organisations, bodies, people) and main contacts (for example active carbons suppliers);

– main procedures for events management;

– main logistic and technical information, such as the localization of possible alternative water supplies and related rate.

## 4.3.3. Decision-making process

According to the experiences gained, it is deemed to be useful to recall, as an example, the decision-making process occurring during the emergency stage and that has to be supported through adequate technical-scientific knowledge:

– situation analysis and hazards objective assessment;

– identification and involvement of key functions in monitoring actions, control over decisions concerning supply methods;

– identification of actions priorities such as the integration of water treatment systems with other practices and technologies;

– definition/selection of possible options and impact analysis in terms of cost-benefit;

– establishment of additional water supply measures;

– alert measures at Civil Defence for water supply during the emergency stage;

– risk mitigation adequate treatments (for ex.: integration with GAC of pre-existing sand filters);

– communication to consumers and users;

– planning of mid-long term actions such as the analysis of factors favouring the algal bloom;

Assessment of risks related to use other than human consumption such as agriculture, fishery,recreational uses and food production.

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# Chapter 5

Conclusions

## 5.1. 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.

In the past few decades, in almost all the Italian regions, problems correlating to developments of cyanobacteria, critical in terms of frequency and scale, have been observed in natural and artificial reservoirs used for the supply of water for human consumption. The production by cyanobacteria of multiple secondary metabolites which are toxic to mammals, noted as cyanotoxins, may represent a considerable health risk, given that the main toxins may travel from the reservoir all along the drinking water chain up to the point of consumption. The potential impact on health linked to the presence of cyanotoxins in water and the lack of harmonisation in managing the proliferation of cyanobacteria in water resources used for producing drinking water has led to countless water emergencies in Italy, with secondary control measures being adopted in line with criteria which are not consistent at national level as well as causing panic and controversy for local authorities and the population groups concerned. The many requests for support from the central health authorities due to cyanobacteria risks in water management systems, and the need to draw up guidelines and reference values for cyanotoxins in water intended for human consumption, as expressed directly by various health authorities and legal bodies regarding possible cyanotoxic contamination of water, indicate that a national reference approach is necessary in order to guide the decision-making, technical management and operational levels of prevention and control of the massive risk posed by cyanobacteria in water intended for human consumption.

In this framework, the present PhD thesis 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.

As the first phase of project, a highly specific and sensitive analytical method based on solid phase extraction, separation and detection in LC tandem mass spectrometry has been developed and validated for the direct simultaneous determination of 21 different cyanotoxins potentially affecting water supply chains in Italy. The method has been proven to be robust, precise and accurate with recovery percentages above 85% and with relative standard deviations $\leq$ 16%, fit for the intended purposes at the concentrations of interest. The LOD obtained applying the procedure SPE-LC-MS/MS were within the range 0.002 to 0.025 μg / L, at least 50-fold lower than the guideline value proposed by the WHO for drinking water (i.e. 1.0 μg / L for microcystin-LR).

The analytical method was then applied for a monitoring plan during a drinking water emergency occurred in Vico Lake. The systematic study of the contamination phenomenon in the drinking water chain has shown in raw water the presence of
[D-Asp3]-MC-RR, MC- RR and [D-Asp3]-MC -LR respectively, at concentrations of up to 1.52, 0.100 and 0.159 μg / L. Other toxins (LW, LY, LF, HilR, WR, HtyR CYP 1007, CYP 1042, Anabaenopeptins, Ana-a and Microginins) have never been detected during the twenty-four sampling months . CYN has been sporadically found.

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.

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

Based on the extensive applicative experience acquired in the course of the thesis, the developed analytical method has proved itself to be an effective instrument for the risk

assessment from exposure to cyanotoxins, in the management of public health emergencies and water crises related to the proliferation of toxic cyanobacteria in surface water destined for human consumption. Thus, the analytical protocol developed during the doctoral research activities is proposed as a reference method for the determination of cyanotoxins in water intended for human consumption in accordance with Legislative Decree no. 31/2001.

According with the pursued objective of the research consisting in assuring the water-supply undertakings meet the cyanotoxin quality standards in drinking water by appropriate water-protection measures at the water environment, and/or by appropriate water-treatment measures to be applied before supply, another important research activity was focused on the development of a new model of intervention criteria targeted on the level of estimated risk in the drinking water reservoir and on the treatment of water before the human consumption.

Practical, technical-scientific tools for risk analysis throughout the chain of production and distribution of water were defined and the critical elements in the management of emergency scenarios, with respect to risk communication, were finely tuned.

This essentially consists of the structuring and implementation of an integrated system based on the principles of Alert Level Framework (ALF) and the Water Safety Plan (WSP) as a comprehensive prevention strategy dealing with the risk management over the entire drinking water supply chain, from the control of the reservoir to the delivery points. Analytical protocols for health Authorities, involving methods and frequency of monitoring targeted to the risk level, and water quality standards were established to assure the prevention and management of emergencies caused by the proliferation of cyanobacteria and the production of cyanotoxin from the water environment to the water tap.

The results achieved by the doctoral studies were shared within an interdisciplinary national working group, with experts from the Health Ministry, regional health and environmental authorities universities and research institutes, thus producing the

"Italian Guidelines for the risk assessment and risk management of cyanobacteria in water intended for human consumption".

The guidelines recommend to adopt a maximum permissible value for cyanotoxins (*i.e*., 1.0 mg/liter for microcystin-LR referred to the total intra- and extra- cellular toxin content and to the sum total of concentrations of the various congeneric elements of microcystin present in the sample, considered to be equivalent to microcystin-LR) within the framework of a preventive approach based on analysis and integrated risk control. The guidelines edited by the Italian National Institute for Health are intended to be useful tools for health and environmental central and local Authorities, managers and operators of water supply systems, as well as for consumers or for interest groups wishing to investigate the issues related to the presence of cyanobacteria in water resources.

All the above activities allowed to propose an interministerial decree for the introduction, in Annex I part B of Legislative decree no 31, of 2 February 2001, of the parameter "Microcystin-LR" and related parameter value, with the aim of improving the level of health protection through the monitoring and control of emerging risk factors, such as cyanotoxins, in water for human consumption.

# Chapter 6

List of publications

# List of publications

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