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# Investigation on cyanotoxins and volatile organic compounds in lake Karaoun (Lebanon) by bioanalytical and mass spectrometry techniques

Noura Hammoud

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# THÈSE

UNIVERSITE DE PAU ET DES PAYS DE L'ADOUR

École doctorale sciences exactes et leurs applications ED211

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**par Noura Alice HAMMOUD**

pour obtenir le grade de docteur

de l'Université de Pau et des Pays de l'Adour

**Spécialité : Chimie analytique de l'environnement**

Investigation of cyanotoxins and volatile organic  
compounds in lake Karaoun (Lebanon) by bioanalytical  
and mass spectrometry techniques

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## ABSTRACT

Karaoun artificial lake (1965) is the largest water body in Lebanon and undergoes successive blooms of two potentially toxic cyanobacteria: *Microcystis aeruginosa* and *Aphanizomenon ovalisporum*. Apart from lake Kinneret, middle-eastern lakes are poorly studied and documented.

Hydropower, irrigation and future water supply to the capital Beirut were the main purposes of the reservoir back in the 60ties when the water quality was outstanding. Since then, untreated industrial water, sewage and agricultural activities (excessive use of fertilizers) have resulted in the degradation of the water quality and lake eutrophication. At present, several activities are prohibited due to the degraded state of the lake and due to the occasional environmental disasters (massive death of fish and goat, the blockage of the river flow by hyper- scums and the bad odor emissions). This thesis addresses the question of assessment of the effects of such blooms and to facilitate the implementation of the adequate future monitoring of the reservoir.

The literature part gives a general overview on cyanobacterial blooms, their driving factors and their potential risks and impacts. It also summarizes the latest techniques implemented in the detection of cyanobacteria and the quantitation of their secondary metabolites. It also describes the site of the Lebanese reservoir Karaoun in the context of its environmental problems.

Nearly monthly field campaigns were conducted between August 2019 and October 2020. Water specimens were directly examined for taxonomic identifications and then processed together with fish samples with adequate preliminary pre-treatments. Molecular examinations using q-PCR, biochemical techniques (ELISA and PPIA), and mass spectrometry (LC-MS/MS, LC-HRMS and HS-SPME-GC-MS) were implemented for a holistic and complementary approach.

Results not only confirmed the dominance of cyanobacteria because of nutrient enrichment in the lake, but also proved its contamination with high levels of cyanotoxins (almost 200 times higher than the WHO guidelines). Microcystins were the most abundant toxins, mainly in October and December with concentrations up to 200 µg/L. Other toxins were also detected (anabaenopeptins), together with some bioactive compounds (microginins, Aeruginosin and Balgacyclamide).

In parallel, a variety of bio-organic taste and odor compounds, e.g. dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide, dihydro 6-ionone, β-cyclocitral, 3 methyl-indole, dihydro β-ionone, β-ionone) were detected in water samples. Samples with sulfur compounds had distinctive “septic”/“cabbage” odors, degrading the quality of water intended for human consumption. Other anthropogenic compounds, such as toluene and trichloromethane, reflecting the various sources of pollution along the stream and the reservoir were also detected.

The results of all the applied methods converged, with mass spectrometry techniques showing the possibility of the identification of the individual species not possible to be obtained by the other techniques.

## RÉSUMÉ

Le lac Karaoun est le plus grand espace d'eau douce au Liban. Le barrage construit en 1965 sur le plus long fleuve du pays (Litani), avait plusieurs objectifs comme production de l'électricité (hydroélectricité), l'irrigation et alimentation de la capitale Beyrouth en eau potable. Au début, le lac bénéficiait d'une excellente qualité d'eau, mais actuellement, il est considéré comme hypereutrophe, suite à l'enrichissement excessive en engrais. Depuis quelques années, le lac subisse d'efflorescences successives des deux cyanobactéries : *Microcystis aeuriginosa* et *Aphanizomenon ovalisporum*.

Aujourd'hui, et suite à la dégradation par les eaux usées / industrielles, les activités agricoles excessives de la région et les incidents occasionnelles de mort de poissons et de chèvres, toute utilisation du lac est devenue interdite. Parfois et due à la prolifération excessive de cyanobactéries, le cours d'eau peut être bloqué. Cette thèse évalue l'impact de ces efflorescences sur la production de cyanotoxines, et propose une approche méthodologique pour la surveillance systématique du réservoir.

Des campagnes d'échantillonnage quasi-mensuelles ont été menées entre en août 2019 et en octobre 2020. Les échantillons d'eau ont été directement examinés pour des identifications taxonomiques. Des examens moléculaires utilisant la q-PCR, des évaluations biochimiques (ELISA et PPIA) ainsi que des techniques de spectrométrie de masse (LC-MS/MS, LC-HRMS et HS-SPME-GC-MS) ont été mis en œuvre pour une approche holistique et complémentaire, afin d'avoir une vue d'ensemble de l'état du réservoir d'eau.

Suite à l'eutrophisation, deux cyanobactéries dominant le lac. Également, les résultats ont confirmé leur sécrétion importante de cyanotoxines et par la suite la contamination du lac par des taux élevés de métabolites secondaires (presque 200 fois plus élevés que les recommandations de l'OMS). Les microcystines sont les plus abondantes, principalement en octobre et décembre avec des concentrations allant jusqu'à 200 µg/L. D'autres toxines ont également été détectées (anabaenopeptides), ainsi que certains composés bioactifs (microginines, Aeruginosine et Balgacyclamide).

Parallèlement, une variété de composés bio-organiques de goût et d'odeur a été détectée dans les échantillons d'eau (comme le disulfure de diméthyle, le trisulfure de diméthyle, le tétrasulfure de diméthyle, la dihydro 6- ionone, le β-cyclocitral, le 3 méthyl-indole, la dihydro β-ionone, la β-ionone). Les échantillons contenant des composés sulfureux avaient des odeurs caractéristiques de "septique"/"chou", dégradant la qualité esthétique des eaux destinées à la consommation humaine. D'autres composés d'activités anthropogéniques et reflétant les différentes sources de pollution le long du cours d'eau et du réservoir ont également été perçus, tels que le toluène et le trichlorométhane.

Toutes les méthodes appliquées ont donné des résultats convergents. La spectrométrie de masse a permis pour la première fois la caractérisation d'un nombre important de cyanotoxines appartenantes à des classes différentes.

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INVESTIGATION ON CYANOTOXINS AND VOLATILE ORGANIC COMPOUNDS IN LAKE KARAOUN  
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## List of abbreviations

- AA- acetic acid
- ACN- acetonitrile
- CID- collision induced dissociation
- CTx- cyanotoxin
- DNA- deoxyribonucleic acid
- EDTA- ethylenediaminetetraacetic acid
- ELISA- enzyme-linked immunosorbent assay
- ESI- electrospray ionization
- FA- formic acid
- GC- gas chromatography
- Glu- Glutathione
- HCD- higher- energy collisional dissociation
- HILIC- hydrophilic interaction liquid chromatography
- HR- high resolution
- HPLC- high performance liquid chromatography
- LOD- limit of detection
- LOQ- limit of quantification
- MCs- Microcystins
- MeOH- methanol
- MS mass spectrometer
- MS<sup>n</sup> - multistage mass spectrometer/spectrometry
- MS/MS- tandem mass spectrometer/spectrometry
- PCR- polymerase chain reaction
- PPIA- Protein Phosphatase Inhibition Assay
- ppm- parts per million
- PSP- paralytic shellfish poison
- Q- Quadrupole (analyzer)
- RF- radio frequency
- RP-LC - reversed phase liquid chromatography

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RNA- ribonucleic acid

SPE- solid phase extraction

SRM- single reaction monitoring

TOF- time of flight (analyzer)

UPLC- ultra performance liquid chromatography

VOCs- volatile organic compounds

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## I. LITERATURE

### 1. Introduction

In a world with growing needs and, consequently, with excessively growing production, agricultural, service and industrial sectors have been intensifying their efforts to respond to human demands [1]. This has considerable environmental implications that would alter vital resources. In times where water sources are becoming scarce, surface waters, including artificial reservoirs are at a high risk of pollution and contamination, be it anthropogenic or natural (domestic and industrial wastes) [2].

Harmful algal blooms caused by cyanobacteria, that have always been present in the environment and are the main atmospheric oxygen producers, are currently on the radar of a big number of scientists and organizations [3]. The two major enhancing factors of their excessive proliferations are nutrient enrichment and climate change [4, 5]. Cyanobacterial blooms do not just alter a certain ecosystem by the formation of colonies, scums and/or by their bad smell emissions (caused by taste and odor compounds) [6, 7] but can also decrease the water transparency, affect the biodiversity, deplete oxygen levels, especially in deep water layers, and impact the economy [8]. They can also be dangerous when they produce and secrete toxic secondary metabolites, such as cyanotoxins [9, 10].

Freshwater cyanotoxins risk effects were first reported by Francis *et al.* in 1878 who described a bloom as “a thick foam like green oil paint with a thickness of two to six inches”; “wild animals that were in contact and drank from this water died painfully and rapidly” [11]. Since then, different classes of cyanotoxins, such as, e.g. microcystins, nodularin, cylindrospermopsin and their variants have been reported and studied [12]. The World Health Organization (WHO) set guidelines to avoid short- and long-term problems (acute intoxication, chronic diseases...) [13]. Therefore, it is important to monitor continuously water bodies that suffer from a blooming episode [14].

Blooms can conveniently be identified and predicted by remote sensing, but the identity of putative toxins cannot be proven without field visits [15]. For this, a number of different analytical methods have been developed for the detection and quantification of cyanotoxins, each having its advantages and limitations. Several bio-chemical assays based on the recognition of cyanotoxins by adequate antibodies have long been used. ELISA tests are commercially available as ready to use kits [16]. Microcystins' activity can be detected by PPIA kits [17]. PCR approaches are widely available to confirm the presence of cyanotoxin-coding bacterial genes [18]. Such methods commonly suffer from over/under- estimations, cross-reactivity's and matrix effects [19, 20].

Mass spectrometry techniques have become increasingly popular since they offer a high degree of accuracy, and the possibility for the simultaneous identification of various cyanotoxin categories and their structural variants [21] as well as other pollutants, such as VOCs. Their limitation is insufficient availability of standards, high cost and non-amenability to field [22]. The successful use is critically dependent on the development

of pre-concentration and purification techniques, such as e.g. solid phase extraction (SPE) [23].

Water is a scarce and highly demanded resource in the Middle-East and artificial lakes are valuable water providers for hydropower, irrigation and drinking water reservoirs. Nevertheless, the consequences of cyanobacterial blooms in this region are poorly documented, except for the Israeli Lake Kinneret [24]. In Lebanon, the artificial lake of Karaoun, created in 1959, is the largest water body playing an important role in the Lebanese hydropower, irrigation and has been under discussion as drinking water supply for the capital Beirut [25]. It is currently drastically polluted (industrial and domestic inputs) and cyanobacterial blooms are frequent, although not well investigated [26].

## 2. Cyanobacterial blooms

### 2.1. Cyanobacteria

Cyanobacteria, also called blue-green algae, are microscopic organisms found naturally in all types of water. They are the oldest photosynthetic prokaryotes (single-cell organisms) and live in fresh, brackish, and marine water<sup>1</sup>. The name of blue-green algae comes from the presence of phycocyanin and chlorophyll-a but they can sometimes be pink, yellow, red, and/or brown, the colors are conferred by the accessory pigments as the red phycoerytherin, and yellow-orange carotenoids [27]. Individually, they are microscopic organisms, but when they proliferate extensively, they can form macroscopic colonies and they can dominate other living organisms [28] (Figure 1).

Cyanobacteria were responsible of the oxidization of earth's atmosphere 3.8 billion years ago. Their long evolutionary history made them one of the most adaptive microorganisms [29]. They are able to acclimate to various environmental changes as rising temperatures, pollution, nutrient enrichment, low water levels salinization and many other surroundings [30]. They can colonize various ecosystems as soil, water and air. Some of them have the ability of fixing atmospheric nitrogen via specialized cells called heterocyst [31]. All these criteria give them a competitive advantage over other living entities and give them the advantage to occupy all kind of climatic zones and all types of water bodies going from the smallest ponds to the biggest oceans [32].

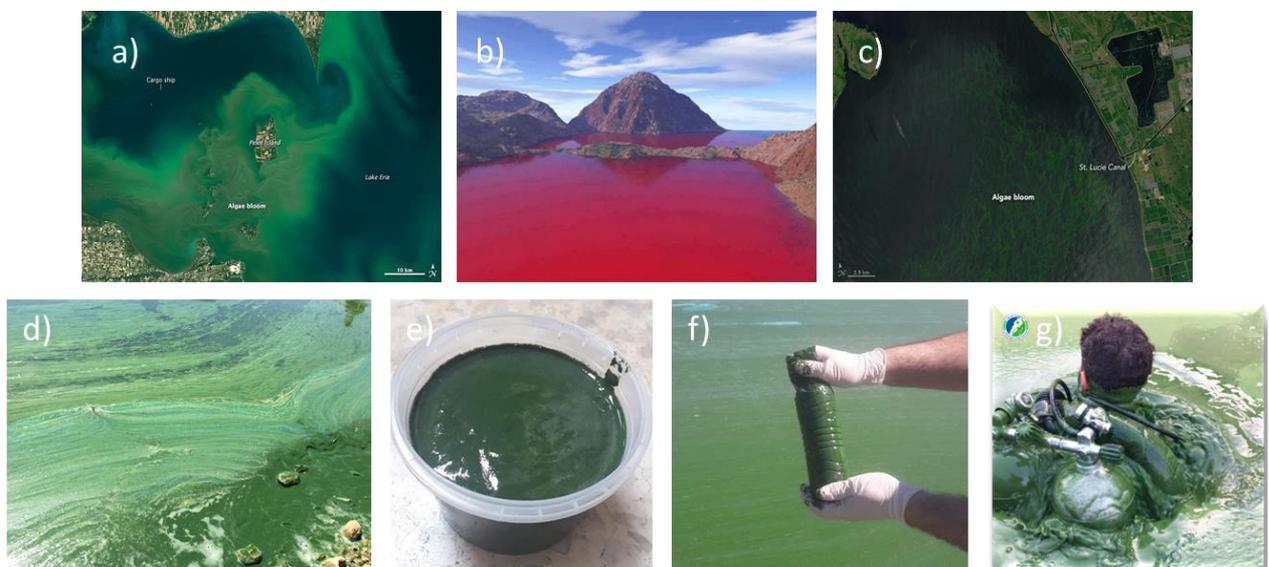


Figure 1: Cyanobacterial blooms: a) Algal blooms in Lake Erie and surrounding waters on August 4, 2015 [33]; b) Fisher Reservoir, San Angelo State Park, TX, Chromatiaceae bacteria on 10 April 2013 [34]; c) An early summer algae bloom in Florida's Lake Okeechobee (July 6, 2016) [35]; d) Lake Karaoun Lebanon on August 2019; e) and f) Scum from a cyanobacterial bloom in lake Karaoun on April 2020; g) Diver in Lake Karaoun during a security check-up on the dam on December the 11<sup>st</sup>, 2020 [36].

Cyanobacteria can be classified into four main orders [37]:

Chroococcales are species of spherical shape, called coccoid. They are unicellular and can form mucilaginous colonies during their maturation but they are never filamentous; colonies create dense masses too obvious to be spotted on the water surface (mostly in freshwater bodies). They reproduce by binary fission. They group some genera as *Microcystis*, *Snowella*, *Synechococcus*, *Synechocystis*, *Chroococcus*, *Cyanodictyon*, *Aphanocapsa*, *Pleurocapsa*, *Merismopedia* and *Aphanothece*.

Oscillatoriales can occupy a wide range of ecosystems, from fresh to seawater. They are filamentous lacking akinetes and heterocysts. They include: *Phormidium*, *Microcoleus*, *Lyngbya*, *Spirulina*, *Trichodesmium* and *Tychonema* and *Planktothrix*

Nostocales can form filamentous heterogeneous structures called trichomes. All cells undergo a division and some of them are able to differentiate into heterocysts with a thick hyaline protoplast that contributes to the fixation of nitrogen, and into akinetes with thick cell walls allowing them to resist extreme hostile conditions. This order includes *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia*, *Anabaena*, and *Anabaenopsis*.

Stigonematales are cyanobacteria which have no planktonic representatives. Their structure is very similar to the *Nostocales* and they group *Stigonema* and *Mastigocladus*.

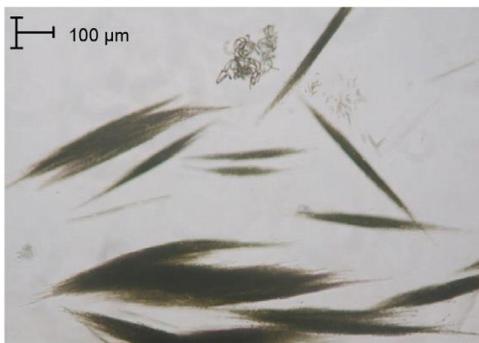
Cyanobacteria by their production of oxygen can support the life of ecosystems and organisms nevertheless in certain conditions they can be harmful and induce degradation and death (Figure 2). Cyanobacterial harmful algal blooms (CyanoHabs), are more and more threatening aquatic ecosystems, when they form, they reduce the transparency and might kill some endogenous species. In some conditions, blooms are so severe, scums can form and bad smells can be emitted and sensed [38, 39].



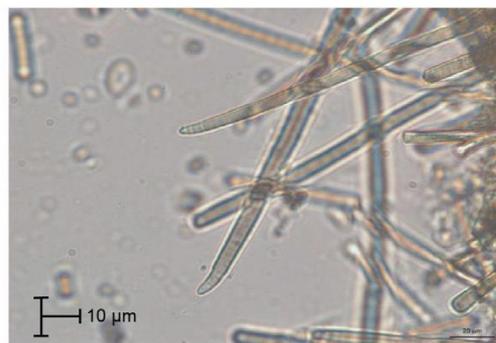
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Genre : *Cylandrospermopsis*.  
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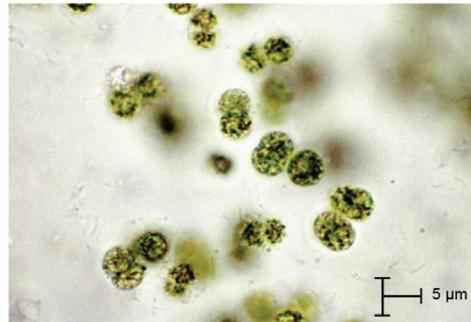
Genre : *Aphanizomenon*.  
© C. Duval, MNHN.



Genre : *Phormidium*.  
© I. Echenique, MNHN.



Genre : *Dolichospermum* (anc. *Anabaena*).  
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Genre : *Microcystis*.  
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Figure 2: Main bloom forming cyanobacteria genera [37].

## 2.2. Causes of cyanobacteria proliferation

### *Nutrient input and eutrophication*

Human development and advanced, intensified activities in agriculture along with industry has drastically increased the inputs of phosphorous and nitrogen via pollutants, pesticides, and fertilizers in waterbodies [40, 41]. Nutrient enrichment of various aquatic ecosystems leads to the well-known phenomenon called eutrophication that will eventually lead to dramatic impacts as enhancing the risk of excessive proliferation of algae and cyanobacteria causing uncontrollable blooms [42, 43]. Although new regulations are to improve eutrophication problems, the damage caused needs more time to be repaired [37].

Phosphorous is a vital element for cyanobacterial cells, it is one of the main constituents of DNA, cell walls and proteins; it also plays an important role in cellular energy and information signals [44]. Orthophosphate is the only bioavailable source of phosphate in the environment, so it can be considered as a limiting nutrient, but cyanobacteria can remain functional to concentrations as low as 0.3 µg/L [4, 44].

Nitrogen is a limiting growth factor used as a nutrient source, and some cyanobacteria have developed what is called heterocyst when nitrogen in the water column is depleted. The heterocyst is a differentiated cell of the colony, that would capture and fix the atmospheric nitrogen as a response to a privation of fixing nitrogen ( $\text{NH}_4^+$  or  $\text{NO}_3^-$ ) [45].

A low nitrogen to phosphate ratio coupled to temperatures above 15°C offers favorable conditions for cyanobacterial blooms [46]. But since worldwide measures were taken to reduce nutrients load in aquatic ecosystems phosphorous concentrations were reduced more effectively than those of nitrogen [30]. The nitrogen to phosphorus ratio is currently increasing and is likely to change the cyanobacterial blooms composition. The non-nitrogen-fixing predominates in the nitrogen-fixing cyanobacteria (i.e.: *Microcystis spp.* and *Planktothrix spp.*) and a rise in nitrogen-rich cyanotoxins as microcystins is observed [47].

### *Climate change*

The rise in water bodies temperatures is one of the leading factors of enhanced and successive cyanobacterial blooms [48]. Climate change be it natural or anthropogenic, is surely affecting the behavior of cyanobacteria their severity and their longevity [5]. Rising temperatures that induce higher evaporation rates and water levels reduction, as well as the shorter cold periods and longer hot seasons permit the persistence of cyanobacterial blooms for long durations which can sometimes last from one season to another [49, 50]. Since cyanobacteria are photosynthetic prokaryotes, long photoperiods offer adequate conditions for the proliferation of cyanobacteria. For maximizing their exposure to sunlight they have developed an ability of buoyancy that allows them to seek and capture light within the water column [37]. The decomposition processes of cyanobacterial blooms lead to high concentrations of methane considered as a greenhouse gas and contribute to global warming [51]. In fact, cyanobacterial blooms are caused by eutrophication and global warming but they are also contributing to these phenomena by two vicious loops: eutrophication / global warming and cyanobacterial blooms [52] (Figure 3).

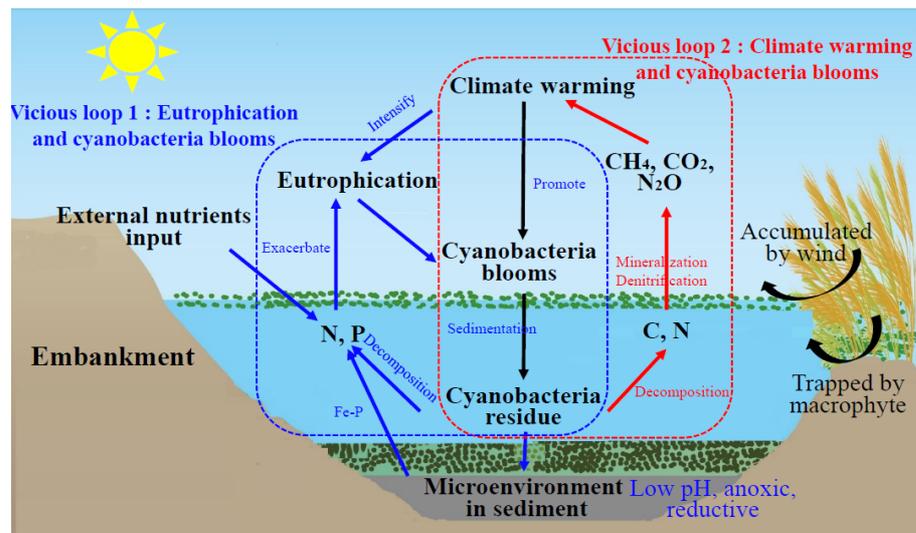


Figure 3: Link between eutrophication/climate change and cyanobacterial blooms [52].

### 2.3. Influence of environmental conditions

#### Light

It plays an important role in the life of photosynthetic prokaryotes such cyanobacteria. It is the source of their energy and is needed to optimize their growth [53]. The composition of cyanobacteria can thus be affected by the light intensity. Some genera as *Anabaena*, *Aphanizomenon* and *Microcystis* prefer intense irradiance conditions and well exposed shallow waters; others, as *Planktothrix*, prefer lower exposures and light intensities [54, 55].

If irradiance is weak, cyanobacteria has developed the ability to increase the number of light harvesting chlorophyll-protein complexes (LHCs) to capture more photons and function as in normal conditions [56, 57]. And if irradiance is too intense, cells and colonies might be subject to photo-inhibition or degeneration so they are able to escape it while moving in the water column and seeking deeper layers [58]. Moreover, they can protect themselves either by the chromatic adaptation known as the possibility to vary the phycobiliprotein composition (phycocyanin/phycoerythrin ratio) that will allow them to regulate the balance of wavelengths absorption [59], either by the production of some quenching agents as carotenoids that would neutralize the effect of highly toxic reactive oxygen species produced by ultraviolet-B radiation or by DNA repairing mechanisms by photoreactivation and light-independent nucleotide excision [58], [60]. Finally, they would secrete some compounds called mycosporine-like amino acids (MAAs) and scytonemin [60]. MAAs are produced for the protection against high levels of solar radiation, they are hydrophilic cyclohexanone or cyclohexenimine chromophores conjugated to nitrogen substituent of an amino-acid or its imino-alcohol [61]. They have a molecular weight of 300 DA and a maximum absorbance from 310 to 360 nm [62]. Different variants are reported in fungi and cyanobacteria: mycosporine-glycine, palythine, palythene, palythanol, asterina-330, porphyra-334 and shinorine [60].

Scytonemin is a cyanobacterial UV sunscreen. It is a yellow brown lipid pigment located in the extracellular polysaccharide sheath, it is of 544 DA and has a maximum absorbance

at 370 nm [63]. Its production can be triggered by other factors than light as salinity, temperature and desiccation. It plays a crucial role in the viability of cyanobacteria in extremely harsh conditions [64].

#### *Temperature.*

Low temperatures in water ecosystems mean a good oxygenation rate and a low solubility of chemical compounds (be it from run off or from sediments). A slight increase in temperatures affects the equilibrium and hence the biodiversity. Each living organism have an optimum, and for cyanobacteria it is 25°C, however they show reproducibility and activity from 15° to 30°C [6].

#### *Stratification / turnover and wind mixing.*

Rising temperatures of water bodies encourage intense stratifications and inhibit turnovers serving as good factors for the development of blooms [65]. The holomixis (the complete turnover) would limit the concentration of cyanobacteria in a certain water layer and would dissociate colonies and the formation of scums [66]. A strong wind activity would create waves or currents helping in the oxygenation of water and also breaking the blooms, however sometimes wind can move superficial cyanobacteria to shores/banks and accumulate them in scums or hyperscums [67]. The latter are intense scums that could reach a thickness of 20 to 30 cm, they look like a thick green paint/paste [49]. In other words, cyanobacteria prefer to proliferate in calm lentic waters, with long residence times and a relatively nutrients content and finally a well light exposed ecosystem [68].

#### *Nutrients and grazing.*

Nitrogen and phosphorous are the main nutrients used by cyanobacterial cells. Even when they are extremely depleted, cyanobacteria can form heterocyst for the capture of atmospheric nitrogen and adapt. They also developed the buoyancy to seek deeper layers searching for nutrients, or the avoidance of grazing by zooplankton and herbivorous fish [44]. Although cyanobacteria are not the most favorite phytoplankton to be consumed, they still submit it and it can affect and reduce their numbers. In the opposite, several studies reported the avoidance of some herbivorous fish areas contaminated by cyanobacteria. It may be due to the high levels of turbidity, to mucilage secreted by cyanobacteria or simply because they can block the gills [69].

### 2.4. Environmental and socio- economic impact

Cyanobacteria can lead to hypoxia via the depletion of oxygen after night respiration, decomposition processes or because of the thick superficial layer that they form. The thick film is a serious barrier that decreases oxygen penetration in water [70, 71]. Depletion of the biodiversity is caused by the high competition and sometimes leads to

the disappearance of some species disequilibrating the food web. The rise in alkalinity may kill fishes, the increase in turbidity and decrease in transparency and light penetration may be also the reason behind the death of aquatic plants [18]. A more important impact is the toxicity induced by the blooms themselves that could be lethal or harmful to any living species [72].

In parallel to the environmental impact, cyanobacterial blooms engender serious economic losses that would have different social aspects. In the USA only, yearly losses due to the consequences of cyanobacterial blooms are estimated for 2 billion dollars and are increasing every year [73].

Tourism and recreational activities are usually banned in contaminated water ecosystems, hence investments in this field might be affected and reduced; in parallel, real estates that are usually of a greater value near rivers, lakes and on seaside may lose their worth when the water quality is altered and may also be abandoned [74, 75].

Riversides and banks are considered of the best agricultural soils, they contribute to the food supply and some countries' (e.g. Egypt) economies are based on such activities. When water is not suitable for irrigation, the usage would be suspended and buying water from private tankers or transferring water from other sources might be very expensive. In addition, fisheries are at the base of livelihood of many people and once fishing is banned or regulated, incomes will be impacted. Long term restrictions, and heavy economic issues would lead to social crisis were people will seek illegal activities as fishing in secret, irrigating with polluted water, and to conflicts among the inhabitants or with the government or public authorities as it was already seen during the field campaigns of this thesis.

On the other side, supervising Cyanohabs might be very costly. It includes the continuous monitoring, the remediation and the control. For monitoring, different approaches are implemented. In-situ, rapid and relatively cheap tests are generally used, but for more accurate investigations laboratory analyses are required. ELISA and PPIA are used as a first scanning, and they can be complemented with molecular techniques and the most accurate approach by liquid chromatography tandem mass spectrometry. All of this testing has a relatively high cost and needs experts.

Some expensive water treatments are necessary to be implemented for the improvement of the water quality; they can be either chemical, either physical or biological [8]. Currently, no method could be considered as the ultimate one. They all have some limitations.

For chemical solutions we can cite the usage of copper sulfate that is being more and more discouraged due to the toxic effect of copper. Although it was a popular approach for the control of CyanoHabs, concerns are rising after the reported negative effects as its persistence in sediments and the alteration of the food web [76]. In addition, we can cite hydrogen peroxide that is more environmental friendly. It can be used for inhibiting blooms and/or their toxic effect. It doesn't affect the biodiversity but the main issue is the optimized dose to be used, also it showed to be ineffective in large waterbodies [77]. Phoslock™ is another technique used for the control of blooms. It is based on a clay of aluminum phosphate that binds to phosphate (the key nutrient of cyanobacteria) and also

able to trap cyanobacteria. The dose is undetermined, it would be used as needed and should be more efficient when phosphorous comes from sediments (internal) rather than coming from the runoff (external) [78]. Another method, costly and time consuming, involves the sediment capping based on a designed capping layer put above the sediment to reduce the release of nutrients favoring blooms [4].

In terms of physical solutions, can be mentioned the construction of screens and barriers to limit the spread of cyanobacterial blooms, the use of surface mixers to avoid the stagnation and accumulation of cells and the formation of scums, and the dredging and/or excavation [79]. A recent alternative consists of the use of ultrasounds to break cyanobacterial cells. However, a problem arises of affecting the biodiversity at its proximity after damaging the cells of other phytoplankton, zooplankton and other living organisms playing an important role in the food web [80, 81].

Biological treatments can go from the use of plant extracts, the bio manipulation of food web (by the removal of some bottom feeder fishes), the growth of aquatic plants, floating island and wetlands to biological treatments [79].

## 2.5. Cyanobacterial blooms in the world

### *General overview on the global occurrence of cyanobacterial blooms*

Rising temperatures, enhanced anthropogenic nutrient loading, increased levels of CO<sub>2</sub>, and vertical stratifications are promoting the worldwide proliferation of cyanobacteria, that are impacting vital water ecosystems that are at the base of dynamism. Blooms can be sensed almost everywhere. They were reported in China in lake Taihu, at the United States of America in Lake Erie [82], in European countries as France [83], Serbia [84] and Poland [85], and they are also occupying waterbodies of all the Mediterranean region. Extensive blooms were seen in Greece in Lake Kastoria [86], lake Pamvotis [87] and Lisimachia. They were also seen in Spain in Gergal reservoir [88], at the Tunisian dam Lebna [89], in Italy [90] and in Turkey [91]. Lastly, middle eastern lakes suffer too, and cyanobacteria was perceived in Lebanon in its largest water body Karaoun [24]. *Microcystis spp.*, *Planktothrix*, *Aphanizomenon* and *Anabaena* are the main and dominant proliferating genera [47].

### *Comparison between Mediterranean lakes affected by cyanobacterial blooms*

*Lake Trichonis* (38°34'N, 21°30'E) is the largest natural lake in Greece in terms of size and water volume (3 × 10<sup>9</sup>m<sup>3</sup>) the lake's perimeter is 51 km, and covers an area of 96.9 km<sup>2</sup>. Its maximum depth is 58 m and the water residence time is approximately 9.4 years. It is considered as a deep monomictic lake. Water level fluctuations are up to 2 m. Trichonis is linked to another lake called Lisimachia (38°35'N, 21°29'E) that is located 2.8 km to the west of Lake Trichonis, has a surface area of 13.2 km<sup>2</sup>, and a maximum depth of 9 m (Tafas and Economouilli,1991). Both lakes are the result of a post-alpine tectonic subduction, and they are both suffering from cyanobacterial blooms (mainly *Microcystis* and *Anabaena* (*Dolichospermum*)).

*El Gergal Reservoir* (37°34'11''N, 6°02'23''W), located in the south-west of Spain, is considered as a medium water body in terms of surface area (2.5 km<sup>2</sup>), with a volume of 35×10<sup>6</sup> m<sup>3</sup>, and a maximum depth of 37.5 m, a mean depth of 15.7 m and an elevation of 26 m above sea level. The reservoir provides a water supply to the city of Seville, but unfortunately suffers from occasional nitrogen fixing cyanobacteria [53]. Recently blooms consist of the 2 buoyant cyanobacteria *Aphanizomenon flos-aquae* and *Anabaena planctonica* accumulating in the surface layers of the reservoir during calm weather, low turbulent mixing periods, while they form stable and dense scums and patches inhibiting the penetration of light and oxygen, having negative impacts on the waterbody [54].

*Lake Kinneret* (32°50'N 35°35'E) located 85 km to the South of Karaoun Reservoir is the only well studied middle-eastern/Mediterranean lake. With a water volume of 4 × 10<sup>9</sup> m<sup>3</sup>, a maximum depth of 43 m. the water body is mainly used for hydropower and domestic water usages. The lake is under the sea levels, and water fluctuations can go up to 5 m. the residential time is of about 5.1 years. The reservoir is considered warm monomictic and stratified from May to December and suffering from cyanobacterial blooms of *Microcystis sp.* that replaced the dinoflagellate, *Peridinium*. All of these changes are induced by natural and anthropogenic factors [55, 56].

**Table 1.** Physical characteristics of Mediterranean lakes

	Lake Trichonis	Lake Lisimachia	El Gergal Reservoir	Lake Kinneret	Lake Karaoun
Volume x10 <sup>6</sup> (m <sup>3</sup> )	2600	53	35	4000	224
Max depth (m)	58	9	37.5	43	60
Area	97	13.2	2.5	167	12

### 3. Cyanotoxins: the principal secondary metabolites of cyanobacteria

Cyanobacterial blooms become toxic when cyanobacteria secrete toxins that are called cyanotoxins; they are secondary metabolites [95]. However, the presence of potentially toxic algal species does not always mean an acute risk of poisoning, because toxins are not always produced or may not be secreted at a high level or concentrations [96]. Although blooms mainly appear in hot seasons, they can occur at any time of the year and produce toxins, but it is not really palpable what the main driver of these secretions is. It can be assumed that the production of toxin can be affected by genetics and by environmental factors, in other words by several factors as nutrients concentrations, pH, water temperatures, illumination, growth rate and cell density and finally the interaction with other aquatic organisms as predation and competition [97].

Cyanotoxins can be classified based on different criteria, whether on their toxic effect, on their molecular structure or on their origin, but the commonly used classification relies on the toxic effect. Based on this principle, cyanotoxins are divided into two main classes cytotoxic and bio-toxic. Cytotoxicity will not be elaborated since it does not show a severe threat however it was used for antimicrobial and anti- fungi activity [98].

#### 3.1. Hepatotoxins

*Microcystins (MC)* represent the most important cyanotoxins in terms of their worldwide existence and impact on water bodies and sanitary effects [57]. Microcystins are normally intracellular toxins (800-1100 Da), as long as the cell remains untouched, and are mainly excreted when the cell disintegrate in other words when the cyanobacteria dies [12]. These toxins are highly stable in the environment; they can last for a long time with an estimated half-life of 10 weeks. They are degraded under extreme conditions, such as temperatures exceeding 40 °C in direct sunlight and in extreme pH conditions (<1 or >9) [58].

Microcystin is a cyclic heptapeptid with over 279 variants (Figure 4). The variability can occur in position 2 and 4 in the ring structure, moreover the fixed chain is composed of 6 amino-acids including four non proteins and two proteins in a ring structure and of a side chain called ADDA (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid). The name given to the microcystin variant is normally based on the two amino acids in the variable positions. The main microcystin in the environment is the variant LR (Leucine-Arginine) or Microcystin-LR. Other known compounds are RR and YR. the ADDA chain is normally constant in all forms and can be used to the quantification of these toxins regardless of the amino- acids in position 2 and 4 [59].

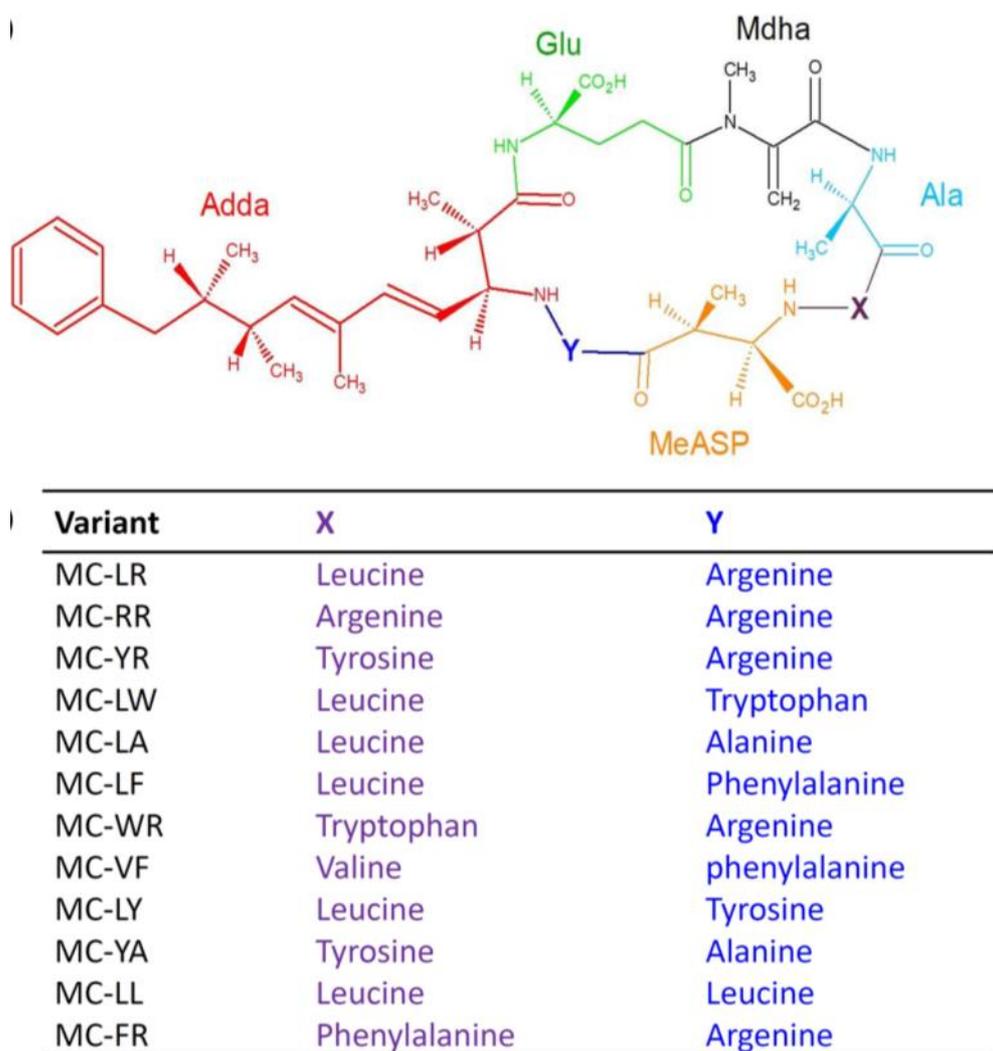


Figure 4: Microcystin and common variants.

In terms of toxicity, all microcystin variants have a similar toxicological effect to the one induced by microcystin-LR, they might just differ in potency. The effects of microcystins depend on many factors. In case of low to medium exposures to these toxins, mammals can suffer from irritation normally coupled to inflammatory reaction in the gastrointestinal and respiratory system and in the skin too [60]. The symptoms can be skin rash, diarrhea and some sore throat. When the exposure is more important in terms of dose and time, and mainly oral intake, the damage will directly affect liver. Liver can show a high failure activity while swelling and releasing liver enzymes in the blood. Symptoms can start from minutes to hours after the exposures and they manifest in extreme levels of hemorrhagic diarrhea and vomiting, depression and finally can lead to coma. On the other side, fish showed to be more resistant to their exposure to cyanotoxins since the intake is in sub-lethal doses for longer periods (days to weeks); moreover, they can cause their liver cells' damage [61]. They can start accumulate from the embryogenic state of fish. It has been noticed that adult fish are more resistant to

cyanotoxins than embryos and juveniles, maybe due to their ability to recognize and escape or avoid areas with cyanobacterial blooms and high concentrations of toxins [62].

Fish accumulate cyanotoxins in their organs, mainly liver, thus the importance of knowing at what doses and which types. During heating or boiling microcystin degrades, the degradation products can be more dangerous since the toxin can be released after breaking its bound with phosphatase [60].

*Cylindrospermopsin (CYN)* is another well-known and potent hepatotoxin, it can be produced by many different bacteria genera as *Aphanizomenon*, *Cylindrospermopsis*, and *Anabaena*.

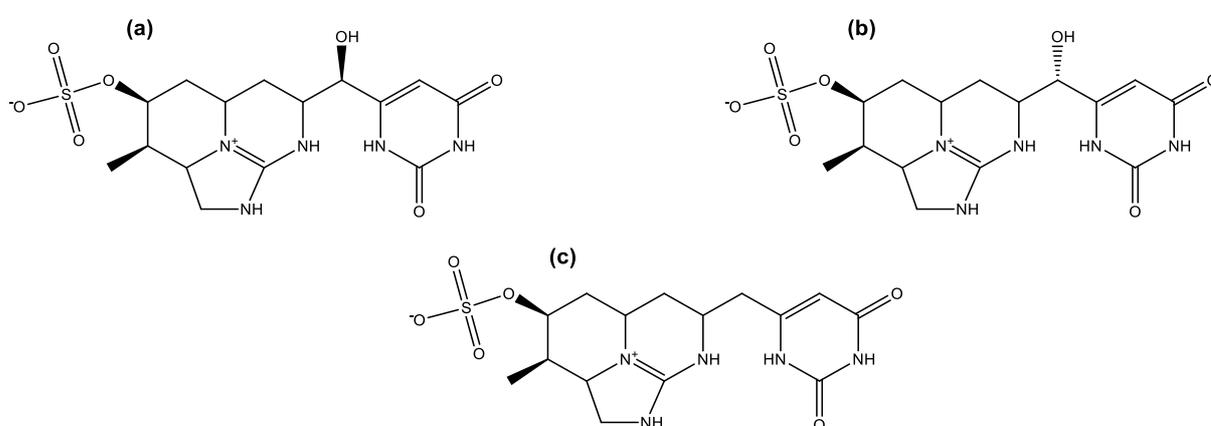


Figure 5: a) Cylindrospermopsin; b) 7-epicylindrospermopsin; c) deoxycylindrospermopsin.

*Cylindrospermopsin* (415 Da) is a tricyclic guanidine alkaloid attached to hydroxymethyluracil. It can have two variants and may differ in toxicity: deoxycylindrospermopsin that is relatively less toxic than the second variant 7-epicylindrospermopsin [63] (Figure 5). *Cylindrospermopsin* used to be characteristic of tropical and subtropical regions, but due to many factors and, especially, to the climate change, its presence is expanding and it can now be found in more temperate regions, as, e.g. Spain and Germany in Europe and Lebanon in Middle-East. In contrast to microcystin, it is hydrophilic and is mainly extracellular. But as microcystin, it is highly resistant to sunlight, extreme pH and temperatures [58].

*Cylindrospermopsin* has very similar toxic effects to those engendered by microcystin; it causes liver and kidney damage, severe gastrointestinal inflammation, hemorrhagic vomiting and diarrhea and could lead to death. In laboratory experiments the damage caused by the toxin is the deterioration of liver and kidneys of fishes. This toxin has the possibility to accumulate in organisms at the base of the food chain as gastropods, crustaceans and bivalve [62]. Both microcystin and *cylindrospermopsin* can induce tumors and particularly in liver.

*Nodularin (NOD)* is a cyclic pentapeptid produced by *Nodularia spp* cyanobacteria, and it is known for inducing hepatic cancer [64]. Seven variants were found and they have a similar effect to microcystins [65]. NOD is a cyclo-(*-D*-erythro- $\beta$ -methylAsp-(iso-linkage)-L-ArgAdda-D-Glu-(iso-linkage)-Mdhb) and its toxicity goes to the ADDA group similarly to microcystins [66] (Figure 6). It is mostly found in sea and brackish water but can also occur in fresh waters and many removal strategies are currently under studies [67].

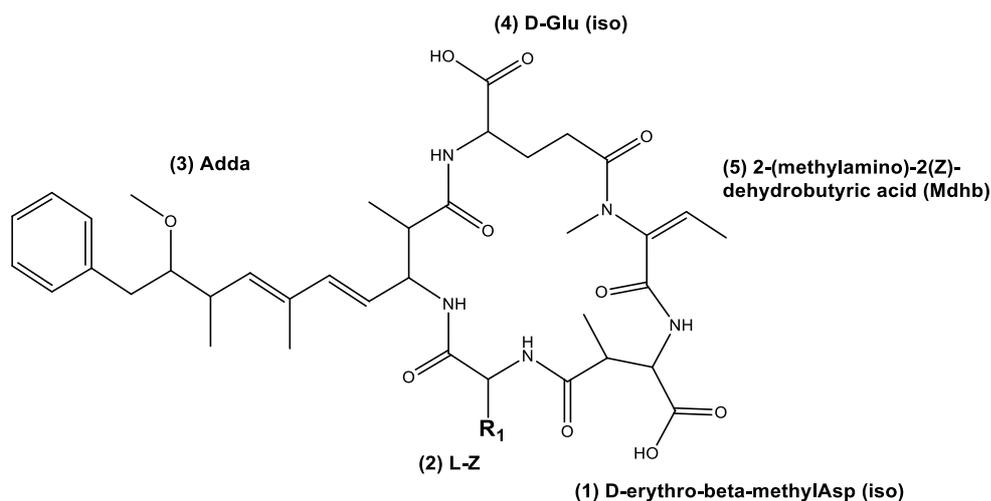


Figure 6: Nodularin.

### 3.2. Neurotoxins

*Anatoxin-a* (Figure 7), is a well-known globally occurring neurotoxin that causes a major public health concern when it comes to the consumption and exposure to contaminated surface waters. The WHO has set 30 and 60  $\mu\text{g/L}$  as guidelines for short-term drinking water and exposure to recreational activities including accidental swallowing respectively.

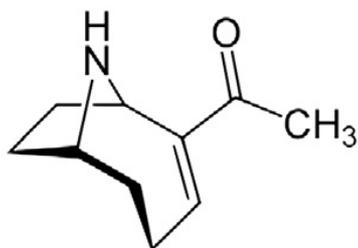


Figure 7: Anatoxin-a.

The toxin is a bicyclic amine alkaloid, produced in freshwater by a high number of cyanobacteria genera as *Aphanizomenon*, *Anabaena*, *Microcystis*,

*Cylindrospermopsis* and others. Intoxication occurs when drinking water or food is already contaminated. But it can also happen due to recreational usages of a lake or water body. Besides its stability in the environment (would last for 10 days in a natural environmental conditions in neutral pH and moderate temperatures and lightning), anatoxin-a can be degraded under the microbial activity.

Resistance to intoxication can differ from a species to another, some more are more vulnerable, the others are more resistant, but its toxic effect has been proved to be sometimes lethal. Symptoms can show from few first minutes to hours after the exposure, for that it is called “Very Fast Death Factor”, so it has a really rapid lethal effect. Normally affected species are dogs, cattle, ducks and other animals [68]. Symptoms can occur after the absorption from the gastrointestinal tract and they can be represented by muscles tremors and loss of coordination, convulsions and respiratory failure. When the respiratory system stops performing, it leads to the loss of control over respiratory muscles and then death.

*Anatoxin-a(s)* is a cyclic *N*-hydroxyguanine with a phosphate ester ( $\text{P}=\text{O}$ ). Mainly produced by in Europe and USA by *Anabaena*, and could be lethal.

The (s) goes back to salivation since following the exposure, the main symptoms are the excess of salivation and lacrimation, sometimes urinary incontinence defecation and convulsions, and when the respiratory system is down and the reduction in the heart rate and blood pressure it can lead to death [68].

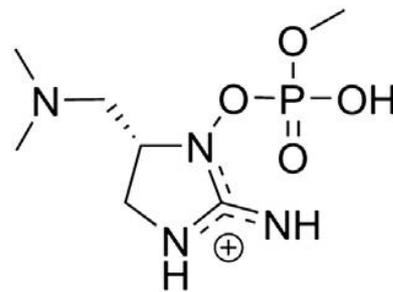
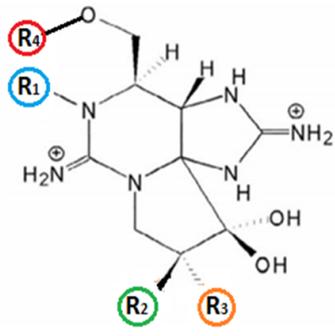


Figure 8: Anatoxin- a (s).

*Saxitoxins*, often called paralytic shellfish poisons (PSP), are produced by marine dinoflagellates and their ingestion could cause an acute intoxication. They can be also produced by cyanobacteria in freshwater such as *Aphanizomenon*, *Cylindrospermopsis*, and *Anabaena*. Saxitoxins are tricyclic perhydropurine alkaloids (Figure 9), with around 57 variants, hydrophilic, highly resistant, and stable in extreme temperatures. They accumulate in freshwater fish tissues and could not be broken or destroyed by cooking process, thus the important hazard they impose. It is noteworthy to mention that they are odor and tasteless [69].



Side Chain	Toxin	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
Carbamoyl-	STX	H	H	H	
	neoSTX	OH	H	H	
	GTX <sub>1</sub>	OH	H	OSO <sub>3</sub> <sup>-</sup>	CO-NH <sub>2</sub>
	GTX <sub>2</sub>	H	H	OSO <sub>3</sub> <sup>-</sup>	
	GTX <sub>3</sub>	H	OSO <sub>3</sub> <sup>-</sup>	H	
Decarbamoyl-	GTX <sub>4</sub>	OH	OSO <sub>3</sub> <sup>-</sup>	H	
	dcSTX	H	H	H	
	dcneoSTX	OH	H	H	
	dcGTX <sub>1</sub>	OH	H	OSO <sub>3</sub> <sup>-</sup>	H
	dcGTX <sub>2</sub>	H	H	OSO <sub>3</sub> <sup>-</sup>	
N-Sulfo-carbamoyl-	dcGTX <sub>3</sub>	H	OSO <sub>3</sub> <sup>-</sup>	H	
	dcGTX <sub>4</sub>	OH	OSO <sub>3</sub> <sup>-</sup>	H	
	B <sub>1</sub> (GTX <sub>5</sub> )	H	H	H	
	B <sub>2</sub> (GTX <sub>6</sub> )	OH	H	H	
	C <sub>1</sub>	OH	H	OSO <sub>3</sub> <sup>-</sup>	CONHSO <sub>3</sub> <sup>-</sup>
	C <sub>2</sub>	H	H	OSO <sub>3</sub> <sup>-</sup>	
	C <sub>3</sub>	H	OSO <sub>3</sub> <sup>-</sup>	H	
	C <sub>4</sub>	OH	OSO <sub>3</sub> <sup>-</sup>	H	

Figure 9: Saxitoxin.

As its name shows, saxitoxins induce paralysis-like symptoms ranging from tingling sensations around the mouth, arms/ hands, and legs/feet to expanded numbness to mouth and upper respiratory tract (throat) causing respiratory failure and finally the muscle paralysis. It all depends on the doses and the time of exposure; symptoms can appear after minutes of exposure and could last for around 3 days.

*β-N-methylamino-l-alanine* (BMAA) is a neurotoxic amino-acid (Figure 10) that is mainly linked to neurodegeneration and was seen to play a potential role in the amyotrophic lateral sclerosis (ALS)/Parkinsonism dementia complex (PDC) [70]. The compound was first detected in Guam, a volcanic American Island, when it accumulates in the Cycads plants used in the

production of tortillas, soups and at the base of the local diet [71].

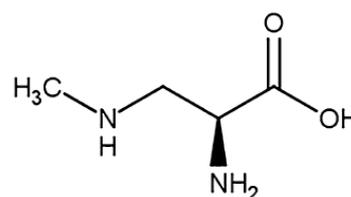


Figure 10: *β-N-methylamino-l-alanine* (BMAA).

The toxin is produced by *Nostoc spp* but seems to be secreted by all cyanobacteria, however none of the hypothesis is confirmed since reports about this cyanotoxin remain-limited. The secondary metabolite is 118 DA could be found free or protein-bound, it can be accumulated and bio-magnified; hence, the importance for further researches [72].

### 3.3. Dermatotoxins and irritants

They are toxins targeting the skin, where the level of exposure could affect more or less the negative effect and irritation caused.

*Lyngbyatoxin* is an indole alkaloid. It is mainly secreted by the filamentous cyanobacteria *Lyngbya* creating important green blooms that could happen anywhere in the world but might be more considerable in sub-tropical and temperate regions. It has three lipophilic variants called a, b and c [73].

It was proved that time of exposure do not affect the severity of symptoms, being exposed even for two minutes is enough to trigger sickness. Another important point is that even without having a contact with water; people could suffer from the symptoms due to the transport in aerosol mechanism. These toxins can be spread from water by wind and cause discomfort.

Casual symptoms could manifest by nausea, headache, vomiting and diarrhea in case of ingestion, and in case of external affection symptoms are burning, blistering, swelling of the skin, commonly called "seaweed dermatitis". Skin uneasiness is more important in ears and eyes areas and in genital and perianal ones. It is also considered as a skin tumor promoter [74].

*Aplysiatoxin* is a phenolic bislactones of a molecular mass is 671 Da. It is mainly produced by *Lyngbya*, and *Planktothrix* genus. They can cause skin irritation and blisters, they can also promote skin cancer, and when inhaled from the wind they cause inflammation of the respiratory system [75, 76].

*Lipopolysaccharides* (LPS) can be usually found in bacterial and cyanobacterial cell walls in complex with proteins and phospholipids. There is a lack of data and knowledge about the main effect of this toxin, due to the difficulties in differentiating the illness caused only by LPS and from the symptoms caused by the cyanotoxins. But some symptoms were attributed to LPS as fever, headaches, dizziness, eye and skin irritation, respiratory complications and blistering of the mucous respiratory membrane [73].

#### 3.4. Other cyanotoxins

*Anabenopeptins* are a group of non-ribosomal oligopeptides (Figure 11), known for their potential inhibition of protein phosphatase, carboxypeptidase and serine-proteases; moreover, they can be secreted by a wide variety of cyanobacterial genera [77]. They are cyclic cyanopeptides, composed of five amino acids including a conserved lysine [78]. To date more than 96 variants were reported (Figure 12 and Figure 13), as well as their co-occurrence with MCs and other CTs. Studies on this group of toxins remain scarce [79].

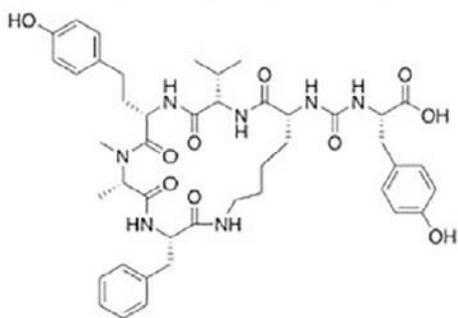


Figure 11: Anabenopeptin- A.

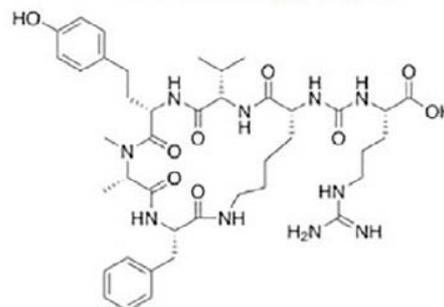


Figure 12: Anabenopeptin- B.

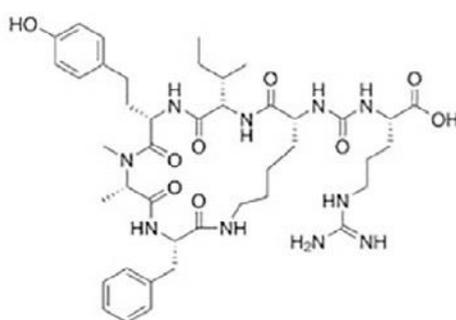


Figure 13: Anabenopeptin- F.

*Microginins* are linear cyanopeptides with 4 to 6 amino-acids where one of them is the typical, conserved, and characteristic Ahda moiety (3-amino-2- hydroxydecanoic acid, or -octanoic acid) that is at the beginning of the chain at the N-terminus [80, 81] (Figure 14). They can be produced by various cyanobacterial genera, (i.e.) *Microcystis sp* and *Planktothrix sp*, and around sixty variants have been reported [82]. They are bioactive compounds known for the inhibition of the angiotensin-converting enzyme (ACE), their activity mimics the most effective treatments for congestive heart failure and hypertension. Therefore it arises questions on the potential use of those toxins as drugs rather than their toxicity avoidance [83]. However final conclusions cannot be drawn due to the lack of assessments regarding environmental impacts and effects [84] especially after a report on their geno- and cytotoxicity [85].

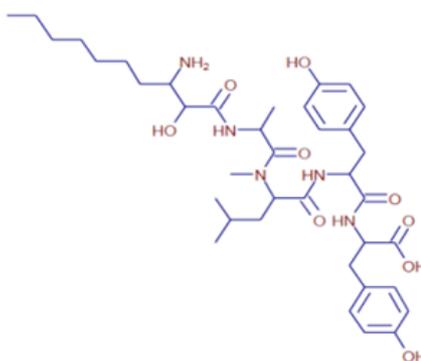


Figure 14: Microginin.

## 4. Other metabolites of cyanobacteria: taste and odor compounds

### *Causes and Impacts of cyanobacterial VOCs*

In addition to the production of cyanotoxins, cyanobacteria are able to secrete a diverse assortment of other secondary metabolites called Volatile Organic Compounds (VOCs) [129], they might belong to various classes as alkanes, alkenes, alcohols, aldehydes, esters, ketones and terpenoids [130]. Till date, no real conclusions are drawn regarding reasons behind the production of these VOCs. Nonetheless, they might be emitted as a response to environmental variations, mainly to the decrease in nutrients, and general stress [131]. They can be produced to inhibit the grazing activity that they are undergoing. Jüttner *et al.* [132] reported that in normal conditions, initial grazer-related would lead to the development of a vigorous  $\beta$ -cyclocitral micro-zone around *Microcystis* colonies; behaving as both a powerful repellent and signal of poor quality food to grazers [132].

VOCs can alter the quality of water that can serve as a drinking supply or for recreations; consequently, they can be also called Taste and Odor (TO) compounds [133]. They might be an early signal of source problems, the inflow of pollutants, a contamination and a proliferation of uncommon living organisms [134].

An outbreak of certain aquatic proliferations would have drastic effects when the water is not accepted because of its smell or taste. The usage of a water body can be omitted and switched to bottled water that is relatively costlier [7]. Lately, climate change and eutrophication has been linked to T&O production and outbreaks. But what does really causes these bad odors?

Sulfur can be present in a variety of compounds as proteins, lipids, polysaccharides, coenzymes, iron-sulfur clusters, secondary metabolites, fermentation products and other biological molecules, and a wide range of biogenic sulfurous compounds; in fact, sulfur is responsible of the emission of swampy/rotten smells in waterbodies that are so rich in nutrients and consequently with a high microbial (bacterial) activity [135].

In 2007, in China, in Lake Taihu the intensive degradation of cyanobacterial bloom material and macrophytes lead to the “Wuxi crisis”, when sulfurous T&O compounds were emitted keeping 2 million residents in severe drinking water shortages [136], [137].

The threat of VOCs and T&O s, is with their olfactory threshold, some of them can be sensed even when in extremely low levels and concentrations, of the order of 1ng/L [7]. Hence, the importance of Sensory analysis (Analysis tools like threshold odor number (TON) and flavor profile analysis (FPA)) that are used for the detection of T&O compounds and assessment of the drinking water quality.

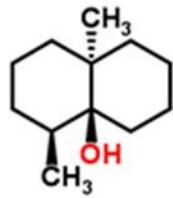
### *Most occurring cyanobacterial T&O*

Despite the wide spread and variety of cyanobacterial taste and odor compounds, some of them are so repetitive and typical. Below is a brief description of the most occurring ones.

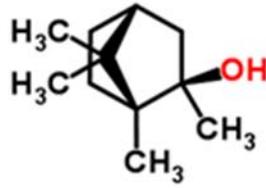
Geosmin, and 2-methylisoborneol (MIB) are earthy and or muddy scent metabolites with low odor threshold concentration (lower than 10 ng/L), they are produced by a range of bacteria. Nevertheless, cyanobacteria are their major producers. Both compounds lead to T&O problems in drinking water, but also have a serious impact on fish worldwide [86], [87]. They are so hard to remove from water via regular and common water- purification procedures. Hence, the continuous research in this field avoiding drastic losses due to shortages in water supply is always performed. For example, *Zamaydi et al.* [88] tried a new UV-H<sub>2</sub>O<sub>2</sub> advanced oxidation and aging processes for their removal from water but were left with no additional effect on the capacity to remove the geosmin and MIB from water. In fact, the earthy smell that is emitted after the rain, is due to these two T&O compounds!

$\beta$ -cyclocitral (2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), has a high odor threshold concentration of 5  $\mu$ g/L with a tobacco-woody odor and usually associated to [89]. It is the result of the oxidation of the carotenoid  $\beta$ -carotene [90]. The compound is used in food and beverage industry, in pharmaceutical products but also fragrances (although  $\beta$ -ionone is more used). It can be extracted from higher plants, from their leaves, their fruits or flowers, can also be extracted from cyanobacteria, algae and freshwater plants. It can also be produced by non -photosynthetic species as fungi. In reality, new studies reported novel considerable roles for  $\beta$ -cyclocitral in cellular signaling, in communication between individuals and in defense mechanisms against abiotic and biotic stressors [91].

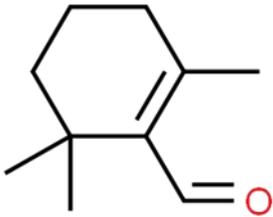
$\beta$ -ionone, has a characteristic flowery-woody odor, showing strong odor thresholds, at a level of 0.007  $\mu$ g/L and commonly occurs in algae and higher plants, and is also produced through the carotenoid cleavage of dioxygenases and its presence has been correlated with the cleavage of *Microcystis* cells. [92]. In lake Taihu (china), Concentrations of taste and odor compounds in the water column were significantly correlated with changes in pH, TN, TP, and NH<sub>4</sub><sup>+</sup> [93]. Below is a brief graphical summary of the most occurring cyanobacterial T&O, and their related consequences in (Table 2).



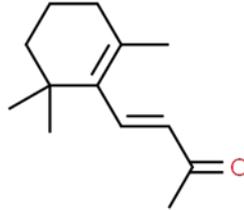
*Geosmin (earthy)*



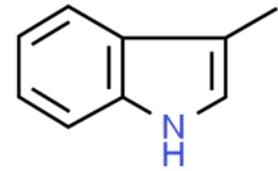
*MIB (musty)*



*beta-cyclocitral (varnish)*



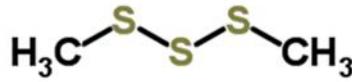
*beta-ionone (floral)*



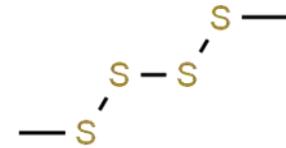
*3-methylindole (fecal)*



*dimethyl disulfide (garlic)*



*dimethyl trisulfide (septic)*



*dimethyl tetrasulfide (sulfurous)*

Figure 15: Most occurring cyanobacterial T&O compounds.

**Table 2.** List of cyanobacterial T&O

Compound	OTC µg/L	Odor
<b>Sulfurous</b>		
Dimethyl trisulfide	0.01	Septic, garlic, putrid, swampy
Dimethyl disulfide	<4.0	Septic, garlic, putrid
Methanethiol	2.1	
Ethanethiol	1	
Propanethiol	0.74	
<i>t</i> -Butylthiol	0.09	
Dimethyl sulfide	1	
Hydrogen sulfide	7.2	
<b>PUFA derivatives</b>		
<i>n</i> -Heptanal	3	Fishy, oily
<i>n</i> -Hexanal	4.5	Grassy, fatty
3-Methylbutyrate	20	Rotten, rancid
<i>n</i> -Pentanal	60	Fishy
<i>trans</i> -2-Nonenal	0.8	Cucumber
1-Penten-3-one	1.25	Pungent; rancid; fishy
<i>trans</i> -2-Hexenal	17	
<i>cis</i> -3-Hexen-1-ol	70	Grassy
2-Methylpent-2-enal	290	Rum, marzipan
<i>trans</i> -2, <i>cis</i> -6-Nonadienal	0.08	Grassy; cucumber
1,3-Octadiene	5600	Earthy/mushroom
<i>trans,cis</i> -2,4-Heptadienal	5	Fishy, oily
<i>trans,cis,cis</i> -2,4,7-Decatrienal	1.5	Fishy, oily
<b>Amines</b>		
Ethanolamine	6.5	Mild ammonia –fish <sup>y</sup>
Isopropylamine	210	Ammonical, amine
Butylamine	80	Sour, ammonical, amine
Propylamine	90,000	Ammonia
Methylamine	21	Ammonia
Trimethylamine	0.21	Pungent, fishy, ammonia
Dimethylamine	47	
<b>Terpenoids</b>		
$\alpha$ -Ionone	0.007	Violets
$\beta$ -Ionone	0.007	Violets
Epoxy- $\alpha$ -ionone	0.007	
Geosmin	0.004	Earthy/musty
3-Methylbut-2-enal	0.15	Rancid, putrid
3-Methyl butanal	0.15	Rancid, putrid
2-Methylisoborneol	0.015	Earthy, musty
Limonene	4	Citrus
Linalool	6	Grassy, floral
Ciencle (1,8)	12	Camphor, spicy, cool
6-Methyl-5-hepten-2-one	50	Fruity, esterlike
$\beta$ -Cyclocitral	19.3	Tobacco, smoky, moldy
Styrene	65	Sweet, balsamic
<b>Pyrazines</b>		
2,6-Dimethyl pyrazine	6	Cocoa, roasted nuts, coffee
3-Methoxy-2-isopropyl pyrazine	0.0002	Earthy/potato bin
2-Isobutyl-3-methoxy pyrazine	0.001	Earthy/potato bin

Note. From Mallevalle and Suffet (1987), Young et al., (1996), and Watson and Ridal (2002).

### *Co-occurrence of CTs and VOCs*

A simple field visit of contaminated surface water with cyanobacterial blooms would be enough to mention that they usually emit bad smells. In addition to the esthetic deterioration of lakes and/or ponds, the general acceptability of the water is affected. Cyanotoxins and taste and odor compounds can both be present [94, 95], however studies related to that subject remain limited.

The co-occurrence of anthropogenic pollutants and cyanobacterial secondary metabolites is poorly documented. A recent publication by [96] points out the issue of the co-existence of different cyanobacterial compounds and anthropogenic pollutants and raises questions about their toxicity and effects [97]. It highlights the different possibilities of their bioactivities and either the result would be synergistic, or additive.

Nevertheless, the relationship between cyanotoxins and T&O is not really established, and a conclusion about their correlation cannot be drawn or confirmed. The development of additional state of arts are required to fill this gap.

## 5. Analytical techniques

### 5.1. Sampling and handling

#### 5.1.1. General considerations

Remote sensing has shown to be a valuable tool or approach for the prediction and visualization of blooms on a global scale [98], but it cannot offer the toxicity assessments. Some buoys with probes were implemented worldwide to measure some basic parameters along with phycocyanin concentration, that are a crucial indicator of blooms [99], [100]. However, field sampling is indispensable for the characterization of the chemistry of cyanobacteria metabolites.

Sample collection represents a health hazard and demanding. It requires “clean” protocols and procedures; that mainly aim to limiting errors and contamination, while being the most representative, and all of that can be achieved by preliminary planning of the sampling site, the material and equipment’s to be used, the appropriate handling of samples and the routine collection of quality- control samples.

Recreational waters, and the one that serves vital activities as drinking are the riskiest exposure with cyanobacterial blooms and the relative secondary metabolites. For that, before every sampling the set of objectives has to be established and questions whether it is done for the taxonomic investigation of cyanobacteria, or qualitative and quantitative studies of their secondary metabolites in the water body, or, it could be the combination of the two approaches [101].

#### 5.1.2. Safety

In view of health hazards that cyanobacterial blooms can impose, sampling in contaminated areas should be done with precautions, using specific material and following certain instructions.

Sampling personal and individuals should be initiated to such activities and well trained. They should be equipped with Personal Protective Equipment (PPE) to protect against minimal effects as skin irritations and rashes to more serious damages as acute intoxications and respiratory issues or chronic disease because of low recurrent exposures [102]. To avoid any contact, sampling must be done with impermeable gloves (nitrile) (Figure 16).



Figure 16: Sampling kit [103].

Shoulder-length gloves might be needed if sampling requires submerging arms and hands. Splashing water on the face should be avoided, to limit all risks of contamination,

and to take all pre-cautionary measures masks and goggles would be really beneficial. However, it is always mandatory not to ingest water and minimize the direct unprotected contact with contaminated waters even via aerosolized water that could for instance come from boat motors when in motion. In case of sampling in open waters, life vests are important. One should never touch mouth or any exposed body surface without washing hands with soap and abundant water. In case if the sampling requires the immersion of feet and legs, plastic tight length boots are recommended for more safety [104] (Figure 17).



Figure 17: Sampling with knee- length boots [105]

### *5.1.3. Sampling protocols and reporting*

Before choosing sampling materials, it might be interesting to assess the volumes needed for the analyses. Water samples can be collected in plastic, glass, borosilicate and dark amber bottles. Tissue samples are to be wrapped with aluminum foil and put in a zip lock bag with all the important notes written. All containers should be checked for safety and cleanliness before usage to avoid the loss and contamination of sample [106].

In the case of a monitoring program or a long term study, it is important to distinguish the main objective or target. It is crucial to decide if the analyses are to monitor the presence of cyanobacteria alone or their secondary metabolite (Figure 18). In the former case, much more sampling is needed, however in the latter case less samplings are required but their associate tests and lab works would be much more expensive and

demanding. In order to be able to know the main aim, the Oregon Public Health Division created this diagram to better approach the toxin- monitoring based program [107].

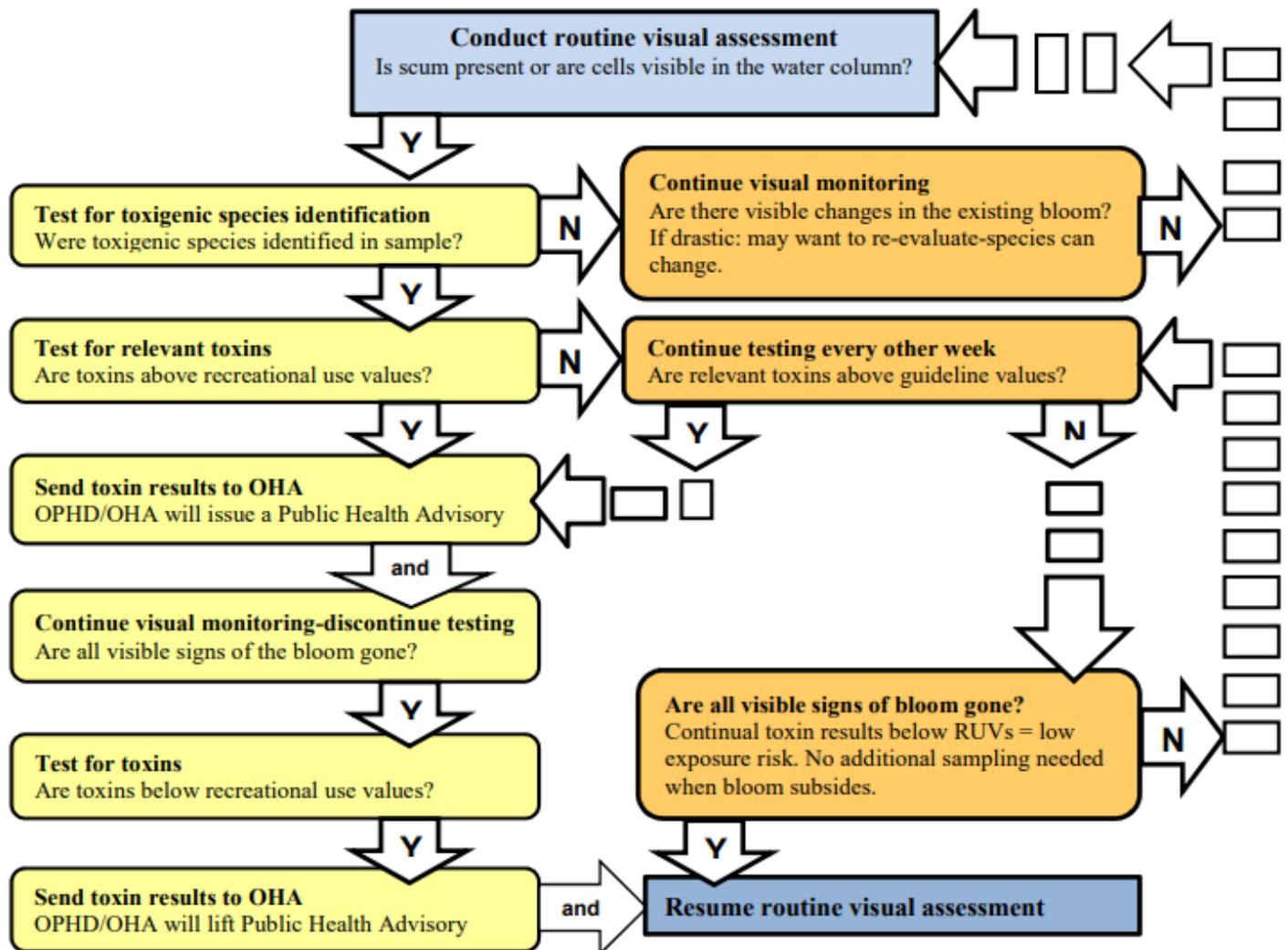


Figure 18: Recommended approach to maintain a toxin- based monitoring program (Health Authority, 2018).

Documenting the state of a field or an area has become easier to trace; taking pictures, coordinates and important data can serve as evidence of field campaigns conditions [107]. A photo album of a certain area would serve as an archive for the comparison of seasonal changes, the recurrences of blooms, their variations through time periods and environmental conditions.

Field documentations can include:

- Date, Time, Coordinates
- Weather conditions and temperature (sunny, cloudy, cold, hot ...)
- Waterbody's conditions (stable, waves, currents ...)

- Estimation of the water level (low, medium, high ...)
- Proofs of recent unusual events (floods, fires, natural disaster...)
- The presence or absence of cyanobacterial blooms, colonies or water coloration
- Water transparency and texture
- Presence/ Absence of scums or hyper-scums
- Smell emissions that could be linked to cyanobacterial blooms

In each sampling point, parameters should be reported along with the date and position. They can include: water temperature, turbidity, transparency by gauging the distance of Secchi disk, pH, conductivity and dissolved oxygen. All of them can be measured with portable equipment.

#### 5.1.4. Water sampling

Cyanobacteria taxonomic identification, their enumeration, and their secondary metabolites analyses should be performed on the same collected sample. Hence, it is important to determine the purpose of sampling in order to determine the needed volume for all the analyses.

Water sampling is preferably to be performed during the day, from 10 am to 3 pm, since the maximal biomass has been proved to occur at noon (12 pm) [102].

Samples can be collected at the surface (0.5- 1.0 m) or from depths usually using Niskin bottles [108] (Figure 19). The bottle is opened from both sides and hanged with a rope where a weight is placed. Once in the water, the weight can be dropped, and when it reaches the bottle, the caps are automatically closed, and the water is trapped [109]. This equipment allows a representative sampling of a targeted water layer for the purpose of the assessment of the quality of water to be ultimately used for drinking or for the general evaluation of the ecosystem.

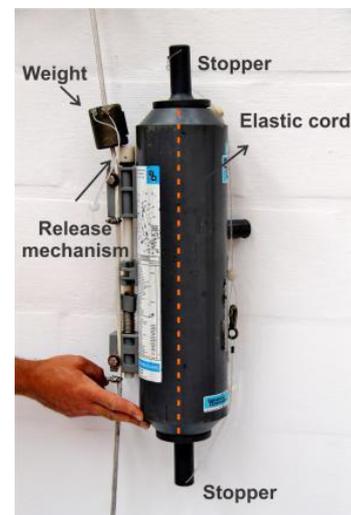


Figure 19: Niskin bottle sampler (Niskin bottle | Flanders Marine Institute, no date).

Manual sampling using a wide opened container (plastic or glass) is recommended for scums that occur close to shorelines. It can be done by simply and carefully collecting the most surface layer of dense floating cyanobacteria. Or, can be done after mixing the scum within the few centimeters of the shallow water; so it could give a general idea of

cyanotoxins concentration in the scenario where kids are playing in recreational waters. For a holistic approach, both types of sampling should be implemented.

Note that some recent studies showed that grab sampling of water is not very representative and opted for new passive sampling techniques [110, 112].

#### *Water preservation.*

Freezing water will cause cyanobacterial cell lysis, so when the identification of cyanobacteria and their enumeration are targeted, avoiding temperatures below 0°C is recommended. For cyanobacterial preservation a solution of iodine (Lugol) is added to reach 1% of the final volume [113, 110, 114]. However, Hawkins *et al.* reported a shrinking of cyanobacteria's size after 2-7 days following the addition and advised just to preserve water at low temperature in the dark [115]. For cyanotoxins analyses, water samples are kept at low temperatures up to 28 days. In such conditions, intracellular and extracellular toxins can be quantified. However, for microcystins, cylindrospermopsins and saxitoxins, optimal results are obtained within 5 days. Samples must be frozen [104]. When water is sampled for molecular approaches, samples should be immediately filtered, at most within 24 h after sampling [113,116].

#### *Water samples preliminary handling.*

Cleanup and pre-concentration steps by solid phase extraction, described in the next paragraphs, is often recommended.

#### *5.1.5. Fish sampling and handling*

Targeted species and their size must be defined prior to sampling, as a function of the project's needs and the local regulations. Normally, 5 to 15 fishes per sampling site are sufficient for analyses, their size usually follows the rule of 75%, it means that, the smallest fish should be at least 75% of the size of the biggest fish caught [102]. If this variety cannot be assured, the edible size should be collected. Each fish is wrapped up in aluminum foil and then put in a zip-locked bag at 4 °C - 6 °C) until it reaches the laboratory. It is then sacrificed, and dissected within 24 h. GPS coordinates of the sampling site are reported. The label on each sample bag has to contain date, time, place, fish species of sampling. All material used in the fish collection (nets, hooks, bags...) should be clean, and washed between samplings.

Before the dissection, fish is described by species name, length, weight, sex, features (eyes, colors, marks, cuts and wounds...) and other (color of organs, freshness of the sample...).

#### *Sample preliminary handling.*

Fish samples are frozen at -80°C, freeze-dried and powdered to facilitate extraction procedures. The main used containers and the initial sample handling steps are shown in (Table 3).

**Table 3.** Samples preliminary handling

Sample	Container and its recommended size	Preservation	Preliminary handling
Water for cyanobacterial identification and enumeration	Amber glass or plastic bottles of 125 mL (or more)	Add 1 mL Lugol to every 100 ml of the sample for minimum 1% Lugol's Iodine concentration	Kept at low temperature
Water cyanotoxins analyses	Amber glass or plastic bottles of 125 mL (or more)	Chill with ice (wet or dry) from 4°C to 6°C	Filtration/ Solid Phase Extraction
Fish tissue samples	5 to 15 fishes per sampling point each wrapped in foil paper and conserved in a zip lock plastic bag	Chill with ice (wet or dry) from 4°C to 6°C	Dissection and freezing. Could be followed by lyophilization

## 5.2. Samples pre-treatment: solid phase extraction (SPE)

### 5.2.1. Principle of solid phase extraction

Solid phase extraction is based on the partitioning of an analyte or of a group of analytes between a solid sorbent and a liquid phase [117]. It serves to reduce sample volume, pre-concentrate analytes and purify them, but it is time consuming and demands a skilled operator. The procedure usually requires four steps [118] (Figure 20):

*Step 1: Conditioning.* The sorbent material is activated by a suitable solvent followed by another solvent that is similar to that in the sample. It will wet the dry solid phase while removing air and impurities and enabling the activation of functional groups.

*Step 2: Loading.* The sample is passed through the sorbent material, by gravity, by pumping or by vacuum aspiration. The percolation or the flow rate should be adapted to give the analytes enough time to be retained, but not too much to avoid blocking. Samples volumes can be from 1 ml to 1 L depending on the system itself and material used. This step will allow the concentration of analytes on the sorbent and its purification from matrix constituents.

*Step 3: Washing.* This is an optional step. It will allow the washing of the sorbent with a low elution strength solvent (usually water), to eliminate matrix impurities, while retaining the targeted analytes. To avoid the presence of water in the final sample/extract, a drying step is recommended and usually done under vacuum.

*Step 4: Elution.* The desired analytes can be washed out / eluted from the sorbent material with an appropriate solvent at a suitable flow rate. The nature of the solvent

and its volume are to be decided based on the chemistry of targeted components and the desired quantifications.

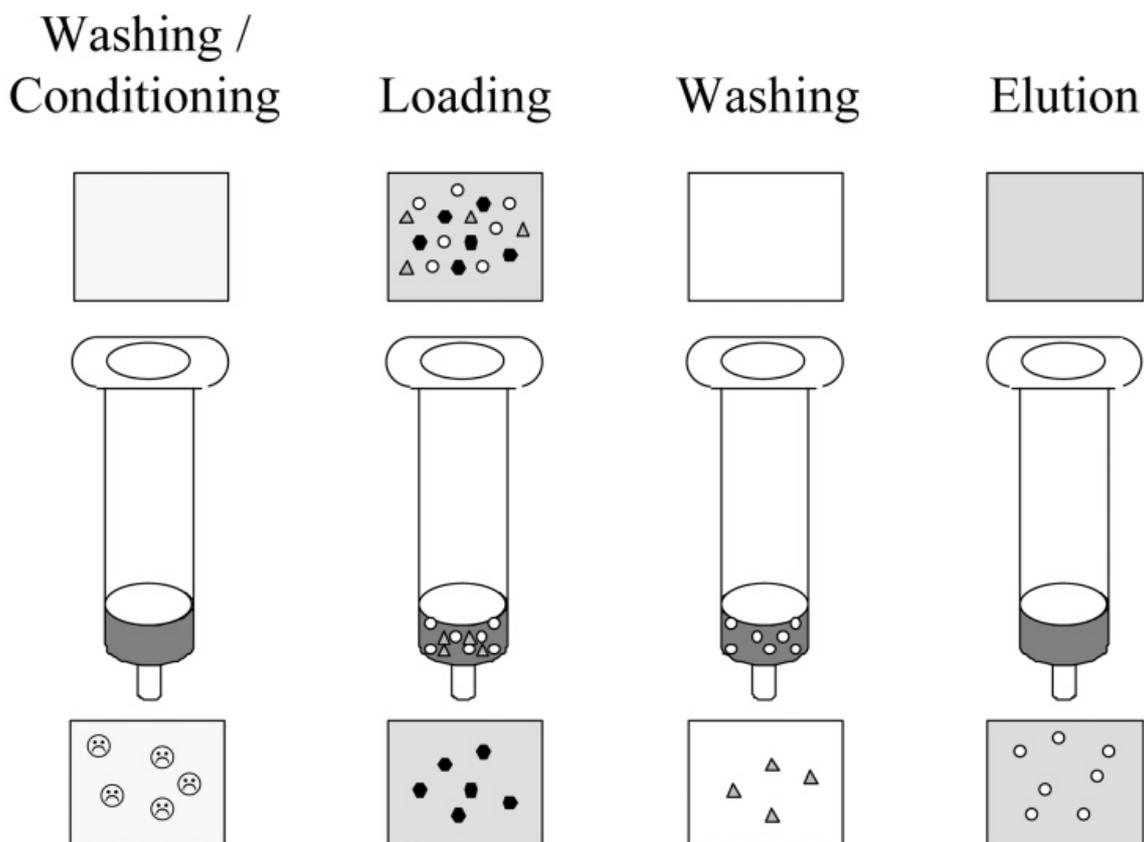


Figure 20: SPE steps [118].

### 5.2.2. System's geometry

SPE sorbent material can be used in different setups. It can be in micro-columns (Figure 21) that could be of different sizes, customized and filled with the desired sorbent material. They are preferably used in on-line systems. They can be reusable, so care must be taken as to their washing protocols [119].



Figure 21: Micro SPE columns [120].

Another SPE format is disposable syringe barrels or cartridges (Figure 22). They cover a large variety of sorbents and are commercially available in different sizes ranging from 1 to 25 mL. The syringe/cartridge is usually made of polypropylene or polyethylene and contains from 100 mg to 1 g of sorbent.

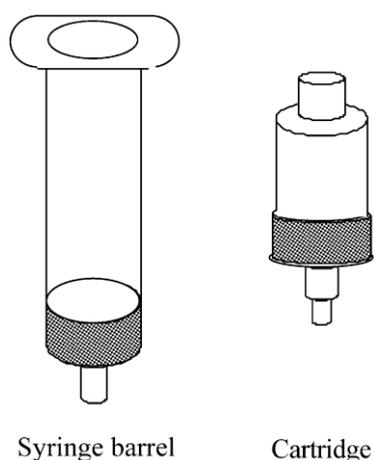


Figure 22: Syringe barrel and cartridge for SPE [118].

Both can fit on SPE manifolds (Figure 23) due to their male Luer at the bottom of the cartridge or barrel that would allow them to operate in vacuum mode. The cartridge, in addition has a female Luer at the top and can be processed under a positive pressure. The disadvantage is the slow sample processing speed [121].



Figure 23: SPE manifold, with vacuum ports [122].

Sorbent material can also come in disk format (Figure 24).



Figure 24: SPE Disks [123].

They are mainly used when a strong interaction between the sorbent and analyte is present [124]. Flat disks come with the inert matrix of glass fiber or polytetrafluoroethylene (PTFE) microfibrils with a ratio of 90:10 with the sorbent material. They are available in different sizes 4 to 90 mm with 47 mm the most frequently used. They are usually used with systems of water filtration. Recently, smaller volume samples disks have emerged and they are similar to the syringes barrels [118].

SPE can be automated and can be coupled to analytical systems, such as liquid chromatography. Such an approach will minimize sample losses, will increase

reproducibility but will not allow the use of extracts resulting from the SPE in different analyses.

In summary, SPE is a convenient tool to pre-concentrate samples, to reduce their volumes while keeping the desired compounds with a high selectivity, and to remove matrix. In addition, the consumption of toxic solvents is much lower than in liquid-liquid extraction (LLE) procedures.

### *5.2.3. Sorbent materials*

The choice of sorbent material in SPE depends on the chemical composition and nature of the sample and of the target compounds. Sorbent materials are divided into two groups: organic and inorganic [118]. The main inorganic sorbents are silica and octadecyl bound to silica materials (C18-silica). Others include polystyrene-divinylbenzene, divinylbenzene-vinylpyrrolidone copolymers, polyurethane polymers, polyethylene polymers, polystyrene polymers, carbon sorbents and others.

In this section, two particular types of sorbents will be discussed, due to their importance in this study. Divinylbenzene-vinylpyrrolidone copolymers (DVB-VP) allow the retention of polar organic compounds (rather than trace elements) in water samples or in other types of extracts. The hydrophobic divinylbenzene allows the retention of analytes in a reversed-phase mode, while the hydrophilic *N*-vinylpyrrolidone permits a good wettability and a good stability on a wide pH range. An example is Oasis HLB sorbents.

The other type is graphitized carbon black (GCB) sorbents which are produced by heating carbon blacks at 2700–3000 °C in an inert atmosphere that give more stability than the activated carbon. The retention is done in reversed phase mode. Activated carbon is known for its high potential for retaining organic and trace elements via van der Waals forces and via hydrogen bonding. The disadvantage is its heterogeneous surface that would affect the reproducibility. In order to cope with the problem of the irreversible retention and the need of backward elution, porous graphitized carbon PGC have been implemented in the extraction of organic compounds, offering a higher stability [125].

### *5.2.4. SPE in the analysis of water samples*

Cyanotoxins occur in waters at low concentration, they could degrade or they could conjugate to other molecules. Solid phase extraction enables their pre-concentration, allows reduction of matrix effects, and improves the LODs (limits of detection) of the analytical method [74]. Several methodologies have been developed:

Draper *et al.* [126] used a C18 cartridge for the concentration of MC/ RR- YR- LR- LA- LF- and LW from water samples, and the recoveries of the arginine containing microcystins ranged from 75% to 90% with the use of 0.1% of TFA in the elution solvent.

Guzmán-Guillén *et al.* [127], developed a method for the SPE of cylindrospermopsin in water samples using a graphitized carbon cartridges that were activated with 10 ml of a mixture of DCM:MeOH (10:90, v/v) 5% formic acid (v/v) and then rinsed with 10 ml of water, and rinsed with the same water after passing the sample and eluted with 10 ml DCM/MeOH (10/90 v/v). the sample was concentrated 800 times after its evaporation and reconstitution in 250µL with pure water. The slight acidification of solvents is crucial for better recoveries (in cylindrospermopsin extractions) that ranged between 38 and 95%.

Yen *et al.* and Zervou *et al.* [74, 125] implemented a dual cartridge assembly in their SPE system, the former used reverse phase C18 cartridge (2000 mg packed in a 12 mL Supelclean\_ LC-18 SPE Tube, Supelco) and a carbon cartridge (500 mg packed in a 6 mL Supelclean\_ ENVI-Carb SPE Tube, Supelco) and eluted them with 40 ml of 90% MeOH/H<sub>2</sub>O, recoveries ranged between 83 and 104%; the latter used the assembly of an Oasis HLB (200 mg, 6 cc, 25–35 µm, Waters Corporation, USA) and HyperSep Hypercarb PGC (porous graphitic carbon, 200 mg, 3 cc, 30–40 µm), and eluted them in a reversed order and eluted them with 10 mL of a mixture of DCM:MeOH (40:60, v/v), containing 0.5% FA. The authors were able to extract 12 variants of MCs, nodularin, cylindrospermopsin, okaidic and domoic acid with recoveries ranging from 62 to 113%.

#### 5.2.5. SPE in the analysis of fish samples

Cyanotoxins ingestion accidents are rare, but their chronic effects may be related to long (term consumption of contaminated food. The common source of cyanotoxins intake is fish. The guideline set by the WHO is 40 ng/kg of body weight as a tolerable daily intake of the LR variant of microcystins (MC- LR) when consuming animal tissues and organs [128].

The analysis of fish samples requires an adequate extraction procedure from organs and tissues, a clean- up step is often desired. SPE is important to preserve chromatographic columns and mass spectrometers from heavy contamination, and helps to reduce the matrix effect in bio-assays [129].

Cyanotoxins in fish can be free or cell-bound and their concentrations can differ as a function of the feeding mode of the species. The levels are usually higher in omnivorous than in carnivorous fish (omnivores > phytoplanktivores > carnivores > zooplanktivores) [130].

Different extraction solvents were proposed. Extraction protocols usually implement sonication and centrifugation steps that enable the cleavage of toxins from the matrix and their collection from the supernatant. Manubolu *et al.* [131] optimized a method for the extraction of MC-LR/RR from fish muscles using 3 different extraction mixtures: (methanol and water) MeOH/H<sub>2</sub>O (80/20), (methanol, water and butanol) MeOH/H<sub>2</sub>O/BuOH (75/20/5) and 5% acetic acid and (Ethylenediaminetetraacetic acid) EDTA (0.01 M), coupled to different SPE sorbents. It was found that the maximum recovery (around 93%), was obtained using methanol:water:butanol solvent system coupled to SPE column (HLB or activated charcoal).

An additional extraction step is sometimes used to remove lipids that can affect liquid chromatography. It can be done by the use of solvents, such as hexane or sorbents, such as silica [132, 133].

Note that all the above cited examples are for the free microcystins. For microcystins covalently bound to protein phosphatase, a more advanced technique is needed. The MMPB method, that is based on the Lemieux oxidation of total microcystins in fish tissues and organs showed to be more significant in terms of microcystins real concentrations. The MMPB method oxidized all microcystin congeners and produced the common fragment from the Adda [(2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4E, 6E-dienoic acid] amino acid: 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) that could be detected and quantified [134].

On the other hand, it was found that fish species, in order to cope with cyanotoxins lethality, have developed an advanced detoxification mechanism based on the 1) excretion, 2) the activation of P450-monooxygenases, or 3) the conjugation of toxins to glutathione, cysteine, S-transferases, and/or other transferases [135]. Hence, for the detection and quantification of the totality of toxins, it would be interesting to evaluate also the covalently bound ones, using an appropriate extraction methodology.

Zhang *et al.* [136] reported the potential conjugation of MC-LR to cysteine and glutathione. They showed that MC-LR-GSH or Cys eluted earlier than MC-LR itself (Figure 25). Consequently, during the detection and quantification of cyanotoxins in fish samples, conjugated and cell-bound microcystins and toxins should be taken into consideration to assess the associated health risks.

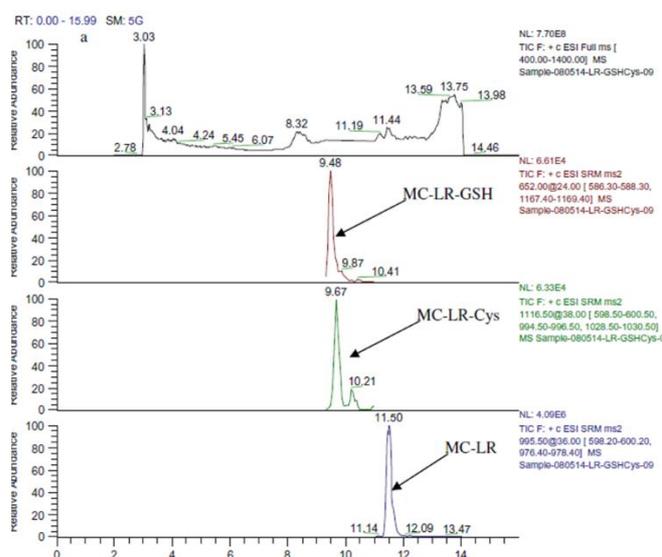


Figure 25: Chromatogram of MC-LR, and the conjugated MC-LR [184].

A succinct summary of studies conducted on fish samples for the detection and quantification of cyanotoxins is shown in (Table 4).

**Table 4.** Fish samples pre-treatments

Study	Cyanotoxins	Sample matrix	SPE system	Elution Solvent
<b>Mekebri <i>et al.</i> 2009</b> [137]	MC/LR- RR- YR- LA- LF- LW	Water, mussels, oysters, rainbow trout	C18, 6cc, 500mg	MeOH/H <sub>2</sub> O (90/10), 0.1%TFA
<b>Hardy <i>et al.</i> 2015</b> [138]	MC/LR- RR- YR- LA- LY	Yellow perch (fish)	C18, 6cc, 500mg	MeOH/H <sub>2</sub> O (90/10), 0.1%TFA
<b>Manubolu <i>et al.</i> 2018</b> [131]	MC/RR- LR	Walleye fish ( <i>Sander vitreus</i> )	Trial of 3 cartridges : HLB, C18 and activated charcoal	25 ml of MeO/H <sub>2</sub> O (80/20)
<b>Haddad <i>et al.</i> 2019</b> [68]	Nod, Atx-a, Cyn, Sax, and MCs	Naïve channel catfish ( <i>Ictalurus punctatus</i> )	A Supelclean ENVI-carb (6cc, 500mg, Supelco Inc., Bellefonte, PA, USA) for Atx-a, Cyn, Sax and an Oasis HLB (6 cc, 200 mg, Waters Corporation, Milford, MA, USA) for Nod and MCs	Envi-carb eluted with 10 ml MeOH/DCM (60/40), 0.5% F.A  And HLB eluted with 10 ml of MeOH, 0.5 % F.A

#### 5.2.6. Sample pre-treatments for volatile organic compound analysis

Investigations on VOCs of cyanobacterial origin can be done by GC/MS or LC/MS or by the use of molecular approaches as polymerase chain reaction (PCR) text, searching for the genes coding for VOCs production. As each kit for molecular analysis has its own protocol to be followed, so the sample pre-treatment should be adapted to it.

In gas chromatography, solid-phase-micro- extraction have largely replaced the traditional extraction techniques. Reports about cyanobacterial T&O compounds have been relatively scarce. *Wang et al.* [139] filtered water samples with glass-fiber filters (GF/C, 1.2 µm, Whatman, UK). The extraction of VOCs was done manually. First, 15 g of NaCl was added, then a double extraction with dichloromethane was performed, followed by a dehydration with 30 g anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporation.

Nowadays, to reduce time and limit solvent consumption, automated extraction can be coupled to GC. This is the case of HS-SPME (head-space solid-phase-micro-extraction). HS-SPME targets the extraction of VOCs that are volatilized in the head-space of the vials. The principle of the extraction is based on exposing a small amount of extracting phase that coats a fiber, to the sample, for a predetermined amount of time under predetermined conditions.

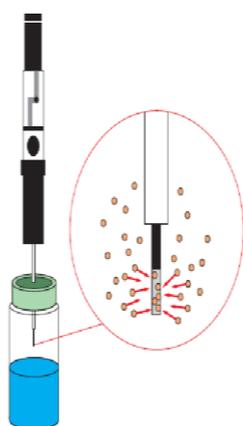


Figure 26: SPME Fiber.

There are two types of SPME coatings: polymeric **films** for absorption of analytes, or **particles** embedded in

polymeric films for adsorption of analytes (Figure 27). The adsorbent type (film) fibers include: polydimethylsiloxane (PDMS), polyacrylate (PA), and polyethylene glycol (PEG). The adsorbent type (particle) fibers contain porous particles such as: divinylbenzene (DVB), Carboxen® adsorbent (CAR) or a combination of both. Usually, PDMS is used as binder. As the adsorption on a particle is a stronger and more efficient consequently, particle fibers were mostly used for trace and ultra-trace analysis. For cyanobacterial VOCs, DVB/CAR/PDMS has been mostly used [186, 187].

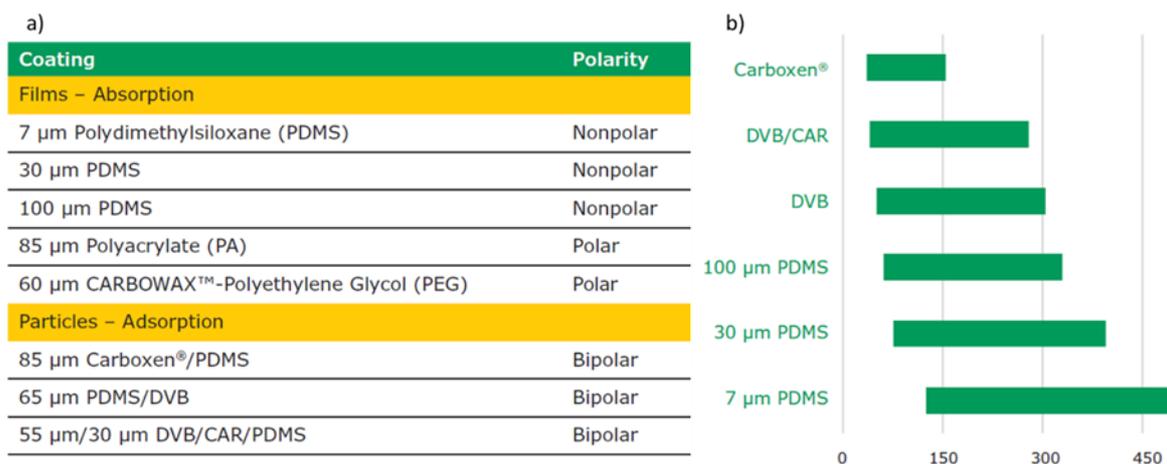


Figure 27: Different types of fibers; a) their polarities, sizes and b) their suitability to analyte molecular mass range (g/mol) [142].

Samples (water) are usually mixed with a certain mass of NaCl that enables the salting-out effect, to ease and maximize the volatilization of compounds during the extraction phase [143]. The headspace extraction is done in different steps [144] (Figure 28):

*Step 1:* the pre-incubation time during which the sample is under controlled parameters as temperature and agitation, to help and facilitate the volatilization of compounds.

*Step 2:* the extraction when the fiber enters the vial, remaining in the headspace for a certain time and under controlled conditions (again temperature and agitation). VOCs will adsorb on the fiber.

*Step 3:* the desorption time when the fiber is moved to the injector of the gas chromatograph, so compounds could be released, separated in the chromatographic column and finally analyzed with an adequate detector.

Extraction conditions: Incubation time, extraction time, desorption time, NaCl content, and fiber content are parameters to be optimized for the maximum extraction of target analytes [141].

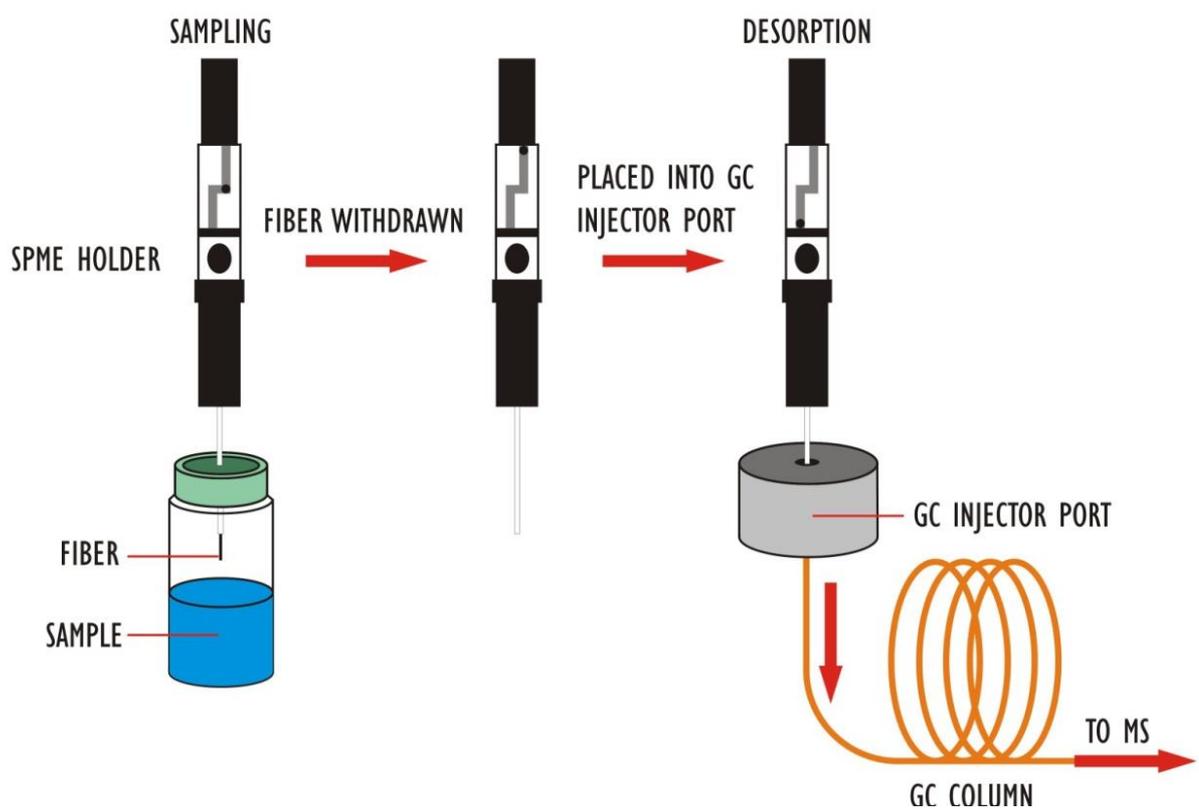


Figure 28: The 3 steps of SPME [144].

### 5.3. Bioanalytical techniques

#### 5.3.1. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) has been commonly used for the detection and quantification of cyanotoxins. It can be used for the preliminary testing for the secondary metabolites in ecosystems [145]. It is based on the principle of antibodies against microcystins, saxitoxins, nodularin and cylindrospermopsin depending on the kit and the objective of the analyses [146]. Kits against MCs (targeting the ADDA group) are the most used.

ELISA offers results within few hours. It can detect MCs, but cannot recognize the different variants. The results are given in MC-LR equivalent. The limits of detection are usually between 0.1 and 10 µg/L [16] and match the WHO guidelines. The limitations of this technique include the overestimation, the cross reactivity, the sensitivity to matrix (matrix-effect), the specificity and the high cost of consumables.

**Table 5.** Benefits and limitations of ELISA

Benefits	Limitations
Fast and easy assay for a primary screening	Relatively high cost of consumables
Suitable for qualitative and/or quantitative detections	Cross-reactivity and matrix effect So positive results require confirmatory alternative methods
Good limits of detection	Kits specific to some cyanotoxins

For instance, Gault *et al.* [147] reported an important overestimation of results, where concentrations of analytes using ELISA were 3 to 8 times higher than the ones quantified with LC-MS/MS. False positives or overestimations can be seen especially at low concentration, whereas with higher concentrations results would correlate with additional methods [148]. Another drawback of the assay is its limitation towards the differentiation among the individual microcystins because the antibodies are for the common ADDA moiety. Bláhová *et al.* reported in 2009 that cylindrospermopsin concentrations measured with ELISA (0.4 - 4.0 µg/L) were higher than those measured with LC-MS/MS (0.01-0.3 µg/L) [149]. In another study, using an in-house developed indirect competitive ELISA, MC-LR at the average level of 0.879 µg/L were reported to be present in various reservoirs in Czech Republic [150].

#### 5.3.2. Protein Phosphatase Inhibition Assay (PPIA)

Protein Phosphatase Inhibition Assay (PPIA) is a test based on the inhibition activity of microcystins and nodularin over the serine and threonine phosphatase enzymes. They are type 1 (PP-1) and 2A (PP-2A) protein phosphatases that are usually in charge of the dephosphorylation of proteins and the regulation of cell age [151].

The assay is widely used as a fast and primary screening method. A kit is commercially available and relies on the colorimetric inhibition of PP2A by the two CTs on the p-nitrophenyl phosphate substrate that are hydrolyzed and liberate the colorimetric product p-nitrophenol [152]. The assay offers results within few hours. It requires only one incubation of the microplate for 30 min at 37 °C [16] within 0.25 and 2.5 ppb as limits of detection matching the WHO guidelines for cyanotoxins in water samples [153].

Some issues are related to matrix effects. When complex matrices, extract purification is recommended by SPE. Since the assay tests the inhibition activity of toxins, it is preferable for more accurate results to complete analyses by mass spectrometry [154].

**Table 6.** Benefits and limitations of PPIA

Benefits	Limitations
Fast and easy assay for a primary screening	positive results require confirmatory alternative methods
Suitable for qualitative and/or quantitative detections	Cross-reactivity and matrix effect
Good limits of detection	Kits specific to some cyanotoxins
Detects the inhibitory activity of microcystins	Cannot differentiate microcystins variants

Simiyu *et al.* [17] determined microcystins in concentrations that ranged between 11 and 109 ng/DW of fish samples but the concentration measured with LC-MS/MS were much lower. Note that concentrations found with ELISA were almost 8 times higher than those found with PPIA ( $R^2= 0.29$ ,  $p<0.0001$ ).

### 5.3.3. Molecular approaches

Molecular approaches in the investigations during cyanobacterial blooms are considered as first probing step for the presence of cyanotoxins as they are rapid and cost effective [155]. The availability of sequenced microbial and cyanobacterial genome and the development of polymerase chain reaction PCR, made it possible to start tracking the presence of cyanobacterial genes in a water sample by pursuing typical cyanobacterial gene sequences as the 16 S rRNA gene fragment, *rpoC1*, *rbcLX*, *rpoB*, *rRNA 16S-23S ITS fragment* and other toxic genes fragments responsible of the production and secretion of CTs (MCs, Nod, STXs, Ana-a, CYN) [116]. Consequently, PCR offers an early toxicity diagnosis of CyanoHabs.

The sample preparation is relatively easy, and it basically relies on the extraction of the genomic material. Following that, samples are amplified in a thermocycler and results are obtained within few hours [156].

Different types of PCR were used during blooms. We can cite the qualitative conventional PCR that targets one gene only and enables the identification of the presence of cyanobacterial genes in a sample. For a quantitative approach q-PCR can be used, where after each amplification cycle the level or amount of genomic material is quantified via fluorescent markers and a fluorescence curve. On the other hand, and for more specificity, the combination of PCR and DNA microarray can be implemented. A specific gene mainly the one responsible for the production of microcystins and nodularin is targeted [157].

The limitation of the technique is the for well-trained personnel [146]. In addition, sometimes, a certain gene can be detected but can be coming from a decaying cyanobacteria, which means that it will not be expressed. The presence of a toxin genes doesn't always mean that the toxin is present in the environment, since it could be degraded [158].

**Table 7.** Benefits and limitations of q-PCR in the investigation on cyanotoxins

<b>Benefits</b>	<b>Limitations</b>
Fast assay for an early and primary screening	Relatively high cost of consumables and apparels
Suitable for qualitative and/or quantitative detections	Requires a very clean and sterile work environment
Covers a wide range of genes	Requires a trained personnel

## 5.4. Gas chromatography

### 5.4.1 Principle of the technique

Gas chromatography coupled to mass spectrometry (GC/MS) separates chemical analytes in the gas phase and identifies them by their molecular mass and/or fragmentation pathway. The gas chromatograph employs a capillary column with specific characteristics (length, diameter, film thickness) with specific phase properties. Heated inert gas (such as helium or argon) carries the analyses through a column with an [159] to the mass spectrometer. Molecules in the mixture are separated as a function of their chemical properties. The separation is done in the chromatographic column, while they are travelling it taking different amounts of time (called the retention time) to come out of (elute from) the gas chromatograph, to finally arrive to the mass spectrometer downstream [160].

Working with certain matrixes, such as cyanobacterial blooms require a minimal sample cleanup. For analyses in the field of cyanobacterial blooms, samples could only be filtered and then diluted in a certain volume of water. If samples are clean this step could be omitted [161, 162].

Mass spectrometers or mass-selective detectors (MSDs) have replaced the classical detectors such as Flame ionization detector (FID) because of their sensitivity and specificity. In a GC/MS analysis, a mass spectrum can be obtained continuously at fixed intervals of time. Therefore, a mass spectrum can be obtained for each chromatographic peak or any point on the chromatogram. Providing a rich data set and a lot of information for results interpretations.

Detection limits can be improved by the use of single ion monitoring (when the mass spectrometer is used for detecting the intensity of one or a few ions), providing the specificity of a mass spectral pattern for response without losses in detection limit.

For better resolutions, analyses could be done in tandem mass spectrometry (GC-MS/MS) that will allow fragmentation of analytes and will have additional confirmatory data [163].

### 5.4.2. Analysis for cyanobacterial VOCs

The implementation of GC-MS in the investigation on VOCs has been widely documented. In (Table 8) are applications of investigations on cyanobacterial VOCs using GC-MS have been summarized.

**Table 8.** Overview of cyanobacterial VOCs

Publication	Sample matrix	Sample Pre-treatment and analyses	SPME Fiber	Species measured
[164]	cyanobacterial culture broth	HS-SPME-GC-MS	DVB/CAR/PDMS	$\beta$ -Ionone, geosmin and 2-MIB
[165]	4 ml of 100 cyanobacterial strains culture	HS-SPME-GC-MS	DVB/CAR/PDMS	Geosmin and 2-MIB
[166]	2 ml of environmental water sample diluted in pure water	HS-SPME-GC-MS	DVB/CAR/PDMS	Geosmin and 2-MIB
[139]	Raw water and finished water samples	Chemical extraction with dichloromethane/GC-MS/MS	-	organic sulfides, aldehydes, benzenes, phenols, ethers, esters, ketones, nitrogenous heterocyclic compounds, 2-methylisoborneol and geosmin.
[141]	Surface water, treatment process water, and tap water	HS-SPME-GC-MS	Trial of several fibers with DVB/CAR/PDMS the best	geosmin, MIB, 2,4,6-trichloroanisole (TCA), 2-methylbenzofuran, 2-isopropyl-3-methoxypyrazine (IPMP), 2-isobutyl-3-methoxypyrazine (IBMP), cis-3-hexenyl acetate, trans,trans-2,4-heptadienal, trans, cis-2,6-nonadienal, and trans-2-decenal

#### 5.4.3. Analysis for cyanotoxins

Studies of cyanotoxins by gas chromatography have been scarce because of their poor volatility; LC has been by far the most widely used technique [22].

In the case of microcystins, an oxidation that splits the Adda ((2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid) side is required before the analyses. It produces the 3-methoxy-2-methyl-4-phenylbutyric acid called MMPB, and the detection and quantification will be for total MCs. This method will not allow the differentiation of variants and the intracellular or extracellular content or even the conjugated MCs. Moreover, results cannot be given in MC-LR equivalents and conversions should be done to fit the guidelines. Thus, it cannot be used in a monitoring plan [167]. In the case of anatoxin, derivatization was needed before GC-MS [168].

## 5.5 Liquid chromatography MS/MS

### 5.5.1. Principle of the technique

Liquid chromatography coupled to mass spectrometry is based on the separation of compounds on the bases of their affinity to a chromatographic stationary phase followed by their ionization at an ion source, such as e.g. APCI (Atmospheric Pressure Chemical Ionization) or ESI (Electrospray Ionization). The produced ions are separated as a function of their mass to charge ratios ( $m/z$ ) and finally detected and counted.

The key benefits of LC-MS are sensitivity, specificity and precision as analysis is done at molecular level, giving important structural details of targeted and untargeted analytes.

Tandem mass spectrometry (MS/MS) is based on the detection of transitions between precursor molecule ion and their specific product ions formed in a collision cell and the liquid chromatography (mainly on a C18 column for cyanotoxins) [23, 169]. The detection and quantification of analytes mainly depends on some basic criteria as: the retention time of the analytes, the mass to charge ratio ( $m/z$ ) and the ion ratios of the fragment ion compared to the standard data [125]. It is able to recognize different variants of cyanotoxins even in trace quantities (low limits of detections and quantifications) and give their structure with a high degree of selectivity and accuracy [154].

LC-MS/MS analyses are rather cost effective, they are in a continuous development for reaching maximal sensitivity and accuracy and they do not pose any ethical considerations. The limitations include the non-availability of certified standards, or the co-elution of several compounds [151]. Moreover, the technique is not able to account for the biological activity of cyanotoxins as the PPIA assay does. Matrix components can foul and ultimately clog the column. A sample cleanup stem is required.

MCs, NODs, CYN, derivatised ANA-a and derivatised BMAA are usually separated on a reverse phase chromatography using a slightly acidified acetonitrile as a mobile phase (for a higher resolution and selectivity). The acidification can be done with Trifluoroacetic acid (TFA) (0.05%-0.1%) or formic acid (FA) (0.1%); for a higher retention on the chromatographic column [59, 126].

For the more hydrophilic toxins, as underivatized ANA-a, CYN, STXs, and BMAA a separation with a HILIC method (hydrophilic interaction liquid chromatography) is more compatible [127, 151, 170].

### 5.5.2. Mass analyzers

In the past, Photodiode-Array (PDA) detector for Ultraviolet (UV) absorbance was the most commonly used technique for the investigations on cyanotoxins with a UV absorption maximum for (MCs) between 222 and 238 nm (Figure 29).

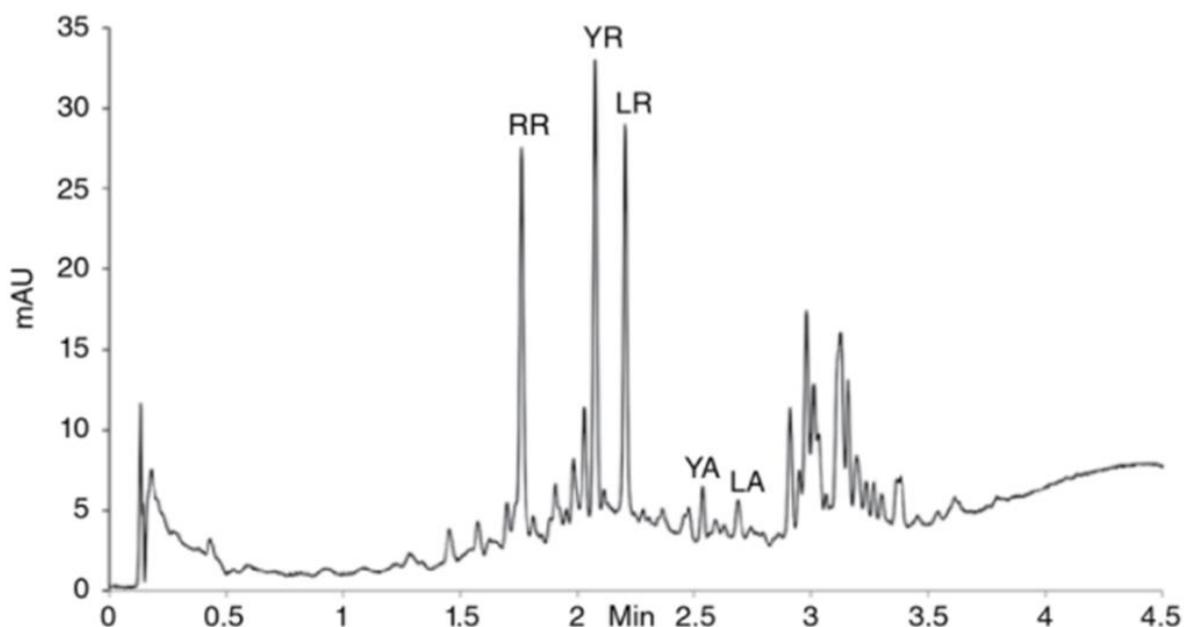


Figure 29: Chromatogram of a water sample with PDA [16].

However, the diode-array detector, neither in UV (ultraviolet) nor VIS (visible) range is specific to cyanotoxins [171]. Therefore, the use of mass spectrometry is mandatory. Different types of mass analyzers can be used, either low or high resolution, and in different configurations, MS or MS/MS.

#### *The Quadrupole Mass Analyzer*

It consists of four rods arranged in a tubular like structure. Where a voltage (radiofrequency (RF) and direct-current (DC) components) is applied on the opposite pairs of poles that are connected electrically.

At a specific amplitude of voltage, ions of a particular  $m/z$  follow a stable typical trajectory through the rods and reach the detector. A mass spectrum is therefore produced by varying the RF and DC voltages in a systematic way to bring ions of

increasing or decreasing  $m/z$  ratios to the detector. It is considered as an ideal detector for chromatography since it is capable of fast scanning and uses low voltages. However, it is classified as a low-resolution device, unable to provide the elemental composition of an ion [172].

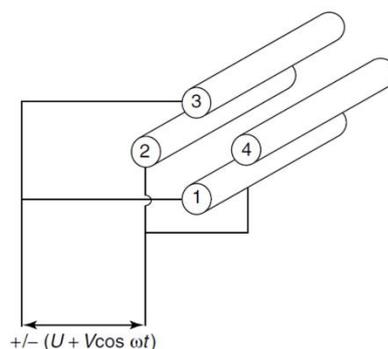


Figure 30: Quadrupole representation [172].

### The Time-of-Flight Mass Analyzer

The principle behind this instrument is to let all ions produced in the source of a MS to travel in a field-free region (the flight tube of the mass spectrometer) to reach the detector with an individual; specific time. Hence, mass spectrum is obtained simply by allowing sufficient time for all of the ions of interest to reach the detector [173]. This analyzer is considered to have a medium resolution.

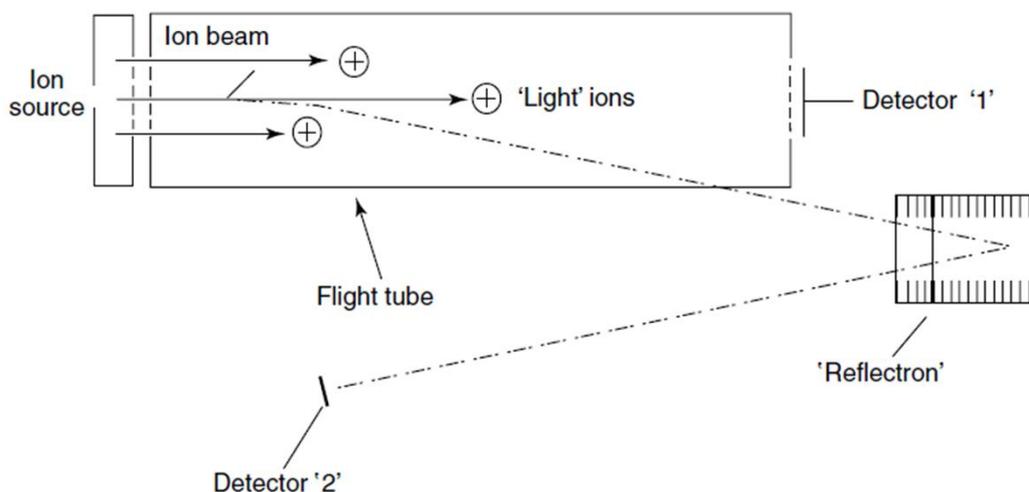


Figure 31: Representation of the time-of-flight mass analyzer [172].

### Orbitrap mass analyzer

It is a high resolution high mass accuracy mass analyzer which allows the determination of the molecular weight to several decimal places. That precision allows the molecular formula to be narrowed down to only a few possibilities.

ESI Orbitrap  $MS^n$  copes with the limitations of LC-MS. It allows analyses even in the absence of standards, by comparing to databases, with a higher selectivity, higher

accuracy and the exploration ability of new cyanotoxins variants [174]. In fact, the high resolution could be higher than 150,000 and mass accuracy is demonstrated to be as low as 0.2 ppm (parts per million) under favorable conditions

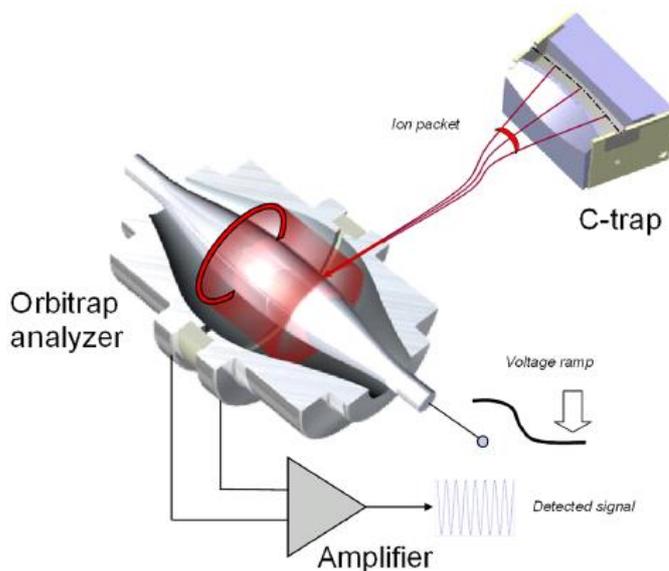


Figure 32: Representation of the Orbitrap mass analyzer [175].

### 5.5.3. Tandem MS

Tandem, means the succession of mass filters, one of them is used as collision cell allowing the fragmentation of precursor ions into product ions specific to targeted compounds. It is a technique that provides further data on analyzed compounds giving more information on their structural characteristics. The typical configurations or modes are shown in (Figure 33).

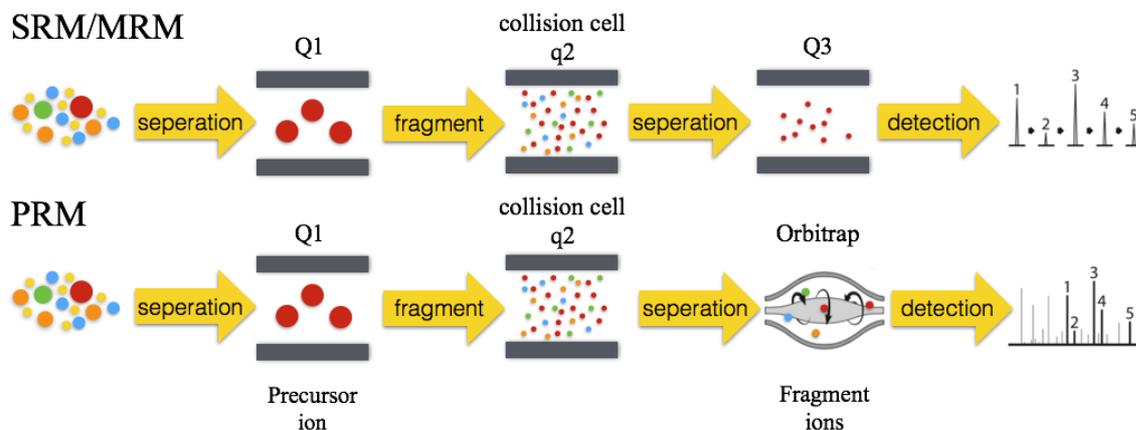


Figure 33: Configuration modes [176].

They include:

#### *The Triple Quadrupole*

Is the one that is the most widely used of MS–MS instruments. It consists of three sets of quadrupole rods in series where the second set of rods is used as a collision cell, where fragmentation of ions transmitted by the first set of quadrupole rods is carried out, and as a device for leading and conducting any product ions into the third set of quadrupole rods. Both sets of rods may be controlled to allow the transmission of ions of a single  $m/z$  ratio SRM (Selective reaction monitoring) or a range of  $m/z$  MRM (Multiple reaction monitoring) values to give the desired analytical information [177].

#### *The Hybrid Quadrupole – Orbitrap Mass Spectrometer*

It is the case when the second quadrupole is replaced by a high resolution instrument, e.g. Orbitrap. Q-Orbitrap as an advanced and sophisticated MS instrument allows the combination of a high-sensitivity MS/MS, multiple MS or  $MS^n$  and accurate mass measurements. It can be used as a qualitative and quantitative approach [178]. It allows the PRM (Parallel reaction monitoring) is based on high-resolution and high-precision mass spectrometry. The principle of this technique is comparable to SRM/MRM, but it is more suitable for delicate analyses [176].

The fragmentation of precursor ions is usually done in collision-induced dissociation (CID) or collisionally-activated dissociation (CAD) mode. This means when an ion and a neutral species (e.g., helium, nitrogen, argon) are forced to collide, and the resulting energy is transferred to the internal energy of the ion, leading to its fragmentation. The product fragments are then accelerated towards the mass analyzer, they then exit the collision cell and are analyzed by a detector. Alternatively, Higher-energy C-trap dissociation (HCD) uses in addition a higher RF voltage to keep fragment ions in the C-trap. The HCD cell fragments the ions, that are cooled down and stored inside of the C-trap later on and they are finally injected and separated inside the Orbitrap based on their rotational frequency differences. This type of fragmentation gives a better signal to noise ratios (S/N) for characteristic fragments ions

#### *5.5.4. Data acquisition: targeted vs. untargeted approaches*

Cyanotoxins are still mostly analyzed in targeted approaches. The fragmentation into product ions when in tandem MS will give more accurate results. The research in this field is kind of advanced with small gaps mainly because of the unavailability of standards. Problem that could be solved with the development of HRMS that will allow untargeted analyses, and the identification of compounds based on structural elucidation and comparison with available libraries and databases.

Untargeted approaches play a key role in the investigation on metabolites. It will allow the identification of novel, unexpected, unknown, uncommon and previously uncharacterized compounds. It will provide measures on 1000 to 10000 chemicals [179],

aiming to acquire data on as many species as possible. While targeted analysis aim to identify and quantify limited and known metabolites [180], untargeted techniques provide data on known and unknown chemicals, based on online libraries and structural elucidations, to be at the base of hypotheses that can be further elaborated with targeted approaches [181]. In conclusion, coupling the targeted and untargeted methodologies, would offer holistic approaches of studies [182] (Figure 34).

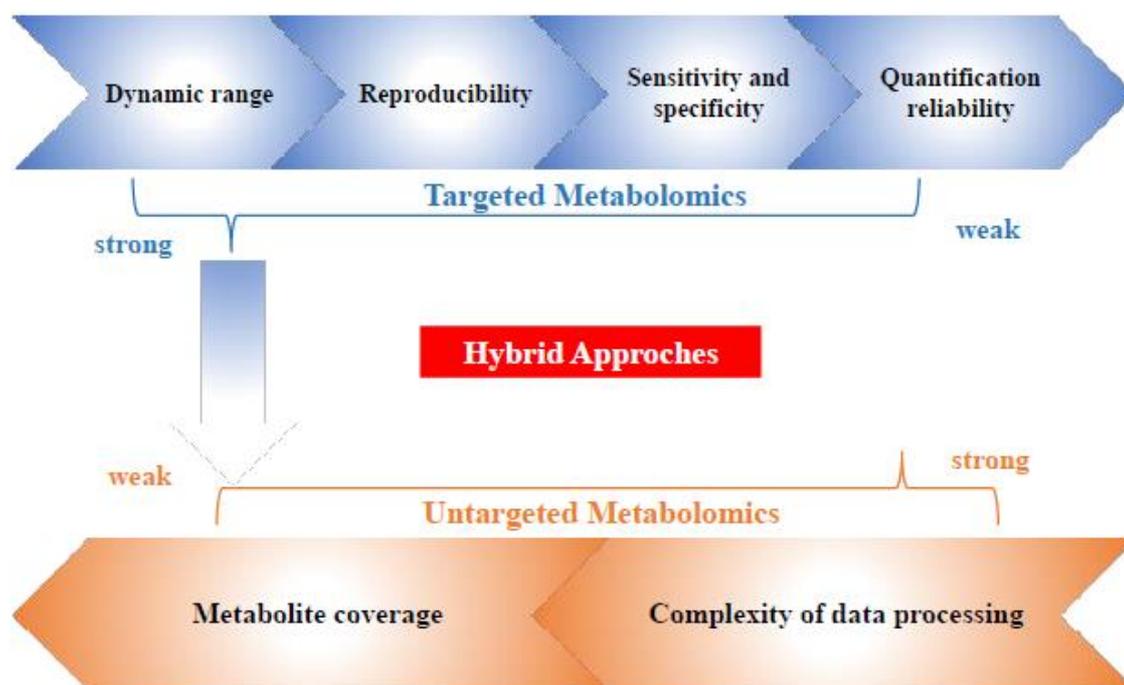


Figure 34: Coupling targeted and untargeted approaches (Li, F and J, 2020).

**Table 9.** Benefits and limitations of HRMS

Benefits	Limitations
Method development simpler and faster. In untargeted analyses, there is no need to develop compound-dependent parameters	HRMS instruments are of a high cost
Untargeted data collection allows the identification of novel unexpected compounds	Data analyses can be complex, since the data collected from a single sample is so big
Possibility of retrospective review of data	In untargeted analyses, a slight sensitivity loss could happen

Data acquisition, it could be data dependent (DDA), when in a full scan only the most intense precursor ions are fragmented or it could be a data-independent acquisition (DIA) where all precursor ions in a selected window of m/z are fragmented but with a high risk of co-eluting compounds and a probability of the matrix effect [183].

To have a higher confidence in the identification of analytes, even in an untargeted screening it should be based on accurate mass. Accurate m/z, charge states, isotopic pattern and fragmentation patterns. Similarly, to LC-MS/MS, a cleanup and pre-concentration step might be essential and helpful before proceeding with analyses [184].

With the unavailability of standards, due to the wide variety and broad number of cyanotoxins, HRMS copes with the limitations of targeted analyses, while it uses exact mass measurements and couples/compares and complement data with available databases and with the use of powerful software's as compound discoverer that permits in reducing the complexity of raw data by using a database and by analyzing the degree of matching and compatibility, hence, this technique allows the accurate and effective identification of potential and unknown compounds [185].

#### 5.5.5. Overview of applications for cyanotoxins

A list of fragmentation data of the most occurring cyanotoxins for the targeted MS/MS analysis is shown in (Table 10).

**Table 10.** Fragmentation data of common cyanotoxins

Cyanotoxins	Structure	Precursor Ion	Collision energy	Product Ion
<b>Cylindrospermopsin</b>	C <sub>15</sub> H <sub>21</sub> N <sub>5</sub> O <sub>7</sub> S	416.1	36, 36, 20	176.2, 194.2*, 336.2
<b>Anatoxin</b>	C <sub>10</sub> H <sub>15</sub> NO	166.1	21, 16, 13	91.0, 131.1, 149.1*
<b>Nodularin</b>	C <sub>41</sub> H <sub>60</sub> N <sub>8</sub> O <sub>10</sub>	825.3	49, 46	134.9*, 227.0
<b>MC-LR</b>	C <sub>49</sub> H <sub>75</sub> N <sub>10</sub> O <sub>12</sub>	995.5	73, 62, 54	104.6, 135.1*, 213.0
<b>MC-RR</b>	C <sub>49</sub> H <sub>75</sub> N <sub>13</sub> O <sub>12</sub>	519.9	39, 38, 26	104.9, 127.1, 135.1*
<b>MC-LA</b>	C <sub>46</sub> H <sub>68</sub> N <sub>7</sub> O <sub>1</sub>	910.4	29, 24, 19	375.2, 402.2, 776.4*
<b>MC-LY</b>	C <sub>52</sub> H <sub>72</sub> N <sub>7</sub> O <sub>13</sub>	1002.4	46, 28, 19	264.9, 494.2, 868.2*
<b>MC-LW</b>	C <sub>54</sub> H <sub>72</sub> N <sub>8</sub> O <sub>12</sub>	1025.4	34, 32	375.1, 446.1*

<b>MC-YR</b>	C <sub>52</sub> H <sub>72</sub> N <sub>10</sub> O <sub>13</sub>	1045.2	63, 62, 52	135.1*, 213.0, 375.1
<b>MC-LF</b>	C <sub>52</sub> H <sub>72</sub> N <sub>7</sub> O <sub>12</sub>	986.2	28, 23, 21	375.1, 478.2*, 852.5*
<b>MC-HiLR</b>	C <sub>50</sub> H <sub>77</sub> N <sub>10</sub> O <sub>12</sub>	1009.6	62, 63, 46	135.0*, 213.3*, 599.5

Microcystins, nodularin, anatoxin and cylindrospermopsin have been on the radar of many scientists worldwide. resuming some of the research work (in low and high resolution) is shown below.

**Table 11.** Examples of studies on cyanotoxins using LC-MS/MS and HRMS

Publication	Sample matrix	Sample Preparation	Species measured	LC-Separation	Limit of detection
<b>Friedman et al. 2018</b> [82]	Water	Freeze-drying, extraction	Microginins	reversed-phase flash column ODS, YMC-GEL,  / reversed phase HPLC column	-
<b>Yen et al. 2011</b> [74]	water	Sonication-Filtration- SPE	Nod, Cyn, Ana-a, MCs-LR/RR/YR/LW/LF/LA	reverse phase C18 analytical columns	2-100 ng/L
<b>Simiyu et al. 2018</b> [17]	Water, fish	Water filtration, fish extraction	MC-YR/LR	HPLC-DAD	0.6 µg/L
<b>Greer et al. 2016</b> [75]	Water	Lyophilization, extraction, Dual cartridge assembly SPE	Nod, Cyn, Ana-a, domoic acid  MCs-LR/RR/YR/LY/LF/LA	ACQUITY UPLC HSS T3 column,	0.3-5.6 ng/L
<b>Zervou et al. 2016</b> [125]	Water	Filtration, SPE	Nod, Cyn, Ana-a, 12 MCs ([D-Asp3]MC-RR/ -RR/-	4 columns were tested, optimal	1-10 ng/L

			YR/-HtyR,[D-Asp3]MC-LR, MC/LR/ -HilR, /-WR/-LA/-LY/-LW and MC-LF), Okadaic acid and Domoicacid.	results with reversed phase- the Atlantis T3 column	
<b>Draper et al. 2013</b> [126]	Water	Freeze thawing, SPE	MC-RR/YR/LR/LA/LF/LW	C8 column	0.03 to 0.04 µg/L
<b>Díez-Quijada et al. 2018</b> [186]	Vegetable matrix (lettuce)	Extraction and SPE	MC-LR/RR/YR/ and Cyn	UPLC on a 100 HSS T3 column	0.06-0.42 ng/g FW
<b>Tokodi et al. 2019</b> [187]	Water and fish	Water, filtration, centrifugation and SPE  Fish extraction	Cyn, MC-dmRR, /RR/dmYR/YR /dmLR/LR/LY/LW /LF	An Ascentis RP-Amide/ STAR RP-18e column,	-
<b>Hormazábal et al. 2000</b> [188]	Fish	Extraction and SPE	Ana-a, MC D3-LR/RR/LR/YR	The analytical column (stainless steel,) was packed particles of PLRP-S polymer adsorbent (Polymer Laboratories, Amherst, MA, USA)	7, 1, 5, 0.5, and 5 ng/g for ATX, D-3, LR, RR, and YR respectively
<b>Guzmán-Gillén et al. 2015</b> [189]	Fish	Extraction and SPE	Cyn	Zorbax Sb-Aq column.	0.07µg/g DW in Liver and 0.002 µg/g DW in muscle
<b>Filatova et al. 2020</b> [174]	Water	Sonication-centrifugation-SPE	Cyn, Ana-a, Nod- MC-LR/RR/YR/LA/LY/LW/LF	C18-reversed phase HPLC column	4-150 pg/L

<b>Dispofi et al. 2020</b> [190]	Water	SPE	21 CTXs (MCs, Cyanopeptolins, anabaenopeptins, microginins )	UPLC C18 column	0.002-0.047 µg/L
<b>Lachapelle et al. 2019</b> [185]	Water	Freeze-thawing cycles, filtration, online-SPE	Cyn, Ana-a, homoanatoxin-a, anabaenopeptins A and B, and 12 MCs (-RR/ [Asp3]-RR/YR/HtyR/LR, [Asp3]-LR/HilR/WR/LA/LY/LW and/LF)	Hypersil Gold C18 column	8 -53 ng/ L
<b>Bogialli et al.2017</b> [178]	Water	-	(M(O)R, MC-FR, MSer7-YR, D-Asp3MSer7-LR, MSer7-LR, dmAdda-LR and dmAdda-YR) and 6 anabaenopeptins (A, B, F, MM850, MM864, oscylamide Y)	Accucore C-18 column	-

Observing all the studies conducted on cyanotoxins, and besides the type of matrix, almost all samples require a pre-treatment. Preliminary clean-ups are mainly done with SPE. Chromatographic separation of cyanotoxins is mostly done in reversed phase using a C18 column. Targeted analyses cover a large list of cyanotoxins, mainly constituted of microcystins variants. Limits of detection are at the range of ng/L.

## 6. Study Site - Lake Karaoun ecosystem

### 6.1. Location, origin and importance of the Karaoun lake

The lake Karaoun is located in the western part of the valley Bekaa (33.34° N, 35.41° E) that is located between the two Lebanese mountainous chains, and covers a surface of 12 Km<sup>2</sup> [191].

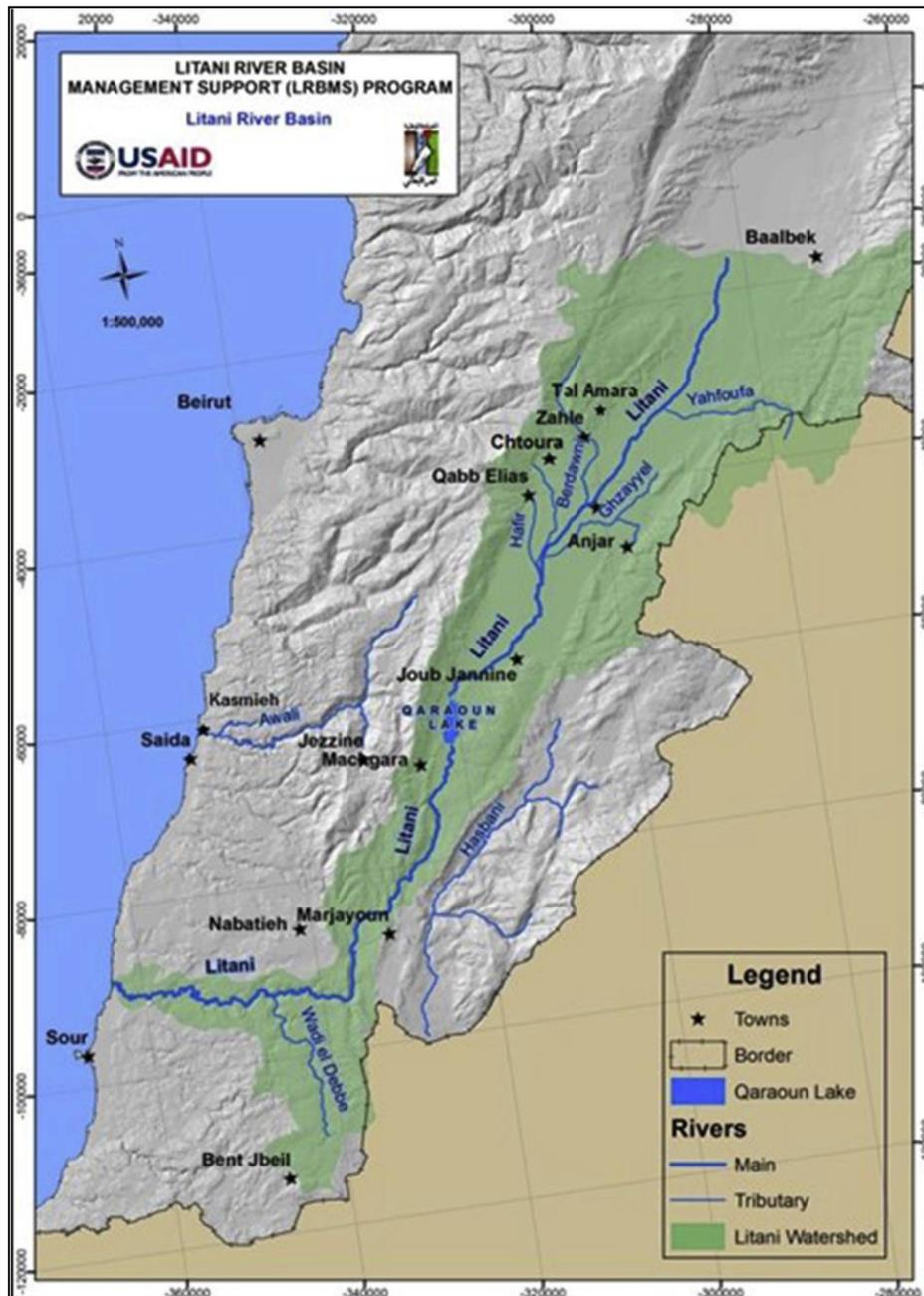


Figure 35: Map of the Litani River Basin [192].

Karaoun lake was created due to the accumulation of the Litani river water at the altitude 800 m behind the Albert Naqash dam after its construction in the early sixties

(1959) close to the village Karaoun; Karaoun is the largest reservoir and biggest freshwater body in Lebanon.

The main objectives for building the dam were to create a real water supply for irrigation thru two canals: Canal 800 and Canal 900, to assure a reliable water source for drinking water in the capital Beirut, to create hydropower and finally to create an attractional site for recreational activities and for encouraging investments in the area [193].

### *Irrigation*

Canal 900 meant to be used for the irrigation of the upper Litani basin at an altitude of 900 m covering an area of 190 Km<sup>2</sup>. The open canal was built in the early seventies it is around 18.5 Km length and would transfer around 4.8 m<sup>3</sup>/s of water from the lake [194]. Due to the current bad water quality in the lake, the canal is abandoned and water is not conducted anymore. In parallel, Canal 800 meant to be an additive drinking and irrigation water supply for villages at the 800m to 400 m altitude, essentially villages in the South and Nabatiyeh districts. Water was supposed to be conducted from Karaoun reservoir to Chaqra village located at 45 km to the southern side of the lake. Canal 800 is still not functional ultimately due to the degraded water quality.

### *Drinking water*

Water from the reservoir was a potential source for drinking water to different regions and to the capital Beirut. It was considered as the solution for the water deficit in the dry season. In 2012, it was decided to construct the system that could conduct and treat 6 m<sup>3</sup>/s of water from Karaoun to the capital [195].

### *Hydropower*

Visioning the hydropower plan from the Litani river between 1962 and 1968 was a successful trial. Seven hydropower units for the Litani River were constructed, in Marakabi in 1961 (Paul Arcache power plant), in Awali in 1964 (Charles Helou power plant) and in Joun in 1967 (Abdel-Aal power plant); the total installed power is 199 MW [196]. In fact, Karaoun contributes in 10% of the total installed power generation in Lebanon. On average, the annual production would record 500 million kW/h and during plentiful years the production could exceed 1 billion KW/h.

### *Recreational activities and fisheries.*

When the dam was constructed, water accumulated and created a new greeting ecosystem that encouraged investments in the area. People capitalized in hotels, restaurants and aquatic activities (boats rental). At the same time the reservoir was home to many aquatic species considered as a valuable source for fisheries. Around 30 fishermen used to fish in the reservoir and the fleet were about 15 small traditional

boats. The total catches could reach 150 tons per year, of two main species: (Cyprinus carpio) and common trout (Oncorhynchus mykiss) [24]. The current situation is different, since all aquatic activities including fishing are completely forbidden due the excessive cyanobacterial blooms. The National Authority of the Litani River banned any usage of the Karaoun water.

## 6.2. Geology and hydrology

Litani watershed dates back to the Mid Eocene, the plain is covered with quaternary alluvium limestone, and chalk thus the high permeability of the catchment [197]. Rocks are susceptible to erosion and are the origin of minerals like calcium magnesium iron and carbonate in the catchment soil and water. Bekaa overlays two active faults: Yammouneh fault and Serghaya fault [24].

The upper part of the reservoir is constituted of cretaceous Cenomanian limestone that is relatively composed of dolomite. To the west, it is mainly constituted of Neocene marls in the upper part and of lower cretaceous limestones in the southern part. To the south and central region of the reservoir, rocks are mainly magnesium and iron formations creating basalt rocks, the southern part of the reservoir is formed by Eocene limestones. At the end and for the eastern part, Senonian marls are taking place [198].

The total capacity is of 220 million m<sup>3</sup> with 60 and 19 m of maximum and mean depth respectively. Water could be completely renewed each 0.77 years [199]. Based on isotopic fractionation studies, lake Karaoun is considered monomictic, the mixing pattern is happening once a year where water blends from the top to the bottom. The concrete-faced rock fill dam of (60 m high, 1090 m long and 6 m wide) was constructed to collect water from the river Litani that is the largest and longest (170 Km) perennial river in Lebanon [191].

Water can be evacuated thru two facilities: the spillway that is a reinforced concrete tower connected to a channel that conducts water to the river stream underneath the dam (at a discharge capacity of 750 m<sup>3</sup>/s); and the two bottom drains that evacuates water at a discharge flow of 60 m<sup>3</sup>/s. these facilities are to assure that the dam doesn't crash and to avoid any flood [200].

Litani River's source is in Olleik village at an altitude of 1800 m, arriving to Karaoun Lake; 1000 m are lost in altitude. Then the flow drops intensely, 600 m on a distance of 30 Km from the reservoir to AL KHARDALI, where now the slope is smoother, it drops 300 m on a distance of 50 km arriving to the Mediterranean Sea [193].

While winters are cold (13 °C as an average temperature) and the wet season extends from November to April marking 700mm as an annual average precipitation; summers are hot and dry and lasts from July to September (with 25- 27 °C as a mean temperature). Hence the area could be considered as arid to semi-arid [15] .

### *Reservoir inflows.*

The first and main source of water in Reservoir Karaoun is the Litani River and its tributaries. The river's stream is contributing in around ( $290 \times 10^6 \text{ m}^3/\text{year}$ ) as an inflow to the lake. The input of springs should not be underestimated since a single spring could be contributing in around  $0.7 \text{ m}^3/\text{s}$ . Precipitations are the second source of input, the average rainfall rate in the catchment of the reservoir is around  $700 \text{ mm}/\text{year}$ , in other words, 543 million m of precipitations are considered as a water supply to the dam [200].

### *Reservoir outflows.*

The dam has two main evacuation amenities: the bell-mouth spillway located close to the dam at an altitude of 859 m and the two bottom drains conducting water for canal 900, but one more is to be mentioned and can be represented by the power generation tunnels holding a total capacity of  $22 \text{ m}^3/\text{s}$ . Power generation tunnels are at 810 m altitude conducting water to hydropower plants [15].

In parallel with evaporation ( $8.42 \text{ mm}/\text{day}$ ) [200], losses can be due to leakage of the dam and to infiltration ( $0.01$  and  $0.03 \text{ m}^3/\text{s}$ ) [24]. Since the reservoir had a good water quality withdrawals volumes were regular and extensive ( $180 \times 10^6 \text{ m}^3/\text{year}$ ), which used to lead to a significant decrease in the water level especially in summer and late summer periods. A dead storage of  $30 \times 10^6 \text{ m}^3$  used to be kept is kept in the reservoir at the end of autumn awaiting the new precipitations events. Due to the actual degraded water quality, water is no longer used in several purposes; thereby the water level is not significantly fluctuating thru seasons.

## 6.3. Sources of contamination and pollution

For a long time, Lebanon was considered as the water tower of the Middle East, mainly due to the numerous and richness of its water resources (40 streams from which 17 are perennial). The total length of streams in Lebanon is around 730 Km, and the catchment area of the Litani River is about 20 % of the Lebanese surface [201]. However, the lack of management and social responsibility put water resources in qualitative and quantitative stresses.

Litani River's and Lake Karaoun catchments are both suffering from various sources of pollution, where untreated industrial effluents, agriculture and agribusiness, untreated domestic sewage, dumpsites and quarries are the main threats to the degradation of the water quality [192].

### *Untreated industrial effluents*

They can be considered as the main source of pollution. The main industrial inorganic pollutants are heavy metals that are potent compounds. The threat can be in their direct presence in the water body but also in their accumulation in consumable products as drinking water, cereals, vegetables, fish ...etc.; other inorganic pollutants could be

chlorine, ammonia, phosphates, sulphates, nitrites and nitrates. Organic pollutants including oils, sugars, proteins, hydrocarbons, phenols, detergents, and organic acids could also be discharged from industries causing a lower risk than the inorganic compounds, but when in great concentrations they alter the system and cause its indirect pollution (Figure 36).

The upper Litani River Basin exhibits approximately 300 industries (plastics, paper, metal work, furniture and woodwork, and construction materials) of which the majority is functioning without environmental guidelines and precautions. Most of these industries, that could sometimes be large factories with extensive productions, release their liquid and/or solid wastes directly in the stream or in the sewage network without prior handling in treatments plants.

#### *Agriculture and agribusiness.*

The Bekaa valley is known as the Lebanese basket, it has a high productivity (agricultural) due to the high fertility of the lands (contributes to 20% of the incomes). It is a leading producer of cereals and feculent, tubercles (potatoes and beetroots) and dairy products. It is very common to see the combination of agriculture and industry, where companies harvest their goods and produce the final added quality products. But farmer's behavior isn't helping in keeping this sector environmentally safe. Fertilizers and pesticides applications are in 90% of the cases much higher than the recommended doses (for fertilizers 1.5 to 3 times higher and for pesticides twice higher) contributing to the nutrient enrichment of water bodies and causing their degradation. Fertilizers are becoming a great environmental concern, they are considered as the main enhancers of cyanobacterial blooms due to the enrichment with nitrogen and phosphate that are an important source of cyanobacteria. When combined to industries agricultural pollution is coupled to industrial pollution.

Dairy, food and beverages, wine and spirits, fruits and vegetables processing, bakery, livestock and poultry productions and others are the main activities in the Litani River and Karaoun basins.

#### *Quarries*

They might be less polluting than the other sources but their impact is still present. They are generally bringing to the water bodies rocks and sand that are in their turn fastening the sedimentation phenomenon. Rocks and deposits could be contaminated; in this case they bring to the reservoir heavy metals, oils, fats and other compounds.

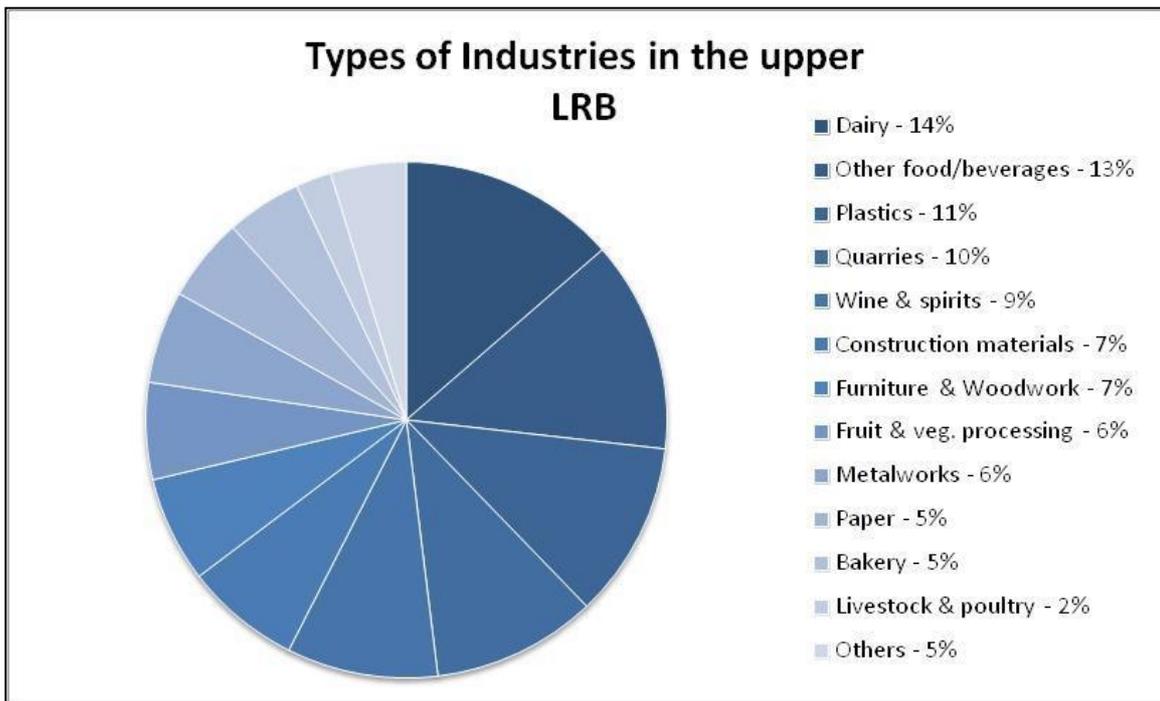


Figure 36: Industries in the upper watershed of Litani River [192].

#### *Untreated domestic sewage*

Until now, there is no real management of sewage and waste water in the area. Hence, houses accumulate their waste in big holes or septic tanks that could be left to drain in the groundwater, or routinely emptied by truck that are in their turn liberating the waste directly in the river. After 2000 many water treatment plants were built in order to clean up water before releasing it in streams, and an objective was set to use this water for irrigation, but the current situation doesn't confirm the proper use of the treatment plants and untreated domestic sewage is still liberated in the water bodies. The danger is real when untreated sewage is coming from hospitals and medical establishments.

#### *Dumpsites*

Bekaa area (Litani River's basin) lack of a good management plan for domestic and general wastes, therefor the presence of dumpsites and littering is common. Due to the lack of knowledge and social responsibility, the bad behavior of burning waste or letting it to leak in streams and groundwater, is contributing to the pollution of the water bodies. The inputs can be solid materials like wood, organic products, batteries, plastics, and they can also be organic and organic pollutants as heavy metals. The burning of dumpsites would lead to the emission or toxic volatile compounds or gases (dioxin...). In such a case it is assured that dumpsites are also contributing to the apparition of cyanobacterial blooms by the enrichment with nutrients.

#### 6.4. State of the art of knowledge about cyanotoxins and other contaminants in the Karaoun lake

Lake Karaoun had been known for its excellent water quality for a long period of time. Hence, it was used for different activities (irrigation, recreation, fishing, hydropower...). However, for several years, this good water quality was subject to various alteration and degradation [15].

During the first years following the dam construction, the reservoir exhibited an important biodiversity. It was rich in phytoplankton; 90 species were identified between 2000 and 2001. Diatoms were one of the dominating species (80% of total phytoplankton); around 60 species were identified. *Aulacoseira granulata* was the dominant diatom while *Ceratium hirudinella* was the dominant dinoflagellate. In 2003 *Ceratium hirudinella* vanished, giving place to the multiplication of numerous filamentous green algae as *Spirogyra lambertiana*, *Oedogonium sp*, *Ulothrix zonata* and *Cladophora glomerata* [24]. These variations could be an early alert of the alteration of the water quality. Few years afterwards, in 2009, blooms of *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* were reported for the first time. The mentioned cyanobacteria were gradually dominating all the other cyanobacterial species. It was noticed that *A. ovalisporum* and *M. aeruginosa* bloom in specific periods in a similar and predictable pattern [26]. Due to its tolerability to lower temperatures, *A. ovalisporum* blooms at the beginning of spring and autumn and would disappear at temperatures higher than 25 °C, while *M. aeruginosa* preferably blooms in summer and hot periods. *M. aeruginosa* blooms are seen in hot waters with temperatures ranging from 27- 32 °C and usually disappear with temperatures lower than 20°C.

Lately mixed cyanobacterial blooms have been noticed during extreme weather conditions. The most logical explanation would be the adaptation of cyanobacteria to the changes in the environment and their tolerating higher temperatures. The second most logical explanation would be the climate change, that is offering cyanobacteria longer warm periods, stimulating their unusual blooms in December or February [25].

Lake Karaoun despite being the largest water body in Lebanon has many utilities and would serve as an important water resource for different usages. Any alteration in the reservoir's water quality would affect lot of sectors causing sanitary, environmental, ecological, and social and many other losses. The advanced degradation that is witnessed today is due to many bad behaviors and lack of management and assessment. Hence it is important to conduct a continuous monitoring of the lake's state, and resources that are still consumed by the population, even sometimes in secret and illegally.

## 7. Thesis' Objectives

Previous studies on lake Karaoun, confirmed the dominance of cyanobacteria and their corresponding cyanotoxins as cylindrospermopsin using ELISA techniques. The objective of this thesis is to assess and confirm the presence of cyanotoxins in Karaoun for the first time using a holistic approach using mass spectrometry (LC-MS/MS) and high resolution mass spectrometry (LC- Orbitrap MS/MS), complemented with fast screenings/biochemical assays, such as ELISA and PPIA that can be used on-site. In addition, a molecular approach using q-PCR that allows the detection of cyanobacterial and toxic genes at the early stages of blooms was investigated.

Moreover, “non- toxic” cyanobacterial secondary metabolites called trace and odor compounds (T&O) that could be co-produced with cyanotoxins and negatively affect drinking water acceptability by consumers could be an early sign of blooms; they can be detected with GC-MS/MS.

The detailed objectives were : (i) Investigation on the occurrence of cyanobacterial toxins in water and in fish (flesh and liver), ii) comparison of different analytical techniques (LC-MS/MS, ELISA, PPIA), (iii) investigation on cyanobacterial 16s rRNA gene and toxins producing genes by q-PCR, iv) investigation on volatile compounds of biological and/or anthropogenic origins using HS-SPME-GC-MS/MS, and v) Exploratory (untargeted) analysis by LC- high resolution (Orbitrap) MS<sup>n</sup>- comparison of different mass spectrometry techniques for the study of already known and new cyanobacterial metabolites

In addition to the analyses of water samples, a goal was to optimize and validate extraction methods of cyanotoxins from fish with development and validation of mass spectrometry protocols that could be applied in future analyses for the determination and quantitation of cyanobacterial compounds.

## II. EXPERIMENTAL PART

## 8. Sampling

All samples analyzed in this thesis were collected from lake Karaoun. Field campaigns covered both wet and dry seasons, 16 water samples along with a total of 57 individuals of *Cyprinus carpio* fish (215 g on average) were collected with the help of the National Litani River Authority that is in charge of the surveillance, protection and legal activities of the river and reservoir (Table 12).

Water samples were collected in polyethylene bottles from 5 sampling points of Lake Karaoun (S1, S2, S3, S4 and S5). S1 is located very close to the river input at the west bank, S2 is on the eastern side of the dam, S3 is close to the river input at the east bank, S4 is at the middle-south part of the lake (where an ultrasound apparel was implemented) and S5 is at the dam (Figure 37).

Water samples were transported in coolers (4°C) for preliminary pre-treatments in Beirut, Lebanon and more analyses in Athens, Greece and Pau, France. Moreover, fish samples were kept on ice and were sacrificed within 24 h. Fish liver and muscle sub-samples were labeled and frozen separately at -20°C. Frozen fish tissue samples were then freeze-dried with a Labconco lyophilizer for 48 h at -84 °C and 0.2 mbar and finally powdered with a pestle and mortar.

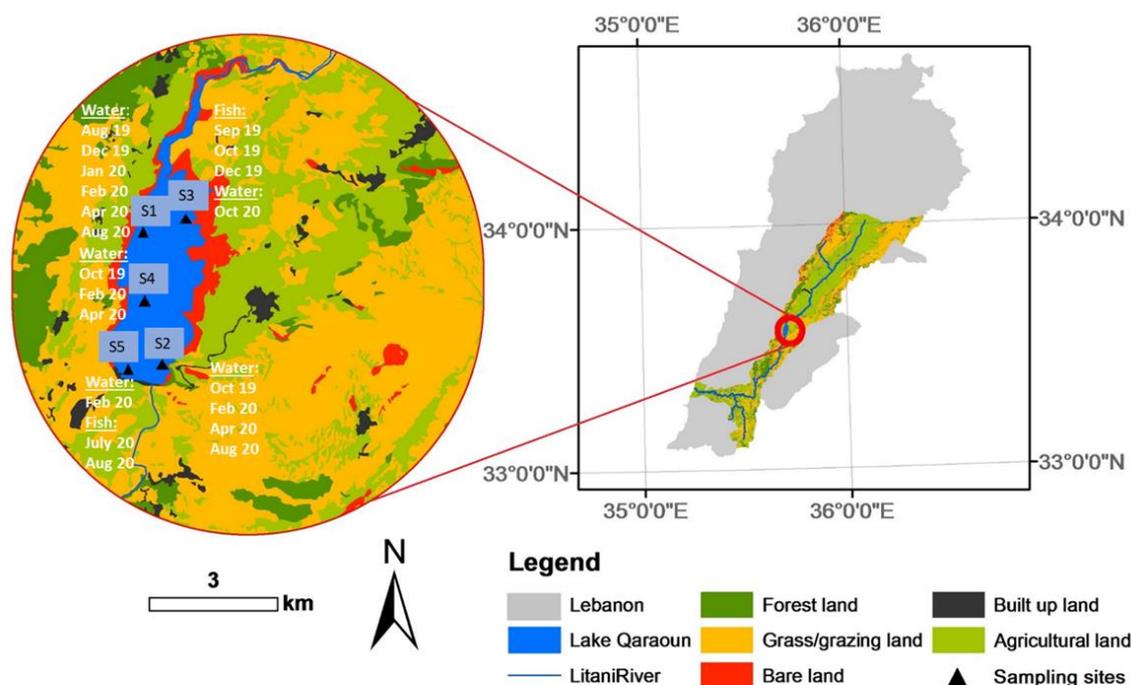


Figure 37: Map of study sites.

**Table 12.** Detailed list of sampling events

Event	Sampling date	Name of sampling site	Location GPS coordinates	Micro-algal composition	Comments
<b>Water</b>					
1	22/08/2019	S1	33°34'40.8"N 35°41'14.9"E	<i>M.a.</i> : 50% <i>A.a.</i> : 49% <i>M.w.</i> : 1%	Bloom
2	02/10/2019	S2	33°32'55.0"N 35°41'50.0"E	<i>M.a.</i> : 5%	Intense scum
3	02/10/2019	S4	33°33'51.5"N 35°41'34.9"E	<i>A.o.</i> : 95%	Bloom
4	22/12/2019	S1	33°34'40.8"N 35°41'14.9"E	<i>M.a.</i> : 4% <i>A.o.</i> : 95% <i>Nostoc sp.</i> : 1%	Bloom not seen
5	15/01/2020	S1	33°34'40.8"N 35°41'14.9"E	<i>A.o.</i> : 100%	Bloom not seen
6	04/02/2020		Unknown	<i>A.o.</i> : 100%	Intense bloom
7	15/02/2020	H.T	33°32'50.2"N 35°41'31.4"E	<i>A.o.</i> : 100%	
8	17/02/2020	H.T	33°32'50.2"N 35°41'31.4"E	<i>A.o.</i> : 100%	At depth 60 m
9	22/02/2020	S1	33°34'40.8"N 35°41'14.9"E	<i>A.o.</i> : 100%	Bloom not seen
10	22/02/2020	S2	33°32'55.0"N 35°41'50.0"E	<i>A.o.</i> : 100%	Bloom not seen
11	15/04/2020		Unknown	<i>A.o.</i> : 100%	Bloom
12	22/04/2020	S2	33°32'55.0"N 35°41'50.0"E	<i>A.o.</i> : 100%	Intense bloom, and dirty water
13	14/08/2020	S1	33°34'40.8"N 35°41'14.9"E	<i>M.a.</i> : 95%	Bloom not seen
14	14/08/2020	S2	33°32'55.0"N 35°41'50.0"E	<i>A.o.</i> : 3% <i>M.i.</i> : 1% <i>W.n.</i> : 1%	
15	2020	Unknown			
16	09/10/2020	S3	33°34'58.2"N 35°42'27.7"E	<i>M.a.</i> : 100%	Intense bloom
<b>Fish</b>					
A	02/09/2019	S3	33°34'58.2"N 35°42'27.7"E	-	<i>Cyprinus carpio</i> fish ±215g
B	02/10/2019	S3	33°34'58.2"N 35°42'27.7"E	-	
C	22/12/2019	S3	33°34'58.2"N 35°42'27.7"E	-	
D	28/07/2020	S3	33°34'58.2"N 35°42'27.7"E	-	
E	14/08/2020	S5	33°32'56.6"N 35°41'04.3"E	-	

## 9. Analyses for cyanotoxins

### 9.1. Materials

#### 9.1.1. Chemicals and reagents

##### Standard solutions

12 microcystins (dm MC-RR, MC-RR, MC-YR, MC-HtyR, dm3MC-LR, MC-LR, MC-HiIR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF), anatoxin-a (ANA), cylindrospermopsin (CYN) and nodularin (NOD) and 9 Paralytic Shellfish Poisoning toxins including 3 saxitoxins (STXs) (STX, neoSTX and dcSTX) and 6 gonyautoxins (GTXs) (GTX1, GTX2, GTX3, GTX4, dcGTX2 and dcGTX3) were targeted. The corresponding standard solutions were prepared (Table 13). They were all of analytical purity.

**Table 13.** Cyanotoxins standards

Cyanotoxin name	Abbreviation	Molecular formula	Molecular weight	CAS Number
Anatoxin-a	ANA	C <sub>10</sub> H <sub>15</sub> NO	165,23	64285-06-9
Cylindrospermopsin	CYN	C <sub>15</sub> H <sub>21</sub> N <sub>5</sub> O <sub>7</sub> S	415,43	143545-90-8
Nodularin-R	NOD	C <sub>41</sub> H <sub>60</sub> N <sub>8</sub> O <sub>10</sub>	824,98	118399-22-7
[D-Asp3] Microcystin-RR	[D-Asp3] MC-RR	C <sub>48</sub> H <sub>73</sub> N <sub>13</sub> O <sub>12</sub>	1024,17	-
Microcystin-RR	MC-RR	C <sub>49</sub> H <sub>75</sub> N <sub>13</sub> O <sub>12</sub>	1038,20	111755-37-4
[D-Asp3] Microcystin-LR	[D-Asp3] MC-LR	C <sub>48</sub> H <sub>72</sub> N <sub>10</sub> O <sub>12</sub>	981,14	-
Microcystin-LR	MC-LR	C <sub>49</sub> H <sub>74</sub> N <sub>10</sub> O <sub>12</sub>	995,17	101043-37-2
Microcystin-YR	MC-YR	C <sub>52</sub> H <sub>72</sub> N <sub>10</sub> O <sub>13</sub>	1045,18	101064-48-6
Microcystin-HtyR	MC-HtyR	C <sub>53</sub> H <sub>74</sub> N <sub>10</sub> O <sub>13</sub>	1059,21	-
Microcystin-HiIR	MC-HiIR	C <sub>50</sub> H <sub>76</sub> N <sub>10</sub> O <sub>12</sub>	1009,20	-
Microcystin-LA	MC-LA	C <sub>46</sub> H <sub>67</sub> N <sub>7</sub> O <sub>12</sub>	910,06	96180-79-9
Microcystin-LY	MC-LY	C <sub>52</sub> H <sub>71</sub> N <sub>7</sub> O <sub>13</sub>	1002,16	123304-10-9
Microcystin-LW	MC-LW	C <sub>54</sub> H <sub>72</sub> N <sub>8</sub> O <sub>12</sub>	1025,19	157622-02-1
Microcystin-LF	MC-LF	C <sub>52</sub> H <sub>71</sub> N <sub>7</sub> O <sub>12</sub>	986,16	154037-70-4
Microcystin-WR	MC-WR	C <sub>54</sub> H <sub>73</sub> N <sub>11</sub> O <sub>12</sub>	1068,22	138234-58-9
Gonyautoxin1	GTX1	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O <sub>9</sub> S	411.35	-

<b>Gonyautoxin2</b>	GTX2	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O <sub>8</sub> S	395.35	-
<b>Gonyautoxin3</b>	GTX3	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O <sub>8</sub> S	395.35	-
<b>Gonyautoxin4</b>	GTX4	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O <sub>9</sub> S	411.35	-
<b>Decarbamoyl-gonyautoxin2</b>	dcGTX2	C <sub>9</sub> H <sub>16</sub> N <sub>6</sub> O <sub>7</sub> S	352.324	-
<b>Decarbamoyl-gonyautoxin3</b>	dcGTX3	C <sub>9</sub> H <sub>16</sub> N <sub>6</sub> O <sub>7</sub> S	352.324	-
<b>Saxitoxin</b>	STX	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O <sub>4</sub>	299.29	-
<b>neo-Saxitoxin</b>	neoSTX	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O <sub>5</sub>	315.2	-
<b>Decarbamoyl-saxitoxin</b>	dcSTX	C <sub>10</sub> H <sub>17</sub> N <sub>6</sub> O <sub>3</sub>	256.26	-

In molecular analyses, yellow NA013 (200 copies/ $\mu$ L or 1000 copies/ reaction) and blue NA014 (2000 copies/ $\mu$ L or 20000 copies/ reaction) were used as standards. If frozen, they were brought to room temperature for at least 15 min, vortexed for 30 s and briefly spun. In between usages, standards must be kept at low temperature around 4 °C.

#### *Solvent solutions*

For the preparation of extraction and elution solvents, in addition to other solutions, the following chemicals were used.

- HPLC Purity Acetonitrile (ACN), Sigma-Aldrich (St. Louis, MO)
- HPLC Purity Methanol (MeOH), Fischer Scientific (Leics, UK)
- Analytical purity hexane, MERCK (Darmstadt, Germany)
- High purity formic acid (HCOOH) (98-100%), Riedel-de Haën (Seelze, Germany)
- High purity acetic acid (CH<sub>3</sub>COOH) (>99.8%), Riedel-de Haën (Germany)
- Ultra-pure water generation device (18.2 M $\Omega$  / cm) of TEMAK device, type: TSDW21 (Greece, 2007)
- EDTA (Ethylenediaminetetraacetic acid / C<sub>10</sub>H<sub>16</sub>N<sub>2</sub>O<sub>8</sub>), Serva, Mr 292.3
- L-cysteine (>99.5%), Fluka analytical/ Sigma-Aldrich (Japan)
- Potassium carbonate (K<sub>2</sub>CO<sub>3</sub>) (>99.5%), Carlo Erba Reactifs-SDS
- Hydrochloric acid (HCl) (37%) (Merck, KGsA, 64271 Darmstadt, Germany)
- Water of PCR grade, Sterility: 0.1 $\mu$ m filtered, Sigma-Aldrich
- Rnase AWAY<sup>®</sup>, decontamination reagent for Rnase, Sigma-Aldrich

### 9.1.2 Laboratory equipment and consumables

#### *Glass and general utensils*

- Automatic pipette 5-40  $\mu\text{L}$ , (Eppendorf (calibrated))
- Automatic pipette 200-1000  $\mu\text{L}$ , (Eppendorf (calibrated))
- Automatic pipette 2-10 ml, (Finnpipette Labsystems)
- Volumetric cylinders 10,100 ml, Class A
- 1 L glass bottles
- Glass vacuum filter device with holder for 47 mm filters, (Millipore)
- Glass Fiber filters with a diameter of 47 mm, pores 0.7  $\mu\text{m}$ , (Millipore)
- Eppendorf 1.5 ml micro-centrifuge tube
- 10 ml glass test tubes
- 16 ml glass vials with PTFE lined caps, (Thermo Scientific)
- Automatic sampler glass vials 1.5 ml
- Inserts for 0.1 and 0.4 ml vials, (Kinesis)
- Nipro syringe 20 mL
- Nipro syringe 10 mL
- Bead Lysis Tubes, BioGX (each tube contains 300 mg glass beads and 500  $\mu\text{L}$  buffer)
- SmartCycler<sup>®</sup> Tube 25  $\mu\text{L}$  (Cepheid, USA)
- Whatman Tweezers (Germany)
- Whatman Filters Nucleopore 25 mm, 0.8  $\mu\text{m}$
- Re-usable filter holder (Whatman)

#### *Laboratory equipment*

- Phase contrast microscope (Olympus, Munster, Germany)
- Freeze dryer (FreeZone 4.5 Liter Benchtop Freeze Dry System form Labconco)
- Ultra-pure water device (18.2 M $\Omega$  / cm) TEMAK, Type: TSDW10 (2007, Greece)
- Analytical balance with accuracy of 5 decimal places Sartorius CP225D (calibrated)
- Magnetic mixer with 6 positions
- Solid phase extraction device (SPE, Supelco) entailing:
  - Vacuum diaphragm pump (KNF Ladoport) and 10 L volume collection bottle
  - Manifold SPE 12 operating positions in vacuum, with Teflon piping
  - 1 L sample glass bottles, calibrated
  - Cartridges:

*Oasis HLB (200mg, 6cc, 25-35 $\mu\text{m}$ , Waters Corporation, USA)*

*Sep-Pak Vac (silica cartridge) (Waters)*

*Supelclean "ENVI"- 18 SPE 500mg*

- Nitrogen device (purity 99.9%) with water bath, 12 positions Organomation N-VAP 111
- Bandelin sonorex super RK 106 ultrasound device
- HARRIER 18/80 Refrigerated SANYO Centrifuge
- Centrifuge (for Eppendorf tubes)
- Miele Professional glass washer, Type: G7883
- Memmert UFE 400 glass oven
- Nahita Mini centrifuge 2507/7 capacity:8x 1.50/0.5/0.2mL, max speed: 7000 rpm
- Bead Bug Microtube Homogenizer

For the determination of 12 MCS, NOD, CYN, and ANA a reversed phase LC column Atlantis T3, 2.1 x 100mm, 3 $\mu$ m (Waters, Ireland) was used.

For the determination of 9 Paralytic Shellfish Poisoning toxins including 3 saxitoxins (STXs) (STX, neoSTX and dcSTX) and 6 gonyautoxins (GTXs) (GTX1, GTX2, GTX3, GTX4, dcGTX2 and dcGTX3) a hydrophilic interaction liquid chromatography column combined to a pre-column were used:

- SeQuant<sup>®</sup> ZIC<sup>®</sup>-HILIC (150mm x 2.1mm, particle size: 3.5  $\mu$ m, Merck)
- Pre-column SeQuant<sup>®</sup> ZIC<sup>®</sup>-HILIC (5  $\mu$ m, 200 Å, 20 x 2,1 mm, Merck)

## 9.2 Instrumentation

### 9.2.1 Mass spectrometers

For LC-MS/MS analyses of 12 microcystins, anatoxin-a, nodularin and cylindrospermopsin, a Finnigan Surveyor LC system, equipped with a Surveyor AS autosampler (Thermo, USA), coupled to a TSQ Quantum Discovery Max triple-stage quadrupole mass spectrometer (Thermo, USA), with electrospray ionization (ESI) source operating in the positive ionization mode was employed (Figure 38).

Xcalibur software 2.0 was used to control the MS parameters for data acquisition and data analysis. High purity nitrogen and argon were used as sheath gas and collision gas, respectively.



Figure 38: Finnigan Surveyor LC system.

For high resolution mass spectrometry, samples were analyzed, in targeted and exploratory approaches, using an Orbitrap Fusion Lumos Tribrid mass spectrometer. Separation of compounds was performed by RP HPLC. Data analysis for the detection and identification of cyanobacterial metabolites was performed with Compound Discoverer software in combination to the latest version of CyanoMetDB mass list [202]. In-silico fragmentation was achieved by Mass Frontier software and fragment ion search (FISH) scoring.



Figure 39: Orbitrap Fusion Lumos Tribrid.

### 9.2.2 Molecular analyses and toxicity measurements

Cepheid Smartcycler™, a real time PCR system was used for reading the relevant dye sets obtained using the Phytoxigene™ CyanoDTec (Diagnostic Technology) assay. The 2 following kits were used: (i) Phytoxigene™ CyanoDTec Total cyanobacteria (16S rRNA and Internal Amplification control test that contains 6 tubes, each one having the all necessary reagents (enzymes, probe, primers and dNTP) for 4 tests), and (ii) the Phytoxigene™ CyanoDTec Toxins genes (microcystin/nodularin, cylindrospermopsin and saxitoxin) test containing 6 tubes with all necessary reagents.



Figure 40: q-PCR apparatus.

### 9.2.3 Enzyme-linked Immunosorbent Assay (ELISA)

The Enzyme-linked Immunosorbent Assay (ELISA) was done using the Microcystins (ADDA)-DM (direct monoclonal) kit from Eurofins Abraxis that contained:

- A microtiter plate (12 X 8 strips) coated with a second antibody (goat anti-mouse)
- Standards (6): 0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb (parts per billion), 1.5 mL each
- Control:  $0.75 \pm 0.185$  ppb, 1.5 mL, prepared from a secondary source, for use as a Quality Control Standard (QCS)
- Sample Diluent, 25 mL, for use as a Laboratory Reagent Blank (LRB) and for dilution of samples above the range of the standard curve
- Microcystins-HRP Conjugate Solution, 6 mL
- Microcystins-DM Antibody Solution (monoclonal anti-Microcystins), 6 mL
- Wash Buffer (5X) Concentrate, 100 mL, that must be diluted prior to use
- Substrate (Color) Solution (TMB), 16 mL
- Stop Solution, 12 mL

#### 9.2.4 Protein Phosphatase Inhibition Assay (PPIA)

The Protein Phosphatase Inhibition Assay (PPIA) was done using the Microcystins/Nodularins PP2A, Microtiter Plate kit from Eurofins Abraxis that contained:

- A microtiter plate (12 X 8 strips)
- Standards (4): 0.25, 0.50, 1.00, and 2.50 ppb each
- 4 vials of Phosphatase
- 1 vial Chromogenic Substrate
- 1 vial Phosphatase Dilution Buffer
- 1 vial Stop Solution

### 9.3 Methods

#### 9.3.1 q-PCR

Molecular analyses were performed to detect the presence of cyanobacteria and their toxicity at the genetic level. In both cases, the thermocycler worked in two stages: Stage 1: Hold 95°C for 120 s, and Stage 2: 2- Temperature Cycle repeat for 40 times (95°C for 15 s and 60°C for 30 s with optics ON). The Dye set used was FCTC25, the number of cycles was 40 and the time was of an average of 45 min.

For performing PCR a volume of each sample (based on the level of bloom) was filtered through a Whatman Filter Nucleopore 25 mm, 0.8 µm (face up) using a syringe and filter holder. The filter was then transferred to a bead lysis tube and bead-lysed for 2 minutes at 4000 speed and centrifuged afterwards for 5 minutes. In the mean-time, an appropriate amount of the PCR mix was reconstituted with PCR water and vortexed.

In each of the spinning tubes, 20 µL of PCR mix was combined with 5 µL of either a blank, a standard or the sample, and the tube was spun with a spinner for 2 minutes. The sequence was initiated and run with a Cepheid Smartcycler.

Quantitation of gene copies was based on calibrations with the PhytoXigene™ CyanoNAS standards 559 (100-1,000,000 copies per reaction).

### 9.3.2 Sample preparation

#### *Water*

Samples for cyanotoxins analyses were processed following Zervou *et al.* methodology [125], as briefly discussed below. A volume of 150 mL of water was filtered with a glass vacuum filter device with holder for 47 mm glass fiber filters. The latter with retained biomass were preserved in low temperatures for further analyses. The pH of the water filtrate was adjusted to 11 with NaOH (2M) (dropwise).

A quality control sample set was prepared with a standard solution of different Cyanotoxins (100 ng/L). SPE was performed using a combination of two cartridges, Oasis HLB (200 mg, 6 cc, 25–35µm, Waters, USA) and HyperSep Hypercarb PGC (porous graphitic carbon, 200 mg, 3 cc, 30–40 µm, Thermo Scientific, UK) using a 12-port SPE vacuum manifold with large volume samplers (PTFE tubes) (Supelco) with a vacuum pump. Cartridges were conditioned passing, in the following order, 6mL dichloromethane (DCM), 6 mL of methanol (MeOH), and 6 mL of water (pH 11) without vacuum. Flow rate was 0.5 ml/min. After passing the samples and 6mL of water cartridges were dried for 15 min (under vacuum). Then the sequence of cartridges was reversed. Elution was done with (60/40 v/v) MeOH/DCM (dichloromethane) with 0.1% formic acid (HCOOH). Eluents were evaporated under a gentle nitrogen stream, then reconstituted with 150 µL of (95/5) H<sub>2</sub>O/MeOH. Quality control samples were reconstituted with 200 µL. Samples were then vortexed, kept in an ultrasonic bath for 5 min, then vortexed again (the concentration ratio was 1:1000). The resulting solutions were transferred into vials for LC-MS analysis.

#### *Biomass*

Filters served as a material for the evaluation of intracellular cyanotoxins within the biomass. Glass filters were extracted with 9mL of 75% MeOH, vortexed, put in an ultrasonic bath for 15 min, vortexed once more, then centrifuged for 10 min at 4000 rpm at 22°C. A 3-ml supernatant aliquot was evaporated, reconstituted with 500 µL 5 % MeOH, vortexed and ultra-sonicated for 5 min. An additional centrifugation may be needed to eliminate suspended particles. The supernatant was finally transferred to the LC-MS vials.

For the investigation on the presence of PSPs, 2 samples (50 ml for S1 and 30 ml for S2) were filtered through glass fiber Millipore 0.45 µm filters. The filters with biomass were placed in Eppendorf tubes with 4.5 ml of 0.2 M CH<sub>3</sub>COOH (acetic acid) in 50 % ACN (acetonitrile) and were vortexed for 30 s. They were sonicated for 15 min, vortexed again for 30 s and centrifuged at 4000 rpm for 10 min at 20°C. The supernatant was separated from the pellet. A volume of 2.5 ml was transferred in a glass tube and dried under a gentle stream of nitrogen at 30°C. The residue was re-dissolved with 500 µL 3mM HCl, sonicated for 5 min, centrifuged at 10000 rpm for 5 min and filtered using PVDF 0.22 µm

Whatman filters. Finally, the samples were transferred to an autosampler glass vial for HILIC-MS/MS analysis.

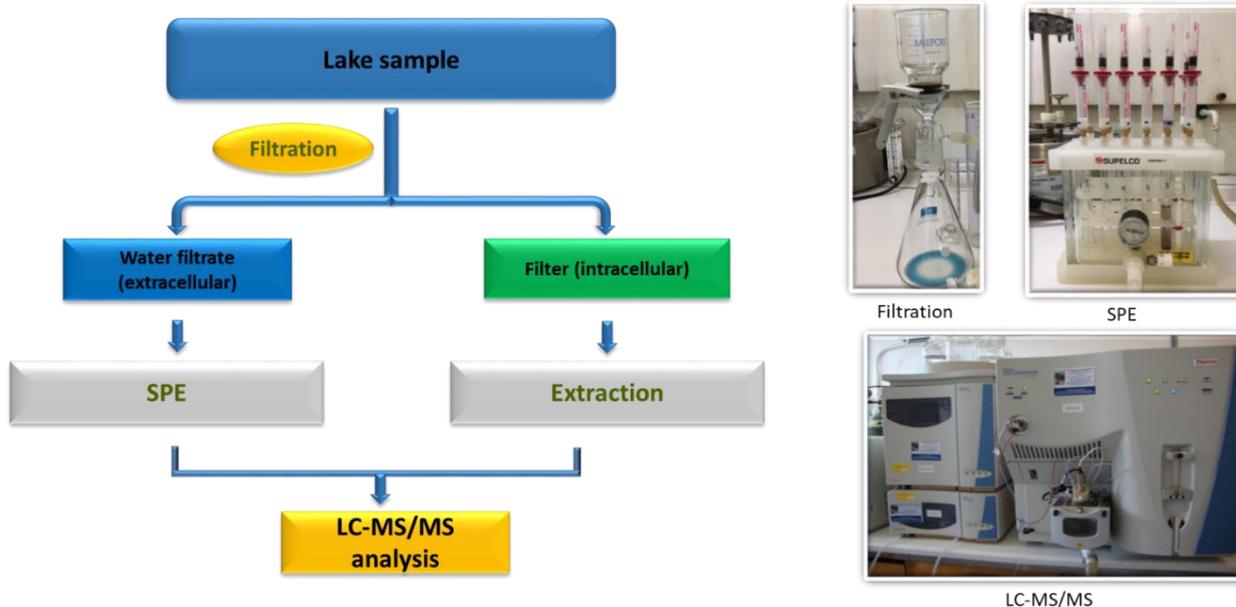


Figure 41: Water samples analysis workflow.

### *Fish tissues*

Fresh fish were transported on ice to the laboratory within 24 h after sampling and sacrificed. Livers and muscles were dissected and frozen separately at  $-20^{\circ}\text{C}$ . Frozen organs and tissues were then lyophilized with a Labconco freeze-dried for 48 h at  $-84^{\circ}\text{C}$  and 0.2 mbar and powdered with a pestle and mortar.

Samples were handled according to the in-house optimized and selected methods. A mass of 0.2 g of lyophilized and powdered muscles was extracted with 5 mL of 0.5% formic acid in 80% methanol, by magnetic mixing for 15 min, followed by ultra-sonication for 30 min. The mixture was then transferred to a Falcon tube and centrifuged for 15 min at 4500 rpm at  $22^{\circ}\text{C}$ . The supernatant was extracted with 1 mL of hexane in a separatory funnel. Hexane was discarded and the bottom part containing the analytes was transferred to a flask and diluted with 100 mL of 0.3% formic acid. The extract was cleaned up with a HLB cartridge activated with 6 mL methanol followed by 6 mL acidified water (0.3% formic acid). After the passage of samples, the cartridge was washed with 6 mL of water, dried for 15 min under vacuum and eluted with 6 mL pure methanol. The eluents were dried in a water bath at  $40^{\circ}\text{C}$  under a gentle nitrogen stream. They were reconstituted with 200  $\mu\text{L}$  of 5 % MeOH, vortexed for few seconds, ultra-sonicated for 5 min, vortexed again, and finally transferred to LC-MS/MS vials. When analyses were not performed directly after the sample preparation, vials were kept in the freezer at  $-20^{\circ}\text{C}$ .

In parallel, for liver samples, 50 mg of lyophilized powder was homogenized with 5 mL of 0.5% formic acid in 80% methanol, by magnetic mixing for 15 min, followed by ultra-sonication for 30 min. The mixture was then transferred to a Falcon tube and centrifuged

for 15 min at 4500 rpm at 22°C. then, the supernatant was extracted with hexane and kept aside. The pellet was re-extracted with 5mL of 5% Acetic Acid containing 0.01M EDTA, following the same procedure, centrifuged and extracted with hexane. The two extracts were then pooled, diluted in 100 mL of acidified water and pre-concentrated on a HLB cartridge. The cartridge was washed with 6mL of water after the passage of the sample, dried under vacuum for 15 min, eluted with 6 mL of methanol and evaporated. The residue was reconstituted with 200  $\mu$ L of (95/5) H<sub>2</sub>O/MeOH.

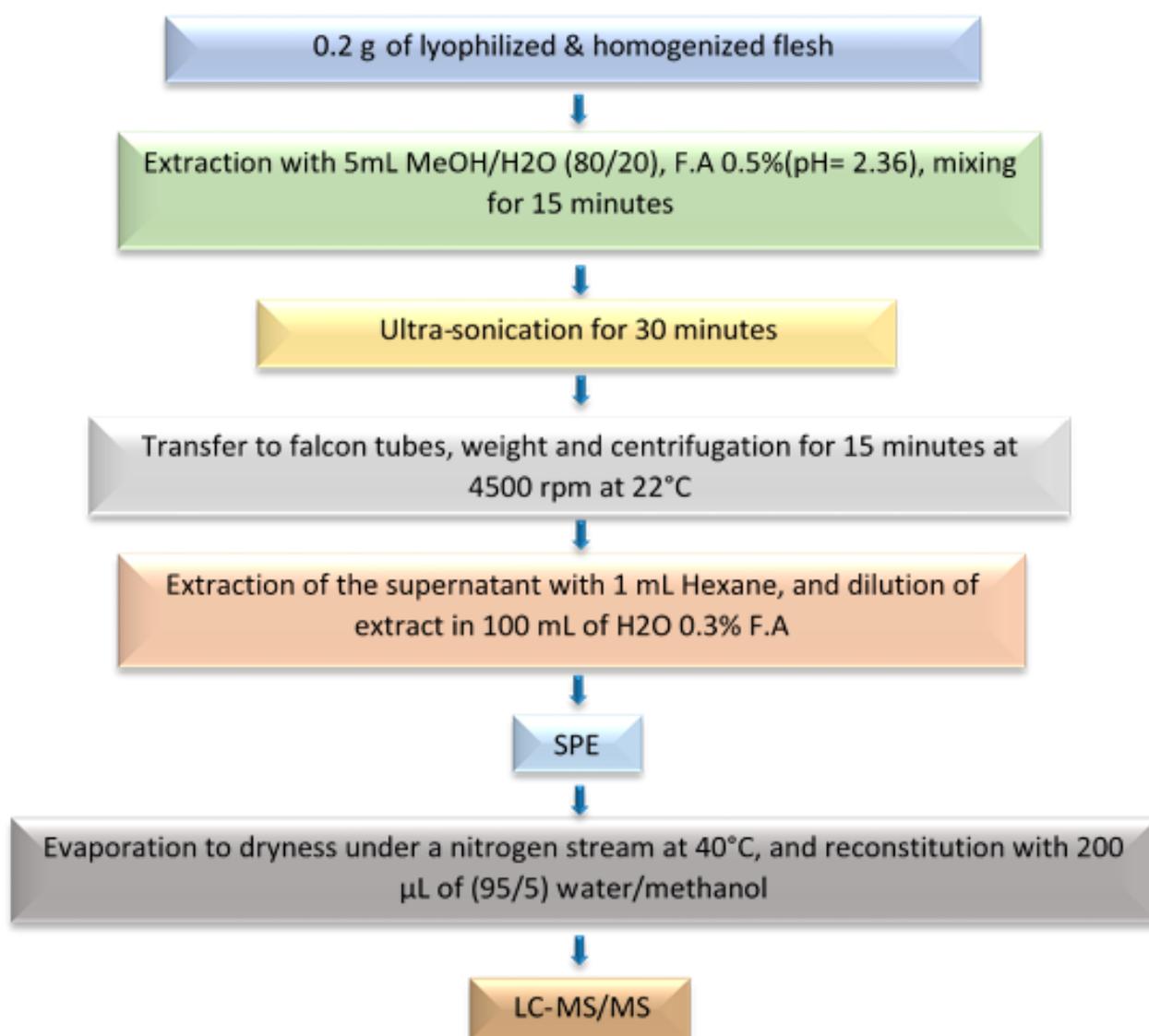


Figure 42: Workflow of cyanotoxins' extraction procedure from fish liver.

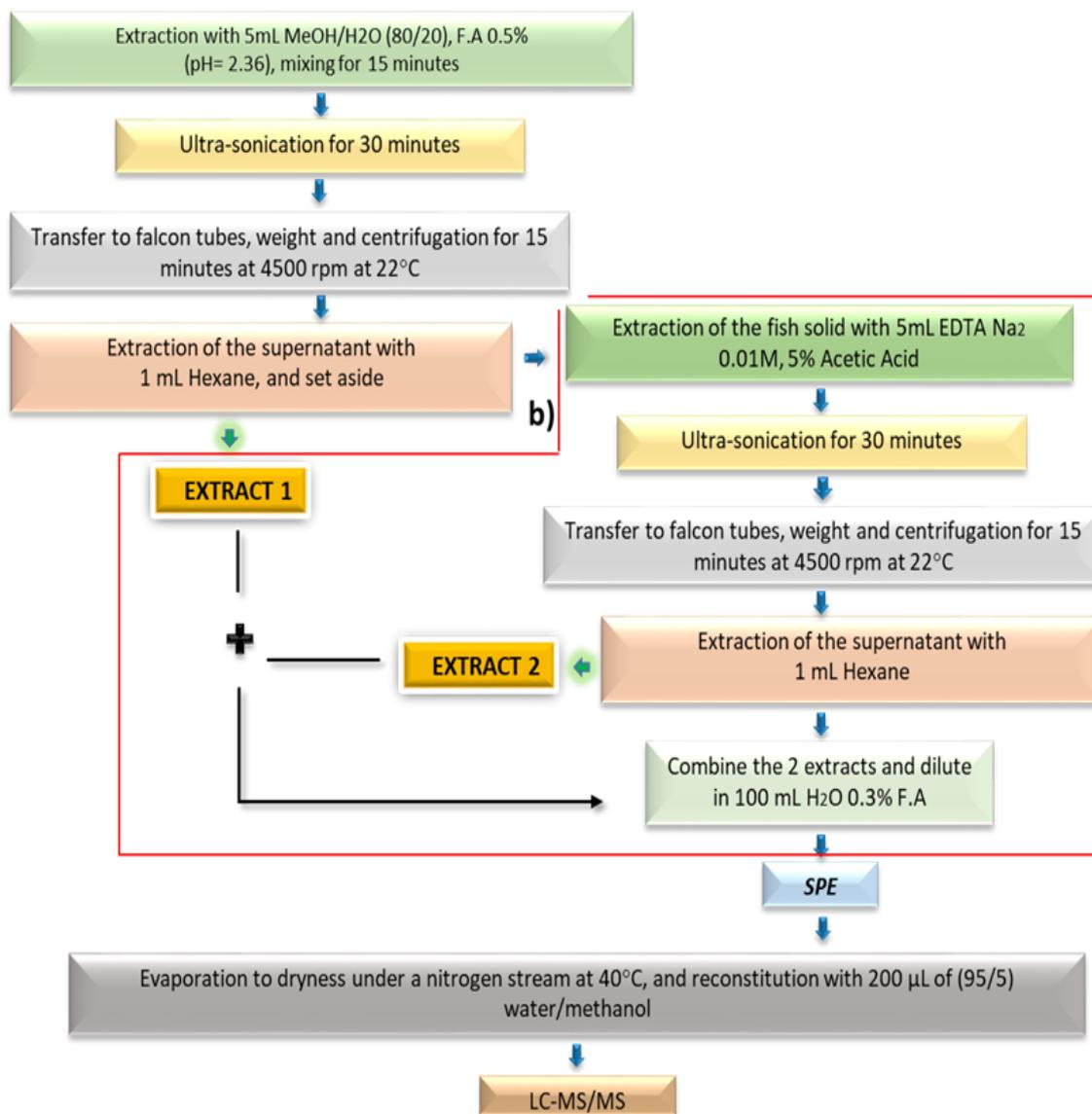


Figure 43: Workflow of cyanotoxins' extraction procedure from fish muscles.

### 8.3.3. LC-MS/MS for cyanotoxins determination

#### Low-resolution mass spectrometry

LC-MS/MS analysis of nodularin and 12 microcystins (dm MC-RR, MC-RR, MC-YR, MC-HtyR, dm3MC-LR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF) were performed using A Finnigan Surveyor LC system, equipped with an AS autosampler coupled with a TSQ Quantum Discovery Max triple-stage quadrupole mass spectrometer with electrospray ionization (ESI) source as described in Zervou *et al.* [125].

In brief, chromatographic separation was performed by reversed phase liquid chromatography (RPLC) column Atlantis T3 (2.1 mm × 100 mm, 3 μm). A gradient elution program was applied with solvents (A) ACN and (B) water, both containing 0.5% formic acid. The gradient started at 5% A (held for 3 min), which increased to 20% A in 1 min (held for 2 min), further to 35% A in 1 min (held for 7 min), 70% A in 14 min and finally 90% in 1 min (held for 3 min). An equilibration time of 10 min was kept after each sample run. Flow rate was set at 0.2 mL min<sup>-1</sup> with 20 μL injection volume. Column temperature was set at 30 °C. Electrospray Ionization (ESI) in positive mode was used.

Detection of cyanotoxins was carried out in a Multiple Reaction monitoring (MRM) mode, using the three most intense and characteristic precursor/product ion transitions for each analyte. Sheath gas was set at 30 a.u (arbitrary unit), auxiliary gas was set at 5 a.u and spray voltage was 4250 V. Capillary ion transfer temperature was set at 325°C and collision gas pressure (argon) was 1.5 mTorr.

The HILIC-MS/MS analysis of 3 saxitoxins (STXs) (STX, neoSTX and dcSTX) and 6 gonyautoxins (GTXs) (GTX1, GTX2, GTX3, GTX4, dcGTX2 and dcGTX3) was performed using a hydrophilic interaction liquid chromatography column, SeQuant® ZIC®-HILIC (150mm x 2.1mm, particle size: 3.5 μm, Merck) combined with a pre-column SeQuant® ZIC®-HILIC (5 μm, 200 Å, 20 x 2,1 mm). A gradient elution program was used with (A) ACN/H<sub>2</sub>O containing 2 mM HCOONH<sub>4</sub> (ammonium formate) and 3,6 mM HCOOH (95:5, v/v) and (B) H<sub>2</sub>O, containing 2 mM HCOONH<sub>4</sub> and 3,6 mM HCOOH. The gradient started at 50% A (held for 9 min), in 1 min was decreased at 30 % (held for 29 min) and in 1 min increased at 40 % (held for 4 min). An equilibration time of 15 min was kept after each sample run. Flow rate was 0.1 mL/min, the column temperature was 25°C and the injection volume was 5 μL and the total run was 60 minutes.

Detection of PSPs (paralytic shellfish poisoning toxins) was carried out in a multiple reaction monitoring (MRM) mode, using the three most intense and characteristic precursor/product ion transitions for each analyte. Sheath gas was set at 20 arbitrary unit (a.u), auxiliary gas was set at 5 a.u and spray voltage was 4200 V. moreover, capillary ion transfer temperature was set at 370°C and collision gas pressure (argon) was 1.5 mTorr.

Identification of target CTs was based on three criteria: (1) retention time (t<sub>R</sub>) of compounds (2) three characteristic precursor/product ion transitions, and (3) two calculated ratios of precursor/product ion transitions.

#### *High resolution mass spectrometry*

The same samples as for low resolution MS were analyzed. Separation was performed with an RP chromatographic column (Atlantis T3, 2.1 x 100mm, 3μm) applying gradient program according to *Zervou et al* [125]. The electrospray Ion Max NG (new generation) with H (heated)-ESI probe functioned in a positive mode, at a temperature of 275°C. For data acquisition, the detector was an Orbitrap scanning for precursors from 400 to 2000 Dalton. Data dependent acquisition (DDA) mode was applied in order to obtain the fragmentation spectra of the 3 most abundant compounds in collision-

induced dissociation (CID) and higher-energy C-trap dissociation (HCD) modes with a 120,000 resolution and 70% for RF lens.

In other words, and in a more simplified way, it was done as the following:

Full MS + 6 ddMS2 scans (CID [3 scans] + HCD [3 scans]) = Top3

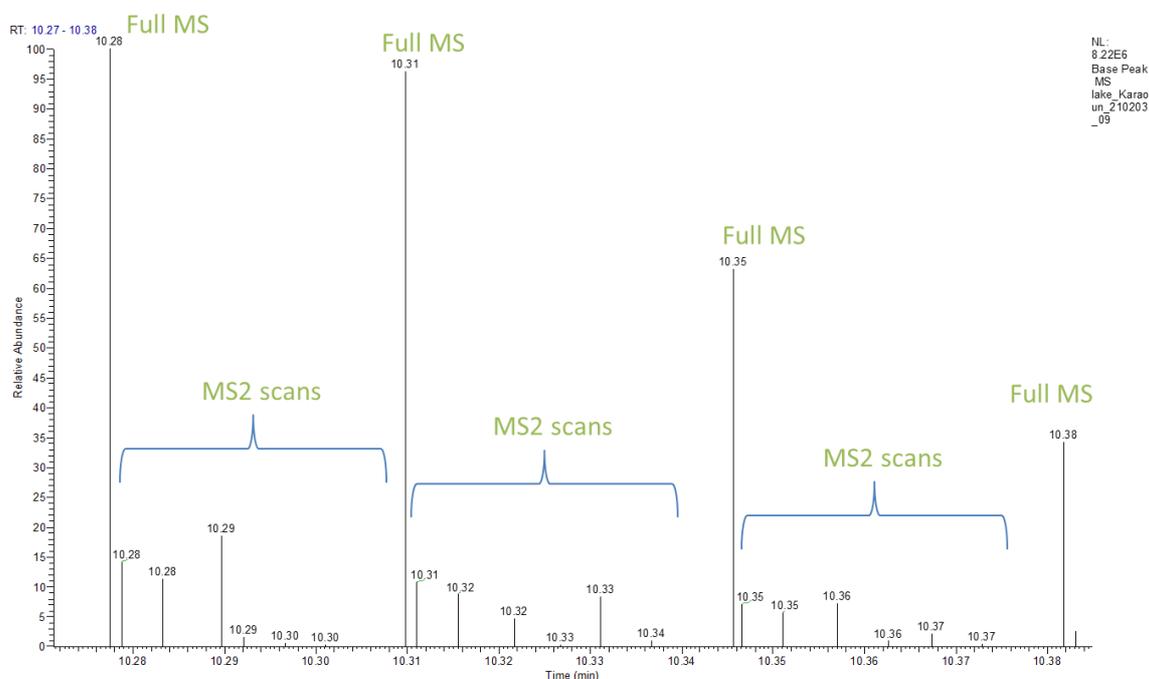


Figure 44: Workflow of mass spectrometry analyses.

However, and in order to maximize the number of data, or cyanotoxins detected, and to avoid underestimating the less abundant compounds that give a lower signal, a variety of filters was implemented as:

- the Mono Isotopic Precursor Selection (MIPS)
- Dynamic exclusion that targets to maximize the number of precursors fragmented by avoiding to repeat already fragmented ones (it means, if a precursor is fragmented for 8 times in 10 seconds, it will be excluded in the coming 10 seconds).
- Targeted Mass Exclusion: fixed list of masses to be ignored in the fragmentation (that could be artefacts from the instrument or common contaminants in solvents)

In order to obtain fragmentation spectra in CID and HCD, Orbitrap was operated with the same resolution as in the full MS, but with a collision energy of 45% in CID and 30, 40 and 50% in HCD.

For data treatment, Compound Discoverer software was used combined to online and published cyanotoxins databases. The implemented workflow (Figure 45) maximized the chances of accurate matching and identification of cyanotoxins, taking into consideration

retention time, the  $m/z$  ratio, the shape of the peak, and the degree of matching with a theoretical compound.

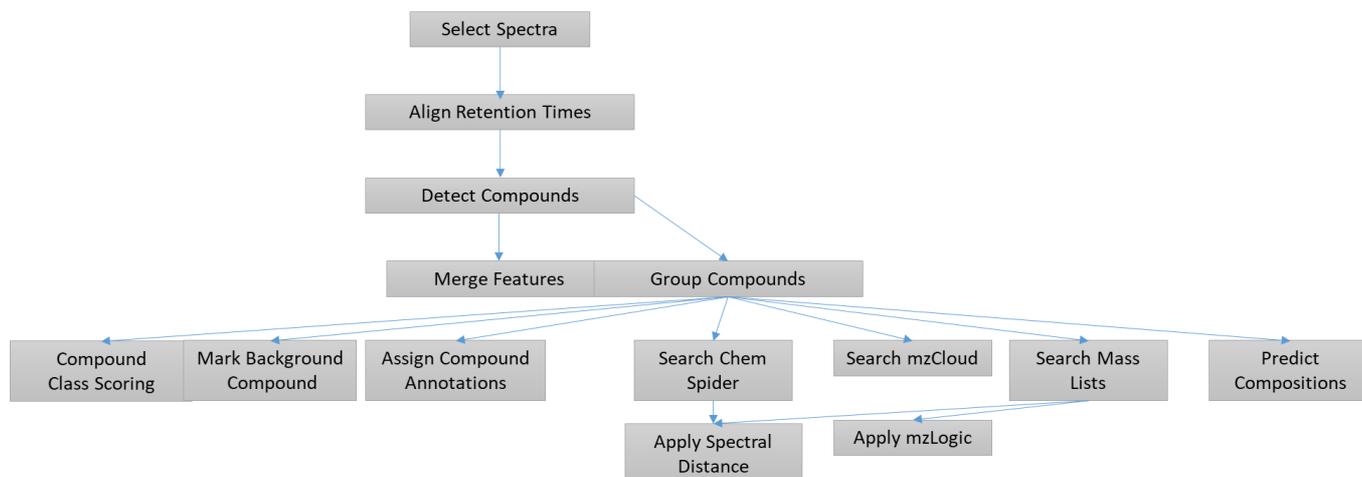


Figure 45: Data treatment workflow using compound discoverer.

#### 9.3.4. ELISA for cyanotoxins determination

Aliquots of 100  $\mu\text{L}$  of standard solutions, samples, and control were placed in duplicates in a 96-plate microtiter plate. Then 50  $\mu\text{L}$  of the enzyme conjugate was added, followed by the addition of 50  $\mu\text{L}$  of the anti-body solution, using a multi-channel pipette. Wells were covered with parafilm. The microplate was agitated by a circular motion on a solid surface or on the benchtop for 30 s followed by 90 min of incubation at room temperature. After the incubation, the cover was removed and the content of the wells was emptied in a sink with strong and successive blotting on towel papers. Strips were washed 3 times, each time with at least 250  $\mu\text{L}$  of diluted buffer and blotting. After the last wash, all the remaining buffer was removed even if it required additional blotting. Again, with a multi-channel pipette, 150  $\mu\text{L}$  of substrate (color) solution were added to wells individually and were followed by another incubation period (at room temperature and away from sunlight) of 20-30 min after covering wells with parafilm. The stop solution (100  $\mu\text{L}$ ) was added after the incubation. The absorbance at 450 nm was read with a microplate ELISA photometer within 15 min following the addition of the stop solution.

#### 9.3.5. PPIA for cyanotoxins determination

In wells A to D, MC-LR 50  $\mu\text{L}$  standard solutions of different concentrations were added in duplicates. In the other wells, the same volume of samples was added also in duplicate. Moreover, 70  $\mu\text{L}$  of phosphatase solution to each well was added followed by 90  $\mu\text{L}$  of chromogenic substrate a brief circular motion agitation and an incubation at 37°C for 30 min. Finally, the stop solution 70  $\mu\text{L}$  was added after the incubation, and the

absorbance at 450 nm was read with a microplate photometer within 15 min following the addition of the stop solution.

## 10. Volatile organic compounds analyses

### 10.1. Materials

#### 10.1.1. Chemicals and reagents

Standards used in untargeted analyses for volatile organic compounds are listed in (Table 14).

**Table 14.** VOCs standard solutions

VOC's name	Retention time with non- polar column
2-Butanone	1.93
1-Pentanol	3.01
Hexanal	3.32
1-Hexanol	4.03
2-Heptanone	4.26
Benzaldehyde	5.12
1-Heptanol	5.15
1-octen-3-ol	5.26
Eucalyptol	5.92
Y-Terpinene	6.18
1-Octanol	6.25
Naphtalene	7.60
1-Butanol	-
Tetradecane	9.49
$\alpha$ -Ionone	9.76
Pentadecane	10.35
2,4-di-tert-butylphenol	10.42
Hexadecane	11.16
Heptadecane	11.93

No solvents were needed for the preparation of water samples during GC-MS/MS analyses. Sodium chloride was added to a certain volume of sample and it was completed to 10 mL with water.

#### 10.1.2. Laboratory equipment

16-ml glass vials with PTFE lined caps were from Thermo Scientific. Measurements of water samples and UP water were done with an automatic 200-1000  $\mu\text{L}$  pipette (Eppendorf) or with an automatic 2-10 ml (Finnpipette Labsystems)

### 10.1.3 Chromatographic columns

The columns used in head space- solid phase micro-extraction gas chromatography tandem mass spectrometry were:

- RXI®- 5 Sil MS, 30 m, 0.25 mm internal diameter and 0.25  $\mu\text{m}$  df (film thickness) (Restek, part number 13623).
- RXI®- 624 Sil MS, 60 m, 0.32 mm internal diameter and 1.8  $\mu\text{m}$  df (film thickness) (Restek, part number 13872)

## 10.2. Instrumentation

### HS-SPME-GC-MS/MS

The analyses of Volatile Organic Compounds (VOCs) were performed with a Bruker 456 GS Gas Chromatograph coupled to a Bruker TQ Triple Quad Mass Spectrometer with a PAL SPME auto-sampler (Figure 46). The fiber used is a 50/30  $\mu\text{m}$  DVB/CAR/PDMS stableflex (Supelco). Data acquisition and instrument control were done with Bruker MSWorkstation version 8.2, 2015 software. Data processing was done with NIST MS Library and AMDIS from NIST 2017.



Figure 46: Bruker 456 GC-Scion TQ MS/MS, PAL SPME Autosampler.

## 10.3. Methods

### 10.3.1. Untargeted screening of cyanobacterial volatile and T&O compounds

A volume (2-10 mL) of sample was transferred into a 20ml SPME glass vial containing 3g NaCl. Then the volume was adjusted to 10ml with water and closed tightly. A fluorobenzene standard solution in methanol (Sigma) spiked at a concentration of 5  $\mu\text{g/L}$  in the samples was used to evaluate the SPME efficiency.

Untargeted screening of cyanobacterial volatiles and T&O compounds was done by a 456 gas chromatograph coupled to TQ mass spectrometer, equipped with an SPME autosampler (Bruker, Germany). The HS-SPME was done by the 2cm divinylbenzene/carboxen/polydimethylsiloxane SPME fiber under the following

conditions: 1) Pre-incubation for 10 min at 60°C with 300 rpm agitation; 2) Extraction (fiber goes into the headspace) for 10 min at 60°C with 300 rpm agitation; 3) Desorption (fiber enters the injector) for 2 min (and stays for 8 more min) with split open.

The separation was done on a non-polar column RXI®- 5 Sil MS, 30 m, 0.25 mm internal diameter and 0.25 µm (film thickness) (Restek). The injector worked in a splitless mode at 250 °C. The flow was 1.0 mL /min and the column oven temperature followed the gradient starting at 50°C for 1 min and rising to 250 °C at 15°C/min, and then kept at 250°C for 5 min. The total run time was 19.3 min. Helium of high purity (99.9999% Air Liquide) was used.

For MS conditions, the electron Impact source (EI) was at 250 °C in a full scan mode from 30 amu to 300 amu (atomic mass units) (to 400 amu when using the polar column) with a positive polarity and a mass resolution of 0.77 amu.

Data were treated and analyzed using MSWS software (Bruker). Mass spectral deconvolution and identification of compounds was carried out with AMDIS (NIST) using the NIST MS library (2015) and retention index calibration with C7-C30 saturated alkanes reference standard (Sigma). Combined matching scores (retention index-RI and mass spectral matching) were used as criteria for identification (RI  $\pm$ 20 of the reference RI and a spectral matching  $\geq$ 80%). Identification was considered definite only if a reference standard of the candidate compound was available and retention times of the candidate and reference compounds matched within  $\pm$  0.01 min, in addition to the matching of the mass spectra.

### *10.3.2 Quantitative determination of VOCs*

The quantitative determination of several VOCs was also carried out by HS-SPME-GC-MS according to EN ISO 17943:2016 (HS-SPME-GC-MS), using 5ml samples [203] . The compounds determined were chloroform, bromoform, dibromochloromethane, bromodichloromethane, benzene, 1,2-dichloroethane, tetrachloroethene and trichloroethene, 2-ethoxy-2-methyl propane (ETBE), bromochloromethane, dibromomethane, toluene, 1,2-dibromoethane, chlorobenzene, styrene, bromobenzene, n-butylbenzene, hexachlorobutadiene, naphthalene, 1,1-dichloroethane, 1,1-dichloroethene, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 1,2-dibromo-3-chloropropane, 1,2-dichloropropane, 1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene, 1,3-dichloropropane, 2-methoxy-2-methyl-butane (TAME), ethylbenzene, n-propylbenzene, o-xylene, p+m-xylenes, sec-butylbenzene and tert-butylbenzene. A VOC 57 standard mix (Supelco) was used for calibration and fluorobenzene, toluene-d8 and benzene-d6 (Sigma) as internal standards [203].

HS-SPME was done using the same fiber but at different conditions: equilibration time/temp. 10min/40°C, headspace extraction time/temp. 10 min/40 °C, agitation 300rpm and desorption time 2min.

The chromatographic conditions using the column RXI®- 624 Sil MS, 60 m, 0.32 mm ID, 1.8 µm df (Restek) were: injector temp. 250 °C, splitless, constant flow 1 ml/min (He), column program: 35 °C (5 min) to 250 °C at a rate of 8 °C/min and at 250°C (10 min). MS

conditions were: EI source (70 eV), selected ion monitoring (3 ions per compound), positive polarity. The LOD of each compound was 0.2 µg/L. Detection and confirmation of compounds was carried out with the ISO criteria for retention time and ion ratios.

## 11. Methods calibration and validation

### 11.1. Determination of cyanotoxins in water samples using LC-MS/MS *ELISA and PPIA*

The method applied in this study for the detection and quantification of cyanotoxins in water samples was previously validated [125]. The method's performance parameters such as specificity, linearity, precision, accuracy, and limits of detection were assessed elsewhere. Quality control procedures including measurements of blank/negative and control/positive samples were followed in each batch of analyzed samples. The method is also accredited by ISO 625 17025 in the NCSR Demokritos laboratory [204]. Validation data of ELISA and PPIA for MCs in water were reported previously and methods were proven suitable for quantitative screening of MCs. Negative and positive control samples were included in each analysis batch.

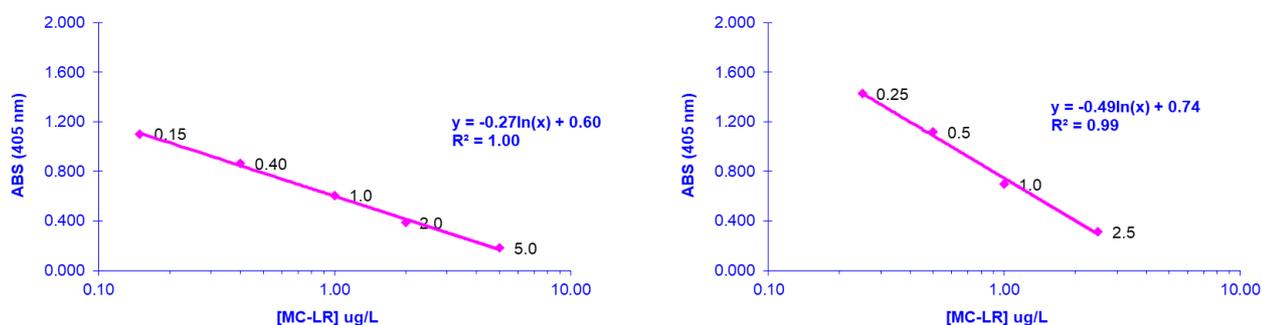


Figure 47: Calibration curves and accuracy of ELISA (left) and PPIA (right) methods.

### 11.2. Determination of cyanotoxins in fish *Cyprinus carpio*

The in-house optimized method for the extraction of CTs from fish and their determination using LC-MS/MS was also validated.

For optimization, several combinations of extraction solvents, extraction/mixing times, clean-up steps and SPE cartridges were tested (Oasis HLB and HLB followed by Sep-Pak Vac, silica) as presented in (Figure 48) and (Figure 49). Optimization experiments were performed with samples spiked with MC-LR and MC-RR at concentration levels of 100 and 400 ng/g dw for flesh and liver, respectively.

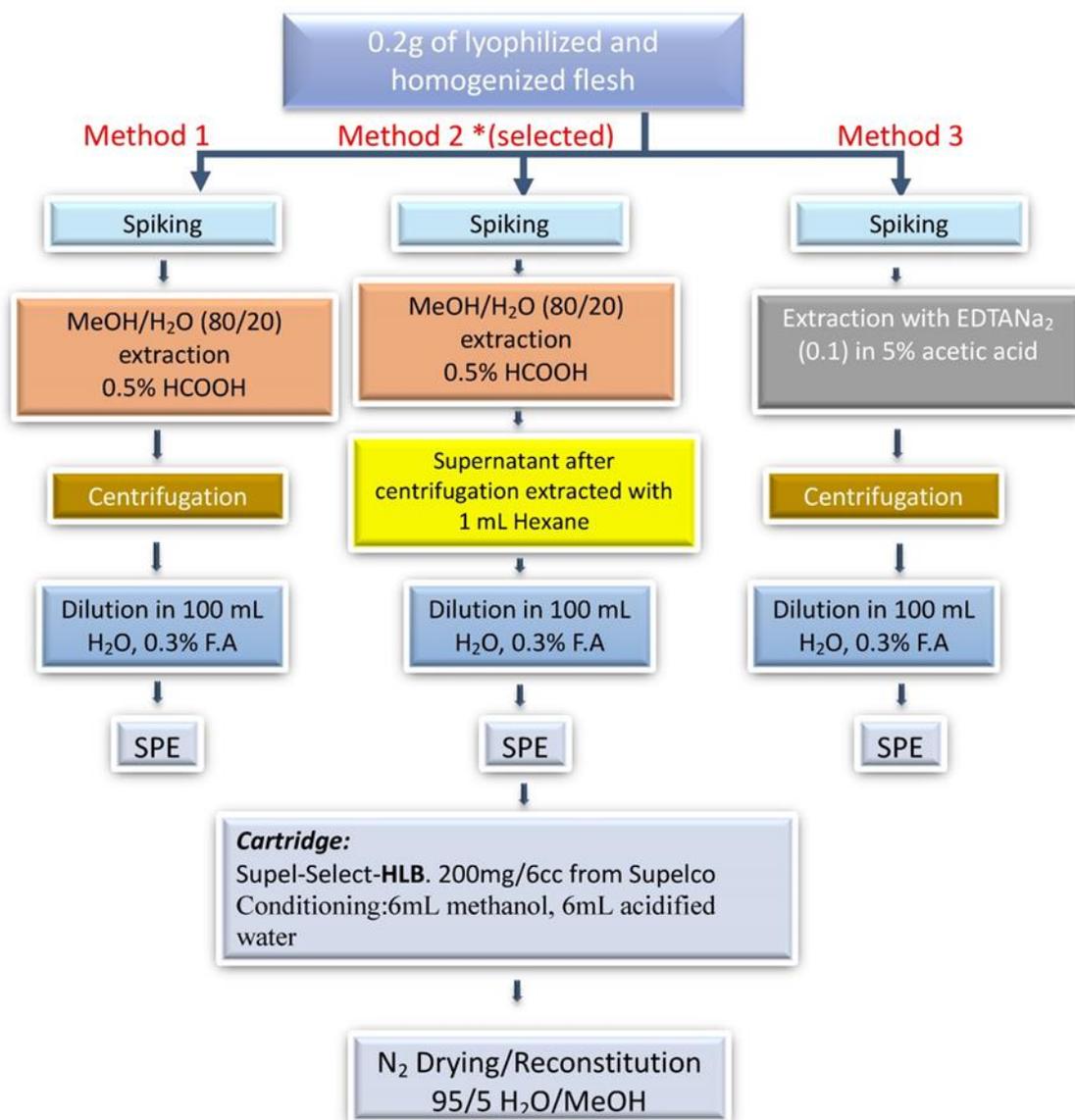


Figure 48: Validated method for fish analyses.

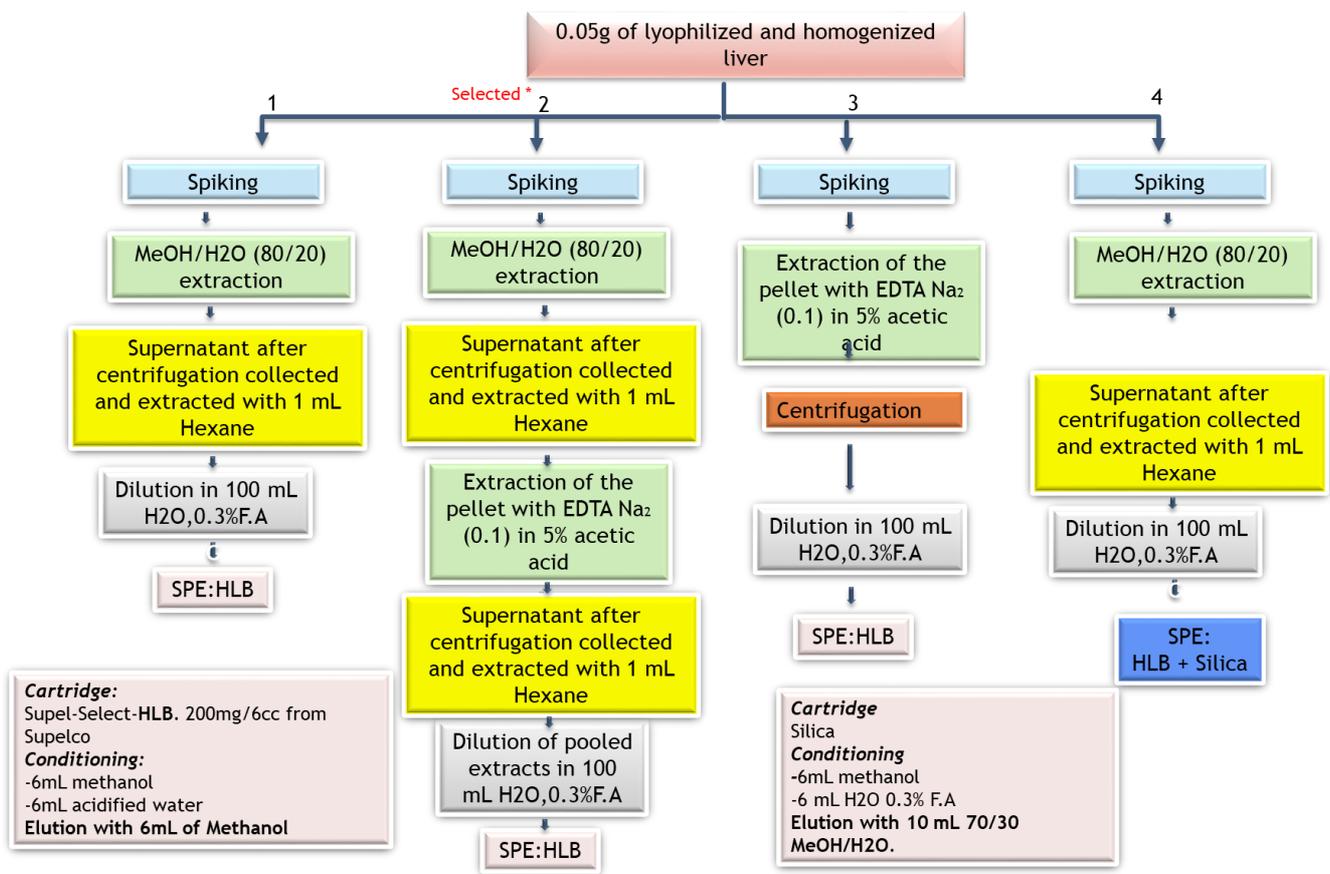


Figure 49: Validated method for fish liver analyses.

The optimized method was selected for maximum of recoveries (Figure 50). Method 2 provided the highest mean recoveries for the two spiked MCs in flesh (78.9% for MC-RR and 79.1% for MC-LR) and liver (77.1% for MC-RR and 75.4% for MC-LR).

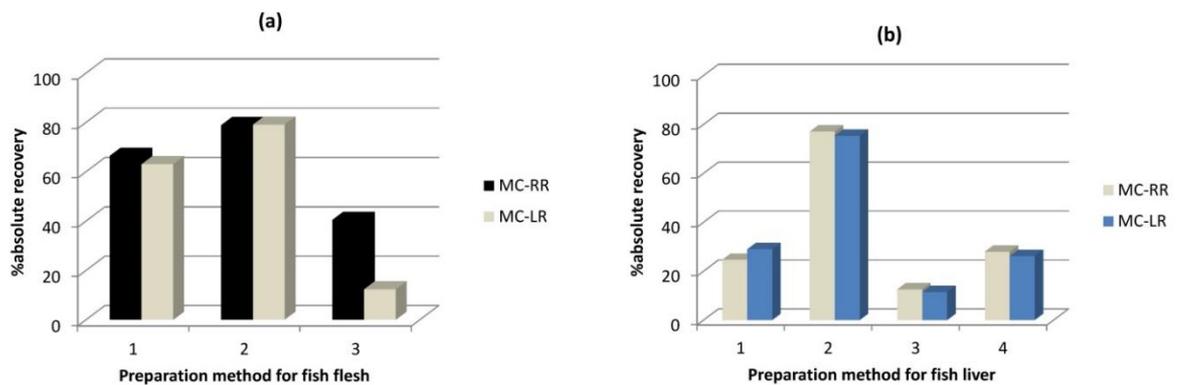


Figure 50: Recovery rates of optimized methods in fish analyses.

Subsequently, the optimized method was validated. Recovery and precision were obtained by analyzing environmental/toxins-free lyophilized flesh and liver fish samples spiked with a mixture of 12 MCs, at concentration levels of 100 and 400 ng/g dw in 3 replicates. Samples were extracted and analyzed as outlined in Chapter 9. The performance characteristics of this method are provided in (Table 15). Mean recoveries of [D-Asp<sup>3</sup>]MC-RR, MC-RR, MC-YR, [D-Asp<sup>3</sup>]MC-LR, MC-LR, MC-HiLR, MC-LA and MC-LY ranged from 68.5% to 81.6% for flesh, while for liver samples, recoveries ranged from 61.5% to 72.2%. Intra-day precision was in the range of 6.8 - 16.5% for flesh and 5.5 – 15.2% for liver. LODs (limits of detection) for fish flesh and liver samples ranged from 1.0 to 7.0 ng/g dry weight and from 0.8 to 5.6 ng/g dry weight, respectively.

**Table 15.** LOD s and LOQ s in fish analyses

Fish Flesh	[D-Asp <sup>3</sup> ]MC-RR	MC-RR	MC-YR	MC-HtyR	[D-Asp <sup>3</sup> ]MC-LR	MC-LR	MC-HiLR	MC-WR	MC-LA	MC-LY	MC-LW	MC-LF
LOD flesh (ng/g dw)	2.0	1.0	4.0	7.0	4.0	4.0	6.0	6.0	3.0	6.0	4.0	5.0
LOQ flesh (ng/g dw)	6.0	3.0	12.0	21.0	12.0	12.0	18.0	18.0	9.0	18.0	12.0	15.0
% Recovery	80.4	81.6	76.8	45.6	72.5	78.8	68.5	10.5	75.4	76.8	22.2	29.5
intra-day % rsd	7.4	6.8	11.4	11.2	11.2	12.2	16.5	26.5	6.5	5.6	23.4	13.5
Fish Liver	[D-Asp <sup>3</sup> ]MC-RR	MC-RR	MC-YR	MC-HtyR	[D-Asp <sup>3</sup> ]MC-LR	MC-LR	MC-HiLR	MC-WR	MC-LA	MC-LY	MC-LW	MC-LF
LOD liver (ng/g dw)	1.6	0.8	3.2	5.6	3.2	3.2	4.8	4.8	2.4	4.8	3.2	4.0
LOQ liver (ng/g dw)	4.8	2.4	9.6	16.8	9.6	9.6	14.4	14.4	7.2	14.4	9.6	12.0
% Recovery	65.5	71.8	71.2	39.9	69.5	70.4	61.5	5.5	72.2	70.1	19.2	16.5
intra-day % rsd	12.2	11.1	5.5	10.3	14.4	15.2	15.5	28.4	9.5	12.2	30.2	33.1

### III. RESULTS AND DISCUSSION

## 12. Survey of cyanobacteria and cyanotoxins in lake Karaoun

### 12.1. Diversity of cyanobacteria in lake Karaoun.

In almost all field campaigns, cyanobacterial blooms were always seen. Microscopic examination of water samples showed that the reservoir was dominated by cyanobacteria.

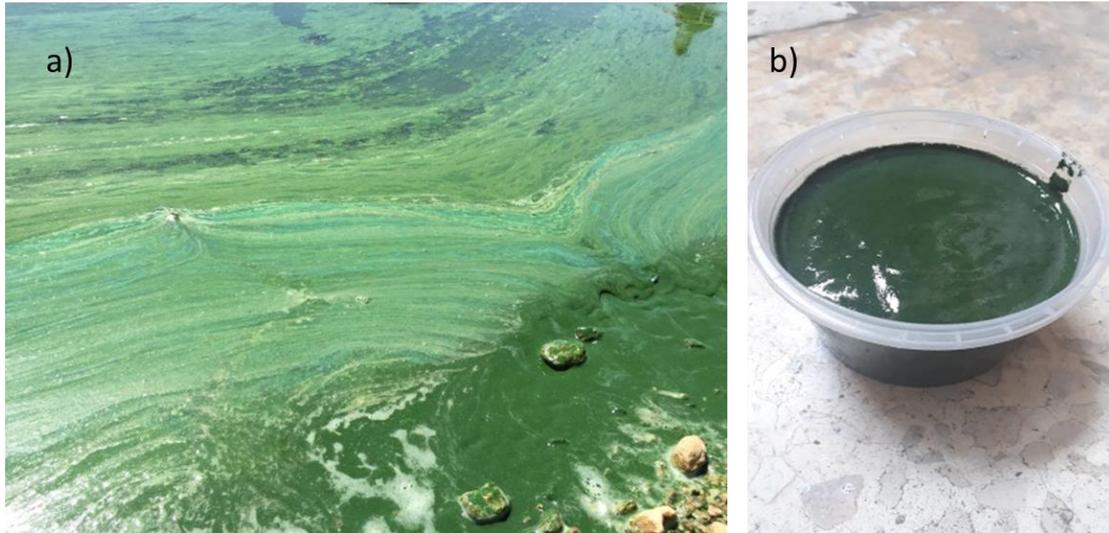


Figure 51: a) Scum in lake Karaoun in August 2019; b) Scum from lake Karaoun in April 2020.

The seasonal dominance of *Aphanizomenon ovalisporum* stretched from October 2019 until April 2020, when it counted for more than 95% of the phytoplankton population. On the other hand, *Microcystis aeruginosa* was the most abundant on July and October 2020. Between the two seasons, in August 2019, both *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* were equally present with abundances of 50% and 49%, respectively (Figure 53).

Occasionally, other phytoplankton species would appear in the reservoir with a very low abundance. The diatom *Aulacoseira granulate* was detected in January 2020 and in July of the same year, *Microcystis ichtyoblable* and *Woronichia naegeliana* were identified in very low abundances, of 1 % each.

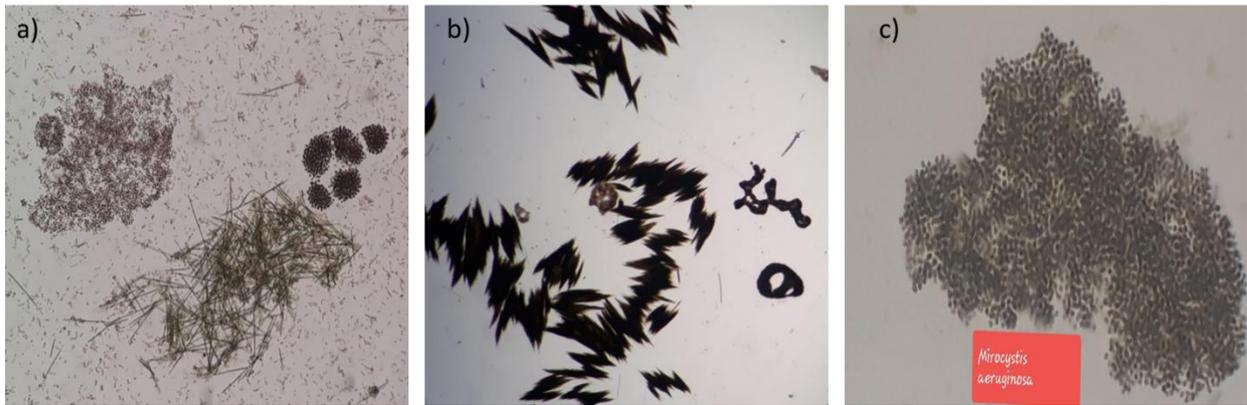


Figure 52: a) *M. wesenbergii* and *Aphanizomenon ovalisporum* in August 2019; b) *Aphanizomenon ovalisporum* in October 2019; c) *Microcystis aeruginosa* in December 2019.

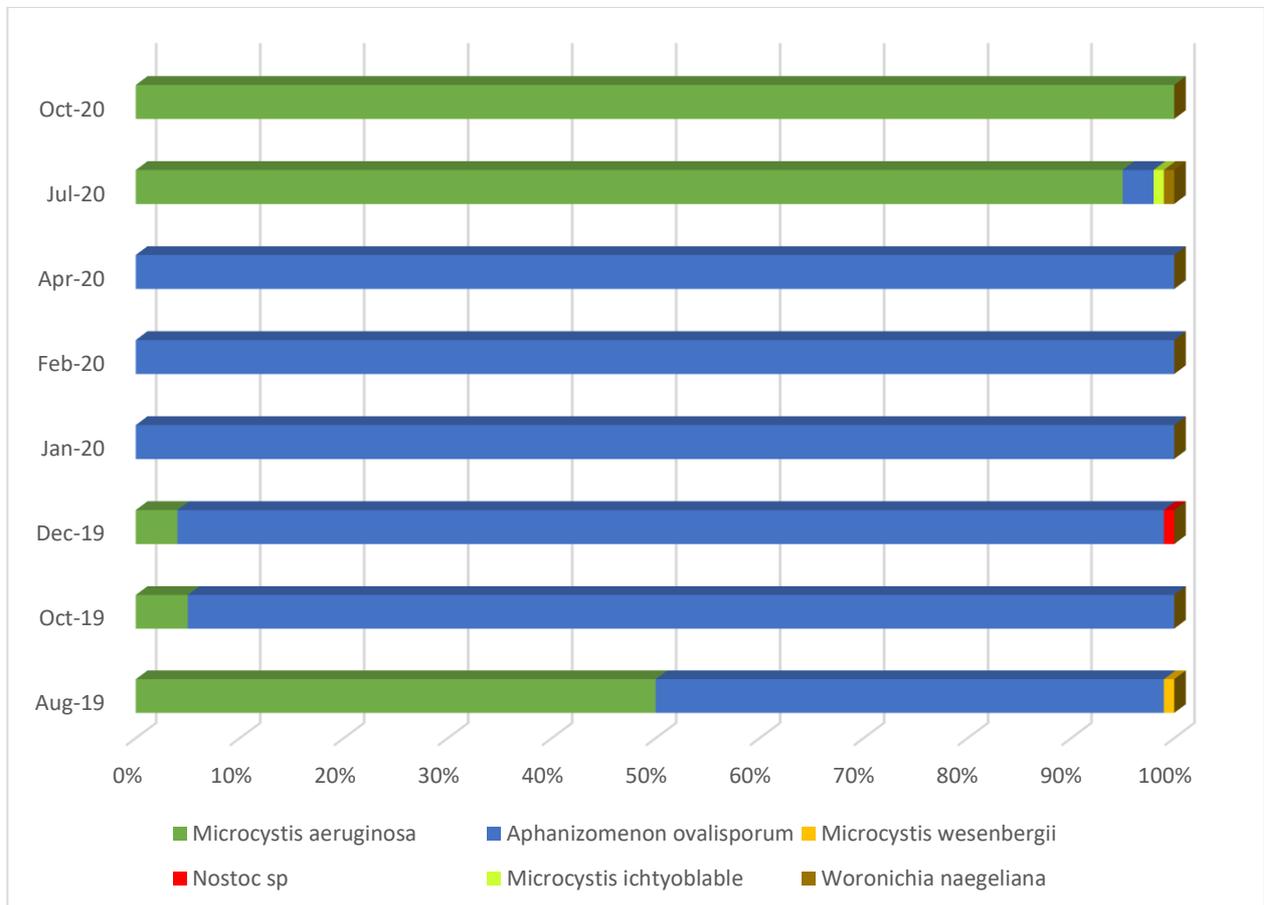


Figure 53: Abundance of cyanobacterial species in Lake Karaoun.

Rarely, and for a short time, some other genera and some zooplankton could appear. On the sampling of 22-12-2019 for example, *Nostoc sp.* and *Chydorus sphaericus* (small crustacean) were seen in the lake in negligible quantities and for a very brief period.

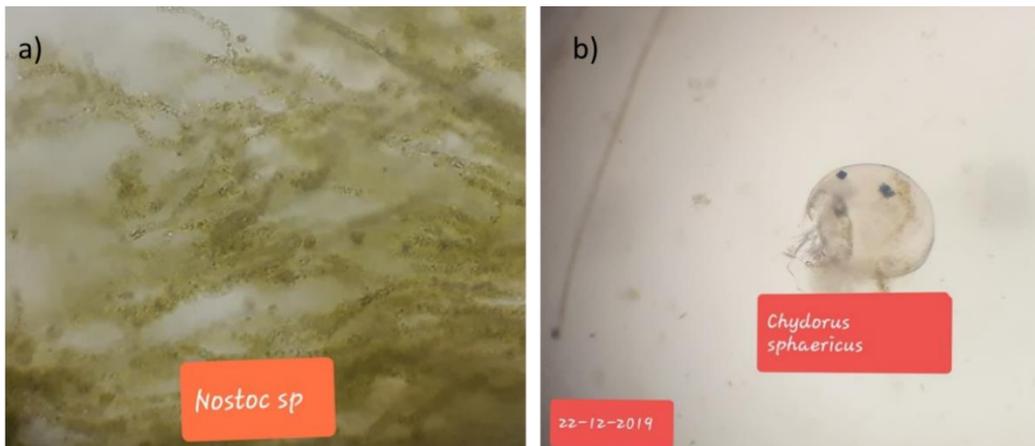


Figure 54: a) *Nostoc sp.*; b) *Chydorus sphaericus* in lake Karaoun in December 2019.

In conclusion, the lake was found to be under the consecutive dominance of the recurrent cyanobacteria *Microcystis aeruginosa* (summer) and *Aphanizomenon ovalisporum* (autumn and spring). Fadel & Slim previously reported that, since 2009, these two species had been constituting >95% of the total phytoplankton biomass. Due to the difference in optimal conditions and temperature of those two cyanobacterial genera, they were proliferating in different seasons.

## 12.2. Microbiological and physico-chemical parameters

The national Litani River Authority (that is in charge of the river and all its contributed usages) provided this project with microbiological and physico-chemical parameters data. After sampling, specimens were measured and analyzed at the authority's laboratory located in Bekaa.

Results showed high concentrations of nitrate, nitrite and phosphate that recorded up to 74, 0.93 and 0.5 118 mg/L, respectively. Those informations confirm once again the hypereutrophic state of the lake declared by Fadel *et al* [24].

The unregulated domestic waste and the direct deposit of ruminants left-overs in the river stream and reservoir were responsible for the presence of *Escherichia coli*, fecal and total coliforms. They were found to be at the levels of 300, 370 and 1400 122 CFU/100 ml, respectively, confirming the extremely poor sanitary conditions of the lake water. The reservoir suffers not only from nutrient enrichment but also forms a great sanitary threat.

**Table 16.** Microbiological and physico-chemical parameters in lake Karaoun

Microbiological parameters	French guidelines for irrigation water	Aug-19	Sep-19	Feb-20	Mar-20	Apr-20	Aug-20
Escherichia coli (CFU/100 ml)	250		-	-	300	0	-
Total coliform (CFU/100 ml)	1000	1400	-	-	425	1100	-
Fecal coliform (CFU/100 ml)	100	370	-	-	-	-	-
Physio-chemical parameters	FAO guidelines for irrigation water	Results					
Conductivity ( $\mu\text{S/m}$ )	30000	310	330	560	410	393	315
Total Dissolved Solids (mg/l)	0-2000	198.4	211.2	-	-	-	189.1
Calcium (mg/l)	0-400	86.7	80.7	-	-	-	-
Magnesium (mg/l)	0-60	17.3	13.9	-	-	-	-
Sodium (mg/l)	0-920	11.4	28.2	-	-	-	-
Bicarbonate (mg/l)	0-610	126.3	117.3	-	-	-	-
Chloride (mg/l)	0-1065	34.4	38.3	-	-	-	-
Sulphate (mg/l)	0-960	-	-	44	54.63	52.31	39.8
Potassium (mg/l)	0-2	3	5	-	-	-	-
pH	6-8.5	8.1	7.9	8.34	8.33	8.09	8.97
Hardness (mg/l)	0-2000	111.6	137.7	-	-	-	-
Nitrate (mg/l)	0-30	12	2.5	15	74	26.62	-
COD (mg/l)	-	-	-	<5	4.59	0	-
BOD (mg/l)	-	-	-	-	2.7	<5	17
Nitrite (mg/l)	-	-	-	0.931	0.177	0.09	0.0597
Phosphate (mg/l)	-	-	-	0.298	0.5	0.26	0.14

### 12.3. Detection of 16S rRNA cyanobacterial and toxic genes

The analyses conducted with q-PCR for the molecular detection of cyanobacteria (16S rRNA) and biosynthetic genes of MCs, NOD, ATX, and Saxitoxins (STX) showed that all samples were found positive for the cyanobacterial ribosomal RNA, proving the presence of cyanobacteria.

In addition, Samples 2 (S2 - 02/10/2019), 4 (S1 - 22/12/2019) and 16 (S3 - 09/10/2020) exhibited the MCs producing genes (*mcyE*). Sample 4 showed the highest abundance in both cyanobacteria and MC genes among all samples.

On another hand, genes responsible of the production of CYN (*cyrA*) and STX (*stxA*) were not found in any of the samples. The results expressed in gene copies per ml of sample, are presented in (Table 17).

**Table 17.** Results of q-PCR analyses

ID	SAMPLE DETAILS	Gene copies/ml			
		16S rRNA (cyanobacteria)	<i>mcyE</i> (MCs & NODs)	<i>cyrA</i> (CYN)	<i>stxA</i> (STX)
1	S1, 22/08/2019	NA	NA	NA	NA
2	S2, 02/10/2019	6951375	69857	ND	ND
3	S4, 02/10/2019	NA	NA	NA	NA
4	S1, 22/12/2019	9765354	127870	ND	ND
5	S1, 15/01/2020	NA	NA	NA	NA
6	S4, 04/02/2020	194446	ND	ND	ND
7	S5, 15/02/2020	9808	ND	ND	ND
8	S5, 17/02/2020	54262	ND	ND	ND
9	S1, 22/02/2020	62344	ND	ND	ND
10	S2, 22/02/2020	5093	ND	ND	ND
11	S1, 15/04/2020	40877	ND	ND	ND
12	S2, 22/04/2020	186812	ND	ND	ND
13	S4, 22/04/2020	15466	ND	ND	ND
14	S1, 14/08/2020	112948	ND	ND	ND
15	S2, 14/08/2020	34032	ND	ND	ND
16	S3, 09/10/2020	171308	6921	ND	ND

ND: Not detected, NA: Not analyzed.

#### 12.4. Targeted LC-MS/MS analyses

Occurrence of CTs (cyanotoxins) in samples from lake Karaoun was assessed by liquid chromatography tandem mass spectrometry (LC-MS/MS) complemented by ELISA, and Protein Phosphatase Inhibition Assay (PPIA).

Based on the state of art of cyanotoxin analyses, liquid chromatography coupled to mass spectrometry was chosen for its specificity, sensitivity and reliability [22]. Water samples were analyzed by LC-MS/MS for the detection and quantitation of extracellular and intracellular cyanotoxins, i.e. anatoxin, cylindrospermopsin, nodularin and 12 microcystins variants.

The water quality of lake Karaoun was once again found alarming. Even if CYN, ATX and NOD were not detected in any of the samples, microcystins were detected in half of the samples (8 out of 16) and their availability extended from 2019 to 2020 samples.

Out of 12 MCs (microcystins) variants, 11 were detected in at least one sample (dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-HiLR, MC-WR, MC-LA, MC-LY, MC-LW and MC-LF). The variant MC-HtyR was not detected in any sample.

Samples 2 (S2-October 19) ,16 (S3-October 20) and 4 (S1-December 19) exhibited the highest number of MCs variants 10, 9 and 5 respectively. Moreover, sample 16 showed the highest concentrations in MCs, where total MC-LR (sum of extracellular and intracellular fractions) reached 211 µg/L and MC-YR 199 µg/L. In this sample, the dissolved (extracellular) amounts of MC-LR and MC-YR were roughly 100-fold of the intracellular amounts. In contrast, in samples 2 (S2-October 19) and 4 (S1-December 19), MC-LR, MC-RR and MC-YR are found mostly as intracellular. An indicative MRM chromatogram (sample 2, S2-02/10/2019 – intracellular fraction) is shown in (Figure 55).

Results of q-PCR were in accordance with those from LC-MS/MS since *mcyE* gene was only detected in samples that showed concentrations of MCs. The absence of cylindrospermopsin and saxitoxins in samples could be explained by the unavailability of their producing genes investigated by the molecular approach.

Variants that were detected in the lake (dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-HiLR, MC-WR, MC-LA, MC-LY, MC-LW, and MC-LF) peaked in October 2019, December 2019 and October 2020 with respective concentrations 146.8, 25.8 and 429.3 µg/L expressed as sum of extra and intracellular fractions (Table 18). MC-LR is the universally most occurring cyanotoxin [57], and in this study was found to be the most abundant followed by MC-YR and MC-RR. On the other hand, CYN, ATX and NOD were not detected in any of the samples.

Although *Aphanizomenon ovalisporum* is a well-known CYN producer, CYN was not detected during the study. The presence of CYN was investigated with ELISA and previously reported during May to November 2012 and March to May 2013. CYN was detected in concentrations ranging from 0.5 to 1.7 µg/L in 2012, and from undetected upto 1.7 µg/L in 2013. However, till now, and to the best of our knowledge, the detection of CYN has never been confirmed by LC-MS/MS.

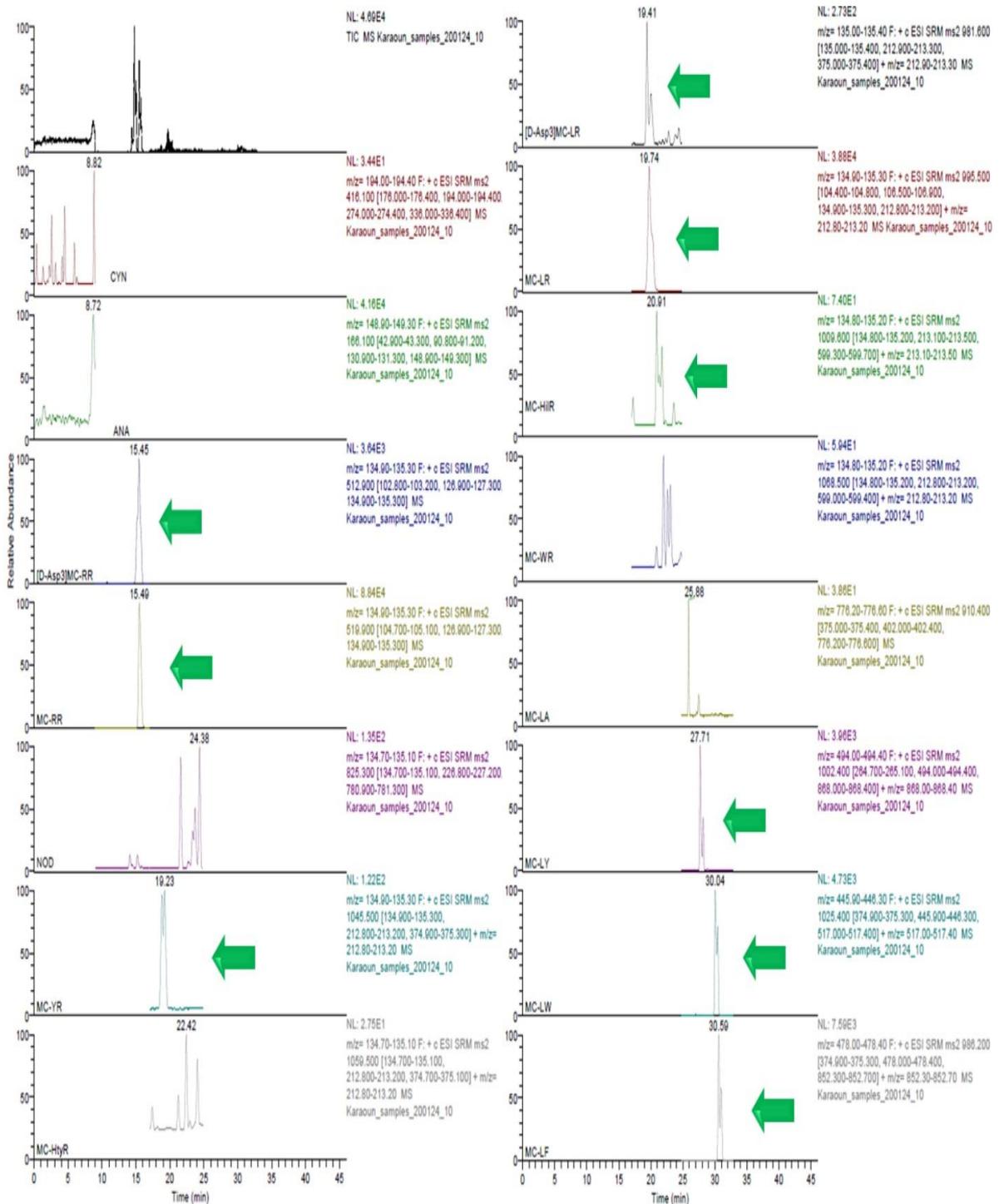


Figure 55: Chromatogram of sample 2 showing peaks of some cyanotoxins.

**Table 18.** Results of LC-MS/MS analyses in water samples; concentrations are expressed in µg/L (ND: not detected, NA: not analyzed)

Sample ID	Sample details	CYN	ATX	dm MC-RR	MC-RR	NOD	MC-YR	MC-HtyR	dm MC-LR	MC-LR	MC-HilR	MC-WR	MC-LA	MC-LY	MC-LW	MC-LF
1	S1, 22/08/19	ND <sup>1</sup> /NA <sup>2</sup>	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA
2	S2, 02/10/19	ND/ND	ND/ND	ND/1.60	0.339/26.4	ND/ND	ND/1.67	ND/ND	ND/1.87	0.438/91.5	ND/1.29	<LOQ <sup>3</sup> /ND	ND/ND	<LOQ/3.29	0.014/10.5	<LOQ/7.48
3	S4, 02/10/19	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
4	S1, 22/12/19	ND/ND	ND/ND	ND/0.53	0.050/7.42	ND/ND	0.187/8.28	ND/ND	0.050/0.70	0.158/8.43	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
5	S1, 15/01/20	ND/ND	ND/ND	ND/ND	0.007/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
6	S4, 04/02/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
7	S5, 15/02/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
8	S5, 17/02/20	ND/ND	ND/ND	ND/ND	0.005/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
9	S1, 22/02/20	ND/ND	ND/ND	ND/ND	0.007/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
10	S2, 22/02/20	ND/ND	ND/ND	ND/ND	0.008/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
11	S1, 15/04/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
12	S2, 22/04/20	ND/NA	ND/NA	ND/NA	0.090/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA
13	S4, 22/04/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
14	S1, 14/08/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
15	S2, 14/08/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
16	S3, 09/10/20	ND/ND	ND/ND	0.069/ND	7.0/0.086	ND/ND	197/1.94	ND/ND	6.9/<LOQ	209/2.18	1.6/ND	ND/ND	3.0/ND	<LOQ/ND	ND/ND	0.021/ND

## 12.5. ELISA and PPIA analyses

As mass spectrometry may not always be available in areas affected by CyanoHabs, this project aimed to demonstrate the possibility to use other fast and efficient techniques in the investigations on cyanotoxins. As targeted LC-MS/MS analysis concerned only a specific number of MC variants (12), the assessment of the presence of MCs was completed by ELISA and Protein Phosphatase Inhibition Assay (PPIA), in order to estimate total MCs concentration based on structural (ELISA) (Figure 56) and functional (PPIA) (Figure 57) similarities.

Samples were analyzed for extracellular (dissolved) and intracellular MCs, similarly to LC-MS/MS. Results of ELISA and PPIA together with the sum of MCs determined by LC-MS/MS, for comparison, are presented in (Table 19). In fact, samples in which MCs were not detected by LC-MS/MS, or in which their concentrations were lower than the LODs of ELISA/PPIA (0.10/0.25 161 µg/L), were negative by ELISA and PPIA. Samples 8 (S5–17/2/2020), 11 (S1-15/04/2020), 12 (S2-22/04/2020) and 13 (S4-22/04/2020) were exceptions.

In sample 13, MCs were detected only by ELISA/PPIA as extracellular, in very close concentrations (2.30/2.32 µg/L eq. MC-LR).

In sample 16 (S3-9/10/2020) MCs were mainly found extracellular by ELISA and PPIA, result compatible with LC-MS/MS, although in this sample ELISA and PPIA gave about 55% and 32% lower concentrations than LC-MS/MS.

The correlation between the three used techniques is shown in (Figure 58). Although they are not strongly correlated, ELISA and PPIA could serve as preliminary and fast screening techniques, since positive results of ELISA and PPIA showed concentrations of CTs with LC-MS/MS.

**Table 19.** Comparison of results from the 3 different techniques

ID	SAMPLE DETAILS	Total Extracellular/Intracellular MCs		
		LC-MS/MS*	ELISA**	PPIA**
		sum of MCs, µg/L	µg/L eq.MC-LR	µg/L eq.MC-LR
1	S1, 22/08/2019	ND/NA	ND/NA	ND/NA
2	S2, 02/10/2019	<b>0.83/146</b>	NA/ <b>97.2</b>	NA/ <b>111</b>
3	S4, 02/10/2019	ND/ND	ND/ND	ND/ND
4	S1, 22/12/2019	<b>0.45/25.4</b>	<b>0.13/30.6</b>	<b>0.25/16.2</b>
5	S1, 15/01/2020	<b>0.007/ND</b>	ND/ND	ND/ND
6	S4, 04/02/2020	ND/ND	ND/ND	ND/ND
7	S5, 15/02/2020	ND/ND	ND/ND	ND/ND
8	S5, 17/02/2020	<b>0.005/ND</b>	<b>0.12/ND</b>	ND/ND
9	S1, 22/02/2020	<b>0.007/ND</b>	ND/ND	ND/ND
10	S2, 22/02/2020	<b>0.008/ND</b>	ND/ND	ND/ND
11	S1, 15/04/2020	ND/ND	<b>0.25/ND</b>	<b>0.39/ND</b>
12	S2, 22/04/2020	<b>0.090/NA</b>	<b>1.80/NA</b>	<b>0.87/NA</b>
13	S4, 22/04/2020	ND/ND	<b>2.30/ND</b>	<b>2.32/ND</b>
14	S1, 14/08/2020	ND/ND	ND/ND	ND/ND
15	S2, 14/08/2020	ND/ND	ND/ND	ND/ND
16	S3, 9/10/2020	<b>425/4.29</b>	<b>190/2.41</b>	<b>290/2.34</b>

ND = Not detected, NA = Not Available. LODs: LC-MS/MS 0.001 µg/L; ELISA 0.10 µg/L; PPIA 0.25 µg/L.  
\* analysis with validated methods, extracellular toxins RSD < 16%, intracellular toxins RSD < 26%; \*\*  
duplicate analysis, RSD < 25%.

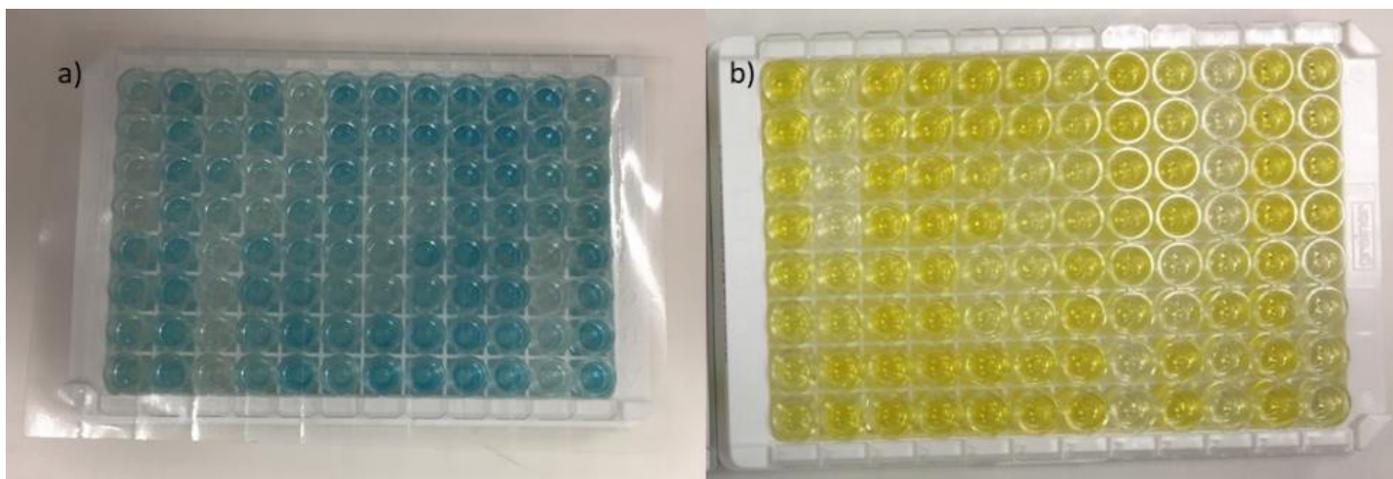


Figure 56. ELISA microtiter plate.

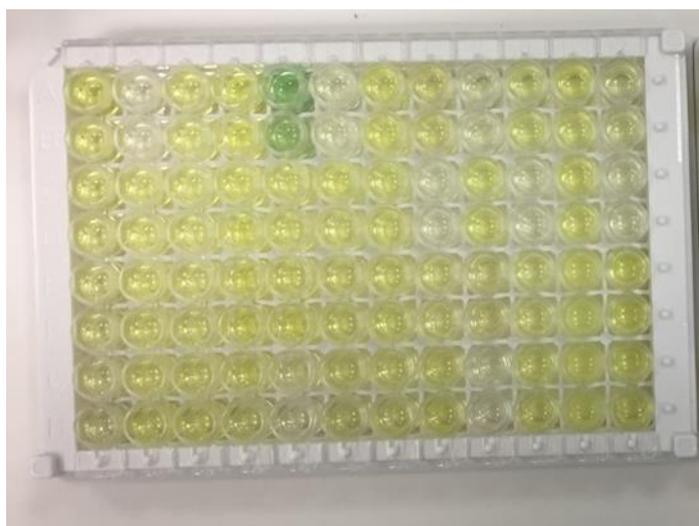


Figure 57: PPIA Microtiter plate.

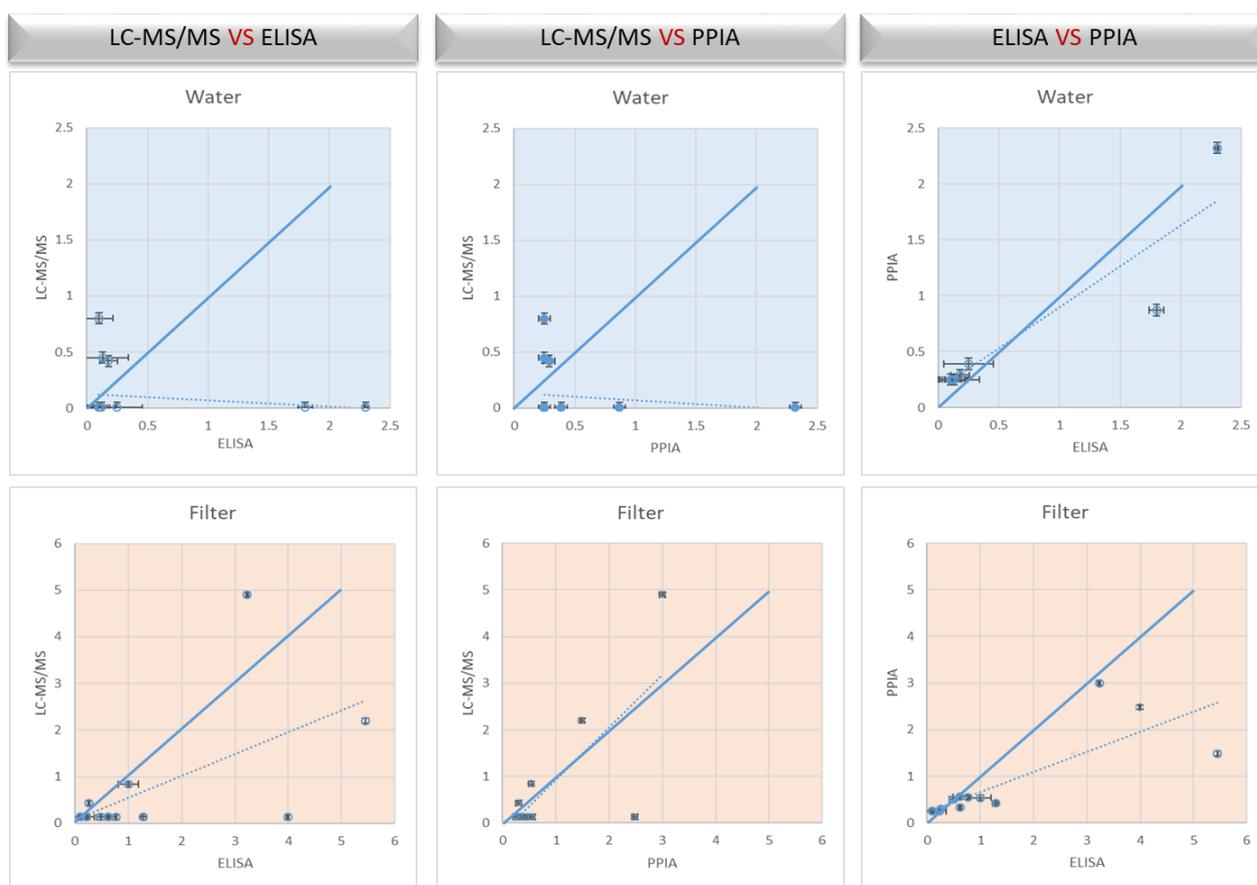


Figure 58: Correlation between the 3 used techniques ELISA,PPIA and LC-MS/MS.

For samples 11, 12 and 13, the higher values for extracellular fraction obtained by ELISA and PPIA may indicate the presence of MC congeners other than those that were targeted by the LC-MS/MS method. This shows the limitation LC-MS/MS caused by the unavailability of MCs reference standards. Therefore, complementary quantitative analysis for total MCs by ELISA and PPIA is important. However, disagreements between ELISA/PPIA and LC-MS/MS that were seen above could also be caused by well documented limitations of ELISA/PPIA. Indeed, ELISA is vulnerable to matrix effects, the variable cross-reactivity of different MC congeners and the response to degradation and/or transformation products of MCs that can result in overestimations. Regarding PPIA which based on the assessment of enzyme activity indicating the overall toxicity, it does not have the same sensitivity to the different MCs and may also be interfered with other unknown compounds present resulting in under- or over-estimation of toxins concentration.

The two developed methods lack the specificity of LC-MS/MS, as they respond to structurally/functionally similar molecules in total, and not to specific MC congeners. ELISA and PPIA can be considered as useful, fast, easy, and accessible approaches for screening surface waters for MCs, especially where advanced laboratory infrastructure (e.g. LC-MS/MS) is not easily available.

## 12.6. Non-targeted LC- MS/MS analyses

### 12.6.1. Detection of new microcystin congeners

Even, if the results from the different techniques were in accordance, some differences were observed. For example, when comparing outcomes of ELISA and PPIA with LC-MS/MS, samples 11, 12 and 13, showed higher values for extracellular fraction obtained by the former methods (where they were not even detected), and that may indicate the presence of MC congeners other than those that were targeted by the LC-MS/MS method used in the study. In other words, results' overestimations in ELISA might be due to the presence of additional variants that were not detected in targeted analyses (LC-MS/MS) [205]. This is a well-documented limitation, caused by the unavailability of commercial MC reference standards, and it underscores the need for complementary detection techniques as the development of non-targeted methods based on high-resolution mass spectrometry and related mass-spectral databases [184].

Therefore, this question was addressed by untargeted high resolution mass spectrometry. Data was interpreted based on the isotopic pattern matching spectrum and FISH scoring together with other indices (as the retention time, and fragment ions). Results of HRMS has shown the presence of 42 additional MCs congeners other than the original 12 targeted ones with LC-MS/MS (Table 20).

**Table 20:** Results of HRMS analyses, showing new MCs variants in water samples

	<i>Name</i>	<i>tR (min)</i>	<i>Calc. MW</i>	<i>FISH Coverage</i>	<i>Presence in Samples</i>
1	MC congener <sup>1</sup>	11.262	1060.52318	-	16extra
2	[D-Aps3]MC-RR <sup>2</sup>	11.80	1023.55035	38.10	2intra, 4intra
3	[DMAdda5]MC-YR	11.857	1030.51250	45.45	4intra, 16extra
4	[Dha7]MC-RR	11.947	1023.55051	32.23	2intra
5	MC-RR <sup>2</sup>	11.89 – 12.28	1037.56607	30.66 – 32.00	2extra, 2intra, 3intra, 4intra, 4extra, 12extra, 16intra, 16extra
6	[DMAdda5]MC-LR	12.271	980.53314	53.67	2intra, 16extra
7	MC congener <sup>1</sup>	12.642	1010.50792	-	16extra
8	[seco-4/5]MC-LR	12.664	1012.55852	42.46	16extra
9	[epoxyAdda5]MC-LR	12.41 – 12.88	1010.54362	43.78 – 63.41	16extra
10	MC congener <sup>1</sup>	12.601	1301.63315	-	2intra
11	MC-M(O)R	13.303	1028.50019	38.79	16extra
12	[NMe-Ser7]MC-YR	13.796	1062.53801	39.67	16extra
13	MC-M(O2)R	14.119	1044.49495	51.50	16extra
14	[MSer7]MC-YR	14.245	1062.53867	48.52	16extra
15	[D-Asp3,(E)-Dhb7]MC-YR	14.393	1030.51300	42.52	4intra, 16extra
16	[dm(1)Adda5]MC-LR	14.403	980.53343	63.30	16extra
17	[ADMAdda5,Mser7]MC-LR	14.506	1040.55399	53.10	16extra
18	MC-Y(OMe)R	14.44 - 14.65	1074.53872	44.95 – 45.13	16extra
19	[D-Ser1,D-Asp <sup>3</sup> ]MC-HtyR	14.615	1060.52300	39.59	16extra
20	[MSer7]MC-LR	14.672	1012.55952	44.71	2intra, 16extra,
21	MC congener <sup>1</sup>	14.683	980.53342	-	16extra
22	[dm(2)Adda5]MC-LR	14.933	980.53343	47.79	2intra, 16extra
23	MC-YR <sup>2</sup>	14.98 – 15.33	1044.52812	44.06 – 61.54	2intra, 4intra, 4extra, 16intra, 16extra

24	[Dha7]MC-YR	15.182	1030.51291	51.10	16extra
25	[D-Ser1]MC-LR	15.389	1010.54350	44.23	2intra, 16extra
26	MC-HtyR <sup>2</sup>	15.795	1058.54389	65.04	-
27	[Dha7]MC-LR <sup>3</sup>	15.88	980.53343	46.82 (m/z Cloud 90.7)	2intra, 4intra, 16intra, 16extra
28	[D-Asp3]MC-LR <sup>2</sup>	15.65 – 16.17	980.53324	44.33 - 58.87	16extra
29	MC-LR <sup>2</sup>	15.34 – 16.34	994.5488	44.80 – 63.30	2intra, 2extra, 4intra, 4extra, 16intra, 16extra,
30	MC congener <sup>1</sup>	17.00 – 17.50	1048.55901	-	16extra
31	[ADMAdda5]MC-HiIR	17.068	103.55925	40.81	16extra
32	MC-HiIR <sup>2</sup>	17.35 – 18.20	1008.5644	46.88 – 65.12	2intra, 16extra
33	[D-Asp3]MC-FR	17.745	1014.51731	53.06	16extra
34	MC-FR	17.952	1028.53304	44.03	16extra
35	MC-WR <sup>2</sup>	18.52 – 18.86	1067.54435	64.71 – 65.38	2intra
36	[D-Ser1]MC-LA	19.640	925.47972	41.75	16extra
37	[D-MeO-Glu6]MC-LR	20.147	1008.56499	50.95	16extra
38	MC congener <sup>1</sup>	22.626	967.49053	-	16extra
39	[D-Asp3]MC-LA	23.154	895.46935	54.45	16extra
40	MC-YA	23.335	959.46361	41.36	16extra
41	MC-VA	23.660	895.46935	43.73	16extra
42	MC congener <sup>1</sup>	23.921	1010.51140	-	2intra
43	MC-LA <sup>2</sup>	24.084	909.48509	44.96	16extra
44	MC-VF	24.336	971.50029	37.40	2intra
45	MC-YAba	24.632	973.47905	47.80	16extra
46	MC-HiIA	24.844	923.50035	61.21	16extra
47	MC-FA	24.883	943.36910	60.00	16extra
48	MC-LY <sup>2</sup>	24.87 – 25.08	1001.51134	48.59 – 55.88	2intra
49	MC-LAba	25.348	923.50035	44.74	16extra
50	[Asp3,Dhb7]MC-LY or [D-Asp3]MC-LY	25.788	987.49535	55.34 (for both)	16extra
51	MC-LM	26.334	969.48833	51.84	2intra,
52	MC-LV	26.504	937.51610	47.38	16extra
53	MC-LW <sup>2</sup>	27.15 – 27.36	1024.52715	41.54 – 51.53	2intra
54	MC-LF <sup>2</sup>	27.67 - 27.92	985.5162	44.23 - 54.05	2intra, 16extra
55	MC-LL	27.56 – 27.97	951.53179	55.03 – 57.33	2intra, 16extra

Consequently, the detection of additional MC congeners seems to be the reason for the difference between the targeted mass spectrometric results and biochemical assessments [206].

Results of the targeted analyses were matched by those obtained with HRMS. Samples 2 (S2, 02/10/2019), 4 (S1, 22/12/2019) and 16 (S3, 09/10/2020) exhibited the presence of different variants of microcystin. The highest number of variants was observed in sample 16 (Figure 60).

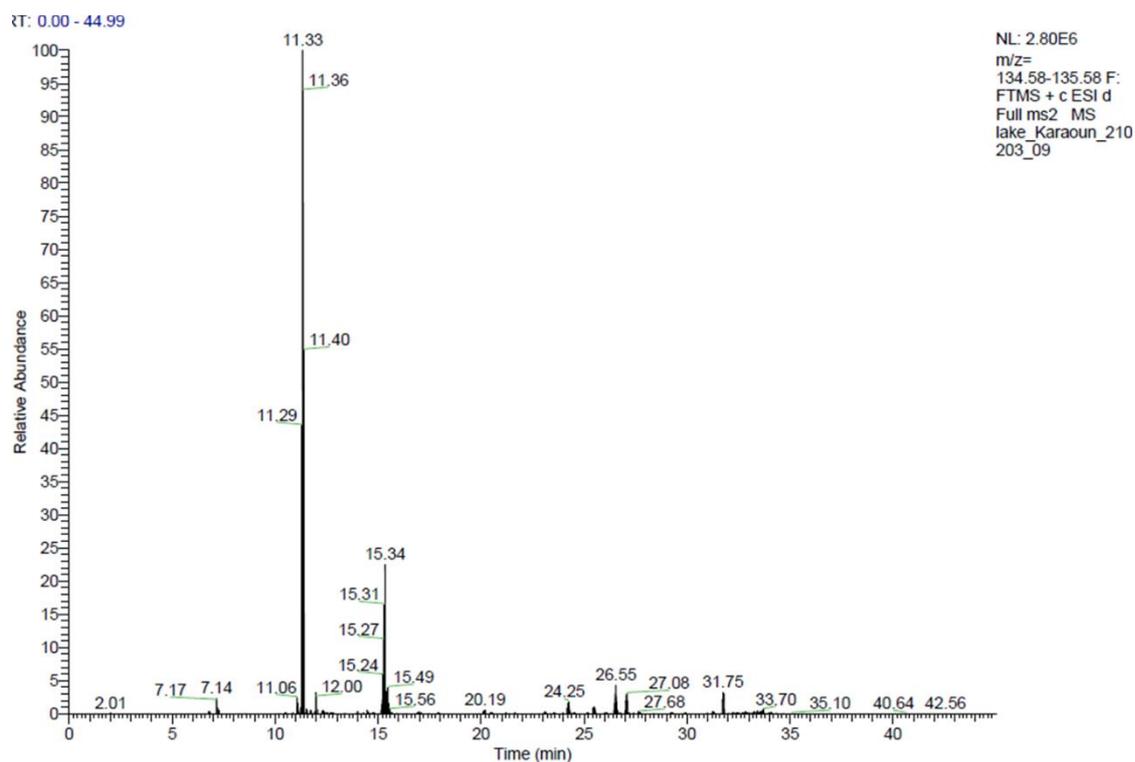


Figure 59: Extracted ion chromatogram of the characteristic MC fragment ion  $m/z$  135.08 of sample 2intra, representing the variety of MC congeners present in the sample.

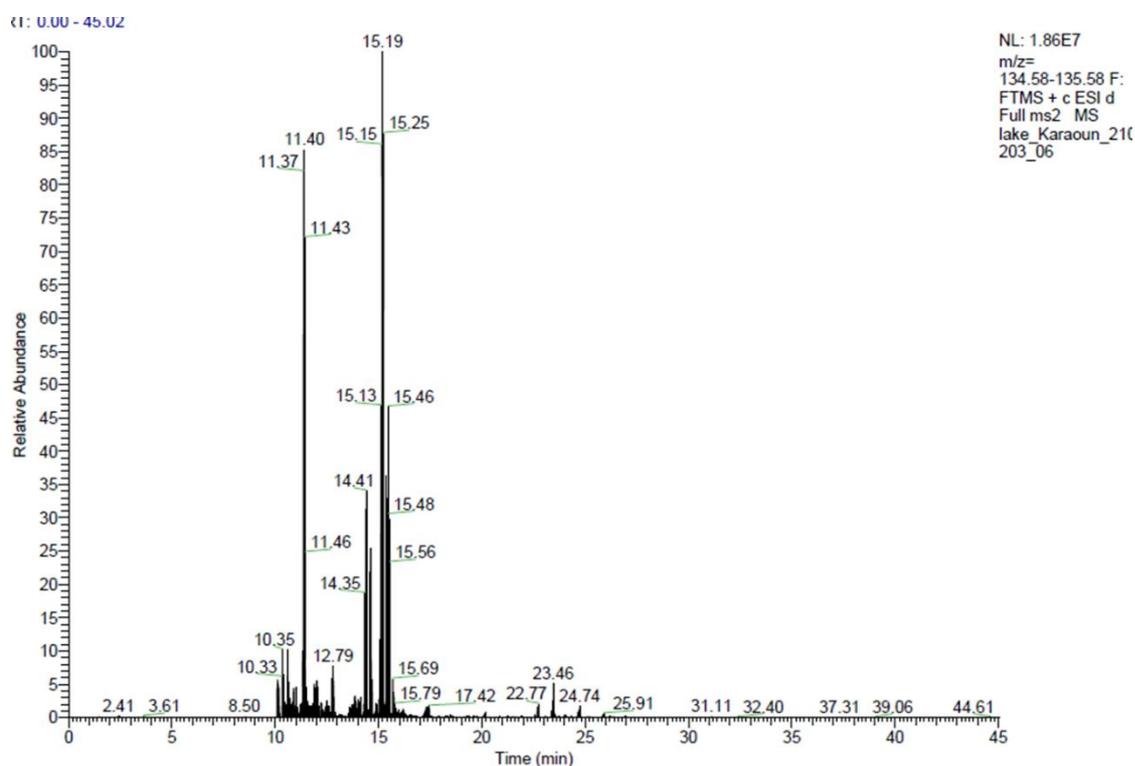


Figure 60: Extracted ion chromatogram of the characteristic MC fragment ion  $m/z$  135.08 of sample 16extra, representing the variety of MC congeners present in the sample.

Sample 3 that was collected on the same day as sample 2 but from a different location (from the middle of the lake), and for the first time showed the presence of intracellular MC-RR. Moreover, 7 novel undetermined MC congeners were detected in samples 2 and 16. The identification and confirmation of a variant was based on the structural elucidation both in CID and HCD spectrums and relying on the FISh score.

### 12.6.2. Identification of Anabaenopeptins

Several studies have highlighted the co-occurrence of MCs and other toxins. For instance, MCs were reported elsewhere to co-occur with cyanopeptolins, microginins, and anabaenopeptins [77].

*Anabaenopeptins* that is a class of cyclic cyanopeptides reported to co-occur with microcystins [207], were detected as summarized in (Table 21). Six congeners were found: AP A, B, F and Oscillamide Y were the most common variants. These toxins were mainly found in October and December 2019 (in samples 2 and 4, respectively). It means that cyanobacteria that were present and detected on these sampling dates, besides producing MCs, produced anabaenopeptins. The latter have the same toxic activity as MCs. They are considered as protein phosphatase inhibitors and a potential source of cancers. However, studies on this class of toxins remain scarce [208, 209], and including them in the future monitoring program of lake Karaoun seems essential and important.

**Table 21.** List of APs identified in samples of Lake Karaoun

	Name	tR (min)	Calc. MW	FISh Coverage	Presence in Samples
1	AP B	10.98 – 11.29	836.4545	40.62-63.43	2intra, 2extra, 4intra, 4extra,
2	AP F	11.29 – 11.66	850.47013	40.34 – 61.51	2intra, 2extra, 4intra, 4extra
3	AP congener <sup>1</sup>	12.716	901.44042	-	2intra, 4intra
4	AP A	14.169	843.41691	46.84	2intra, 4intra
5	Oscillamide Y	15.480	857.43290	45.62	2intra, 2extra, 4intra
6	AP 842	21.326	841.43767	68.27	2intra

<sup>1</sup> Compound belonging to APs class based on the presence of characteristic fragment ion (i.e.,  $m/z$  84) in its spectra but the structure confirmation was not able due to the low signal on the other fragment ions.

Based on the presence of the characteristic fragment ion (i.e.,  $m/z$  84) in its spectra, a novel AP congener was detected in samples 2 and 4 but the unambiguous structure confirmation was not possible since the other fragment ion showed a low signal.

### 12.6.3. Detection of Microginins

Microginins (9 variants) were discovered by untargeted analyses (Table 22). They are linear cyanopeptides, containing between 3 and 6 amino acids [83]. This group of cyanotoxins is of a great environmental importance since MGs are bioactive compounds [80]. They are considered as effective treatments for hypertension and congestive heart failure, as they inhibit the activity of angiotensin-converting enzyme (ACE). Hence, they can be considered as potential valuable metabolites to be used as antihypertensive drugs. Nevertheless, information about these bioactive compounds remain scarce. The determination of their relevant environmental concentrations along with their environmental effects and impacts are still to be elaborated.

**Table 22.** List of MGs identified in samples of Lake Karaoun

	Name	tR (min)	Calc. MW	FISh Coverage	Presence in Samples
<b>1</b>	MG 713B	11.571	713.36357	83.82	2intra
<b>2</b>	MG FR3	11.761	727.37902	69.00	2intra, 12extra
<b>3</b>	Cyanostatin B	13.346	753.43132	72.89	4intra
<b>4</b>	MG 743	13.414	743.41061	65.51	2intra, 4intra
<b>5</b>	MG 741 C	13.993	741.43138	52.75	2intra, 4intra
<b>6</b>	MG 578 A	14.052	578.36811	71.43	2intra, 4intra
<b>7</b>	MG FR1	14.200	727.41561	61.61	2intra, 4intra
<b>8</b>	MG 606 A	14.245 – 14.576	606.39934	66.04-66.60	4extra, 12extra
<b>9</b>	MG 741 A	15.140	741.43126	76.38	2intra, 4intra

#### 12.6.4. Other secondary metabolites

Other cyanobacterial metabolites belonging to different classes than MCs, APs and MGs were also detected in samples from Lake Karaoun. They are summarized in (Table 23) with the relevant FISh coverages.

Spumigins are thrombin inhibitors. As thrombin, a trypsin-like serine protease, plays a significant role in thrombosis and platelet aggregation, they can be used as treatments to avoid blood coagulation [210]. Spumigins belong to the family of Aeuriginosins which are known for their inhibition of proteases in the nano-molar range and being toxic to grazers [81], [211]. Aeuriginosin KB676 was found in 7 samples (in samples 11, 14 and 15). The presence of this toxin in unusual times might be due to its persistence in the environment. However, this assumption is so fragile and needs further investigations.

Early discoveries of Balgacyclamide recorded a certain antimalarial activity, so they might be potential compounds of a great therapeutic importance [212].

**Table 23.** List of cyanobacterial metabolites belonging to other classes than MCs, Aps and MGs identified in samples of Lake Karaoun

	<i>Name</i>	<i>tR (min)</i>	<i>Calc. MW</i>	<i>FISH Coverage</i>	<i>Presence in Samples</i>
<b>1</b>	Dehydroradiosumin	7.601	430.22167	49.60	2extra, 6extra
<b>2</b>	Spumigin J	11.051	612.31578	60.20	2extra
<b>3</b>	Aeruginosin KB676	11.568-11.720	676.39486	64.22 - 64.79	2intra, 4intra, 4extra, 7extra, 11extra, 14intra, 14extra, 15intra, 15extra, 16extra
<b>4</b>	Microcyclamide MZ568	13.397	568.26807	62.37	2intra, 4extra, 5extra, 10extra, 12extra
<b>5</b>	Microcyclamide MZ602	13.517	602.25224	64.57	4intra, 4extra, 11extra, 16extra
<b>6</b>	Balgacyclamide C	13.528	584.24170	75.76	4intra, 4extra
<b>7</b>	Balgacyclamide B	16.240	550.25734	76.09	2intra, 16intra, 16extra
<b>8</b>	Planktocyclin	22.700	800.42582	65.70	2intra, 4intra

#### 12.7. Cyanotoxins in muscle and liver of *Cyprinus carpio*

None of the targeted toxins was detected in the samples of muscle and liver of *Cyprinus carpio*) although the developed method was proven to be adequate. An example chromatogram of a fish liver sample is shown in comparison with a multi-toxin standard, that confirms the absence of all toxins (Figure 61).

The high concentrations of cyanotoxins in water but their absence in fish samples were previously documented in several studies [213, 214]. It is known that liver organs accumulate toxins more than muscle tissues do. But in some cases, toxins would be absent in the whole fish body even if they are collected during cyanobacterial blooms. It could be due to a variety of reasons, such as local bloom conditions, fish feeding habits and ecosystem characteristics. The occurrence of blooms and the presence of microcystins in water are temporally and spatially variable, leading to different exposure of fish to microcystins. Inter-species differences in fish are also important factors for the intake and accumulation of microcystins in their tissues. For example, zooplanktivores fish are more likely to concentrate microcystins than omnivorous fish like the studied *Cyprinus Carpio*. Also, the mechanisms of MC excretion and the timescales for MC elimination may differ considerably among different fish species. Some fish they just avoid areas affected by blooms.

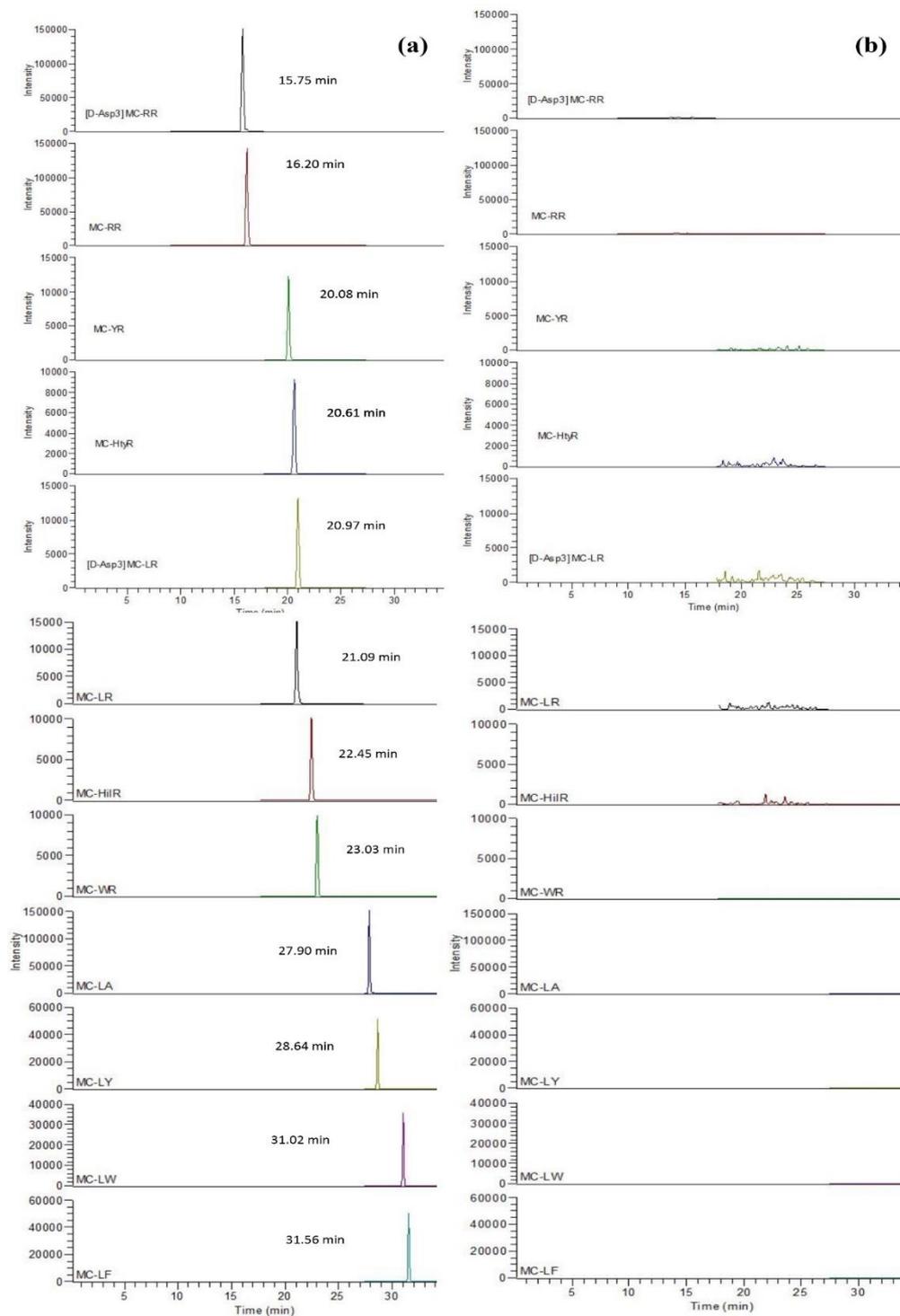


Figure 61: Chromatogram of a fish sample in comparison with a standard showing the absence of cyanotoxins.

This adaptation technique was described in the study of Godlewska *et al.* [215] demonstrating that the number of fish caught in gillnets within the bloom area was about half that caught outside the bloom area.

It was also reported elsewhere that 67% of absorbed or ingested cyanotoxins were bound in fish tissues, which means that for a good extraction and an accurate assessment an oxidization is required [216]. Many studies implemented the Lemieux oxidization with the MMPB method to have more precise results and to confirm the suitability of fish for consumption [217].

## 13. Volatile Organic Compounds in lake Karaoun's water

### 13.1. Targeted analyses

Targeted quantitative analyses for 36 VOCs that are industrial pollutants resulted in the detection of up to ten compounds in at least one sample.

Dichloromethane was found in all samples in concentrations that ranged from 25.1 to 34.0 µg/L. it is a common industrial solvent used in several chemical processes. Toluene was present in 12 samples at concentrations up to 0.81 µg/L. Trichloromethane (chloroform) was detected at low concentrations in samples 11 and 16 (0.40 and 0.50 µg/L respectively). Traces (<0.25 µg/L) of trichlorobenzenes were detected in sample 6. Sample 16 contained three compounds of the "BTEX" (benzene, toluene, ethylbenzene, xylenes) family that are frequently used as an index to assess the impact of pollution caused by spills or leaks from fuel storage tanks into water bodies. Sample 16 contained traces of 1,2-dichloropropane, benzene, *o,m,p*-xylenes and styrene in addition to the already cited ones above. Trichloromethane that was found in two samples in concentrations up to 0.5 µg/L is a proof of the lake's contamination with waste water since trihalomethanes, are commonly present in chlorine-treated water

**Table 24.** Results of quantitative determination of VOCs in targeted screening approach

Sample ID	Sample details	Concentration (µg/L)										
		1,2,3-trichlorobenzene	1,2,4-trichlorobenzene	dichloromethane	toluene	1,2-dichloropropane	benzene	trichloromethane	<i>o</i> -xylene	<i>m+p</i> -xylene	styrene	
1	S1, 22/08/19	ND	ND	31.0	0.72	ND	ND	ND	ND	ND	ND	ND
2	S2, 02/10/19	ND	ND	28.8	0.65	ND	ND	ND	ND	ND	ND	ND
3	S4, 02/10/19	ND	ND	28.1	0.60	ND	ND	ND	ND	ND	ND	ND
4	S1, 22/12/19	ND	ND	29.1	0.25	ND	ND	ND	ND	ND	ND	ND
5	S1, 15/01/20	ND	ND	29.0	<0.25	ND	ND	ND	ND	ND	ND	ND
6	S4, 04/02/20	<0.25	<0.25	27.2	<0.25	ND	ND	ND	ND	ND	ND	ND
7	S5, 15/02/20	ND	ND	28.2	ND	ND	ND	ND	ND	ND	ND	ND
8	S5, 17/02/20	ND	ND	26.3	<0.25	ND	ND	ND	ND	ND	ND	ND
9	S1, 22/02/20	ND	ND	34.0	<0.25	ND	ND	ND	ND	ND	ND	ND
10	S2, 22/02/20	ND	ND	29.4	<0.25	ND	ND	ND	ND	ND	ND	ND
11	S1, 15/04/20	ND	ND	27.2	ND	ND	ND	0.40	ND	ND	ND	ND
13	S4, 22/04/20	ND	ND	30.2	ND	ND	ND	ND	ND	ND	ND	ND
14	S1, 14/08/20	ND	ND	32.3	0.46	ND	ND	ND	ND	ND	ND	ND
15	S2, 14/08/20	ND	ND	33.6	0.35	ND	ND	ND	ND	ND	ND	ND
16	S3, 09/10/20	ND	ND	25.1	0.81	<0.25	<0.25	0.50	<0.25	<0.25	<0.25	<0.25

### 13.2. Untargeted analyses

Untargeted analyses of environmental water samples, using Headspace Solid Phase Micro-extraction coupled to Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) and the Automatic Mass Spectral Deconvolution and Identification System (AMDIS) enabled the detection and identification of 20 compounds belonging to different volatile chemicals categories.

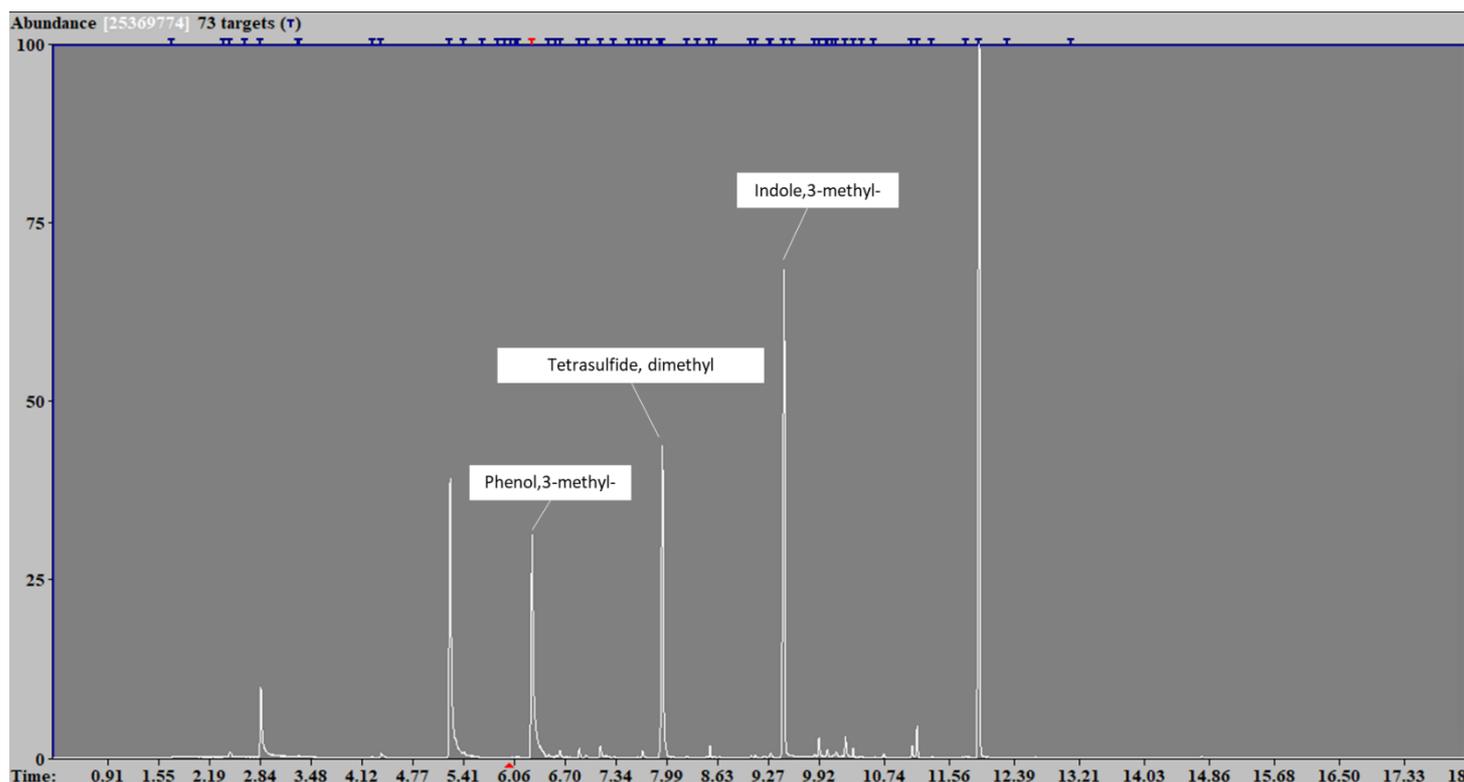


Figure 62: A chromatogram of sample 4.

Terpenes/terpenoids, hydrocarbons, aldehydes/ketones, phenols, phthalates, alkyl sulfides and indoles were found.  $\beta$ -cyclocitral (tobacco/woody odor) and  $\beta$ -ionone (floral odor), are two terpenoids detected in samples 2, 4 (Figure 62) and 16 and 4, 12 and 16 respectively. Nonanal, the known aldehyde with a “fishy odor” was seen in 9 samples. Dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide (which are alkyl sulfides) were detected in 4 so smelly samples (swampy/septic). Skatole or 3-methylindole that is a fecal compound with a typical smell (Mahmoud 2018) was detected in samples 2 and 4. All of the above detected compounds are usually associated to algae and are produced by cyanobacteria. CyanoHabs are generally accompanied by bad smell emissions that can be caused of one of the mentioned T&O compounds or other odorous metabolites.

Moreover, several hydrocarbons were detected, with 2,2,4,6,6-pentamethyl heptane found in all samples. The renown industrial pollutants: 2,4-di-tert-butylphenol, diisobutyl phthalate and p-cresol were detected in samples 10, 4 and 2, respectively.



strong odors described as swampy/septic, rotten, rancid and stinky, with very low odor threshold concentration levels of ng/L or less [38]. Dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide that were detected in sample 4 that had a strong swampy/septic odor. Organosulfur compounds can be produced by myriad of biotas regardless the oxygen availability (in oxic and anoxic conditions) via different biochemical pathways, enzymes and precursors. Another compound responsible for the septic odor in samples 2 and 4 is the 3-methylindole or “skatole” that is a fecal compound with a characteristic odor that was previously reported to occur in algal cultures and field samples [7]. The combination of the 2 above mentioned T&O gave sample 4 an extreme swampy/septic, that would irritate the eyes and respiratory system.

Among the hydrocarbons detected, 2,2,4,6,6-pentamethyl heptane (all samples) and straight-chain hydrocarbons hexadecane (2 samples) and heptadecane (6 samples), could generally be of anthropogenic or biogenic origin. 2,2,4,6,6-pentamethyl heptane has many industrial uses in anti-freeze products, coatings, fillers, lubricants, or greases. Biogenic hydrocarbons, represented exclusively by straight-chain components, were reported elsewhere [221]. Other compounds, such as 2,4-di-tert-butylphenol (antioxidant), diisobutyl phthalate (plasticizer) and p-cresol were detected in samples 10, 4 and 2, respectively. They are known as common industrial pollutants, used in fuels, plastics, and production of chemicals.

The confirmation of compounds' presence was done on different levels and was complemented by the availability of some standards. Table 25 summarizes the VOCs found.

On an opposite note, the most common cyanobacterial T&O compounds, geosmin and 2-methylisoborneol, were not detected in any of the samples. In Figure 64, structures of discovered compounds are shown.

The co-occurrence of cyanotoxins and T&O compounds has been previously discussed, although the number of reports is limited. Some strains of cyanobacteria as *Microcystis* produce microcystins together with  $\beta$ -cyclocitral and alkyl sulfides [221], but the presence of T&O compounds does not necessarily mean the presence of toxins [222]. In the case of Karaoun, the co-presence of VOC, TO and cyanotoxins was observed but the correlation was not significant. Nevertheless, since T&O can be sensed by the human nose at very low concentrations, they can serve as an early warning indicator for further investigations on the presence of toxic cyanobacteria. The cyanobacterial/algal T&O detected in this study can serve as an initial list of compounds to be screened in future T&O episodes in Beirut's drinking water supply.

**Table 25.** Table showing the results of untargeted analyses in HS-SPME-GC-MS

No	Compound	Identification level	Chemical group	tr (min)	Number of Samples	% peak area
1	$\alpha$ -Thujene	A	Terpenes/Terpenoids	4.82	2	0.05 – 0.08
2	2,2,4,6,6-pentamethyl heptane	B	Hydrocarbons	5.43	16	0.03- 0.80
3	2,2,6-trimethyl-cyclohexanone	A	Aldehydes/Ketones	5.95	1	0.004
4	p-Cresol	B	Phenols	6.28	2	0.04 – 5.55
5	2,4-Dimethyldecane	A	Hydrocarbons	6.59	1	0.17
6	Nonanal	A	Aldehydes/Ketones	6.64	9	0.06 – 0.43
7	4,7-Dimethylundecane	A	Hydrocarbons	7.62	1	0.01
8	$\beta$ -Cyclocitral	B,C	Terpenes/Terpenoids	7.90	3	0.05 – 0.16
9	2,6,6-Trimethyl-1-Cyclohexene- 1-acetaldehyde	A	Terpenes/Terpenoids	8.25	1	0.007
10	6,10-Dimethyl-5,9-Undecadien-2-one	B	Aldehydes/Ketones	9.92	1	0.35
11	$\beta$ -Ionone	B,C	Terpenes/Terpenoids	10.25	3	0.04 – 0.38
12	Pentadecane	B,C	Hydrocarbons	10.35	1	0.14
13	2,4-di-tert-butylphenol	A,C	Phenols	10.43	10	0.01 - 39
14	Hexadecane	B,C	Hydrocarbons	11.16	2	0.20 – 0.60
15	Heptadecane	B,C	Hydrocarbons	11.94	6	0.07 – 33
16	Diisobutyl phthalate	B	Phthalates	13.11	4	0.003 – 1.5
17	Dimethyl disulfide	C,D	Alkyl sulfides	5.81	1	0.02
18	Dimethyl trisulfide	C,D	Alkyl sulfides	12.35	1	0.64
19	Dimethyl tetrasulfide	D	Alkyl sulfides	17.68	1	0.78
20	3-methylindole	C,D	Indoles	20.21	2	0.005 – 0.02

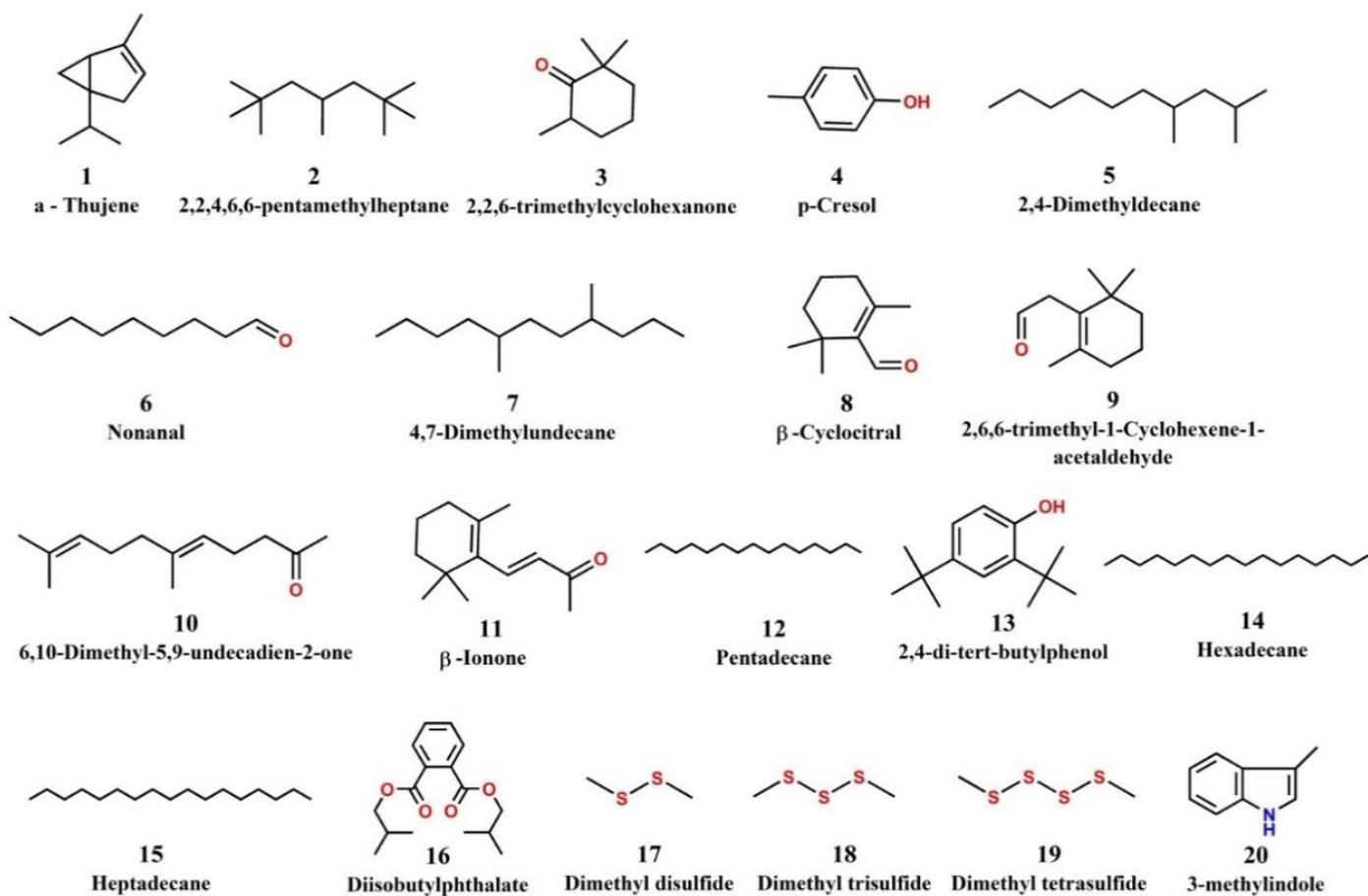


Figure 64: Structural representation of T&O in water samples.

## 14. CONCLUSION

Comparable to other Mediterranean lakes, Karaoun reservoir is under the dominance of two main cyanobacterial species *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* and suffers from recurrent severe CyanoHabs.

A holistic approach with a complementary set of analytical methods was implemented for the first time to investigate and confirm the occurrence of cyanotoxins in the reservoir. A definite determination of 12 MC congeners, NOD, ATX and CYN was conducted by LC-MS/MS, complemented by ELISA and PPIA for MCs and qPCR for MC, CYN and STX producing genes.

Results confirmed the presence of myriad variants of microcystins in the lake with high concentrations mainly during October 2019 and 2020. Monitoring studies of lakes and reservoirs in the Middle East are very limited and scarce, except the natural lake, Lake Kinneret (Israel) that is well documented and on the radar of many scientists. The latter is known to be less eutrophic than Karaoun reservoir, however has been suffering with CyanoHabs since late 1960s when blooms of *Microcystis* sp. have been reported (ref). Then, *Aphanizomenon ovalisporum* and *Cylindrospermopsis raciborskii* were first seen in September 1994 and summer 1998, correspondingly (ref). Cylindrospermopsin is the most occurring and reported toxin in lake Kinneret, while MCs are also present.

Even if cylindrospermopsin was not detected in the waterbody, the presence of its producing cyanobacteria (*Aphanizomenon ovalisporum*), the similarities that the lake shares with lake Kinneret and for the fact that they are both in the same arid to semi-arid area, would leave the former one vulnerable and susceptible to the contamination with CYN that must be included in future monitoring programs to have a better surveillance, and be able to evaluate the associated risks.

Moreover, NOD is certainly and mainly associated to brackish water cyanobacteria with little reports on its occurrence in freshwater bodies. Generally, this toxin is not predicted to occur in lake Karaoun, but could be included in future monitoring programs since the CT has structural similarities with MCs. However, if ATX producing cyanobacteria are present in the lake, the toxin must be targeted and further investigated to avoid its lethal consequences.

The study confirmed the threat of blooms but also highlighted the occurrence of novel bioactive compounds with potential therapeutic characteristics. For instance, spimugins could be used as thrombin inhibitors to avoid blood coagulation. Or Balgacyclamide that could be potential antimalarial agents.

Furthermore, the investigations on the reason behind the bad smells emitted by the water body, demonstrated that cyanobacteria also produce Taste and Odors compounds from different classes. They are generating swampy septic odors via sulfurous compounds and 3-methyl indole but they are also producing the typical  $\beta$ -cyclocitral and  $\beta$ -ionone that could be used in fragrances manufactory. Targeted gas chromatography, investigated on 36 VOCs and detected 10 of them in at least one sample (toluene, fchloromethane) confirming the pollution of the Litani river and Karaoun reservoir by

industrial and domestic wastes. The effect of their co-occurrence is still to be elaborated, since research in this area remains limited.

A comparison between different applied methods showed that there is no ideal and unique one. Consequently, future monitoring programs should rather envisage a holistic approach with the implementation of different techniques. Each one has its limitations but also has its advantages. Molecular (q-PCR) data can be considered as a reliable early warning system, together with biochemical assessment that are used as fast and easy screening techniques. Mass spectrometry coupled with chromatographic separation techniques (liquid and gas) offers unmatched potential for the analysis for individual and unknown species. On another note, high resolution mass spectrometry proved to be a valuable tool in environmental investigations. It would detect analytes in high precision and accuracy, and will also cope with limitations of other techniques. The unavailability of a variety of standards could be solved when such an approach is implemented.

These early findings give significant and noteworthy information on the occurrence, numerous variants and levels of occurring CTs and they can be used for the implementation of consistent future monitoring programs to support the right management of the lakes usages taking into consideration cyanobacteria, cyanotoxins, T&O compounds but also anthropogenic pollutants that are co-existing.

Cyanobacterial blooms had myriad impacts on the area, a lot of economic losses were witnessed and finally, the accumulative concerns lead to social conflicts. Besides all the efforts of LRA to cope with this environmental catastrophe, domestic and industrial waste deposit in the river stream continues and natural disasters are still happening. In April 2021, a massive fish death occurred followed by a significant media hype. Lake Karaoun is at a great risk of those continuous blooms, taking into consideration its location in arid to semi-arid zone and its long summers. The recurrent, successive and excessive CyanoHabs in Karaoun need a close surveillance, a specialized emergency team, and the execution of a future monitoring program especially as it is intended to be implemented in myriad usages and vital activities.

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**ANNEXE III: Published paper***Communication*

# Investigation of the Occurrence of Cyanotoxins in Lake Karaoun (Lebanon) by Mass Spectrometry, Bioassays and Molecular Methods

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**Abstract:** Lake Karaoun is the largest artificial lake in Lebanon and serves multiple purposes. Recently, intensive cyanobacterial blooms have been reported in the lake, raising safety and aesthetic concerns related to the presence of cyanotoxins and cyanobacterial taste and odor (T&O) compounds, respectively. Here, we communicate for the first time results from a recent investigation by LC-MS/MS covering multiple cyanotoxins (microcystins (MCs), anatoxin-a, cylindrospermopsin, nodularin) in water and fish collected between 2019 and 2020. Eleven MCs were identified reaching concentrations of 211 and 199 µg/L for MC-LR and MC-YR, respectively. Cylindrospermopsin, anatoxin-a and nodularin were not detected. The determination of the total MCs was also carried out by ELISA and Protein Phosphatase Inhibition Assay yielding comparable results. Molecular detection of cyanobacteria (16S rRNA) and biosynthetic genes of toxins were carried out by qPCR. Untargeted screening analysis by GC-MS showed the presence of T&O compounds, such as β-cyclocitral, β-ionone, nonanal and dimethylsulfides that contribute to unpleasant odors in water. The determination of volatile organic compounds (VOCs) showed the presence of anthropogenic pollutants, mostly dichloromethane and toluene. The findings are important to develop future monitoring schemes in order to assess the risks from cyanobacterial blooms with regard to the lake's ecosystem and its uses.

**Keywords:** cyanotoxins (CTs); microcystins (MCs); volatile organic compounds (VOCs); taste and odor (T&O) compounds; SPE-LC-MS/MS; HS-SPME-GC/MS

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**Key Contribution:** First report confirming the occurrence of MC congeners and cyanobacterial/algae T&O compounds in lake Karaoun; Lebanon

## 1. Introduction

Cyanobacteria are photosynthetic microorganisms commonly found in surface waters. They can produce a large variety of secondary metabolites, including toxic compounds, known as cyanotoxins (CTs). CTs have various chemical structures and modes of toxicity (Figure S1). Microcystins (MCs) [1] and Nodularin (NOD) [2] are cyclic peptides, both containing the unique L-amino acid Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), which is responsible for their hepatotoxic activity [3,4]. Cylindrospermopsin (CYN) is an alkaloid cyanotoxin with cytotoxic, dermatotoxic, hepatotoxic and possibly carcinogenic potency [5]. The alkaloid Anatoxin-a (ATX), also known as "Very Fast Death Factor", is a bicyclic secondary amine (2-acetyl-9-azabicyclo(1,4,2)non-2-ene) with acute neurotoxicity [6]. Among CTs, MCs is the most frequently reported class [7,8].

Under favorable environmental conditions of temperature, presence of nutrients and light, cyanobacteria can proliferate excessively and form blooms [9]. The presence of CTs in toxic cyanobacterial blooms poses a significant risk to aquatic ecosystems and drinking water sources, while it has been associated with lethal poisonings of wild animals, livestock and humans [10]. The World Health Organization (WHO) established guideline values for MCs, CYN and ATX for drinking-water (lifetime, short-term or acute) and recreational exposure and published guides to hazard identification and management of risks posed by cyanobacteria and their toxins at each step of the water-use system [11].

Lake Karaoun (Qaraoun) is the largest water body in Lebanon and has multiple uses. The reservoir was created in 1959 and is intended for irrigation, hydropower, and recreational activities, as well as an

anticipated drinking water supply to the capital, Beirut [12]. However, nutrient enrichment due to the excessive use of fertilizers in the lake's watershed, untreated sewage and industrial waste water runoffs, are possibly contributing to the degradation of the reservoir's water quality [13]. In 2009, the cyanobacteria *Aphanizomenon ovalisporum* and *Microcystis aeruginosa* were first reported and were seen to dominate the lake's phytoplankton. Their proliferation was attributed to eutrophication and climate change resulting in the extension of dry periods and the rise in water temperature, as well as the pollution from multiple-industrial and agricultural sources [14]. In recent years, the blooms have become more intense, forming thick scums and emitting unpleasant odors in the area.

There is limited information on toxic cyanobacterial blooms in Lake Karaoum [14–16], with a previous study reporting the presence of CYN produced by *Aphanizomenon ovalisporum*, however, not confirmed by mass spectrometry [17]. There are no published data regarding the presence of MCs in the reservoir, especially during incidents of cyanobacterial blooms. Due to the multiple uses of the reservoir, including the supply of drinking water, there is an urgent need to definitively assess and confirm the presence of CTs in Lake Karaoum, in order to enable the design of future monitoring programs and to develop related management strategies.

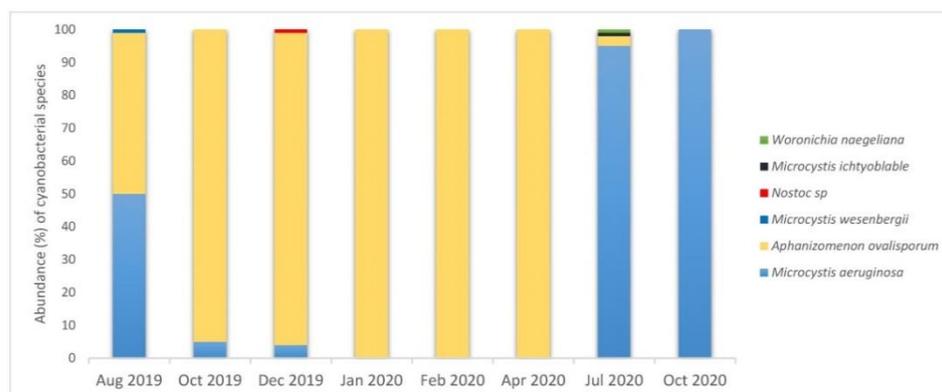
In addition to CTs, cyanobacteria produce a plethora of volatile secondary metabolites, with several of them having a strong taste and odor (T&O). This can make reservoirs unacceptable as drinking water sources due to consumer complaints and can negatively impact recreational activities and tourism [18,19]. Indeed, concerning Lake Karaoum, there has been circumstantial reporting of bad smells, but the presence of T&O compounds has never been investigated and it is not clear if the origin of T&O is from cyanobacteria or from industrial pollution. T&O compounds can be present at very low concentrations and although they are generally not toxic, they are responsible for the unpleasant smell of drinking water. Their removal may require re-planning and additional investments in water treatment [20].

The primary aim of this communication is to report for the first time conclusive results of the presence of CTs (MCs, CYN, ATX) in Lake Karaoum, using mass spectrometric analytical techniques, in addition to bioassays and molecular methods. A further aim is to investigate the presence of T&O compounds, in order to understand whether they originate from cyanobacterial metabolism or from anthropogenic pollution. To meet these objectives, a set of complementary methods were used to produce reliable results for a variety of cyanotoxins and T&O compounds. Since Middle-Eastern lakes (with the exception of Lake Kinneret [21]) are under-studied in regard to toxic cyanobacteria, the results presented give important information on the presence of CTs and T&O compounds in the area, enabling future monitoring programs and management strategies for Lake Karaoum.

## 2. Results

### 2.1. Water Quality and Diversity of Cyanobacteria in Lake Karaoum

During the period of field campaigns (2019–2020), the diversity of cyanobacteria in the reservoir was limited (Figure 1). *Aphanizomenon ovalisporum* was the dominant species (>95%) from October 2019 to April 2020, while *Microcystis aeruginosa* was the most abundant on July and October 2020. In August 2019, both *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* were equally present with abundances of 50% and 49%, respectively. In addition, in July of the same year, *Microcystis ichtyoblabile* and *Woronichia naegeliana* were identified in very low abundances, of 1% each. A microscope image of the dominant cyanobacterial species, i.e., *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* is shown in Figure 2.



**Figure 1.** Abundance (%) of cyanobacterial species in Lake Karaoun per sampling month (August 2019–October 2020).



**Figure 2.** Dominant cyanobacteria species in Lake Karaoun: *Microcystis aeruginosa* and *Aphanizomenon ovalisporum*.

Physico-chemical parameters of the lake's water were also monitored during the sampling period by the National Litani River Authority, which is in charge of the river management (Table S5). In most of the sampling dates, concentrations of nitrate and phosphate exhibited elevated values up to 16.72, 0.28 and 0.16 expressed as mg/L of  $\text{NO}_3\text{-N}$ ,  $\text{NO}_2\text{-N}$  and  $\text{PO}_4\text{-P}$ , respectively. This is in agreement with the assessment of the ecological status of the Karaoun reservoir by Fadel et al., classifying the lake as hypereutrophic, with low phytoplankton biodiversity and regular blooms of toxic cyanobacteria [15,16]. Degradation of the reservoir's water quality is considered to be mainly due to the significant loads of untreated sewage water and the discarding of agricultural and livestock remnants into the river stream.

## 2.2. Occurrence of Cyanotoxins (CTs) in Lake Karaoun

Occurrence of CTs in samples from Lake Karaoun was assessed by liquid chromatography tandem mass spectrometry (LC-MS/MS), complemented by ELISA, Protein Phosphatase Inhibition Assay (PPIA) and molecular detection of cyanobacteria and toxin genes by qPCR.

### 2.2.1. LC-MS/MS Analysis of Water Samples

Water samples were analyzed by LC-MS/MS for detection and quantitative determination of extracellular and intracellular CTs, i.e., ATX, CYN, NOD and 12 MC variants (Table 1). Cyanotoxins were found in 50% of samples (8 out of 16), extending over 2019 and 2020. CYN, ATX and NOD were not detected in any of the samples. On the other hand, 11 out of 12 MC variants i.e., dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-HiLR, MC-WR, MC-LA, MC-

LY, MC-LW and MC-LF were detected in at least one sample, while MC-HtyR was not detected in any samples. The samples with the largest diversity of MCs were sample 2 (S2-October 19, 10 variants), sample 16 (S3-October 20, 9 variants) and sample 4 (S1-December 19, 5 variants). MC-RR was the most frequently detected toxin (8 out of 16 samples). The sample with the highest concentrations of MCs was sample 16 (S3-October 20), where total MC-LR (sum of extracellular and intracellular fractions) reached 211 µg/L and MC-YR 199 µg/L. In this sample, the dissolved (extracellular) amounts of MC-LR and MC-YR were roughly 100-fold of the intracellular amounts, in contrast to samples 2 (S2-October 19) and 4 (S1-December 19), where MC-LR, MC-RR and MC-YR are found mostly as intracellular. The high proportion of extracellular MCs in sample 16 can possibly be attributed to a decaying phase of the bloom, with disruption of cyanobacteria cells and release of MCs into water. An indicative MRM chromatogram (sample 2, S2-02/10/2019—intracellular fraction) is presented in Figure S2.

**Table 1.** Concentrations ( $\mu\text{g/L}$ ) of extracellular/intracellular cyanotoxins (CYN, ATX, NOD, 12 MC variants) in water samples collected from Lake Karaoun during 2019–2020, analyzed by LC-MS/MS.

Sample ID	Sample details	CYN	ATX	dm MC-RR	MC-RR	NOD	MC-YR	MC-HtyR	dm MC-LR	MC-LR	MC-HiLR	MC-WR	MC-LA	MC-LY	MC-LW	MC-LF
1	S1, 22/08/19	ND <sup>1</sup> /NA <sup>2</sup>	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA
2	S2, 02/10/19	ND/ND	ND/ND	ND/1.60	0.339/26.4	ND/ND	ND/1.67	ND/ND	ND/1.87	0.438/91.5	ND/1.29	<LOQ <sup>3</sup> /ND	ND/ND	<LOQ/3.2 <sub>9</sub>	0.014/10.5	<LOQ/7.48
3	S4, 02/10/19	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
4	S1, 22/12/19	ND/ND	ND/ND	ND/0.53	0.050/7.42	ND/ND	0.187/8.28	ND/ND	0.050/0.70	0.158/8.43	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
5	S1, 15/01/20	ND/ND	ND/ND	ND/ND	0.007/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
6	S4, 04/02/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
7	S5, 15/02/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
8	S5, 17/02/20	ND/ND	ND/ND	ND/ND	0.005/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
9	S1, 22/02/20	ND/ND	ND/ND	ND/ND	0.007/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
10	S2, 22/02/20	ND/ND	ND/ND	ND/ND	0.008/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
11	S1, 15/04/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
12	S2, 22/04/20	ND/NA	ND/NA	ND/NA	0.090/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA	ND/NA
13	S4, 22/04/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
14	S1, 14/08/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
15	S2, 14/08/20	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND	ND/ND
16	S3, 09/10/20	ND/ND	ND/ND	0.069/ND	7.0/0.086	ND/ND	197/1.94	ND/ND	6.9/<LOQ	209/2.18	1.6/ND	ND/ND	3.0/ND	<LOQ/ND	ND/ND	0.021/ND

<sup>1</sup> ND: Not detected; <sup>2</sup> NA: Not analyzed; <sup>3</sup> <LOQ: Values higher than limit of detection (LOD) and lower than limit of quantitation (LOQ); LODs and LOQs are given in Table S1.

## 2.2.2. ELISA and PPIA Analysis of Water Samples

Since LC-MS/MS analysis targeted only a limited number of specific MC variants (12), assessment of the presence of MCs was complemented with ELISA and Protein Phosphatase Inhibition Assay (PPIA), in order to estimate total MCs concentration based on structural (ELISA) and functional (PPIA) similarities. Samples were analyzed for extracellular (dissolved) and intracellular MCs, similarly to LC-MS/MS. Results of ELISA and PPIA, together with the sum of MCs determined by LC-MS/MS, for comparison, are presented in Table 2. In general, samples in which either MCs were not detected by LC-MS/MS or their concentrations were lower than the LODs of ELISA/PPIA (0.10/0.25 µg/L), were negative by ELISA and PPIA with the exception of samples 8 (S5–17/2/2020), 11 (S1–15/04/2020), 12 (S2–22/04/2020) and 13 (S4–22/04/2020). Especially, in the latter sample, MCs were detected only by ELISA/PPIA as extracellular, in comparable concentrations (2.30/2.32 µg/L eq. MC-LR). MCs were found mostly as extracellular in sample 16 (S3–9/10/2020) by ELISA and PPIA, in agreement to LC-MS/MS, although in this sample ELISA and PPIA gave about 55% and 32% lower concentrations than LC-MS/MS.

**Table 2.** Total Extracellular/total intracellular MCs concentrations in water samples from Lake Karaoun by LC-MS/MS (sum of 12 extracellular MCs/sum of 12 intracellular MCs, µg/L), ELISA (µg/L MC-LR equivalents) and PPIA (µg/L MC-LR equivalents).

SAMPLE DETAILS	Total Extracellular/Intracellular MCs		
	LC- MS/MS * sum of MCs, µg/L	ELISA ** µg/L eq.MC-LR	PPIA ** µg/L eq.MC-LR
S1, 22/08/2019	ND/NA	ND/NA	ND/NA
S2, 02/10/2019	0.83/146	NA/97.2	NA/111
S4, 02/10/2019	ND/ND	ND/ND	ND/ND
S1, 22/12/2019	0.45/25.4	0.13/30.6	0.25/16.2
S1, 15/01/2020	0.007/ND	ND/ND	ND/ND
S4, 04/02/2020	ND/ND	ND/ND	ND/ND
S5, 15/02/2020	ND/ND	ND/ND	ND/ND
S5, 17/02/2020	0.005/ND	0.12/ND	ND/ND
S1, 22/02/2020	0.007/ND	ND/ND	ND/ND
S2, 22/02/2020	0.008/ND	ND/ND	ND/ND
S1, 15/04/2020	ND/ND	0.25/ND	0.39/ND
S2, 22/04/2020	0.090/NA	1.80/NA	0.87/NA

S4, 22/04/2020	ND/ND	2.30/ND	2.32/ND
S1, 14/08/2020	ND/ND	ND/ND	ND/ND
S2, 14/08/2020	ND/ND	ND/ND	ND/ND
S3, 9/10/2020	425/4.29	190/2.41	290/2.34

ND = Not detected, NA = Not Available. LODs: LC-MS/MS 0.001 µg/L; ELISA 0.10 µg/L; PPIA 0.25 µg/L. \* analysis with validated methods [22,23], extracellular toxins RSD < 16%, intracellular toxins RSD < 26%; \*\* duplicate analysis, RSD < 25%.

### 2.2.3. Molecular Detection of Cyanobacteria and Cyanotoxin Genes with qPCR

Molecular detection of cyanobacteria (16S rRNA) and biosynthetic genes of MCs, NOD, CYN, and Saxitoxins (STX) was carried out by qPCR. All samples were found positive for the presence of cyanobacteria. Samples 2 (S2—02/10/2019), 4 (S1—22/12/2019) and 16 (S3—09/10/2020) were found positive for MC producing genes (*mcyE*), with sample 4 being the most abundant in both cyanobacteria and MC genes among all samples. Genes associated with production of CYN (*cyrA*) and STX (*stxA*) were not detected in any of the samples. Results (gene copies per ml of sample) are presented in Table 3.

**Table 3.** Detection of cyanobacteria genes (16S rRNA) and biosynthetic genes of MCs and NOD (*mcyE*), CYN (*cyrA*) and STX (*stxA*) in water samples from Lake Karaoun (gene copies/mL).

SAMPLE DETAILS	Gene Copies/mL			
	16S rRNA (Cyanobacteria)	<i>mcyE</i> (MCs & NODs)	<i>cyrA</i> (C Y N)	<i>stxA</i> (S TX)
S1, 22/08/2019	NA	NA	NA	NA
S2, 02/10/2019	6951375	69857	ND	ND
S4, 02/10/2019	NA	NA	NA	NA
S1, 22/12/2019	9765354	127870	ND	ND
S1, 15/01/2020	NA	NA	NA	NA
S4, 04/02/2020	194446	ND	ND	ND
S5, 15/02/2020	9808	ND	ND	ND
S5, 17/02/2020	54262	ND	ND	ND
S1, 22/02/2020	62344	ND	ND	ND
S2, 22/02/2020	5093	ND	ND	ND

S1, 15/04/2020	40877	ND	N D	ND
S2, 22/04/2020	186812	ND	N D	ND
S4, 22/04/2020	15466	ND	N D	ND
S1, 14/08/2020	112948	ND	N D	ND
S2, 14/08/2020	34032	ND	N D	ND
S3, 09/10/2020	171308	6921	N D	ND

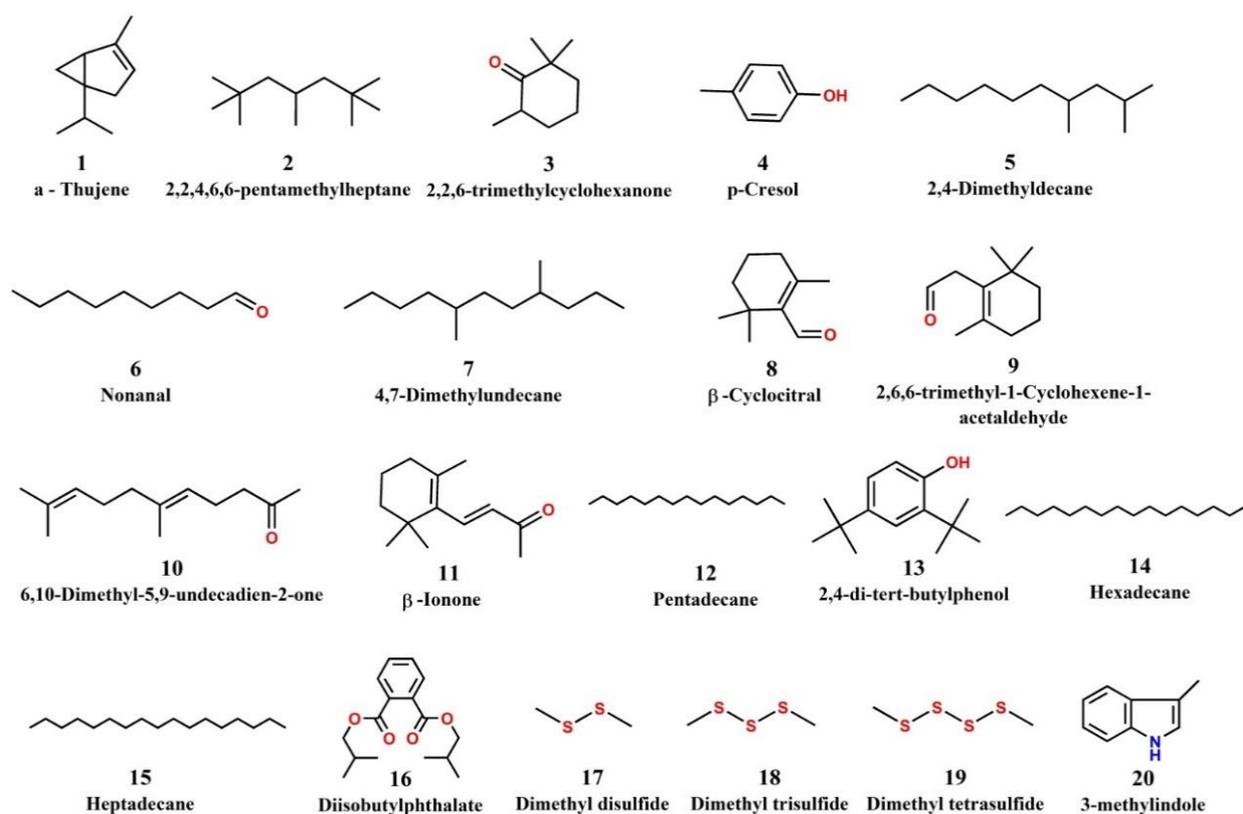
NA: not available, ND: not detected.

#### 2.2.4. LC-MS/MS Analysis of MCs in Fish Samples

Fish (*Cyprinus caprio*) flesh and liver samples were also analyzed for 12 MC variants by LC-MS/MS, using an in-house method. MCs were not detected in the analyzed samples. An indicative LC-MS/MS MRM chromatogram of a fish liver sample along with the chromatogram of a multi-toxin standard (Figure S3), shows the absence of MCs.

#### 2.3. Taste and Odor (T&O) and Volatile Organic Compounds (VOCs)

Untargeted screening of volatile compounds by Headspace Solid Phase Microextraction coupled to Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) led to the detection and identification of 20 compounds belonging to the following chemical groups: terpenes/terpenoids, hydrocarbons, aldehydes/ketones, phenols, phthalates, alkyl sulfides and indoles (Table 4). The chemical structures of the identified volatile compounds are presented in Figure 3. Two typical cyanobacterial terpenoids T&O compounds,  $\beta$ -cyclocitral (tobacco/woody odor) and  $\beta$ -ionone (floral odor), were detected in samples 2, 4 and 16 and 4, 12 and 16, respectively (Table S2). Nonanal, a known cyanobacterial/algal aldehyde with a fishy odor [24], was detected in 9 samples. Dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide, which are alkyl sulfides associated with algae/cyanobacteria were detected in sample 4, which had a strong swampy/septic odor. Samples 2 and 4 also contained 3-methylindole (skatole), a fecal compound with a characteristic odor. A number of hydrocarbons were also detected, with 2,2,4,6,6-pentamethyl heptane being the most common, as it was found in all samples. Industrial pollutants 2,4-di-tert-butylphenol, diisobutyl phthalate and p-cresol were detected in samples 10, 4 and 2, respectively. Results of untargeted HS-SPME-GC/MS screening per sample are given in Table S2.



**Figure 3.** Chemical structures of the volatile compounds identified.

**Table 4.** Compounds detected by untargeted HS-SPME-GC/MS screening, identification level of analysis, chemical group and retention time ( $t_R$ ) of detected compounds, number of samples in which they have been identified and their % peak areas.

No	Compound	Identification Level	Chemical Group	$t_R$ (min)	Number of Samples	% Peak Area
1	$\alpha$ -Thujene	A	Terpenes/Terpenoids	4.82	2	0.05–0.08
2	2,2,4,6,6-pentamethyl heptane	B	Hydrocarbons	5.43	16	0.03–0.80
3	2,2,6-trimethyl-cyclohexanone	A	Aldehydes/Ketones	5.95	1	0.004
4	p-Cresol	B	Phenols	6.28	2	0.04–5.55
5	2,4-Dimethyldecane	A	Hydrocarbons	6.59	1	0.17
6	Nonanal	A	Aldehydes/Ketones	6.64	9	0.06–0.43
7	4,7-Dimethylundecane	A	Hydrocarbons	7.62	1	0.01
8	$\beta$ -Cyclocitral	B,C	Terpenes/Terpenoids	7.90	3	0.05–0.16
9	2,6,6-Trimethyl-1-Cyclohexene-1-acetaldehyde	A	Terpenes/Terpenoids	8.25	1	0.007
10	6,10-Dimethyl-5,9-Undecadien-2-one	B	Aldehydes/Ketones	9.92	1	0.35
11	$\beta$ -Ionone	B,C	Terpenes/Terpenoids	10.25	3	0.04–0.38
12	Pentadecane	B,C	Hydrocarbons	10.35	1	0.14
13	2,4-di-tert-butylphenol	A,C	Phenols	10.43	10	0.01–39
14	Hexadecane	B,C	Hydrocarbons	11.16	2	0.20–0.60

15	Heptadecane	B,C	Hydrocarbons	11.94	6	0.07–33
16	Diisobutyl phthalate	B	Phthalates	13.11	4	0.003–1.5
17	Dimethyl disulfide	C,D	Alkyl sulfides	5.81	1	0.02
18	Dimethyl trisulfide	C,D	Alkyl sulfides	12.35	1	0.64
19	Dimethyl tetrasulfide	D	Alkyl sulfides	17.68	1	0.78
20	3-methylindole	C,D	Indoles	20.21	2	0.005–0.02

A: Retention Index (RI)  $\pm$  20, spectral matching  $\geq$ 80%; B: RI  $\pm$  20, spectral matching  $\geq$ 90%; C: matching with a reference standard; D: spectral matching  $\geq$ 80%.

Quantitative determination of 36 VOCs (given in 5.8.2) was carried out by HS-SPME-GC/MS. The targeted compounds are typical industrial pollutants of anthropogenic origin. Concentrations of VOCs which were detected in at least one sample are presented in Table 5. All samples were found to contain dichloromethane (25.1–34.0  $\mu\text{g/L}$ ). Toluene was present in 12 samples at concentrations up to 0.81  $\mu\text{g/L}$ . Trichloromethane (chloroform) was detected at low concentrations in samples 11 and 16 (0.40 and 0.50  $\mu\text{g/L}$ , respectively). Traces (<0.25  $\mu\text{g/L}$ ) of trichlorobenzenes were detected in sample 6, and traces of 1,2 dichloropropane, benzene, xylenes and styrene in sample 16.

**Table 5.** Concentration ( $\mu\text{g/L}$ ) of VOCs detected in at least one sample by targeted HS-SPME-GC/MS analysis.

Sample ID	Sample Details	Concentration ( $\mu\text{g/L}$ )									
		1,2,3-Trichlorobenzene	1,2,4-Trichlorobenzene	Dichloromethane	Toluene	1,2-Dichloropropane	Benzene	Trichloromethane	o-Xylene	m+p-Xylene	Styrene
1	S1, 22/08/19	ND	ND	31.0	0.72	ND	ND	ND	ND	ND	ND
2	S2, 02/10/19	ND	ND	28.8	0.65	ND	ND	ND	ND	ND	ND
3	S4, 02/10/19	ND	ND	28.1	0.60	ND	ND	ND	ND	ND	ND
4	S1, 22/12/19	ND	ND	29.1	0.25	ND	ND	ND	ND	ND	ND
5	S1, 15/01/20	ND	ND	29.0	<0.25	ND	ND	ND	ND	ND	ND
6	S4, 04/02/20	<0.25	<0.25	27.2	<0.25	ND	ND	ND	ND	ND	ND
7	S5, 15/02/20	ND	ND	28.2	ND	ND	ND	ND	ND	ND	ND
8	S5, 17/02/20	ND	ND	26.3	<0.25	ND	ND	ND	ND	ND	ND
9	S1, 22/02/20	ND	ND	34.0	<0.25	ND	ND	ND	ND	ND	ND
10	S2, 22/02/20	ND	ND	29.4	<0.25	ND	ND	ND	ND	ND	ND
11	S1, 15/04/20	ND	ND	27.2	ND	ND	ND	0.40	ND	ND	ND
13	S4, 22/04/20	ND	ND	30.2	ND	ND	ND	ND	ND	ND	ND
14	S1, 14/08/20	ND	ND	32.3	0.46	ND	ND	ND	ND	ND	ND
15	S2, 14/08/20	ND	ND	33.6	0.35	ND	ND	ND	ND	ND	ND
16	S3, 09/10/20	ND	ND	25.1	0.81	<0.25	<0.25	0.50	<0.25	<0.25	<0.25

ND: Not Detected. Sample 12 was not available for analysis.

### 3. Discussion

A complementary set of analytical methods was used to investigate and confirm the occurrence of cyanotoxins in Lake Karaoun for the first time. Unambiguous determination of 12 MC congeners, NOD, ATX and CYN was carried out by LC-MS/MS, complemented by ELISA and PPIA for MCs and qPCR for MC, CYN and STX producing genes. Results confirm the presence of several congeners (variants) of microcystins in the lake at elevated concentrations during October 2019 and 2020. These early findings give important information on the occurrence, variants and levels of occurring CTs, and they can be used for the establishment of reliable future monitoring programs to support management of cyanobacteria, cyanotoxins and T&O compounds in the context of the multiple uses of the lake.

LC-MS/MS analysis confirmed the presence of dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-HiLR, MC-WR, MC-LA, MC-LY, MC-LW, and MC-LF, with concentrations at 146.8, 25.8 and 429.3  $\mu\text{g/L}$  in October 2019, December 2019 and October 2020, respectively, expressed as the sum of extra and intracellular fractions. MC-LR was the most abundant MC congener followed by MC-YR and MC-RR. CYN, ATX and NOD were not detected in any of the samples.

Results from the analysis of water samples using ELISA and PPIA for total MCs concentration were in general agreement with those obtained from LC-MS/MS (Table 2). In samples 11, 12 and 13, the higher values for extracellular fraction obtained by ELISA and PPIA may indicate the presence of MC congeners other than those that were targeted by the LC-MS/MS method used in the study. This limitation, caused by the unavailability of commercial MC reference standards is well known in the literature, and it underscores the need for complementary quantitative analysis for total MCs (e.g., ELISA, PPIA), or for the development of non-targeted methods based on high-resolution mass spectrometry and related mass-spectral databases [25]. However, discrepancies between ELISA/PPIA and LC-MS/MS could also be attributed to the well-documented weaknesses of ELISA/PPIA. In particular, the shortcomings of ELISA are its high susceptibility to matrix effects, the variable cross-reactivity of different MC congeners and the response to degradation and/or transformation products of MCs that can result in overestimations [26]. In addition, PPIA based on the assessment of enzyme activity indicating the overall toxicity, does not have the same sensitivity to the different MCs and may also interfere with other unknown compounds present in the sample, thus resulting in under- or over-estimation of the concentration of toxins [27]. Furthermore, ELISA/PPIA lack the specificity of LC-MS/MS, as they respond to structurally/functionally similar molecules in total, and not to specific MC congeners. ELISA/PPIA are useful, quick, easy approaches for the screening of surface waters for MCs, especially where advanced laboratory infrastructure (e.g., LC-MS/MS) is not easily available. However, they must be considered as quantitative screening techniques for the detection of cyanotoxins [28,29].

Molecular analysis (qPCR) confirmed the presence of cyanobacteria (16s rRNA gene) in all the water samples collected. The gene *mcyE* which is associated with the production of MCs was found in 3 samples, i.e., samples 2 (S2, October 2019), 4 (S1, December 2019 and 16 (S3, October 2020), where the presence of MCs was also confirmed by LC-MS/MS, ELISA and PPIA. The gene responsible for the production of CYN (*cyrA*) was not detected in any of the samples (Table 3). This is in agreement with LC-MS/MS analysis where CYN was also not detected (Table 1).

Although significant concentrations of target MCs were detected in water samples, MCs were not detected in fish samples (flesh or liver). Previous studies also referred to cases where very low concentrations or total absence of MCs were reported in fish tissues collected during toxic cyanobacterial blooms [30,31]. It was shown that concentrations of MCs in fish tissues is strongly affected by local bloom conditions, fish feeding habits and ecosystem characteristics [32]. The occurrence of blooms and the presence of MCs in water are temporally and spatially variable, leading to different exposure of fish to MCs [33,34]. Inter-species differences in fish are also important factors for the intake and accumulation of MCs in their tissues. For example, zooplanktivorous fish were more likely to accumulate MCs than fish of other feeding guilds e.g.,

omnivorous, such as the studied *Cyprinus Carpio* [35]. The mechanisms of MC excretion and the timescales for MC elimination can also differ considerably among different fish species [36].

In the course of this study, *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* were almost unique cyanobacterial species that were observed in autumn/spring and summer, respectively. These results are in agreement with a previous study reporting *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* to be the most frequently encountered bloom-forming species in Lake Karaoun, either separately or together [14]. Furthermore, low phytoplankton biodiversity in Lake Karaoun was reported by Fadel and Slim who found that, since 2009, these two species had constituted >95% of the total phytoplankton biomass [16]. It was also reported that in Lake Karaoun, *Aphanizomenon ovalisporum* blooms formed both in spring and autumn, while *Microcystis aeruginosa* blooms formed at higher water temperatures observed during the summer months [15]. Although, *Aphanizomenon ovalisporum* is a well-known CYN producer, CYN was not detected during this study. On the contrary, the presence of CYN was previously reported during May to November 2012 and March to May 2013. The determination was performed with ELISA showing concentrations ranging from 0.5 to 1.7 µg/L in 2012, and from undetected to 1.7 µg/L in 2013 [17]. However, to date, the detection of CYN has never been confirmed by LC-MS/MS.

Monitoring studies of lakes and reservoirs in the Middle East are rare, with the exception of the closest natural lake, Lake Kinneret (Israel). Although Lake Kinneret is less eutrophic than the Karaoun reservoir, blooms of *Microcystis sp.* have been reported since the late 1960s [37], while *Aphanizomenon ovalisporum* and *Cylindrospermopsis raciborskii* were first detected in September 1994 and summer 1998, respectively [38,39]. The most frequently found cyanotoxins in Lake Kinneret have been CYN and MCs [21]. Despite the fact that CYN was not detected in this study, it is proposed that CYN should be included, together with MCs, in future monitoring programs to better evaluate the associated risks, since *Aphanizomenon ovalisporum* blooms continue to occur in lake Karaoun. NOD is mostly associated with brackish water cyanobacteria with a limited number of studies to report its occurrence in freshwater bodies [4,40]. Therefore, its presence is generally not expected in Lake Karaoun, while it can be determined along with MCs due to its structural similarity with them [41] in future monitoring programs. The presence of ATX should further be investigated, in cases where known cyanobacteria producers are present [4].

Screening for volatile compounds showed the presence of T&O cyanobacterial/algal terpenoids β-cyclocitral (in 3 samples) and β-ionone (in 3 samples). In particular, the presence of β-cyclocitral was reported to be associated with the occurrence of strains of *Microcystis* [42,43] which has a tobacco-woody odor with a rather high odor threshold concentration of 5 µg/L [44]. It was shown that β-cyclocitral is rapidly produced upon *Microcystis* cell rupture via a carotene oxygenase reaction and it subsequently affects grazer behavior, acting as a repellent and signal of low-quality food to grazers [43]. β-Ionone, commonly occurring in algae and higher plants, is also produced through the carotenoid cleavage of dioxygenases [45]. It has a characteristic flowery-woody odor, showing strong odor thresholds, at a level of 0.007 µg/L [46]. The release of β-ionone in lake water was positively correlated with microcystis biomass [47]. The common cyanobacteria/actinomycetes T&O compounds, geosmin and 2-methylisoborneol were not detected in any of the samples.

Nonanal, a known cyanobacterial/algal aldehyde with fishy odor [24] and an odor threshold of 1 µg/L in water [48] was detected in 9 samples. Aldehydes are mostly derivatives of polyunsaturated fatty acids and are common causes of odor in surface waters [18]. Dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide were detected in sample 4 that had a strong swampy/septic odor. Such undesirable odors have been a major concern for drinking water in several countries. In particular, dialkyl sulfides present strong odors described as swampy/septic, rotten, rancid and stinky, with very low odor threshold concentration levels of ng/L or less [49–51]. These and other organosulfur compounds can be produced both under oxic and anoxic conditions by a diversity of biota, biochemical pathways, enzymes and precursors [52]. 3-methylindole (skatole) was detected in samples 2 and 4. In particular, sample 4 had a strong swampy/septic odor due to the presence of dimethyl sulfides and skatole. The latter is a fecal

compound with a characteristic odor that was previously reported to occur in algal cultures and field samples [18].

Several species of cyanobacteria can produce cyanotoxins and T&O compounds. For example, some strains of *Microcystis* produce microcystins together with  $\beta$ -cyclocitral and alkyl sulfides [42]. However, cyanobacterial T&O do not inevitably indicate the occurrence of cyanotoxins [53]. Nevertheless, since T&O can be sensed by the human nose at very low concentrations, they can serve as an early warning indicator for further investigations into the presence of toxic cyanobacteria [54]. The cyanobacterial/algal T&O detected in this study can serve as an initial list of compounds to be screened in future T&O episodes in Beirut's drinking water supplies.

The hydrocarbons detected, such as 2,2,4,6,6-pentamethyl heptane (all samples) and straight-chain hydrocarbons hexadecane (2 samples) and heptadecane (6 samples), could generally be of anthropogenic or biogenic origin. The hydrocarbon 2,2,4,6,6-Pentamethyl heptane has many industrial uses in anti-freeze products, coatings, fillers, lubricants, greases, etc. [55]. On the other hand, cyanobacteria and algae have long been known producers of alkanes, and their potential for biofuel production has been an area of increased research interest [56]. A study of volatile compounds associated with cyanobacteria and algae in freshwater by Juttner et al., [42] reported that biogenic hydrocarbons were represented exclusively by straight-chain components. Other compounds detected, such as 2,4-di-*tert*-butylphenol (antioxidant), diisobutyl phthalate (plasticizer) and *p*-cresol are common industrial pollutants, with uses in fuels, plastics, and production of chemicals. These were identified in samples 10, 4 and 2, respectively.

Quantitative determination of VOCs showed the presence of dichloromethane in all samples, at concentrations up to 34  $\mu\text{g/L}$ . Dichloromethane is a common industrial solvent used in many chemical processes. Toluene was detected in the majority of samples at concentrations of up to 0.81  $\mu\text{g/L}$ , while in sample 16 it co-occurred with traces of benzene and *o,m,p*-xylenes. These compounds are found in fuels and petroleum products and are also common industrial solvents. The "BTEX" volatiles (benzene, toluene, ethylbenzene, xylenes) are frequently used as an index to assess the impact of pollution caused by spills or leaks from fuel storage tanks into water bodies. Trichloromethane, detected in two samples at concentrations of up to 0.5  $\mu\text{g/L}$  could indicate contamination of the lake with wastewater, since trihalomethanes<sub>7</sub> are commonly present in chlorine-treated water [57].

The above findings imply that, besides cyanobacteria and their metabolites, cyanotoxins and T&O compounds, anthropogenic pollution can also be a concern for Lake Karaoun, regarding its use as a drinking water reservoir, supporting the emerging need for studies and impact assessment of the co-occurrence of toxic cyanobacteria with other anthropogenic pollutants [58].

#### 4. Conclusions

The results of this study demonstrate for the first time the presence of multiple MC congeners in Lake Karaoun, i.e., dmMC-RR, MC-RR, MC-YR, dmMC-LR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW, and MC-LF, with total MCs reaching up to 429  $\mu\text{g/L}$  (sample 16). Additionally, T&O compounds such as  $\beta$ -cyclocitral,  $\beta$ -Ionone, nonanal and dimethylsulfides were identified, while industrial pollutants of anthropogenic origin including dichloromethane and toluene were determined up to 34 (sample 9) and 0.81 (sample 16)  $\mu\text{g/L}$ , respectively. Complementary methods were applied aiming at the reliable determination of cyanotoxins and T&O compounds in the lake. Since blooms of *Microcystis aeruginosa* and *Aphanizomenon ovalisporum* continue to occur in Lake Karaoun, monitoring of cyanobacterial blooms is necessary in the future, for the assessment of risks related to the intended uses of the lake. With regard to the cyanotoxin analysis, the results show that MCs should be a monitored target, especially when blooms of *Microcystis aeruginosa* occur. Quantitative screening by ELISA or PPIA could be used for the monitoring of MCs if LC-MS/MS facilities and expertise are not available. It is, however, recommended to confirm the findings by LC-MS/MS which has the advantage of being compound-specific. Despite the fact that CYN was not detected in this study, CYN should be

included in the ongoing monitoring schemes, especially in the presence of *Aphanizomenon ovalisporum* in the lake. Whenever incidents of unpleasant odors occur in the lake or in water supplies, further analysis of T&O and VOCs should be carried out to identify the source of T&O.

## 5. Materials and Methods

### 5.1. Study Site and Sampling

The Middle-Eastern Lake Karaoun (Qaraoun) is the largest water body in Lebanon, located in the western part of the valley Bekaa (33.34° N, 35.41° E) (Figure 4). Lake Karaoun is an artificial reservoir, created in 1959 by construction of a dam on the Litani River. It has a surface of 12 km<sup>2</sup> at a full capacity of 220 million m<sup>3</sup>, a maximum depth of 60m and a mean depth of 19m [16]. The lake is considered to be located in a vulnerable arid to semi-arid zone, with winters being moderately cold (13 °C average temperature), the wet season extending from November to April, and summers being hot and dry, lasting from July to October [15,59].

During field campaigns that covered the wet and dry periods, 16 water samples along with a total of 9 specimens of *Cyprinus carpio* fish (weighing 215 g on average) were collected mainly by the national Litani River Authority. All water samples were collected in polyethylene bottles from 5 sampling points of Lake Karaoun (S1, S2, S3, S4 and S5). S1 was located close to the river input at the west bank, S2 was east of the dam, S3 was close to the river input at the east bank, S4 was in the middle of the lake and S5 was at the dam (Figure 4). Water samples were transported in coolers (at a temperature of 4 °C) for laboratory analysis in Beirut, Lebanon and Athens, Greece. The fish were kept on ice and were sacrificed within 24 h. Fish liver and muscle sub-samples were labeled and frozen separately at −20 °C. The frozen fish tissue samples were then lyophilized with a Labconco freeze-dryer for 48 h at −84 °C and 0.2 mbar and powdered using a pestle and a mortar.

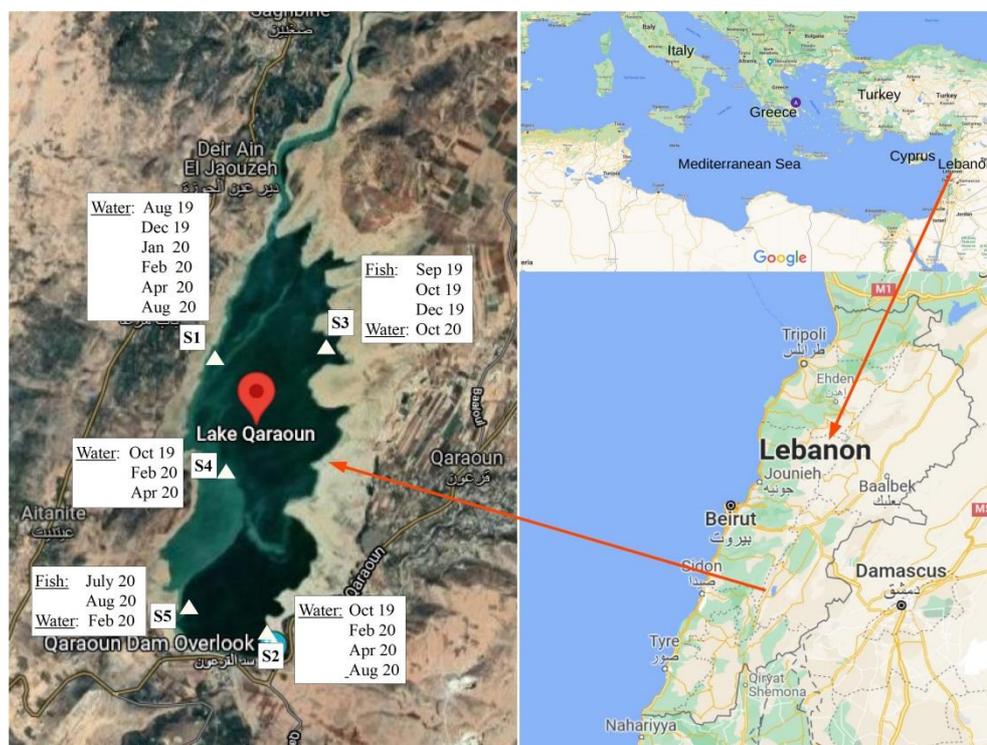


Figure 4. Map and sampling points of Lake Karaoun, Lebanon.

### 5.2. Chemicals and Reagents

Cyanotoxin standards of MC-RR, MC-LR, MC-YR, MC-LA and NOD were purchased from Sigma-Aldrich (Steinheim, Germany), [D-Asp3]MC-LR, [D-Asp3]MC-RR, MC-WR, MC-HtyR, MC-HilR, MC-LY, MC-LW and MC-LF from ENZO Life Science (Lausen, Switzerland), CYN from Abraxis (Warminster, PA, USA), and ATX fumarate from TOCRIS Bioscience (Bristol, UK). All toxin standards had purity >95%. A VOC 57 standard mix (44926-U) was purchased from Supelco (Darmstadt, Germany).  $\beta$ -Cyclocitral (C<sub>10</sub>H<sub>16</sub>O) ( $\geq$ 95.0%),  $\beta$ -ionone (C<sub>13</sub>H<sub>20</sub>O) (purity  $\geq$  97.0%), pentadecane (C<sub>15</sub>H<sub>32</sub>) (purity  $\geq$  99.8%), 2,4-di-*tert*-butylphenol (C<sub>14</sub>H<sub>22</sub>O) (99.0%), hexadecane (C<sub>16</sub>H<sub>34</sub>) (purity  $\geq$ 99.8%), heptadecane (C<sub>17</sub>H<sub>36</sub>) (purity  $\geq$  99.5%), dimethyl disulfide (CH<sub>3</sub>SSCH<sub>3</sub>) ( $\geq$ 99.0%), dimethyl trisulfide (purity  $\geq$  98.5%) and 3-methylindole (C<sub>9</sub>H<sub>9</sub>N) (98.0%), were supplied by Sigma Aldrich (Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) of HPLC grade ( $\geq$ 99.9%) as well as dichloromethane (DCM) and hexane (HXN) of analytical grade (99.9%) were supplied by Fisher Chemical (Loughborough, UK). High purity formic acid (HCOOH) (98–100%) and acetic acid (CH<sub>3</sub>COOH) (>99.8%) were obtained from Riedel-de Haën (Seelze, Germany). Sodium chloride (NaCl) of analytical purity (99.5%) and fuming (37%) hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) of analytical grade was supplied from Serva Electrophoresis. Sodium hydroxide (NaOH) 2M solution used for adjustment of pH was prepared from NaOH pellets (purity 98%) purchased from Sigma-Aldrich (Steinheim, Germany). Potassium carbonate (K<sub>2</sub>CO<sub>3</sub>) (>99.5%) was supplied from Carlo Erba Reagents. High purity water (18.2 M $\Omega$  cm at 25 °C) was produced in-house using a TEMAK TSDW10 water purification system (TEMAK, Athens, Greece).

### 5.3. Microscopic Examination

Microscopic examination of samples was carried out within 24 h on 20 mL water samples collected during field campaigns with a phytoplankton net and kept at low temperature (4 °C). Examination was carried out in the LAEC laboratory in Beirut, Lebanon using a phase contrast microscope (Olympus, Munster, Germany), under a  $\times$ 40 objective and  $\times$ 100 immersion. Identification of cyanobacteria was based on taxonomic keys as cells structures and dimensions, mucilage features and the form of colonies. Estimation of cyanobacterial abundances was performed as described elsewhere [17].

### 5.4. LC-MS/MS Analysis of Cyanotoxins

#### 5.4.1. Sample Preparation of Water Samples

Preparation of water samples for LC-MS/MS analysis of cyanotoxins was based on the method of Zervou et al. [22]. For the determination of extracellular toxins, 150 mL of sample was filtered using a glass fiber filter (Millipore, Ireland). After adjusting the pH of the filtrate to 11, solid phase extraction (SPE) was performed using a dual cartridge assembly with a Supel-Select HLB (bed wt. 200 mg, volume 6 mL, Supelco) and a Supelclean ENVI-Carb (bed wt. 250 mg, volume 3 mL, Supelco) on a 12-port SPE vacuum manifold (Supelco) connected with a vacuum pump. Conditioning of cartridges was carried out with 6mL DCM, followed by 6 mL of MeOH and 6 mL of pure water (pH 11). Sample passed through the two tandem cartridges at a 0.5 mL/min flow rate. After sample passing, cartridges were dried for 15 min (air under vacuum) and the sequence of cartridges in the assembly was reversed. Elution was done with (60:40) MeOH/DCM having 0.1% HCOOH. Eluents were evaporated to dryness under a gentle nitrogen stream, reconstituted with 150  $\mu$ L of 5% (*v/v*) MeOH, and transferred into vials for LC-MS/MS analysis. For the determination of intracellular cyanotoxins, after sample filtration the filters were extracted, according to Chistophoridis et al. [23], with 9 mL 75% (*v/v*) MeOH. A 3-mL aliquot of the filtered supernatant was evaporated to dryness and the residue was re-dissolved with 500  $\mu$ L of 5% (*v/v*) MeOH for LC-MS/MS analysis.

#### 5.4.2. Sample Preparation of Fish Samples

Subsamples of 0.2 g of lyophilized powdered flesh or 0.25g liver were extracted with 5 mL 80% MeOH containing 0.5% HCOOH by stirring for 15 min, followed by ultra-sonication for 30 min (Bandelin Sonorex Super RK106). The mixture was then transferred to a Falcon tube and centrifuged for 15 min at 4500 rpm (DuPont RMC-14 Refrigerated Micro-Centrifuge, Sorvall Instruments, Newtown, CT, USA). The supernatant was extracted three times with 1 mL of hexane. The hexane phase was discarded, and the bottom layer was transferred to a flask and diluted with 100 mL of water containing 0.3% formic acid. The extract was cleaned-up by SPE with a Supel-Select HLB cartridge (bed wt. 200 mg, volume 6 mL, Supelco) conditioned with 6 mL MeOH followed by 6 mL acidified water (0.3% HCOOH). After extraction, the cartridge was washed with 6 mL of water, dried for 15 min under vacuum and eluted with 6 mL MeOH. The eluents were dried in a water bath at 40 °C under a gentle nitrogen stream. Reconstitution was carried out with 200 µL of 5% MeOH, followed by vortexing and sonication for 3 min, prior to LC-MS/MS analysis.

#### 5.4.3. Determination by LC-MS/MS

A Finnigan Surveyor LC system, equipped with an AS autosampler (Thermo, MA, USA) coupled with a TSQ Quantum Discovery Max triple-stage quadrupole mass spectrometer (Thermo, MA, USA) with electrospray ionization (ESI) source, was used for LC-MS/MS analysis. Data was acquired and processed by Xcalibur software. Targeted analysis of CYN, ATX, NOD and 12 MCs (dmMC-RR, MC-RR, MC-YR, MC-HtyR, dmMC-LR, MC-LR, MC-HilR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF) was performed as described in a previous study [22]. Detection of CTs was carried out in multiple reaction monitoring (MRM) mode, using the three most intense and characteristic precursor/product ion transitions for each toxin. Confirmation of identity was based on criteria for retention time ( $t_R$ ), characteristic precursor/product ion transitions and two calculated ratios of precursor/product ion transitions. LC-MS/MS detection parameters of targeted cyanotoxins are given in Table S3. An example of MC-LR identification in a sample (S2, 2 October 2019) from Lake Karaoun is shown in Figure S4.

#### 5.5. ELISA for Microcystins

ELISA was carried out with the Microcystins-ADDA ELISA kit (Eurofins—Abraxis, Warminster, PA, USA) in 96 well microplates, using an Infinite M200 reader (Tecan, Männedorf, Switzerland). The kit was used according to the manufacturer's instructions and concentrations of toxins were calculated using calibration curves based on the absorbance at 450 nm. For the determination of extracellular MCs, water samples were filtered through 47 mm glass fiber filters (Millipore) and analyzed without any further treatment. Dilutions with ELISA sample diluent were carried out when samples exceeded 5 µg/L MC-LR equivalents. For the analysis of intracellular MCs, after sample filtration (see 5.4.1) the filter was extracted with 9 mL of 75% MeOH. Then, 3 mL of the extract was evaporated to dryness and the residue was re-dissolved in water to avoid false results due to the high percentage of MeOH [41]. Finally, samples were analyzed in duplicate and mean values were reported when RSD < 25%.

#### 5.6. Protein Phosphatase Inhibition Assay (PPIA) for Microcystins

PPIA was carried out using the Microcystins/Nodularins PP2A kit (Eurofins—Abraxis) in 96 well microplate using an Infinite M200 reader (Tecan, Männedorf, Switzerland) for measurements at 405 nm, according to manufacturer's instructions. Dilutions with ultrapure water were carried out when samples exceeded 2.5 µg/L MC-LR equivalents. Sample preparation prior to PPIA was the same as in ELISA. Samples were analyzed in duplicate and mean values were reported when RSD < 25%.

#### 5.7. qPCR Assay for Total Cyanobacteria and Cyanotoxin Genes

The PhytoxiGene™ CyanoDTec (Diagnostic Technology, Belrose, Australia) multiplex quantitative real-time PCR assay was applied to determine the gene copies of the 16s rRNA gene

(total cyanobacteria) and the *mcyE*, *cyrA*, *sxtA* genes (microcystins, cylindrospermopsin, saxitoxins, respectively). The kit was used according to the manufacturer's instructions and PCR was carried out in a Smartcycler II system (Cepheid). In brief, a volume of water sample (1 to 15 mL, depending on visual cell density) was filtered through a Nucleopore 25 mm, 0.8 µm filter (Whatman, Little Chalfont, UK) using a syringe and filter holder. DNA extraction of filters was carried out using BioGx bead lysis tubes (Diagnostic Technology) in a BeadBug bead beater (Benchmark Scientific). Extracts were centrifuged (MicroCL 21, Thermo Fisher Scientific, Waltham, MA, USA) and proceeded to qPCR. Quantitation of gene copies was based on calibrations with the PhytoxiGene™ CyanoNAS standards (100–1,000,000 copies per reaction).

### 5.8. GC-MS Analysis of Volatile Compounds

Analysis of volatile compounds was carried out in order to (a) screen samples to detect and identify typical cyanobacterial volatile and T&O compounds (untargeted method) and (b) to quantitatively determine a range of anthropogenic VOC pollutants (targeted method).

#### 5.8.1. Untargeted Screening of Cyanobacterial Volatiles and T&O Compounds

Headspace Solid Phase Microextraction coupled to Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS) was used to screen water samples for volatile compounds with focus on cyanobacterial T&O. A 456 GC coupled to TQ mass spectrometer and equipped with an SPME autosampler (Bruker Daltonics, Bremen, Germany) was used. A (2–10 mL) aliquot of the sample was transferred into a 20 mL SPME glass vial containing 3 g NaCl, then adjusted to 10mL with ultrapure water and tightly sealed. HS-SPME was carried out automatically under the following conditions: 2 cm Divinylbenzene/Carboxen/Polydimethylsiloxane SPME fiber (Supelco, Bellefonte, PA, USA), equilibration 10 min at 60 °C, headspace extraction: 10 min at 60 °C, agitation 300 rpm and desorption time 2 min. GC analysis was carried out using a column RXI®- 5 Sil MS, 30 m, 0.25 mm ID, 0.25 µm df (Restek, Bellefonte, PA, USA). GC conditions were injector temp. 250 °C, splitless, constant flow 1 mL/min (He), column program: (a) 50 °C (1 min) to 250 °C at a rate of 15 °C/min, 250 °C (5 min) and (b) 35 °C (5 min) to 250 °C at a rate of 8 °C/min, 250 °C (5 min). MS conditions were: EI source (70 eV), scan 30–300 m/z, positive polarity. Fluorobenzene standard solution in methanol (Sigma – Aldrich, St. Louis, MO, USA) spiked at a concentration of 5 µg/L in the samples was used as a surrogate for evaluation of SPME efficiency.

Mass spectrometry data were processed using MSWS software (Bruker). Mass spectral deconvolution and identification of compounds was carried out with AMDIS (NIST, Gaithersburg, MD, USA) using the NIST MS library (2015) and retention index calibration with C7-C30 saturated alkanes reference standard (Sigma). Combined matching scores (retention index-RI and mass spectral matching) were used as criteria for identification (RI ± 20 of the reference RI and a spectral matching ≥80%). Identification was considered definite only if a reference standard of the suspect compound was available in the lab and retention times of the suspect and reference compounds matched within ±0.01min, in addition to mass spectral matching.

#### 5.8.2. Quantitative Determination of VOCs

Quantitative determination of a number of VOCs was carried out by HS-SPME-GC-MS according to EN ISO 17943:2016 (HS-SPME-GC-MS), using 5ml samples [60]. The compounds determined were chloroform, bromoform, dibromochloromethane, bromodichloromethane, benzene, 1,2-dichloroethane, tetrachloroethene and trichloroethene, 2-ethoxy-2-methyl propane (ETBE), bromochloromethane, dibromomethane, toluene, 1,2-dibromoethane, chlorobenzene, styrene, bromobenzene, n-butyl benzene, hexachlorobutadiene, naphthalene, 1,1-dichloroethane, 1,1-dichloroethene, 1,1,2-trichloroethane, 1,1,2,2-tetrachloroethane, 1,2-dibromo-3-chloropropane, 1,2-dichloropropane, 1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene, 1,3-dichloropropane, 2-methoxy-2-methyl-butane (TAME), ethylbenzene, n-propylbenzene, o-xylene, p+m-xylenes, sec-butylbenzene and tert-butylbenzene. A VOC 57 standard mix (Supelco) was used for calibration

and fluorobenzene, toluene- $d_8$  and benzene- $d_6$  (Sigma) as internal standards. HS-SPME conditions were: 2 cm Divinylbenzene/Carboxen/Polydimethylsiloxane SPME fiber (Supelco), equilibration for 10 min at 40 °C, headspace extraction for 10 min at 40 °C, agitation 300 rpm and desorption time 2 min. GC conditions were: column RXI®- 624 Sil MS, 60 m, 0.32 mm ID, 1.8  $\mu$ m df (Restek), injector temp. 250 °C, splitless, constant flow 1ml/min (He), column program: 35 °C (5 min) to 250 °C at a rate of 8 °C/min and at 250 °C (10min). MS conditions were: EI source (70 eV), Selected Ion Monitoring (3 ions per compound), positive polarity. LOD of each compound was 0.2  $\mu$ g/L. Confirmation of determinations was carried out with the ISO criteria for retention time and ion ratios [60].

### 5.9. Method Validation and Quality Control Procedures

The methods applied in this study were validated either previously or in the frame of the study and method performance parameters such as specificity, linearity, precision, accuracy, and limits of detection have been assessed. Furthermore, quality control procedures including measurements of blank/negative and control/positive samples were followed in each batch of analyzed samples.

Validation data of the method applied for the determination of CTs in water by LC-MS/MS are reported in previous studies with RSD values of detected CTs being <16% and <26% for extra- and intra-cellular fractions, respectively [22,23]. This method is also accredited by ISO 17025 in the NCSR Demokritos laboratory [61].

The LC-MS/MS method for determination of CTs in fish was developed, optimized and validated in the frame of this study. For optimization, various combinations of extraction solvents, extraction/mixing times, clean-up steps and SPE cartridges were tested (Oasis HLB and HLB followed by Sep-Pak Vac, silica) as presented in Figures S5 and S6. Optimization experiments were performed with samples spiked with MC-LR and MC-RR at concentration levels of 100 and 400 ng/g dw for flesh and liver, respectively. Selection of the optimized conditions was based on maximization of % recoveries. As shown in Figure S7, method 2 provided the highest mean recoveries for the two spiked MCs in flesh (78.9% for MC-RR and 79.1% for MC-LR) and liver (77.1% for MC-RR and 75.4% for MC-LR). Subsequently, the optimized method was validated in-house. Recovery and precision were evaluated by analyzing toxins-free lyophilized flesh and liver fish samples spiked with a mixture of 12 MCs, at concentration levels of 100 and 400 ng/g dw in 3 replicates. Samples were extracted and analyzed as outlined in Section 5.4.2. Table S4 provides performance characteristics of this method. Briefly, mean recoveries of [D-Asp<sup>3</sup>]MC-RR, MC-RR, MC-YR, [D-Asp<sup>3</sup>]MC-LR, MC-LR, MC-HilR, MC-LA and MC-LY ranged from 68.5% to 81.6% for flesh, while liver recoveries ranged from 61.5% to 72.2%, with intra-day precision in the range of 6.8–16.5% for flesh and 5.5–15.2% for liver. LODs for fish flesh and liver samples ranged from 1.0 to 7.0 ng/g dry weight and from 0.8 to 5.6 ng/g dry weight, respectively.

In-house validation and method performance data of ELISA and PPIA for MCs in water were reported previously [41] and methods have been proven suitable for quantitative screening of MCs. Negative and positive control samples were included in each analysis batch.

The qPCR assay for total cyanobacteria and cyanotoxin genes included evaluation of negative and positive control samples in each batch of samples. The Phytoxigene™ CyanoNAS standards used for calibration and accurate quantitation were commissioned and developed by the National Measurement Institute (NMI), of the Australian Department of Industry which participates in the International Bureau of Weights and Measures (BIPM) Consultative Committee for Amount of Substance (CCQM).

The untargeted HS-SPME-GC-MS screening method for detection and identification of cyanobacterial volatile compounds has been tested with a wide range of compounds that are available in the lab of EYDAP SA and has been shown to be capable of detection/identification at concentrations generally <1 $\mu$ g/L. The method has also been tested successfully in interlaboratory trials for unknown odorous compounds in water. The targeted HS-SPME-GC-MS method for

VOCs in water has been fully validated in-house, is accredited by ISO 17025 and has successfully been evaluated in interlaboratory tests.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1. chemical structures of studied cyanotoxins, Figure S2. LC-MS/MS MRM chromatogram of sample S2, 02/10/2019 (intracellular fraction) from Lake Karaoun, Figure S3. MRM chromatogram of (a) a standard solution of 12 MCs at a concentration level corresponding to 50 ng/g dw and (b) liver sample from *Cyprinus Carpio* fish collected in September 2019, Figure S4. example of identification of MC-LR in sample S2, 2 October 2019 (intracellular fraction) from Lake Karaoun, Figure S5. Experimental procedures tested in order to optimize the extraction of MCs in fish flesh, Figure S6. Experimental procedures tested in order to optimize the extraction of MCs in fish liver, Figure S7. Selection of method for (a) fish flesh and (b) fish liver: obtained recoveries of spiked MCs using different treatment processes; Table S1. Method LODS and LOQs for each cyanotoxin analysed in this study using LC-MS/MS, Table S2. Results of untargeted HS-SPME-GC/MS screening per sample, Table S3. LC-MS/MS detection parameters of targeted cyanotoxins, Table S4. Performance characteristics of the method for the analysis of target MCs in fish flesh and liver. Table S5: Physicochemical parameters of Lake Karaoun.

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