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Suspect screening of natural toxins in surface water reservoirs

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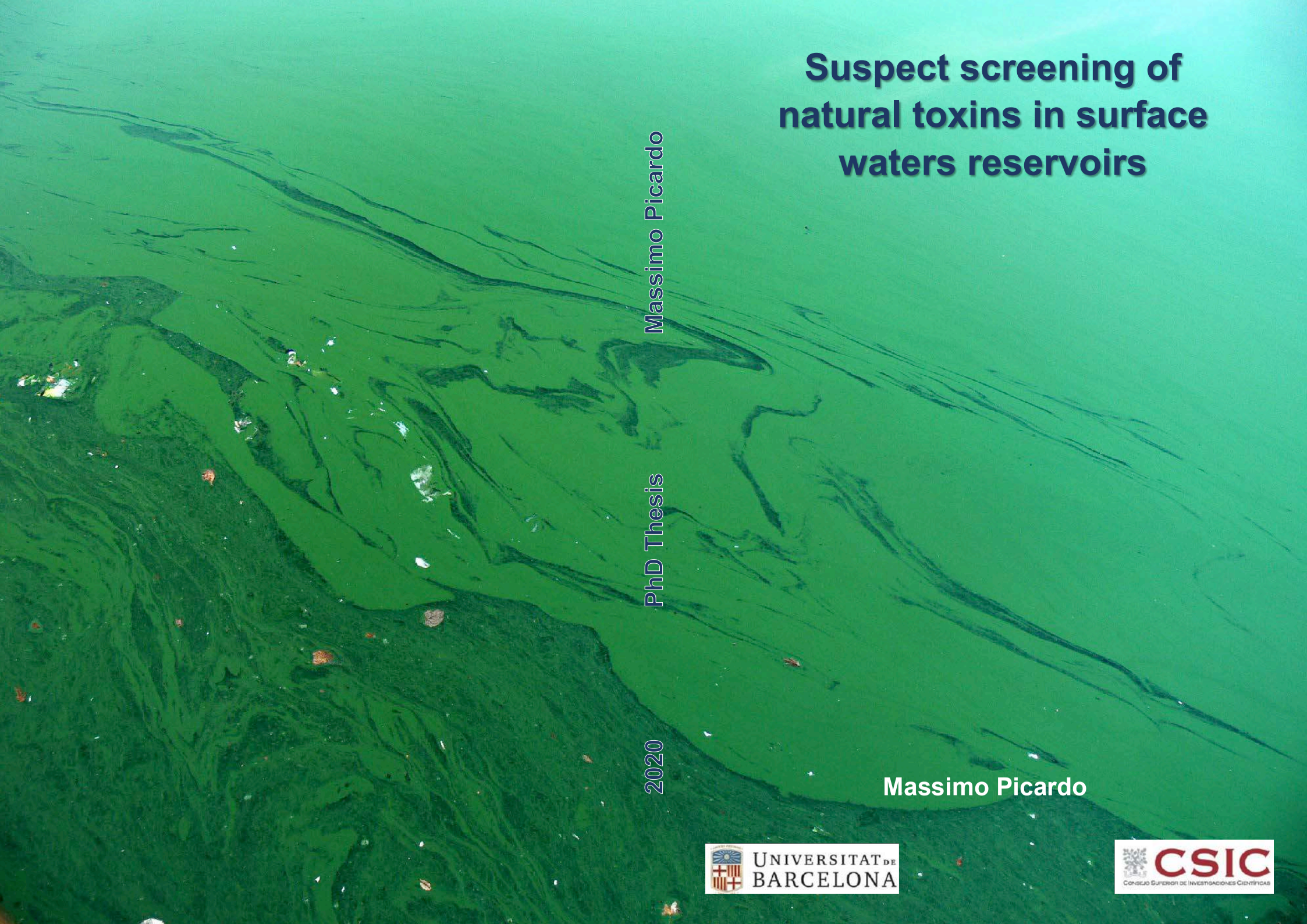

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Massimo Picardo

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Suspect screening of natural toxins in surface water reservoirs

Memoria de tesis presentada para optar al grado
Doctor por la Universidad de Barcelona a

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La Dra. Marinella Farré, científica titular del instituto de Diagnóstico Ambiental y Estudios del Agua, IDAEA y el Dr. Oscar Nuñez Burcio, Profesor agregado del Departamento de Ingeniería Química y Química Analítica de la Universidad de Barcelona,

Certifican:

Que la presente memoria presentada para optar al título de Doctor, titulada "***Suspect screening of natural toxins in surface water reservoirs***" ha sido relaizada bajo nuestra dirección por el Sr. **Massimo Picardo** en el Instituto de Diagnóstico Ambiental y Estudios del Agua (IDAEA), perteneciente al Consejo Superior de Investigaciones Científicas (CSIC), y el departamento de Química Analítica de la Universidad de Barcelona, y que todos los resultados presentados son fruto del trabajo experimental realizado por el mencionado doctorando.

Barcelona, 1 de diciembre 2020

Dra. Marinella Farré

Dr. Oscar Nuñez Burcio

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Rare sono le persone che usano la mente
poche coloro che usano il cuore
e uniche coloro che usano entrambi.
(Rita Levi Montalcini)

A scientist in his laboratory is not only a technician,
he is also a child faced with natural phenomena
that impress him like a fairy tale.
(Marie Sklodowska-Curie)

Le verità scientifiche
non si decidono a maggioranza
(Galileo Galilei)

Per voi che c’eravate,
ma soprattutto,
per voi che ci siete
e che ci sarete sempre.

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Gracias a Gabri y Nico sin vosotros ahora la tesis probablemente seguiría pareciendo un borrador. ¡Habéis siempre estado para darme consejos sobre cualquier tema, no tenéis ni idea de la ayuda que me habéis dado durante estos años!

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Abbreviations

7-epi-CYL	7-epi-cylindrospermopsin
7-deoxy-CYL	7-deoxy-cylindrospermopsin
ADDA	(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
AFL	Aflatoxin
ANA	Anatoxin
Ab	Antibody
Ag	Antigene
Chl-a	Chlorophyll-a
CLIA	Chemioluminescence
CYL	Cylindrospermopsin
Da	Dalton
DIC	Dispersed Inorganic Carbon
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
FIA	Fluoro-immunoassay
GNP	Gold nanoparticles
HAB	Harmful Halgal Bloom
HA	Health Advisories
IA	Immunoassay
LC-HRMS	Liquid Chromatography High Resolution Mass Spectrometry
LFAA	Lateral Flow Aptamer Assay
LFIA	Lateral Flow Immuno-Assay
LIA	Liposome immunoassay
LOD	Limits of Detection
LOQ	Limits of Quantification
LPS	Lipopolysaccharides
MAA	Mycosporine amino acids
MC	Microcystin
MW	Molecular weight
NOD	Nodularin
NOAEL	No Observed Adverse Effect Level
OTA	Ochratoxin A
RIA	Radioimmunoassay
SDWA	Safe Drinking Water Act
STX	Saxitoxin
TDA	Total Daily Intake
UV	Ultra violet
VFDF	Very Fast Death Factor
WHO	World Health Organization
TRFIA	Time-resolved fluorescence immunoassay

Abstract

Natural toxins include a multitude of toxic secondary metabolites produced by animals, bacteria, fungi algae, and plants that are generally not intended as environmental contaminants of concern for water quality.
However, the presence of anthropogenic waste in the aquatic environment, both with the climate changes and the increase of temperatures, is creating favourable conditions for the development of undesired organisms able to produce natural toxins that sum with other well-known anthropogenic pollutants such as pesticides.
Among them, natural toxins produced by algae (cyanotoxins), fungi (mycotoxins), and plants (phytotoxins) are the most encountered in surface water environments. Some of them have various toxic effects on the human body. Carcinogenic, dermotoxic, and neurotoxic effects are generally associated with these compounds. Concern about the effects of cyanobacteria on human health has recently grown in many countries. Several human and livestock poisoning events, due to the contamination of water sources (especially lakes) attributed to toxic cyanobacterial bloom, resulted in a dramatic rise of interest in these toxins.
Despite that, their presence in surface water was not evenly regulated, and only the highly toxic microcystin-LR was limited at 1 µg/L by the Drinking Water Directive *(Consolidated text: Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption*) of the World Health Organization.
Additionally, studies on their occurrence, chemical persistence, buoyancy, and the algal blooms onset prediction in the environment are still scarce. The environmental risk assessment is difficult and the degree of toxicity into surface water ecosystems is still poorly studied. Their fate in the environment can be described by distribution coefficients such as the octanol-water partitioning (Kow). Other data able to define other distribution and partition parameters are generally obtained using *in-silico* prediction tools. The identification and quantification are still difficult due to the availability of certified standards.
Moreover, these compounds are found mixed with other groups of chemicals at low concentrations. Most of the published methods for their determination are specifically designed to identify a single compound or a group of toxins with similar characteristics, making stressful and time-consuming the process to identify other *known-unknown* compounds using non-targeted approaches.
Since the dangerousness of natural toxins in surface water was recently reported by different authors, valuable data were produced and published to assess their presence and concentration in surface water environments. Several analytical techniques have been reported for their qualitative and quantitative analysis, among them MALDI-TOF/MS, LC-ESI/HRMS, LC-ESI-QqQ are the most used. However, due to the thousands of compounds and their extreme heterogeneity, suspect screening has taken a central role as screening purposes.
Over the years, the suspect screening involved the use of in-silico approaches for the tentative identification of natural toxins, becoming one of the preferred methods for the suspect analysis of *known-unknown* compounds in surface water.

It is of primary importance to develop and optimize new methods able to identify natural toxins in a various range of polarities, ranging from the most hydrophobic to the highest water affine. The screening approaches are generally focused on cyanotoxins with no implementations regarding plant toxins and mycotoxins which are very poorly studied in surface water.

In this framework, this thesis has aimed to increase the knowledge about natural toxins in surface water. The first part of the study was focused on the recent advances in the detection of natural toxins retrieving a multitude of analytical approaches for their determination in surface and drinking water.

Finally, two suspect screening approaches using suspect and target analysis with data-dependent (DDA) and data-independent acquisition (DIA) methods exploring the performances for the determination of multitargeted compounds have been proposed. The tentative compounds have been firstly filtered using a suspect list of 2784 compounds retrieved by different databases available in the literature. The validated methods were then applied to analyse surface water samples coming from different sites in Europe.

The presence of natural toxins produced by plants, fungi, and cyanobacteria is a reality in different water environments showing their presence at low levels also in unexpected zones with not favorable conditions for their production.

Almost 80% of the natural toxins encountered were phytotoxins, while a small number of cyanotoxins and mycotoxins have been tentatively identified. The chemical diversity is generally driven by the botanical diversity and the anthropisation of the area. Besides, the seasonality and the precipitations are crucial parameters to understand the presence of phytotoxins respect to the cyanotoxins which have been encountered also in unexpected seasons. However, the quantification was not possible for all natural toxins, since only 32 standards were available in our study.

In conclusion, the work presented in this thesis, allowed to fill some of the gaps in the analysis of natural toxins presenting two approaches to increase the knowledge about the identification of natural toxins in surface waters. In the future, should be crucial to update the latest determination approaches with the newest quantification strategies to finally implement the suspect screening approaches and reach both determination and quantification of suspect compounds when standards are not available.

Resum

La presència de residus antropogènics al medi aquàtic, tant amb els canvis climàtics com a l'augment de les temperatures, crea condicions favorables per al desenvolupament d'organismes no desitjats capaços de produir toxines naturals que es combinen amb els altres contaminants antropogènics coneguts.
Les toxines naturals inclouen multitud de metabòlits secundaris tòxics produïts per animals, bacteris, algues fongs i plantes que no solen estar concebuts com a contaminants ambientals que preocupin la qualitat de l'aigua. Entre elles, les toxines naturals produïdes per algues (cianotoxines), fongs (micotoxines) i plantes (fitotoxines) són les més freqüents en entorns d'aigües superficials. Alguns d'ells expliquen diversos efectes tòxics contra el cos humà. Els efectes cancerígens, dermotòxics i neurotòxics s'associen generalment a aquests compostos. La preocupació pels efectes dels cianobacteris sobre la salut humana ha crescut en molts països en els darrers anys per diversos motius. Aquests inclouen casos d'intoxicacions atribuïdes a cianobacteris tòxics i la consciència de la contaminació de les fonts d'aigua (especialment els llacs) que es tradueix en un augment del creixement de la cianobacteria.
Malgrat això, la seva presència a les aigües superficials no estava regulada ni limitada de manera uniforme, i només la microcistina-LR altament tòxica estava limitada a 1 µg / L per la Directiva sobre aigua potable (Text consolidat: Directiva 98/83 / CE del Consell, de 3 de novembre de 1998 sobre la qualitat de l'aigua destinada al consum humà) de l'Organització Mundial de la Salut.
A més, els estudis sobre la seva aparició, persistència química, flotabilitat i predicció de l'aparició de les flors d'algues al medi ambient encara són escassos. L'avaluació del risc ambiental és difícil i el grau de toxicitat en els ecosistemes d'aigües superficials encara està poc estudiat.
La identificació i quantificació són difícils a causa de la manca d'estàndards certificats disponibles i el seu destí a l'entorn es pot descriure mitjançant coeficients de distribució com el repartiment octanol-aigua (K_{ow}). Altres dades capaces de definir altres paràmetres de distribució i partició s'obtenen generalment mitjançant eines de predicció in silico.
A més, aquests compostos es troben barrejats amb altres grups de productes químics a baixes concentracions. La majoria dels mètodes publicats per a la seva determinació estan dissenyats específicament per identificar un sol compost o un grup de toxines amb característiques similars, cosa que fa que el procés d'identificació de diferents compostos sigui estressant i requereixi temps mitjançant enfocaments específics.
Atès que s'ha determinat la perillositat de les toxines naturals a l'aigua superficial, es van produir i publicar dades valuoses per avaluar-ne la presència i la concentració. S'han informat de diverses tècniques analítiques per a la seva anàlisi quantitativa, entre les quals s'utilitzen MALDI-TOF/MS, LC-ESI/HRMS, LC-ESI-QqQ. No obstant això, a causa dels milers de compostos i de la seva extrema heterogeneïtat, el

“suspect screening” ha tingut un paper central com a propòsit de identificació de compostos desconeguts.
Al llarg dels anys, el “suspect screening” va implicar l’ús de metòdiques “*in-silico*” per a la identificació provisional de toxines naturals, convertint-se en un dels mètodes preferits per a l’anàlisi sospitós de compostos desconeguts en aigües superficials.
És de primera importància desenvolupar i optimitzar nous mètodes capaços d’identificar toxines naturals en diversos rangs de polaritats, des de les més hidròfobes fins a les més afines a l’aigua. Les proves es centren generalment en cianotoxines sense implementacions quant a toxines vegetals i les micotoxines que estan molt poc estudiades en aigües superficials.
En aquest marc, aquesta tesi té com a objectiu augmentar el coneixement sobre les toxines naturals de les aigües superficials. La primera part de l’estudi es va centrar en els recents avenços en la detecció de toxines naturals que recuperen multitud d’enfocaments analítics per a la seva determinació en aigua superficial i potable.
Finalment, s’han introduït dos enfocaments de detecció de sospitosos que utilitzen anàlisis de sospitosos i objectius amb mètodes d’adquisició dependents de dades (DDA) i d’adquisició independent de dades (DIA) que exploren el rendiment per a la determinació de compostos multi target. Els compostos provisionals s'han filtrat en primer lloc mitjançant una llista sospitosa de 2784 compostos recuperats per diferents bases de dades disponibles a la literatura. A continuació, es van aplicar els mètodes validats per analitzar mostres d’aigua superficial procedents de diferents llocs d’Europa.
La presència de toxines naturals produïdes per les plantes, els fongs i els cianobacteris és una realitat en diferents ambients aquàtics que mostren la seva presència a nivells baixos també en zones inesperades amb condicions no favorables per a la seva producció.
Gairebé el 80% de les toxines naturals trobades eren fitotoxines, mentre que només s’han identificat provisionalment poques cianotoxines i micotoxines. La diversitat química es basa generalment en la diversitat botànica i l’antropització de la zona. A més, l'estacionalitat i les precipitacions són paràmetres crucials per entendre la presència de fitotoxines respecte a les cianotoxines que s'han trobat també en estacions inesperades. No obstant això, la quantificació no va ser possible per a totes les toxines naturals, ja que només hi havia 32 estàndards disponibles al nostre estudi.
En conclusió, el treball presentat en aquesta tesi va permetre omplir alguns dels buits de l’anàlisi de toxines naturals presentant dos enfocaments per augmentar el coneixement sobre la identificació de toxines naturals en aigües superficials. En el futur, hauria de ser crucial actualitzar el darrer enfocament de determinació amb mètodes de quantificació més nous implementant les metòdiques analítiques fent possible la caracterització y la quantificació amb el mateix mètode.

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1 INTRODUCTION

1.1 Historical notes on natural toxins

The term poison has been used since very ancient times, and It describes a substance or a mix of substances with natural or anthropogenic origins, which can produce an adverse response in a biological system [1]. The term toxin is different, and it was introduced between 1870 and 1905 to describe infections and diseases that were due to microorganisms. Before that period, the occurrence of adverse conditions in the human body was believed to be produced by other sources. During the Franco-Prussian war, Edwin Klebs (1834-1913) undertook research on dead soldiers and discovered that many deaths were due to septicemia caused by organisms. He also stated that the fever was caused by the production of bacterial metabolites but at that time he did not name these compounds. The first researcher to understand the production and toxic effect of these metabolites was Richard Pfeiffer (1858-1945) in 1892 [2]. He observed that *Vibrio cholera* produced toxins in the culture, he also demonstrated the presence of proteins attached to the bacterial wall which exerted a very intense toxic effect. Thanks to this finding, he named this protein expression *"endotoxin"*.

A multitude of living organisms can produce toxic compounds, and poisonous plants have been used since ancient historical times for their properties. The Greeks were the first to discover a poison in hemlock plants that causes "an easy and painless end" to life. These plants are part of the *Umbelliferae* family, which is a very large and widespread group [3]. Other poisonous plants are still being used by indigenous populations to produce poisonous arrowheads. Curare is one of the most ancient alkaloids to be extracted from aconitum and this was extracted using water. Arrow tips were then dipped in the toxic solution and used against humans or animals [4].

The castor plant (*Ricinus communis* L., *Euphorbiaceae*.) is another invasive plant that has been known since very ancient times and it is used in the traditional medicine of eastern cultures. D'Errico et al. reported the presence of ricinoleic acid or ricin which is derived from castor beans and was discovered by archaeologists on a wooden stick that has been dated as 24000 old [5]. Moreover, the same castor bean oil was also used by Egyptians as a laxative, an abortifacient, and a remedy to treat various illnesses [6,7].

Furthermore, among the very extended list of organisms that can produce toxic compounds in water environments, cyanobacteria are considered to be the oldest creatures that have been recognised to be present on Earth for about 3.5 billion years. These organisms are photosynthetic prokaryotes that are living in aquatic and terrestrial environments which are spread throughout the entire world. To date, at least 2600 cyanobacterial species have been described, but many unknown species remain undiscovered [8]. Cyanobacteria have also been recognised as producers of secondary metabolites called cyanotoxins, which are harmful compounds with various dangerous effects [9].

Due to their ancient uses or toxicity, natural toxins have long been recognised and studied throughout human history. Thanks to this knowledge, it is possible to find a very large amount of information on toxins, especially regarding the presence of

natural toxins in food and feed. However, their presence in water environments, especially for phytotoxins and mycotoxins, remains poorly studied.

1.2 Overview of water resources and water contamination

Water is necessary to sustain life, but oceans and seas cover 70 % of the world's surface and comprise over 97 % of the total water around the world, which is not suitable for human consumption. The remaining 1 % is freshwater, which is considered suitable for human consumption after potabilization treatments [10]. This water is contained in aquifers, lakes, dam reservoirs, and rivers (**Figure 1**).

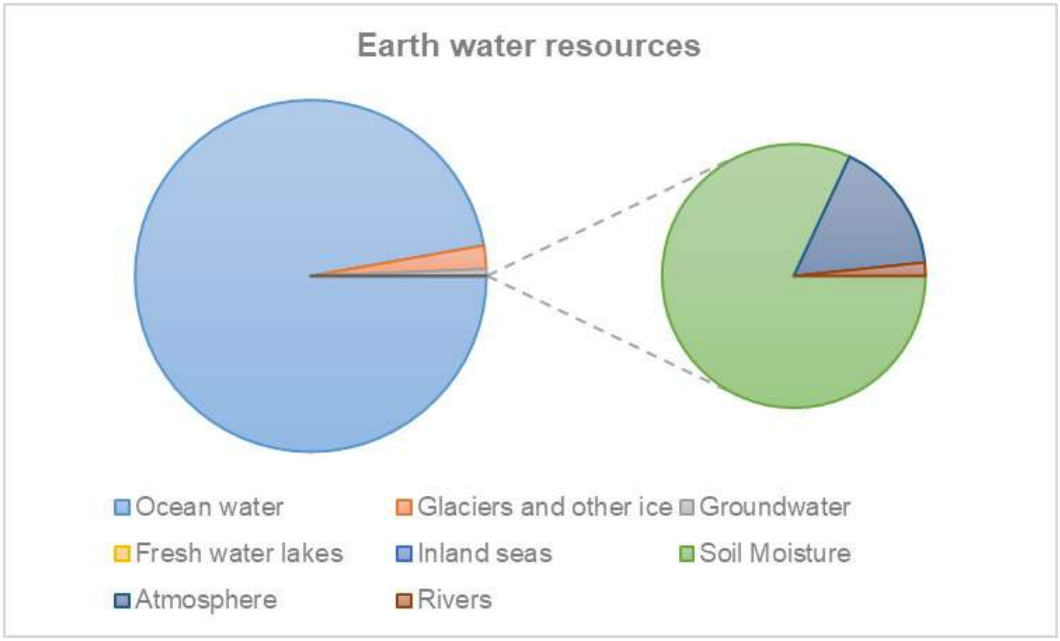


Figure 1: Total amount of water on Earth (Source: Nace, USGS, 1967 and The Hydrologic Cycle (Pamphlet), USGS, 1984).

The total quantity of freshwater has remained stable for centuries thanks to the constant recycling through the water cycle, but during the last 3 decades the world's population has increased dramatically. Each year is characterised by the search for a clean supply of water for drinking, cooking, bathing, and sustaining human activities. However, quantity of the water used for drinking and agricultural purposes is hardly increasing. Notwithstanding, human activities are worsening the environmental conditions regarding the water quality with various contamination sources that can be categorised as follows:

Physical contaminants affect the physical properties of water (sediment or organic material produced by soil erosion).

Chemical contaminants may occur after human activities or be due to natural phenomena (nitrogen, phosphorous, pesticides, heavy metals, natural toxins).

Biological contaminants that are produced by organisms. They are referred to as microbiological contaminants (pathogenic bacteria, protozoan, and parasites).

Radiological contaminants are considered to be radioactive particles (caesium, plutonium, and uranium) [11].

Various sources can affect the hydrological environment via direct and indirect contamination. Direct contamination can be traced to the source (for example, wastewater treatment plants). Indirect contamination is an unknown pollution source due to the geographical and anthropological factors in the area (for instance, agriculture, zootechnics, roads, etc.) [12].

Besides the extremely high number of contaminants that are produced by humans and released into water environments (pesticides [13], plastics and microplastics [14], litter and garbage [15], flame retardants [16], and many others, including heavy metals, organic matter, endocrine disruptors [17]), natural toxins represent a substantial part of the total amount of these dangerous compounds. Natural toxins are comprised of bioactive compounds from different kingdoms (plants, fungi, monera), and they are usually classified as mycotoxins (produced by fungi), bacterial toxins, phycotoxins (from algae), phytotoxins (from plants), and zootoxins (from animals). However, the term “natural toxins” can be ambiguous due to the very extended meaning which also includes the toxins produced by animals and insects [18], parasites [19], and pathogenic bacteria [20]. Among these natural toxins, only a few have been studied for their presence in water environments and most of them are produced by bacteria [21,22], cyanobacteria [23,24], plants [25,26], and fungi [27,28]. These groups are the most studied researchers have an increasing interest to elucidate the structure and the occurrence, that can potentially deplete the available potable water resources. Figure 2 shows a scheme of the natural toxin groups that are considered in this thesis and the potential adverse effects that are produced lead by their presence in contaminated water.

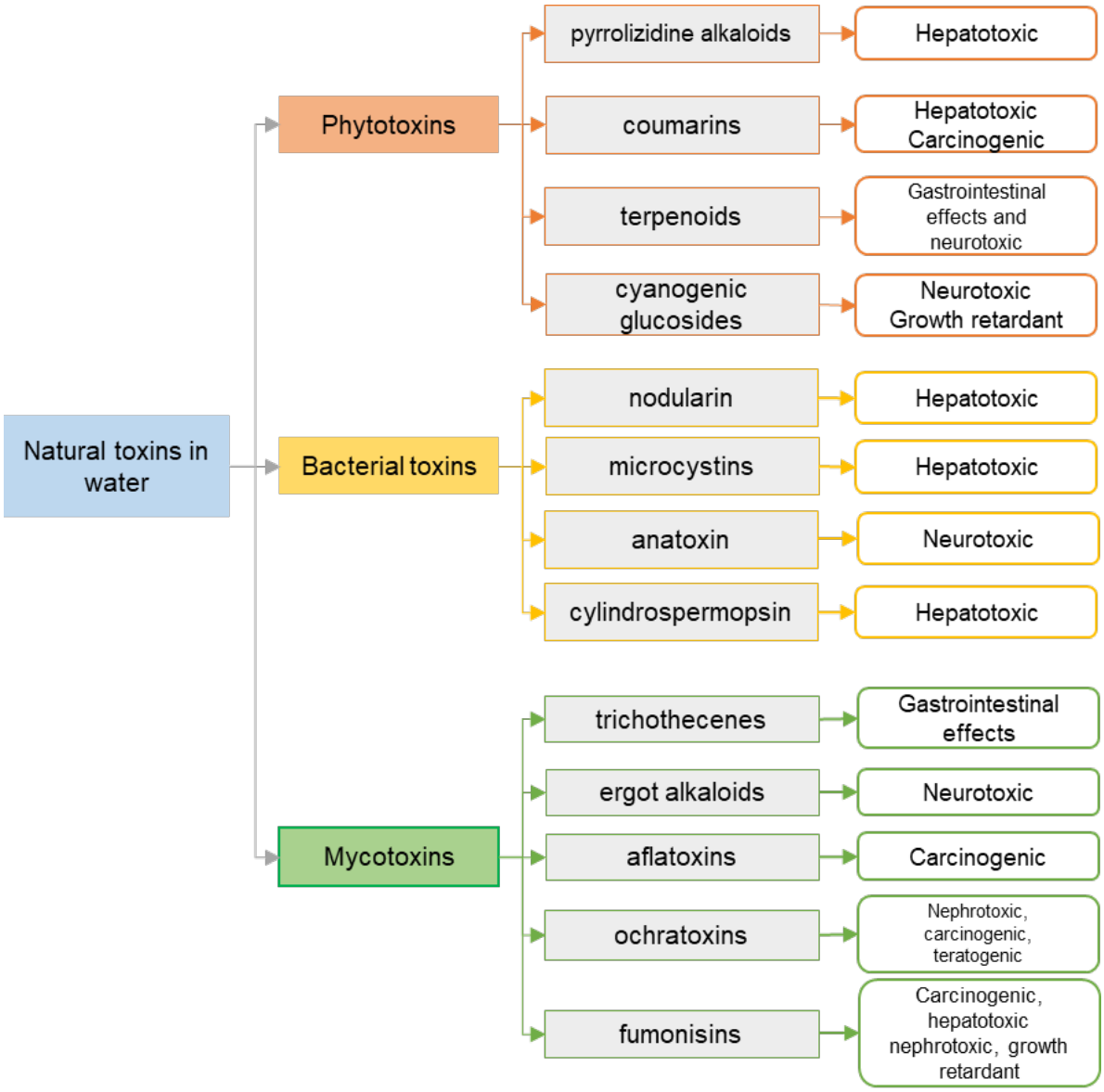


Figure 2: Most common groups of natural toxins in natural water

1.3 Bacterial toxins (cyanotoxins)

Bacterial toxins include very extensive lists of compounds that can be potentially found in water. They are produced by different organisms, and some of them are generally found in food (pathogenic bacteria) while others are mainly present in water (Cyanobacteria). Cyanobacteria have been recognised as producers of Harmful Algal Bloom (HAB). Also, because of the environmental anthropization and reinforcement due to climatic change, the HAB phenomenon is seriously increasing in temperate climates [29] and also in extreme climate regions [30,31].

The most common cyanotoxins in freshwater are produced by Cyano-HAB formed by planktonic species or mats of benthic cyanobacteria: *Microcystis* spp., *Cylindrospermopsis raciborskii, Planktothrix* (syn. *Oscillatoria*) *rubescens*, *Synechococcus* spp., *Planktothrix agardhii, Gloeotrichia* spp., *Anabaena* spp., *Lyngbya* spp., *Aphanizomenon* spp., *Nostoc* spp., some *Oscillatoria* spp., *Schizothrix* spp., *Nodularia, Raphidiopsis*, and *Synechocystis* spp [23,32]. From these species, different groups have been identified, such as Microcystins, Anatoxins,

Cylindrospermopsins, and Nodularins (**Figure 2**). Moreover, these cyanotoxins present a classification that is based on their adverse effects on human health, such as hepatotoxins, neurotoxin, and dermatoxins. Cyanobacterial hepatotoxins include microcystins (MCs) and nodularins (NODs). On the other hand, cylindrospermopsin (CYL) and anatoxins (ANAs) are cytotoxic and neurotoxic compounds [23]. These cyanotoxins are generally intracellular, and this means that the contamination occurs after the cellular lysis with their release into the environment [33].
In the following sections, the chemical-physical properties of cyanotoxins in water will be discussed.

1.3.1 Chemical-physical characteristics of cyanotoxins

Based on their chemical composition, cyanotoxins can be grouped into cyclic peptides (MCs and NODs), alkaloids (ANAs and CYL), and lipopolysaccharides.

Cyclic peptides

Cyclic peptides can present 7 or 5 amino acids that can vary between MCs and Nods congeners, respectively. The cyclic peptides are considered to be large natural products, that are defined by molecular weight (MW) in the range of 800 to 1100 Da, although this is small compared with many other cell oligopeptides and proteins with MW > 10000 Da.
MCs consist of a cyclic structure that is constituted by five non-protein amino acids and two protein amino acids. These two amino acids named Z and Y respectively, distinguish the different microcystins variants. The single-letter amino acid nomenclature is used to name each microcystin with a selected name depending on the amino acids [34]. **Figure 3** shows the structure of MCs with the Z and Y sites that change in the various congeners.

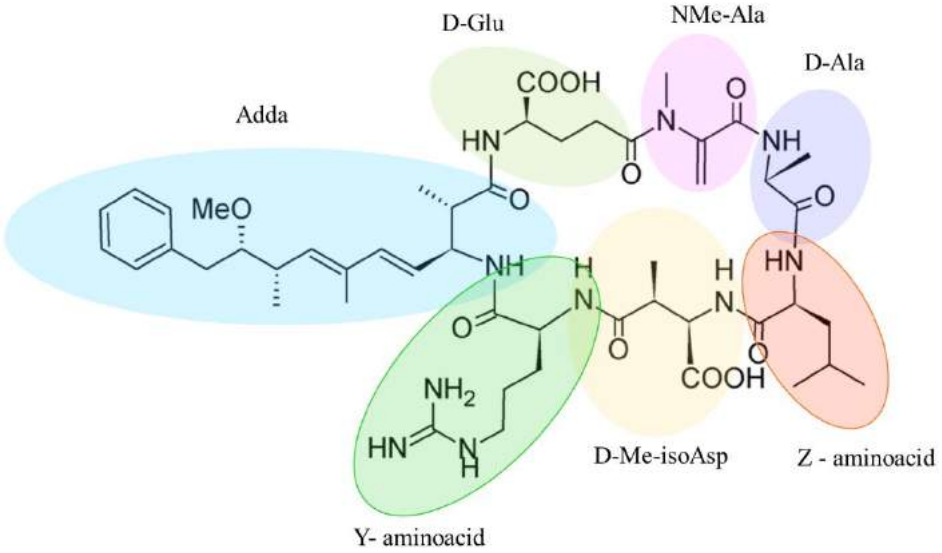


Figure 3: Structure of MCs reporting the 7 amino acids and the Z and Y sites.

The Y and Z sites can be substituted with various amino acids. Table 1 summarises the amino acid codes for one and three letters, taken from the Food and Agriculture Organization (FAO) of the United Nations [34]. The most common and toxic

microcystin-LR contains the amino acids Leucine (L) and Arginine (R) in these variable positions.

Table 1: Amino acidic codes from FAO varying in the MCs sites.

Amino acid	Three letter code	One letter code
alanine	ala	A
arginine	arg	R
asparagine	asn	N
aspartic acid	asp	D
asparagine or aspartic acid	asx	B
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glutamic acid	glx	Z
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S
threonine	thr	T
tryptophan	trp	W
tyrosine	tyr	Y
valine	val	V

Nodularins, instead, are exclusively produced by *Nodularia spumigena*. The general structure (**Figure 4**) is a cyclic pentapeptide presenting the general structure cyclo-(D-MeAsp-L-arginine-Adda-D-Glu-Mdhb), in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid.

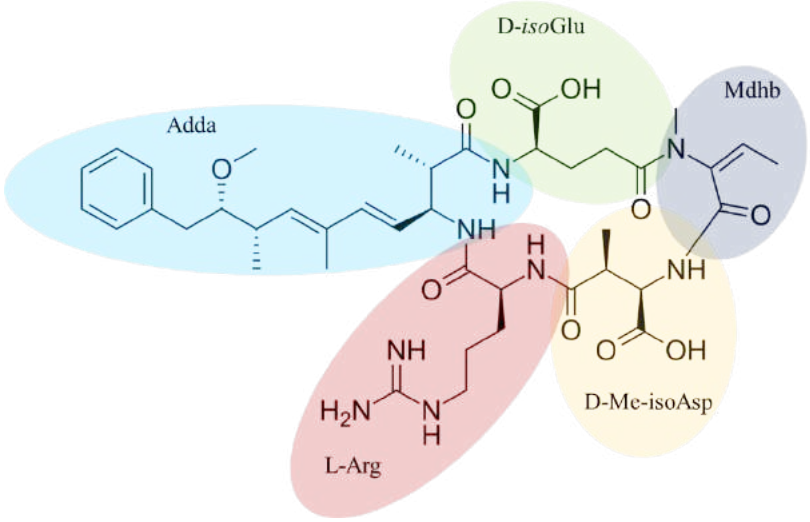


Figure 4: Nodularins structure with the typical pentacyclic configuration

In total, more than 80 microcystin variants have been identified and reported by the World Health Organization (WHO) [35], however, a total of 246 MCs variants and 10

NODs were collected and reported by Spoof and Arnaud [36]. Some modifications in the structure of other amino acids include the demethylation of Masp and Mdha and methyl-esterification of D-Glu sites.

Alkaloids

Alkaloids are a huge group of naturally occurring organic compounds that contain nitrogen atoms. Depending on the type and structure, the properties of different alkaloids can also vary. Most alkaloids have a complex cyclic structure. Six alkaloid cyanotoxins are introduced here, including CYNs, saxitoxins (STXs), ANA-a, anatoxin-a(s) (ANA-a(s)), lyngbyatoxins and aplysiatoxins. However, only ANA-a, ANA-a(s), and Cyl have been reported as being harmful contaminants in surface water. Neurotoxic ANAs are mainly isolated from cyanobacteria *Anabaena flos-aquae*. However, different studies have reported their production by *Anabaena, Aphanizomenon, Arthrospira, Cuspidothrix, Cylindrospermum, Dolichospermum, Oscillatoria*, and *Phormidium*.

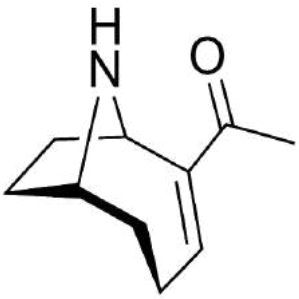


Figure 5: Anatoxin-a structure

ANA-a is a bicyclic amine alkaloid, containing 2-acetyl-9-aza-bicyclo(4-2-1)non-2-ene (Figure 5) [37]. It is also named as Very Fast Death Factor (VFDF) due to its acute neurotoxicity and the higher affinity of the cell receptors for ANA than for acetylcholine blocking post-synaptic depolarization [35]. At high concentrations, ANA can affect the nervous system, causing paralysis and finally death, however, it has low UV and pH stability under acidic conditions. Therefore, these compounds present a short half-life in the environment as a consequence of their fast degradation, resulting in less toxic products [37].

CYL has emerged as an important toxin in freshwater. It is an alkaloid exotoxin produced by several filamentous cyanobacteria, including *Aphanizomenon ovalisporum*, *Cylindrospermopsis raciborskii, Oscillatoria sp., Anabaena bergii, Umezkia nathans, Aphanizomenon flos-aquae* [38], Anabaena lapponica [39], and Lyngbya wollei [40]. To date, there are only three known naturally occurring CYL variants: CYL, 7-epi-cylindrospermopsin (7-epi-CYL), and 7-deoxy-cylindrospermopsin (7-deoxy-CYL). However, it is still not clear if these two compounds can be assumed to be precursors or degradation products [41]. Toxic effects have been observed in various organisms. The main target of CYL was seen to be the liver. Other organs such as kidney, thymus, intestinal tract, lungs, glands, and heart can potentially be affected. Notwithstanding, genotoxicity has been shown to be another toxicological effect from CYL ingestion [42].

Lipopolysaccharides

Lipopolysaccharides (LPSs), are glycolipids that are produced by Gram-negative bacteria and some cyanobacteria [43]. They are situated on the outer cell membrane. LPSs consist of long chains of sugar moieties (the polysaccharide part) that are covalently connected to lipids (**Figure 6**).

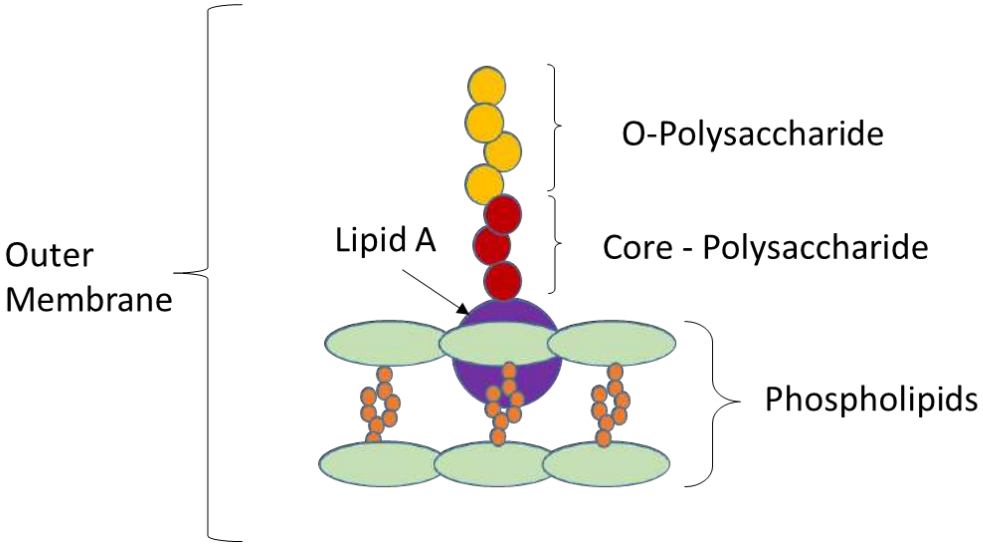


Figure 6: Lipopolysaccharide structure

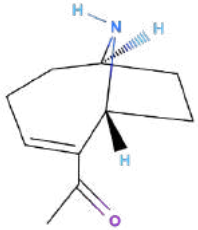
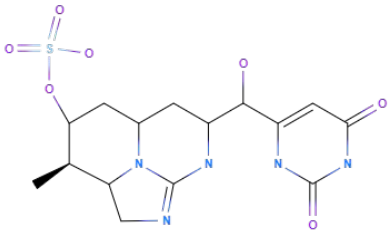
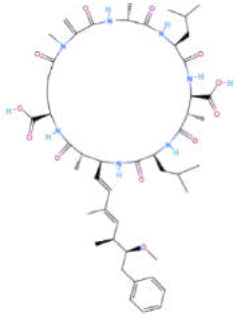
These linked chains form a dense network that acts as a shield to the outside membrane of bacteria, forming a gelatinous layer. LPS is produced due to enzymes, and its function is to maintain the direct outside of bacteria moist and slightly negatively charged and to shield off compounds that may damage the cell, while the layer is loose enough to let nutrients pass. The real physical border that separates the inside of a bacterial cell from the outside world is its membrane, which usually consists of three major portions: lipid A, core oligosaccharide, and O-antigen. The toxicity of LPS is mainly due to this lipid A. At the same time, the polysaccharides are less toxic. In Gram-negative bacteria, LPS is anchored to the outer membrane via lipid A. LPS can be released into the environment, while this layer is constantly renewed to maintain its integrity. Humans have evolved in the presence of bacteria, and since LPS covers the outside of many bacteria, all other living organisms have learned to deal with LPS. In the gut, intestinal phosphatase is produced, which detoxifies lipid A by the removal of phosphate. Some immune cell types of mammals have learned to recognise lipid A and can strongly react to its presence, thus causing an immune response [44]

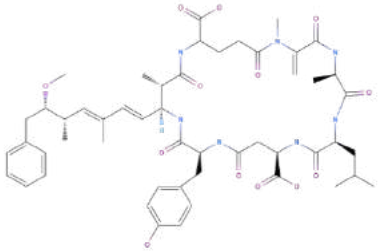
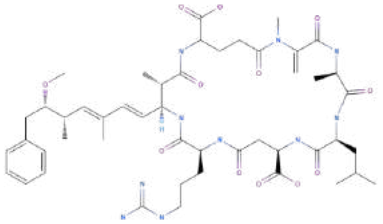
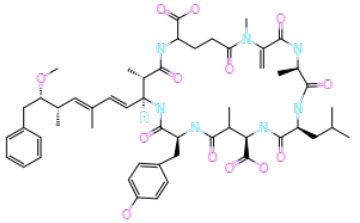
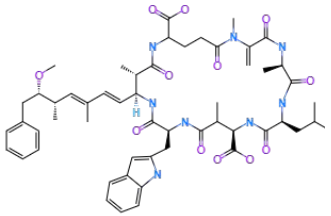
Endotoxins are heat-stable and acid/alkali-stable molecules, and their biological activities and inflammation responses survive even during extreme environmental conditions, such as changes in temperature and pH, and they are neither removed nor destroyed by distillation or sterilisation.

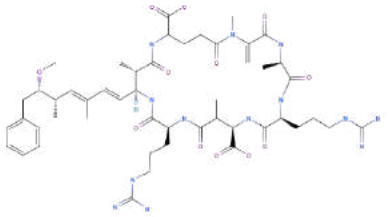
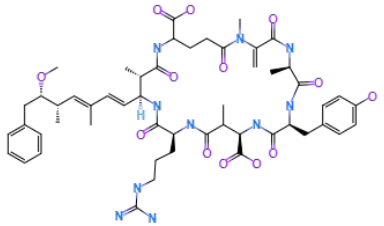
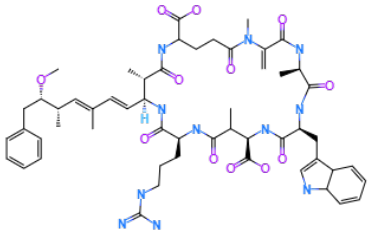
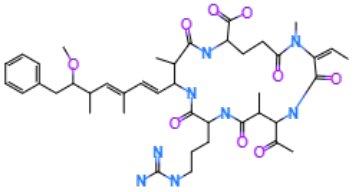
For these reasons, LPS can be easily found in the water environment. The existing forms of endotoxin in the water environment are intricate as endotoxins self-assemble into aggregates with many shapes, which are dependent on the quality of the water environment.

In **Table 2**, a list of the most important cyanotoxins that have been reported, with the producing bacteria, exact mass, and structural conditions for harmful cyanobacterial bloom.

Table 2: List of the most important cyanotoxins reported in surface water

Toxin name	Toxigenic genera	Molecular formula	Exact mass	$[M+H]^+$ *m/z*	Structure
Anatoxin-a	Aphanizomenon, Anabaena, Raphidiopsis, Oscillatoria, Planktothrix, Cylindrospermum	$C_{10}H_{15}NO$	165.1148	166.1226	
Cylindrospermopsin	Cylindrospermopsis Aphanizomenon, Anabaena, Raphidiopsis, Umezakia.	$C_{15}H_{21}N_5O_7S$	415.1162	416.1234	
MC-LL	Microcystis spp. Anabaena, Nostoc, Planktothrix, Anabaenopsis, Hapalosiphon	$C_{49}H_{73}N_7O_{12}$	951.5322	952.5390	

MC-LF	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{52}H_{71}N_7O_{12}$	985.5166	986.5233	
MC-LR	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{49}H_{73}N_{10}O_{12}$	993.5414	994.5482	
MC-LY	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{52}H_{71}N_7O_{13}$	1001.5109	1002.5182	
MC-LW	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{54}H_{71}N_8O_{12}$	1023.5196	1024.5264	

MC-RR	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{49}H_{75}N_{13}O_{12}$	1037.5663	1038.5731	
MC-YR	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{52}H_{72}N_{10}O_{13}$	1044.5285	1045.5353	
MC-WR	Microcystis spp. Anabaena, Nostoc Planktothrix, Anabaenopsis, Hapalosiphon	$C_{54}H_{73}N_{11}O_{12}$	1067.5445	1068.5513	
Nodularin	Nodularia spp, Nostoc	$C_{41}H_{60}N_8O_{10}$	824.4426	825.4505	

1.3.2. Conditions for harmful cyanobacterial bloom

Human activities and anthropisation of the environment are increasing the availability of nutrients that can be used by cyanobacteria for their growth. The eutrophication associated with the increased global human population has stimulated the occurrences of harmful algal blooms of cyanobacterial species, presenting an enhanced adaptation that permits them to use the nutrients which are dispersed in water, and light.

Phosphorus and nitrogen

Cyanobacteria contribute to the fixing of atmospheric carbon and nitrogen [45]. For this reason, inorganic nitrogen (N), together with phosphorus (P), temperature, light intensity, and water turbidity are important parameters in the regulation of cyanobacteria proliferation [46-48]. The affinity of many cyanobacteria for N and P is higher than for many other organisms that are present in the water environment. This means that they can out-compete other phytoplankton organisms under conditions of phosphorus or nitrogen limitation. Further, cyanobacteria can store enough phosphorus to carry out 2-4 phases of mitosis, leading to a huge increase in biomass. Finally, eukaryotic algae have the optimum N/P ratio of 32:1, while bloom-forming cyanobacteria have an N/P ratio of 16:1 [47].

Temperature

The rising temperature of water leads to cyanobacterial bloom development. Further, rising temperature may change various characteristics of water. The optimal temperature for the growth of cyanobacteria is 25 ºC. As an example, the increment in water temperature decreases the viscosity leading to a better dispersion of nutrients in the water column and promoting the buoyancy of the cyanobacterial cells [49,50]. These factors can lead to an advantage for cyanobacteria versus phytoplankton with a less efficient buoyancy system. Furthermore, the global climate is changing, with an observed increase in water temperature. In these conditions, it is also clear that cyanobacteria will be the first organisms to take advantage of a higher growth rate compared to other water organisms [48].

pH and Dispersed Inorganic Carbon (DIC)

The chemistry of aquatic environments can be seriously affected by the increase of CO_2. Freshwater pH is directly connected to the dissolved inorganic carbon such as carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), or carbonate (CO_2^{3-}), and the pH is generally between 7.5 and 8, whereby the inorganic carbon is mainly in the form of HCO_3^-. It has been shown that marine ecosystems show a better capacity to buffer possible changes in the pH, while freshwaters are more affected by CO_2 changes [48]. Also, the pH in freshwater can vary depending on the season, and even daily variations have been reported [51]. The capacity of cyanobacteria to grow in the higher water column closer to the atmospheric CO_2 can lead to a better diffusion and promote their multiplication. This can be explained by the ability of cyanobacteria to compete against eukaryotic algae under high pH and low CO_2 conditions [51]. Raven

et al. [52], reported another interesting mechanism for cyanobacterial supremacy. Eukaryotic algae and cyanobacteria have in their cells a carbon concentration mechanism (CCM). Further, cyanobacteria present a more efficient CCM than the other organisms in water, leading to an easier dominance with low CO_2 conditions.

UV irradiation

Light intensity is an important factor that affects the growth of cyanobacteria, being photosynthetic organisms that possess chlorophyll-a (Chl-a) and -b pigments. Depending on the light intensity and the species, the algae will be at maximum productivity [53]. The cyanobacterial photosynthetic mechanism is different from that of the eukaryotic algae. It is a fast-changing light absorption system that contains phycobiliproteins, which permits to absorb wide range of the light spectrum. Cyanobacteria are also equipped with a protection mechanism against excessive UV light using mycosporine amino acids (MAAs) and scytonemin, which help to absorb the excess UV light, thus allowing them to survive with a high level of irradiance [54].

Wind

Cyanobacteria, and consequently cyanotoxins, appear in water bodies that can permit their growth but they are also in clean waters, such as high-alpine waters near pristine catchments [47].

Cyanobacteria can be extremely competitive over other organisms thanks to their adaptation. During favorable environmental conditions, the Cyano-HAB is formed with a dense algal bloom that can present different distributions in the water column. Distribution can vary depending on the climatic conditions and the concentration of cyanobacterial cells in the HAB. Regarding other organisms, cyanobacterial cells possess an empty vesicle filled with air that allows the organism to regulate its buoyancy to reach the optimal conditions for multiplication. However, the climatic condition can also enhance the accumulation of Cyano-HAB in the water surface after a turbulent condition, by increasing the dimensions of the internal vesicle. This can lead to a massive bloom in the water surface, thus increasing the concentration of cyanotoxins in a small part of the water column. Similarly, windy conditions may also lead to moving the algal bloom along the water surface and reaching the beach or the coastal borders, thus causing very dense agglomerations and, consequently, very high concentrations of cyanotoxins [47]. However, this cyano-HAB can be easily removed or newly mixed by wave or wind movement. The dispersion can lead to disruption of the cell membrane, whereby cyanotoxins are released into the surrounding medium via secretory pathways, senescence, or lyses of the internal cell, causing massive contamination with very high concentrations of toxins creating a significant risk to public and environmental health.

Figure 7 reports a concise example of the possible distribution of cyano-HAB along the water surface.

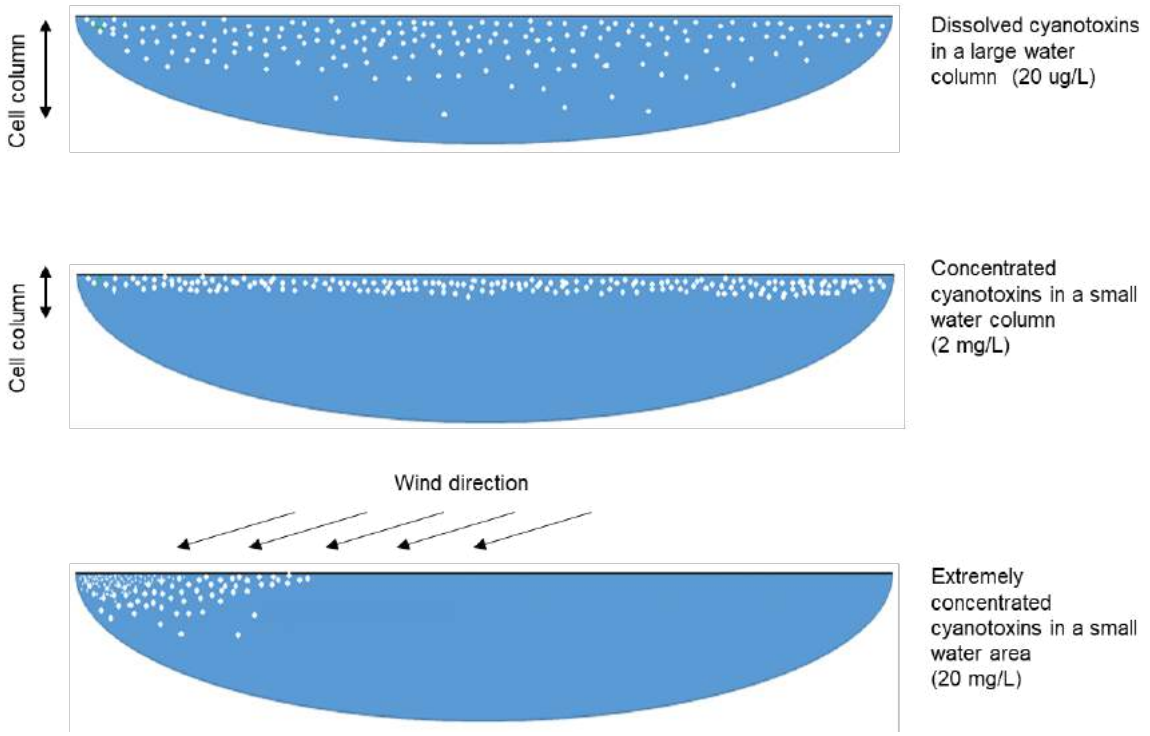


Figure 7: Accumulation of algal cells in the water column depending on the quality of the environment and the wind parameters

1.4 Regulation, guidelines, and health alert levels of cyanotoxins in drinking water

To protect human health, the World Health Organization (WHO) has considered the Microcystin-LR (MC-LR) that is suitable for proposing guideline values for drinking water. This resulted in a deep study for 13 weeks on the effects of using MC-LR in mice. In this study, hepatotoxicity was determined with a No Observed Adverse Effect Level (NOAEL) of 40 µg/kg BW/day and a Total Daily Intake (TDI) of 0.04 µg/kg BW/day. The resulting guideline value for total microcystin-LR (free plus endogenous) is 1 µg L^{-1} in drinking water [55].

Following this proposal, many countries around the world followed the WHO advice by setting regulations for a single class of cyanotoxins, while others are specific for microcystin-LR.

Table 3 reports the latest regulations around the world regarding the presence of MCs in surface and drinking water. In Europe, cyanotoxins in drinking and surface water have been mentioned in a proposal for a directive of the EU parliament on the quality of water intended for human consumption [69] and the EU Bathing Water Directive 2006/7/EC [70] with a limit of 1 µgL^{-1}. As a result, the Czech Republic, France, Finland, Italy, Portugal, and Spain have used this limit as a standard in their national regulation.

Several states in the USA have adopted their own regulations on drinking water safety while many directives were revised in response to the amendment of the Guidelines for Drinking Water Quality of the World Health Organization (WHO).

Australia, Brazil, Singapore, Uruguay, South Africa, and Canada set their maximum acceptable concentration at 1.5 µg L^{-1} for MC-LR. The United States approved the Safe Drinking Water Act (SDWA) to protect public health setting limits to be followed by public water suppliers. Some countries also set health advisory (HA) values for other cyanotoxins such as cylindrospermopsins (US-EPA; 0.7 µg/L for children and 3 µg/L for adults). However, several states in the USA, such as California, Florida, Ohio, Oregon, and Minnesota, do have various values that serve as guidelines or thresholds for certain management actions. Also, in June 2015 under the SDWA, the US Environmental Protection Agency (EPA) released the Drinking Water Health Advisories for microcystins and cylindrospermopsin.

Table 3: Regulations and proposals for cyanotoxins in surface and drinking water [56,57]

Country/source	Cyanotoxin	Limit
Australia	Microcystin	1.3 µg L^{-1} expressed as microcystin-LR toxicity equivalents
	Nodularin	40 000 cells mL^{-1} or a biovolume of Nodularia spumigena
	Saxitoxins	Health Alert of 3 µg L^{-1}
Argentina	Microcystin-LR	1 µg L^{-1}
Brazil	Cyanobacteria	10 000 - 20 000 cells/mL
	Microcystins	1 µg L^{-1}
	Cylindrospermopsin	15 µg L-1
	Saxitoxin	3 µg L-1 (STX equiv.)
California	Microcystins (LA, LR, RR, YR) for human	ND
	Microcystins (LA, LR, RR, YR) for cattle	0.9 µg L-1
	Cylindrospermopsin for cattle	5 µg L-1
Canada	Anatoxin-a for cattle	40 µg L^{-1}
	Microcystin-LR	1.5 µg L^{-1}
	Anatoxin-a	3.7 µg L^{-1}
Czech Republic	Cyanobacteria in raw water	2 000 cells/mL
	Microcystin-LR in treated water	1 µg L^{-1}
Denmark	It is not relevant to regulate for these toxins as they only use groundwater for drinking water.	-
France	Microcystins (sum of all variants)	1 µg L^{-1}
Finland	Potentially toxic Cyanobacteria in raw water	>5000 cells mL^{-1} or
	Microcystins (sum of all variants) in raw water	> 1 µg L^{-1}
	Microcystins (sum of all variants)in finished drinking-water	>10 µg L^{-1}
	Microcystins	1 µg L^{-1}
Florida	On this basis, where cyanobacteria do occur, the WHO rules can be applied for	

	microcystins. National guidance for substances with incomplete toxicological evidence proposes	
Germany	Drinking-water legislation includes "biological parameters" to be monitored by microscopy, e.g cyanobacteria	-
Hungary	Microcystin-LR (sum of all variants in MC-LR equivalent)	1 µg L^{-1}
Italy	Microcystins	1 µg L^{-1}
Netherlands	Microcystis	WHO guidance, < 4700 cells/mL;
	Microcystins	1 µg L^{-1}
New Zealand	Microcystins (as MC-LR equivalents)	1.3 µg L^{-1}
	Cylindrospermopsin	1 µg L^{-1}
	Saxitoxin	1 µg L^{-1}
	Anatoxin-a	6 µg L^{-1}
	Homoanatoxin-a	2 µg L^{-1}
	Nodularin	1 µg L^{-1}
Ohio	Microcystins	1 µg L^{-1}
	Anatoxin-a	20 µg L^{-1}
	Cylindrospermopsin	1 µg L^{-1}
	Saxitoxin	0.2 µg L^{-1}
Poland	Regulation based on European Directive 98/83/EC does not state MCs requirements. However, some surface water treatment plants monitor the microcystin occurrence using the WHO limit for microcystin-LR.	1 µg L^{-1}
Portugal	Microcystin-LR	1 µg L^{-1}
Spain	Microcystin-LR	1 µg L^{-1}
Turkey	Sum of all microcystins (expressed in MC-LR equivalents)	1 µg L^{-1}
Uruguay	Microcystin-LR	1 µg L^{-1}
Singapore	Microcystin-LR	1 µg L^{-1}
South Africa	Microcystin-LR	1 µg L^{-1}
United Kingdom	Regulation is unclear, water must not contain any micro-organism, parasite, or substance at a concentration or value that constitutes a potential hazard to humans.	-
US-EPA	Cylindrospermopsin	0.3 µg L^{-1} for children; 1.6 µg L^{-1} for children >6 years and adults
	Microcystins	0.7 µg L^{-1} for children; 3 µg $L^{-1}$for children >6 years and adults

1.5 Mycotoxins

Mycotoxins are secondary metabolites that are synthesized by a variety of fungi. These are low molecular organic compounds that are toxic to vertebrates and other animal groups in low concentrations. Twenty-five percent (25 %) of the world's food supply may be contaminated with mycotoxins and climate change is predicted to increase the contamination of human foods and animal feeds with mycotoxins. However, fungal compounds have been intensively studied in food and feed, but very few publications are available for their identification in surface and drinking water. The most important toxigenic fungal species are *Aspergillus, Fusarium,* and *Penicillium.* Their presence is generally dependant on abiotic conditions, water activity, substrate, and temperature.

1.5.1 Chemical structure and physic-chemical characteristics

Mycotoxins can be grouped according to their different chemical structures, properties, and toxicity. Primary groups are aflatoxins, fumonisins, trichothecenes. In **Figure 8,** the main structures of mycotoxins are shown.

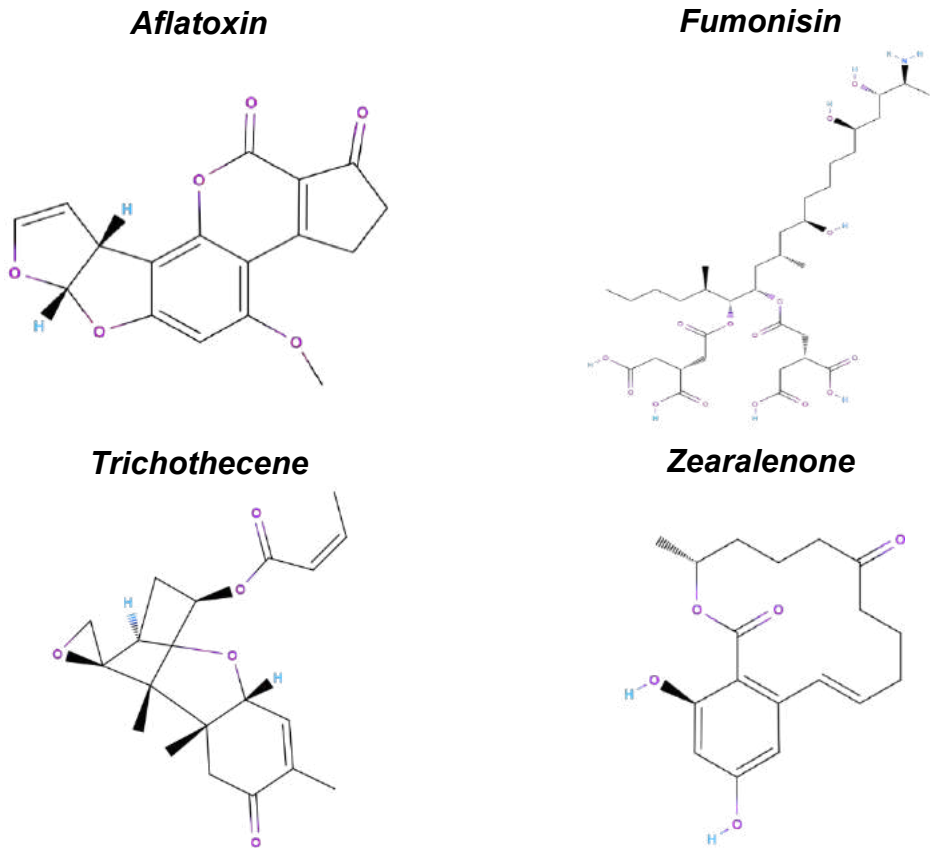


Figure 8: Mycotoxin structures

Aflatoxins (AFLs) are some of the most potent natural carcinogens from *A. flavus, A. parasiticus, A. nomius,* among others. They are difuranocoumarocyclopentenones and difuranocoumarolactones that originate the AFLs -B_1, -B_2, -M_1, -M_2, and the AFLs -G_1 and -G_2, respectively. B and G groups differ regarding their fluorescence under UV light, emitting blue light and green light, respectively. AFLs are stable to heat treatments and are considered an important human health threat due to their toxicity. AFLs are related to human diseases such as cancer, liver neoplasia, immunological suppression, and growth impairment.

Fumonisins are polyketides, being the largest class of fungal secondary metabolites. Since their discovery, the number of identified fumonisins has increased to more than a dozen compounds [58]. They contain 2 propane-1,2,3-tricarboxylic acid side chains which are esterified to an amino polyol backbone. Fumonisins are mainly produced by *Fusarium* species (*verticilloides, moniliforme, proliferatum, oxysporum, globosum*), among others [59]. Since they are structurally similar to sphingosine they inhibit sphinganine N-acetyltransferase, which makes fumonisins a potential human carcinogen [60].

Trichothecenes are represented by more than 200 compounds, formed by a tricyclic 12,13-epoxytrichothec-9-ene structure. Four groups (types A, B, C, and D) can be differentiated based on their substitutions. Hydroxy-trichothecenes form the type A group, while type B shows a keto group such as nivalenol and deoxynivalenol. As depicted in **Figure 8**, type C trichothecenes can be distinguished by the presence of an epoxy group, while type D trichothecenes have an additional ring in their structure. Trichothecenes are mainly produced by *Cephalosporium, Fusarium, Spicellum, Myrothecium, Stachybotrys, Trichoderma,* and *Trichothecium* [61].

Zearalenone is an estrogenic mycotoxin, and its structure is a macrocyclic β-resorcyclic acid lactone that is produced by a Fusarium species such as *F. culmorum, F. roseum, F.graminearum,* among others [62]. Toxicological properties have been reported to be related to their estrogenic activity as endocrine disruptors.

1.6 Plant toxins (phytotoxins)

Phytotoxins are bioactive compounds that are produced by plants via their metabolism as secondary products, which are used to defend themselves from predation. Their occurrence in the environment has been well described, but data regarding their occurrence and dangerous effects in drinking and freshwater are still missing. Phytotoxins can be emitted in the water environment, where they can contribute to increasing the potential exposure of humans to natural toxins.

A complete compilation of plant toxins and their producers has previously been reported by Quattrocchi et al. [63], including the description of toxic plants and their metabolites, but not their occurrence around the world. However, Bucheli et al. [64] presented some compendiums of natural toxins and toxic plants that are found in the body of literature, demonstrating that most toxic plants belong to the angiosperms with a range of 250000 - 400000 species.

Among the secondary plant metabolites that are used for plant metabolism, plant development, hormones, etc., some compounds are produced to defend the

organism against physicochemical and/or biological stress. Plant toxins are composed of several classes such as terpenes, polyketides, tannins, cyanogenic glucosides, coumarins, quinones, flavonoids, and pyrrolizidine alkaloids.
Many of them can produce adverse effects as pollutants on humans, animals, and other organisms, while others are used in the agricultural and pharmaceutical fields for their helpful effects.
Thanks to their properties, phytotoxins have also been considered as natural pesticides, and many of them are being artificially synthesized, being that they are no longer intended as natural compounds. Phytotoxins are also included in the EPA definition as “Biopesticides, also known as biological pesticides, that are pesticides derived from natural materials such as animals, plants, bacteria, and certain minerals” [65].
Plant toxins with pesticide potential can be placed under five categories: herbicides, insecticides, fungicides, nematocides, and rodenticides. ***Herbicides*** reduce the growth of plants and the production of other compounds, making them able to compete for supremacy, in a given area, against other plants. An example is cinmethylin, which is artificially converted into a commercial product “Toxaphenereg”, although is no longer used. Other examples are triketone herbicides, which were developed from leptospermone (*Leptospermum scoparium*), and sesquiterpenoid lactone artemisin (*Artemisia annua*), reported as a plant growth inhibitor. ***Insecticides*** have been historically obtained from plants. The most famous is the pyrethrins, with insecticidal properties, that are extracted from several *Chrysanthemum* species, Meliaceae, and Rutaceae. It is well known that ***Fungicides*** are produced by plants to protect themselves from microorganisms like fungi. For example, several flavonoids from soybeans and other fruits are used against fungi. ***Nematicides*** have also been obtained by starting from plant extracts, such as Tobacco (*Nicotiana tabacum*), clove (*Syzygium aromaticum*), betelvine (*Piper betle*), and sweet flag (*Acorus calamus*) extracts, which are the most effective in killing plant nematodes with an EC_{50} that is 5-10 times more effective than the EC_{50} of the synthetic pesticides chlorpyrifos, carbosulfan, and deltamethrin [66]. ***Rodenticide*** properties from Calendula, sumac, Damsissa, lemongrass, wormwood, Duranta, and camphor have also been investigated regarding *Rattus norvegicus* population reduction. In this study, calendula was the deadliest compound with a population reduction percentage of 67.7 %. The assessment of the phytochemical constituents of the same plant extract reported glycosides, flavonoids, saponins, tannins, triterpenes, and sterols that are responsible for the observed rodenticide effect [67].
Poisonous plant intoxications after the ingestion of aerial parts, fruits, and roots have been reported and they are associated with several constituents. Further, dietary supplements and traditional herbal medicine increased the probability of connecting with phytotoxins. Levels of toxic substances vary considerably in plants depending on genetic, genotypic, and ecotypic factors.
With these considerations, phytotoxins must be considered as pollutants that are of particular relevant to humans, with respect to their chemical parameters, environmental exposure, fate, and behaviour.

1.7 Analysis of natural toxins in freshwater environments.

Chemical analysis, which is intended for the screening and identification of natural toxic compounds in water bodies, presents several challenges that may be connected to the complexity and the intrinsic characteristics of each compound.
Analytical methods can be classified under the biochemical, chemical, and molecular approaches.

1.7.1 Immunochemical methods

Antibodies (Ab) are immunoglobulins which act as a critical part of the immune response by specifically recognising and binding particular antigens. They are a Y-shaped protein that is composed of heavy and light chains (**Figure 9**), with a specific part (*epitope*) that is the antigen's recognition and binding site. Its binding is reversible and based on electrostatic forces, Van Der Waals forces, and hydrophobic interactions following the law of mass action. Immunochemical methods or immunoassays (IAs), based on the binding properties of an Ab to an antigen (Ag), have been used for the development of a variety of analytical techniques that are applicable in fast analysis.
IAs offer many advantages over conventional methods because they can provide fast, simple and cost-effective detection, with sensitivity, in most cases, comparable to conventional techniques and requiring no or minimal volumes of solvent, with a minimal sample pre-treatment. Besides, more advanced formats can be designed to operate on-site in the field. The main limitations that are encountered with these techniques are sometimes poor thermal and chemical stability of reagents, cross-reactivity between structurally-related compounds, and possible matrix effects. Current development is, therefore, focused on the application of new materials to improve the stability and the specificity of immunoreagents [68]
IAs present various methodologies. ranging from radioimmunoassay (RIA), fluoro-immunoassay (FIA), chemiluminescence (CLIA), liposome immunoassay (LIA), and enzyme-linked immunosorbent assay (ELISA) [69], which is one of the most used approaches, following the most advanced approaches, such as immunosensors [70].

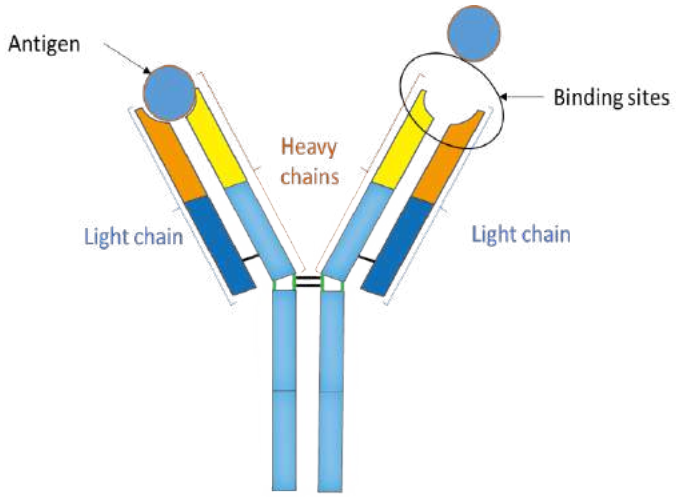


Figure 9: Structure of an antibody

The most common classes of Abs used for the development of IAs are the following:

- **Polyclonal antibodies** are a heterogeneous mix of Abs, derived from the immune response of multiple B-cells. Each one recognizes a different epitope on the same antigen. Because polyclonal antibodies are composed of a mixture of antibodies that represents the natural immune response to an antigen, they are prone to a higher risk of batch-to-batch variability than monoclonal antibodies.
- **Monoclonal antibodies** come from a single B-cell parent clone and, therefore, they only recognize a single epitope per antigen. These B-cells are immobilised by fusion with hybridoma cells, allowing for long-term generation of identical monoclonal antibodies. Because monoclonal antibodies specifically detect a particular epitope on the antigen, they are less likely than polyclonal antibodies to cross-react with other proteins.
- **Recombinant monoclonal antibodies** are developed in vitro using synthetic genes. The encoding sequences can be carefully controlled, allowing for optimised binding and improved reproducibility over monoclonal antibodies that are produced from a hybridoma.

During recent years, different immunoassays have been developed for the rapid determination of some groups of natural toxins, in particular for cyanotoxins in water and a few for mycotoxins.

IAs to determine Cyanotoxins; Due to their variability, the analysis of cyanobacterial toxins is quite demanding. MCs alone consist of more than 246 congeners while NOD congeners are 10 to date [36]. The common molecule present in both MCs and NODs is the ADDA fragment *[(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid]*. This fragment is currently used as an antigen-antibody reaction in methods for the detection of MCs and NODs.

Both MCs and NODs have been detected, using a non-competitive immunoassay, by Akter et al. [71]. The detection was based on the unique combination of an anti-immunocomplex and a monoclonal antibody which were affine to the ADDA part of the molecules. With this method, 11 MCs (MC-LR, -dmLR, -RR, -dmRR, -LA, -LY, -LF, -LW, -YR, -WR, and Nod-R) have been detected in ranges between 0.026 and 0.1 µg L^{-1}.

The identification of different congeners is challenging. Variations in the MC structure is common and generally occurring in the Adda position. The most common of these occur at C-9 [72], with substitution of the methoxy group with a hydroxy or acetyloxy group [73]. Here, an IA method to determine four 9-*O*-desmethylAdda (DMAdda) and 9-*O*-acetylDMAdda (ADMAdda) [ADMAdda^5]MC variants was developed. Finally, this method was applied to determine these poorly studied variants [74]

Similarly, MC variants have been detected using chemical engineering. MCs and NODs were conjugated to carrier proteins using a one-step active ester method and multistep thiolene click chemistry and glutaraldehyde method. The resultant antibodies showed good affinity in a range of $0.23 \leq IC_{50} \leq 0.68$ ng mL^{-1}. Conditions have been further optimised and, one indirect competitive enzyme-linked immunosorbent assay was developed based on mAb for the detection of MC-LR and NOD, with limits of detection of 0.16 and 0.10 µg L^{-1} and recovery of 62-86 %, with a coefficient of variation below 12.6 % in water samples [75]. A monoclonal antibody specific for cylindrospermopsin was produced and characterised as a direct competitive time-resolved fluorescence immunoassay (TRFIA) [76]. The detection limit was 0.02 µg L^{-1}, below the WHO guideline value of 1 µg L^{-1}. Also, a comparison between TRFIA and the current ELISA kit lead to find a good correlation between the two methods, showing that the novel immunoassay was reliable for the detection of CYN in water and algal samples.

For the determination of anatoxin in water, an immunoassay based on fluorescent polarization [77] was developed. A receptor for acetylcholine and nicotine was labeled with a fluorescein derivative. Anatoxin is a neurotoxin that can affect the polarisation of acetyl-cholinergic receptors. The data showed a direct relationship between the concentration of a sample and the polarisation degree of the light, which indicates the specific interaction between the two molecules.

IA for the determination of plant toxins: Due to the extremely high number of secondary metabolites produced by plants, few immunoassays have been developed for their detection in water.

However, an electrochemical immunosensor was developed for the determination of 7-hydroxycoumarin [78]. This approach employed horseradish peroxidase-labelled anti-7-hydroxycoumarin, with the enzyme-catalyzed reaction involving the reduction of hydrogen peroxide in the presence of a mediator compound (hydroquinone). The 7-hydroxycoumarin antigen that was bonded to a film of carbon surface was immobilised with a protein layer. The competitive assay involved the substitution of the labelled anti-7-hydroxycoumarin with the free 7-hydroxycoumarin. This increased

the voltage, which was inversely proportional to the concentration of the analyte, resulting in a detection limit of 24 μM.
Then, an immunoassay based on magnetic nanoparticles coated with silica was developed for ricin toxin [79]. The scope was to combine the catalytic properties of gold nanoparticles with the separation properties of silica-coated nanoparticles for ricin electrical detection. Compared with conventional colorimetric ELISA using similar antibodies, an array of microelectrodes-based approach was more sensitive and rapid for the electrical detection of ricin. In this approach, the nanoparticles were labelled with a specific ricin antibody to capture free ricin A, and gold nanoparticles labelled with anti-ricin B antibody were used as detectors. The electrical signal resulted after the binding of the silver nanoparticles-antibody-gold nanoparticles that enhanced the electric signal after deposition on a microelectrode array (MEA).
Water samples have also been screened to determine ricin through the A-chain, using commercial ELISA kit, thus obtaining detection limits of 3 μg L^{-1} but with a long analytical time. A less time-demanding analysis involved the use of an optic fibre biosensor, thus obtaining higher detection limits (10 and 60 μg L^{-1}) [80].

IAs for the analysis of Mycotoxins; A very high number of immunoassays have been proposed for the detection of dangerous mycotoxins in food and feed. However, regarding the water environment, few methods employing immunoassays have been reported.
The latest approaches to determine mycotoxins in water include the use of portable smartphone-based imaging systems. The use of gold nanoparticles (GNPs) for the detection of $AflB_1$ by monitoring the visible colour change of the strip from red to purple-blue, has a lower detection limit of 2 ng mL^{-1} in 96-well plates. The strips were analysed using a portable smartphone strip reader, achieving a detection limit of 0.3 μg kg^{-1} [81].
Yan et al. optimised a GNP-based multiple Lateral Flow Immuno-Assay (LFIA) in samples of drinking water [82]. Differently coloured GNPs were prepared to attach antibodies selective for $AflB_1$, to obtain dual qualitative LFIA devices. Thus, the test lines present different colours depending on which analytes and their number are present. Finally, the combination of EDTA pre-treatment and the use of a smaller strip allowed to achieve a visual LOD of 0.5 ppb of $AflB_1$ in water samples.
Similarly, a Lateral Flow Aptamer Assay (LFAA) integrated into a smartphone-based portable device for highly sensitive and precise detection of multiple targets, using aptamers functionalised the use of multi-coloured conversion nanoparticles as probes. With this approach, it was possible to determine small molecules, ions, and bacteria. However, for this thesis, the most important aspect was the possibility to analyse the ochratoxin A (OTA). By using the competitive format, OTA can be determined from the colour intensity of the corresponding coloured band in a range of 0.01–50 μg mL^{-1} and LOD of 3 ng mL^{-1} in water samples [83].

1.7.2 Biosensors

A biosensor is an analytical device composed of a biological recognition element (enzymes, antibodies, aptamers, whole cells, nucleic acids, among others), immobilised in intimate contact with a physic-chemical transducer. In this manner, a biological signal is converted to a primary signal (electrical, optical, thermal, etc.) that can be filtered, amplified, quantified, sent, and stored (**Figure 10**).

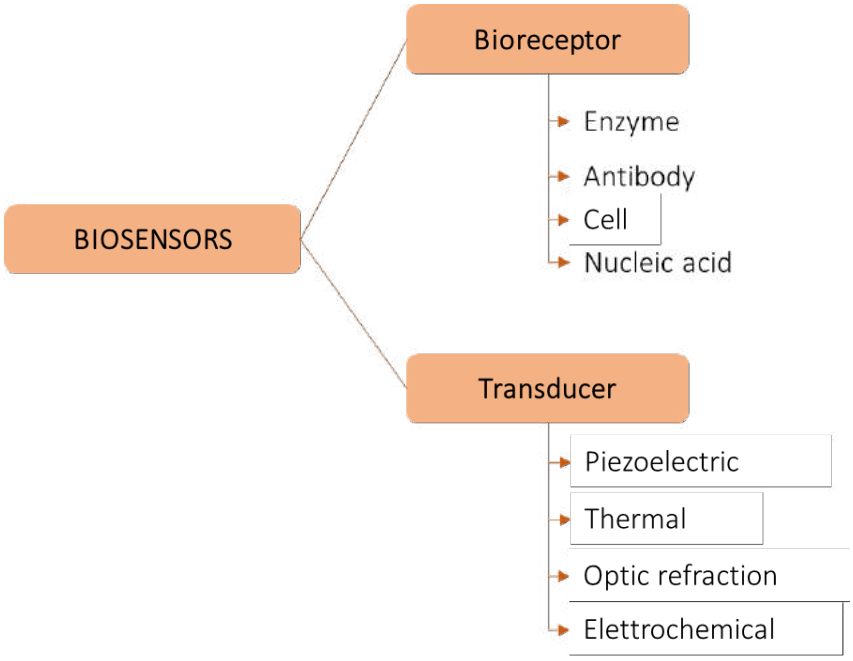


Figure 10: General classification of biosensors

A general scheme is shown in **Figure 11**.

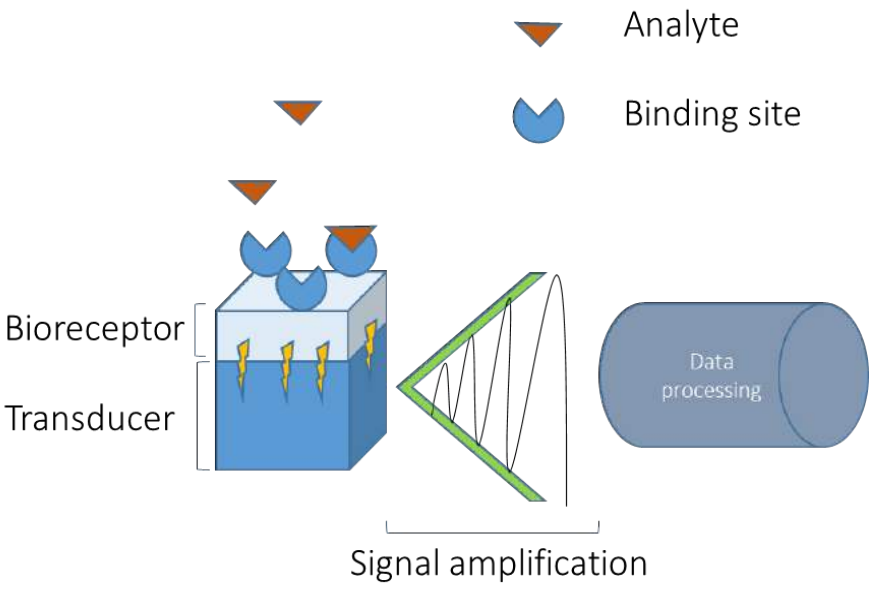


Figure 11: Scheme of a biosensor

The main advantages of current biosensor devices are the rapid and cost-effective responses that can be miniaturised and used on-site, even in unmanned configurations. The potential to assess marine biotoxins on site with remote devices has been exploited during recent years, in particular for seawater or in surface waters of lakes [84]. However, there are some limitations, such as the lack of stability of some biological elements, sometimes some cross-reactivity between structurally related compounds, and under some configurations, matrix effects can lead to over or underestimation.

One of the biosensors developed for the analysis of natural toxins is the immunosensor. In this case, both sensitivity and specificity are directly proportional to the immobilised antibody. For example, a portable sensing system based on electrochemical impedance spectroscopy (EIS), for the simultaneous quantification of free and total microcystin-LR in freshwaters was recently presented by Dos Santos et al. [85].

The performance of the immunosensors was evaluated by electrochemical impedance spectroscopy, showing a linear dynamic range between 3.3×10^{-4} and 10−7 g L^{-1} and a limit of detection of 5.7 × 10−10 g L^{-1}. The results demonstrate its suitable applicability for the analysis of MC-LR at regulated levels for drinking water. In addition, this is the first described system that can differentiate between intracellular and extracellular concentrations of MC-LR. This novel electrochemical sensing platform avoids the multiple processing steps, that are typically needed for standard MC-LR analysis in the laboratory, and provides an early warning system for MC-LR remote monitoring in water. In another example, an electrochemical immunosensor was proposed by Lu and Gunasekaran for the detection of mycotoxins, fumonisin B_1, and deoxynivalenol [86]. In this approach, an electrode with siloxane channels was immobilised on a glass coated with indium-tin-oxide. The electrode was modified with their correspondent antibodies and gold nanoparticles. When forming a complex, a concentration-dependent signal response was produced. Detection of 97 and 35 ng L^{-1} was achieved for fumonisin B_1 and deoxynivalenol, respectively.

Another promising group of biosensors for natural toxins is based on nucleic acids and aptasensors. Among them, recently, Chinnappan et al. developed a biosensor, based on graphene oxide, that was used as the fluorescence-sensing platform for probing the high affinity of the short aptamer that was derived from the wild-type long aptamer-CYN sensing. The platform construction involved two steps: firstly, quenching the fluorescence of fluorescent-labelled truncated aptamer using graphene oxide as a quencher and, secondly, fluorescence recovery in the presence of CYN by competitive binding between the target and the graphene oxide. The sensor specifically detects CYN among other potential interfering toxins. The performance of the sensor was validated using CYN-spiked tap water and showing excellent recovery rates [87]. In another recent example, fluorescence and surface-enhanced Raman spectroscopic dual-modal aptasensor for the detection of cyanotoxins were developed [88].

DNA aptamers were also employed for the fabrication of a label-free electrochemical biosensor to determine okadaic acid (OA), a lipophilic marine biotoxin that accumulates in shellfish [89]. OA was detected in water samples using the aptamer immobilised on an electrode. Its binding induced the alteration of the aptamer conformation causing a decrease in the electron-transfer, that was monitored by electrochemical impedance spectroscopy (EIS). A detection limit of 70 ng L^{-1} was obtained.
Other biosensors have been based on other types of recognition, for example, the potential of plant toxin ricin to bind cell-surface oligosaccharides [90]. Sugar-probes having lipoic acids as anchor functions were immobilised on the sensor. The following surface plasma resonance analysis reached detection limits of 10 ng L^{-1}.

1.7.3 Chemical methods

The chemical methods used for the determination of natural toxins in waters are mainly based on High-Performance Liquid Chromatography (HPLC) combined with ultraviolet (UV), fluorescence (FLD), or mass spectrometry (MS) detection. UV-based techniques are non-specific, due to the presence of similar compounds with similar absorbance, making it impossible to characterise structures and isomers. For this reason, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using quadrupole mass analysers, (LC-MS/MS), Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight (MALDI-TOF) MS, and liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS), either using TOF or Orbitrap mass analysers, attaining high sensitivity and selectivity for the unequivocal identification and quantification, are among the most used methodologies for the determination of natural toxins in water samples.
When working with instrumental chemical methods, the matrix effect is the major concern that can be resolved in a decrease or increase of the signal because of the components of the sample. The complexity of the matrix has always been a challenge to achieve low interferences and detection limits to determine compounds at trace levels. Therefore, sample pre-treatments to isolate, concentrate, and purify the analytes are required.
The analytical procedure follows the same steps starting from the sample treatment to the separation, detection, and finally the interpretation of the result. Here, a resume of the most important steps with previous approaches for the determination of natural toxins using instrumental chemical methods is described.

- **Analyte diversity:** When faced with the analysis of natural toxins, we need to consider several parameters to create a compromise between sample preparation, chromatography, and detection. Some compounds are hydrophobic, while others are easier to dissolve in aqueous solvents, some toxins can be separated with C_{18} columns while others show a better resolution with hydrophilic-lipophilic columns (HILIC), and finally, some toxins are better ionised in positive (+) rather than in negative (-) mode, or vice versa. Therefore, the complexity of the different natural toxins includes thousands of variants and isomers of the same compound, which are

difficult to identify since standards are limited and frequently they are extremely expensive. For instance, more than 85 variants of microcystins have been discovered having similar retention times, fragmentation behaviour with similar parent ions coming from the same structure, making them difficult to be characterised.

- **Matrix diversity:** water has a complex structure. Depending on the type of water (drinking or surface water) it is possible to face problems connected to the presence of chlorine, salts, disinfectants, humic acids, organic particles, and other organic interferents that need to be removed or, at least, reduced. Due to the presence of the organic matrix, it is necessary to perform an extra step to clean the sample, to avoid matrix interferences or matrix effects. The most common matrix effect is the ion suppression or enhancement that is frequently experienced in liquid chromatography-mass spectrometry (LC-MS) techniques when employing electrospray as an ionization source (ESI). Ions suppression/enhancement generally occurs when the ionisation is affected by the presence of other interferent compounds in the ionisation source, causing a decrease or increase in the signal detected by the detector. Sample dilution or reducing the volume of sample injected may give a reduction of ion suppression by reducing the quantity of interfering species present, although the number of analytes of interest will also be reduced, making this an undesirable approach for trace analysis. However, it is not always possible to decrease the ion suppression by sample preparation. When this happens, it is recommendable to compensate for the effects of ion suppression on accuracy and precision using calibration strategies (standard addition and matrix-matched calibration).

- **Sampling, preservation, and degradation during storage:** Sampling is one of the most important steps when setting a screening method. Due to the degradation of some compounds that may occur in a short time, it is important to control the chemical parameter of samples (temperature, conductivity, pH, salts). Moreover, the sample container can also adsorb some polar or non-polar analytes depending on its material, such as glass or plastic.
Surface water samples are commonly manually collected at 0.3 and 1 m depth using inert amber glass containers, in order to not deplete the analytes dissolved in the water due to the adsorption by plastic bottles. Finally, storage is the following step until analysis, which consists of freezing water samples at -40 ºC.

1.7.3.1 Sample treatment

Because of the matrix, an extraction and clean-up step is generally required before each analysis [91]. Sample pre-treatments allow us to extract the analytes from the water matrix to obtain an enrichment and at the same time, reduce possible interferences. Liquid-liquid extraction (LLE), Solid-Liquid Extraction (SLE) and Solid Phase Extraction (SPE), QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methods, Solid Phase Microextraction (SPME) and other Dispersive Liquid-Liquid Micro Extraction techniques (DLLME) are frequently employed [92-97]. Several works can be found in the body of literature employing the different methodologies that are commented on for the determination of natural toxins, including the most frequent sample treatments. However, their applications for the identification and quantification in water environments are still limited. Some of them will be commented on next.

DLLME consists of a mixture of three solvents, that are not miscible between them, to extract analytes from aqueous samples [98]. It works by injecting into an aqueous medium a volume of an organic and dispersive phase to create an emulsion. This allows us to separate the analytes and collect them after phase separation. This approach has been used in the analysis of water samples for the detection of MC-LR, MC-RR, NOD, okadaic acid, and domoic acid [99] achieving good recoveries between 48 % and 118 %. As well as for MCs, DLLME was also optimised for the determination of Fusarium mycotoxins enniatins (ENs) and beauvericin (BEA) in different types of water [100]. Mycotoxins were efficiently extracted from water into carbon tetrachloride by the DLLME technique using acetonitrile as a disperser solvent. Acceptable recoveries were obtained in a range of 78.5 and 100.1 % with relative standard deviations of <14 %. The method was successfully applied for the analysis of surface, ground, tap, and bottled water. Similarly, zearalenone, zearalenone, α-zearalanol, β-zearalanol, α-zearalenol, and β-zearalenol have been determined in environmental water samples using DLLME-LC–ESI–MS/MS [101], with recoveries ranging from 81 to 118 %, demonstrating the suitability of this extraction method for both cyanotoxins and mycotoxins. No other applications regarding the use of DLLME for the identification of plant toxins in water have been found.

LLE is like DLLME and it is based on the partition coefficient of a binary aqueous and organic solvent mixture which is not miscible, and where the analytes can be partitioned in the organic solvent. Further, to increase the partition of the analytes in the organic phase (salting out), these approaches are generally coupled with the addition of salts in the aqueous sample. LLE approaches are not generally eco-friendly, involving the use of high volumes of solvents. However, some studies reported the use of LLE for the determination of several cyanotoxins in highly saline water samples [102] obtaining LODs ranging from 1.0 and 0.02 to 3.4 μg L^{-1} with recoveries higher than 77.0 %. Also, resorcinol and other phenolic compounds have

been extracted from water using the ionic liquid, 1-ethyl-3-methylimidazolium bis(fluorsulfonyl)imide [103].

SPE is the most used sample preparation technique for natural toxins. Almost all analytical methods that involve a chromatographic separation and MS detection include a clean-up and concentration step. Among the most used SPE cartridges for multiclass analysis, immunoaffinity columns (IAC), polar and non-polar sorbent phases, ion exchange columns, graphitised carbon (GCarb), and hydrophilic-lipophilic balance (HLB) are the most used. However, depending on the optimal conditions (pH, T, salts, organic matter) they can be more or less selective with broad recovery ranges. The most common clean-up step for the determination of cyanotoxins is the HLB sorbent, which has been used for most of the latest analytical methods. An on-line SPE-UHPLC-HRMS was developed and validated for the screening of cylindrospermopsin, anatoxin, homoanatoxin-a, anabaenopeptins A and B, and twelve MCs (-RR, [Asp^3]-RR,-YR,-HtyR,-LR, [Asp^3]-LR,-HilR,-WR,-LA,-LY,-LW and-LF) in recreational lake waters. Hypersil Gold C_{18} was used to achieve enrichment, resulting in good extraction and separation efficiency with limits of detection between 8 and 53 ng L^{-1} [104]. SPE has also been reported to be suitable for retaining a wide range of MCs variants. Yilmaz et al. [105] described a method to isolate more than 36 microcystin variants of *Microcystis aeruginosa* strains in a Turkish lake. Further, unreported MC-(H_2)YR, [epoxyAdda5]MC-LR, [DMAdda5]MC-RR, and [Mser7]MC-RR were also detected. Their isolation was achieved using a polymeric strata-X solid-phase extraction cartridge with recovery close to 100 %. However, the most used SPE sorbent for environmental purposes is the HLB, which was employed in a wide number of applications. For example, cyanotoxins clean-up in water has often been performed with Oasis HLB [106]. Among the latest methods, Filatova et al. [107] described the use of a triple-stage solid phase extraction procedure (HLB to isolate different cyanotoxins, MC-LR, MC-RR, MC-YR, MC-LA, MC-LY, MC-LW, MC-LF) with recoveries between 9 and 87 % in surface water [107].

1.7.3.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

Among the analytical approaches that are described in the literature, LC is the most reported, duue to its versatility as a separation method. LC is generally coupled with UV or DAD detectors which allows a fast qualitative/quantitative analysis of most known compounds. However, characterisation and identification of the newest natural toxins, especially when standards are not available, are required. Recent developments in such new analytical instrumentation have coupled separation methods with HRMS, which are now allowing an added outcome regarding structure characterisation of unknown and known compounds. HRMS permit resolutions up to 140,000, reaching high accurate mass measurements that permit to introduce the concept of “exact mass”. HRMS permits the discrimination of two compounds with isobaric masses, having an accuracy of 5 ppm, which is of extreme importance when structural characterization is needed.

Some of the latest approaches that are reported in the literature for the determination of natural toxins analysis are summarised in Table 6. Most of them are based on LC coupled with HRMS in the tandem mode, which allows them to reach very low detection limits with reliable identification of toxins structures. The most used mass spectrometers range from the low-resolution mass spectrometers such as triple quadrupole (QqQ) and ion trap (ITMS) to the high-resolution mass spectrometers, such as Time-of-Flight (TOF) and Orbitrap analysers. Their applications are aimed at discovering new natural toxins structures, that have been possible thanks to the very high resolution and the low trace detection limits that these instruments can reach. Low ng L^{-1} levels have frequently been reported for natural toxins in water environments. As reported in **Table 4**, MCs are the most detected natural toxins in water. Their detection was reported mostly with TOF and QExactive Orbitrap while minor publications were aimed to detect mycotoxins and plant toxins in water.

Here, the latest analytical techniques which were extracted from the body of literature on the determination of natural toxins in surface water have been reviewed. For this purpose, analytical methods from the end of 2018 to date have been the focal point. Previous methods were described in a peer-reviewed article by Picardo et al. [133]; included in this chapter (**Scientific Publication 1)**.

Table 4: Latest analytical approaches for the analysis of natural toxins in water

Toxins	Aim	Sample pretreatment	Recovery %	Instrumentation	LOD	LOQ	Reference
MCs, NOD, DA OA	Identification; Quantification	DLLME	82 and 118	LC-HRMS (TOF)	0.22–1.5 ng mL^{-1}		[99]
MCs	Identification; Quantification	DLLME	45.0 to 109.7	LC-PDA-HRMS (ITMS)	0.005 and 0.003 µg L^{-1}		[108]
MCs	Identification; Quantification	Preparative HPLC Supelcosil LC_{18} DB		LC-HRMS (ITMS)			[109]
Enniantin, Beauvericin	Identification; Quantification	DLLME	78.5 and 100.1	LC-MS/MS (LTQ)	0.06–0.17 µg L^{-1}	200 µg L^{-1}	[100]
Zearalenone, zearalanone, α-zearalanol, β-zearalanol, α-zearalenol and β-zearalenol)	Identification; Quantification	DLLME	81 to 118	LC–MS/MS (LTQ)	4–20 ng L^{-1}	8 to 40 ng L^{-1}	[101]
MC-RR, MC-YR, MC-LR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF and nodularin	Identification; Quantification	LLE	77.0 (except for MC-RR and NOD which were 53.2% and 54.3, respectively)	UPLC-DAD and UPLC-MS/MS (TQ)	0.02 to 0.11 µg L^{-1}	8–11 µg L^{-1}	[102]
Phenol; (b) *p*-cresol; and (c) resorcinol.	Identification; Quantification	LLE (ionic solvents)	79-93	COSMO-RS method			[103]
ANA, CLD, MCs, and NOD	Identification; Quantification	SPE (HLB and ENVI-carb)	53-103	LC-MS/MS (TQ)	10-80 ng L^{-1}	10-280 ng L^{-1}	[110]
MCs; Anabaenopeptins; Microginins; Cyanopeptolins; NOD	Identification; Quantification	SPE (Carbograph 4)	85	UPLC-HRMS/MS (QTOF)	0.002 and 0.047 µg L^{-1}		[111]

CYN, ANA-a, HANA-a, AP-A, AP-B and 12 MCs ([Asp3]RR, RR, YR, HtyR, [Asp3]LR, LR, HilR, WR, LA, LY, LW and LF	Identification; Quantification	Online SPE (C18)	72 to 102	SPE-LC-HRMS/MS (Q-Exactive)	8-53 ng L^{-1}	27-176 ng L^{-1}	[104]
Saxitoxin	Identification; Quantification			Optical biosensor	0.5 µg L^{-1}		[112]
MCs, NOD, CYN	Identification; Quantification	SPE (HLB)	64 and 115	UPLC-MS/MS (TQ)	0.04-0.05 µg L^{-1}	0.2-3 µg L^{-1}	[113]
MC-LR, ANA	Degradation; Removal		Degradation efficiency 35 – 53.6	UV-LED/TiO_2 Oxidation			[114]
MC-LR	Degradation; Removal		100% MC-LR removal after hour	Graphene oxide-based Z-scheme photocatalysts; Ag_2CO_3-GO nanoparticles	0.012-0.025 µg L^{-1}	0.040–0.083 µg L^{-1}	[115]
NOD	Removal	Ultrasound-assisted dispersive SPE with tire-based activated carbon (WTAC)		LC-PDA.	12 ng L^{-1}	40 µg L^{-1}	[116]
DON, 15-ADON, and NIV	Identification; Quantification		97.2, 88.4 and 87.9%	GC/MS			[117]
FOR, equol, COU, DON, DAI, BIO, ZON, and GEN.	Identification; Quantification	SPE (Oasis HLB)	43 (Mean)	LC-MS/MS	BIO (0.5), COU (2.8), DAI (1.8), equol (0.6), FOR (0.8), GEN (2.2), DON (1.5), ZON (0.7) µg L^{-1}		[118]

Zearalenone (ZEN) and its derivatives (i.e. zearalanone (ZAN), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL))	Identification; Quantification	Multiwalled carbon nanotubes microdispersive SPE (μ-dSPE)	77 - 120	LC-MS/MS (IT)	1.68-41 μg L^{-1}	3.9-115 μg L^{-1}	[119]
Fungal phenols, alcohols, alkenes, monoterpenes, aldehydes, and alkanes	Identification; Quantification	Liquid-liquid extraction		GC/MS			[120]
Saponins	Identification; Quantification	2D TLC		HPLC			[121]
Amygdalin, prunasin, neoamygdalin, and sambunigrin	Identification; Quantification	Liquid-liquid extraction		Micellar capillary electrophoresis	5 μM		[122]
Linamarin; thiocyanate	Identification; Quantification	SPE (C_6)	91	Enzymatic hydrolysis (linamarase) UV detection			[123]
MC-LA, -LY, -LW and -LF; MC-(H2)YR (dihydrotyrosine at position-2), [epoxyAdda5]MC-LR, [DMAdda5]MC-RR and [Mser7]MC-RR	Identification; Quantification		70-150	LC-MS/MS; LC-UV-MS	0.05-0.11 μg L^{-1}		[105]
MC-RR, MC-YR, MC-LR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF and nodularin	Identification; Quantification	SALLE	54-77	UHPLC-MS/MS and UHPLC-DAD	1.0 to 3.4 μg L^{-1} (DAD); 0.02 to 0.11 μg L^{-1} (MS/MS)		[102]
MCs, SAX,BMAA, ANA	Identification; Quantification	SPE (Oasis HLB)	20-40	ELISA	0.1 μg L^{-1} (MCs and ANA); 0.015 μg L^{-1} (SAX); 4 μg L^{-1} (BMAA)		[124]

MC-LR, MC-RR, MC-LA, MC-LY, MC-LF, LC-LW, MC-YR, MC-WR, [Asp3] MC-LR, [Dha7] MC-LR, MC-HilR, and MC-HtyR and NOD	Identification; Quantification		80 to 110	UHPLC-MS/MS		0.04 to 0.64 µg L^{-1}	[125]
MC-LR	Degradation; Removal		70% reduction	UV-UV lamp	< 0.5 µg L^{-1}		[126]
CYN, ANA, HANA	Identification; Quantification	SPE (Oasis HLB)	9 % (CYN); 64 and 57.6% (ANA and HANA)	UPLC- MS/MS	0.6 to 15 ng L^{-1}	0.03 ug L^{-1} (CYN); 0.1 ug L^{-1} (ANA); 0.03 ng L^{-1} (HANA)	[127]
MCs; Lyngbyatoxin A	Identification	Direct injection		LC-HRMS/MS (MALDI-TOF)			[128]
MC-LR, MC-RR, MC-YR; MC-LF; MC-LY; MC-LW; ANA	Identification; Quantification	SPE (Oasis HLB)		LC-HRMS/ (LTQ OrbiTrap XL)	0.01–0.6 µg L^{-1}		[129]
Biochanin A; daidzein; equol; formonetin; genistein; coumestrol	Identification; Quantification	SPE (Oasis HLB)	63-133	LC–MS/MS	0.4-11 ng L^{-1}		[130]

1.7.3.3 Scientific publication I

In this chapter the first review that resumed the latest analytical methods until 2018 for the determination of natural toxins in surface water environment has been reported. Here a classification of the most encountered natural toxins in water was reported. Then, the analytical methods used for their determination have been reviewed considering the sample preparation, the analytical performance, the detection limits, and the application for the different groups of toxins.

Recent advances in the detection of natural toxins in freshwater environments

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Recent advances in the detection of natural toxins in freshwater environments

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ABSTRACT

Natural toxins can be classified according to their origin into biotoxins produced by microorganisms (fungal biotoxins or mycotoxins, algal and bacterial toxins), plant toxins or phytotoxins and animal toxins. Biotoxins are generated to protect organisms from external agents also in the act of predation. Among the different groups, bacterial toxins, mycotoxins and phytotoxins can produce damages in the aquatic environment including water reservoirs, with the consequent potential impact on human health.

In the last few decades, a substantial labour of research has been carried out to obtain robust and sensitive analytical methods able to determine their occurrence in the environment. They range from the immunochemistry to analytical methods based on gas chromatography or liquid chromatography coupled to mass spectrometry analysers.

In this article, the recent analytical methods for the analysis of biotoxins that can affect freshwater environments, drinking water reservoirs and supply are reviewed.

1. Introduction

Mycotoxins, algal toxins, bacterial toxins, and plant toxins are the main natural toxin groups that can be present in the aquatic environment. Due to their toxicity and potential damages to human health [1,2] through the diet, an important labour of research has been carried out during the last decades in the field of food analysis. Several review articles have summarised their toxic effects [1], the different analytical approaches [3–8] and occurrence [9], especially in food [10,11]. Marine biotoxins have been very much studied because these toxins can be bioaccumulated on edible parts of seafood [11,12]. In contrast, in freshwater environments, the cyanotoxins are the more prominent group. However, other groups of natural toxins that could as well affect water reservoirs have been almost not studied until now, and the development of new analytical methodologies is still needed.

This article reviews the main analytical methods, in particular, those based on liquid chromatography coupled to mass spectrometry (LC-MS) to assess the presence of natural toxins that can affect freshwater environments.

Organisms producing biotoxins affecting aquatic environments and drinking water reservoirs have been shown to be dependent on different environmental factors. Water contamination, the increase of organic material with the subsequent eutrophication processes [13] and the global warming that conducts to the increasing nutrient enrichment, increasing temperature and extreme precipitation in combination with prolonged drought which are all factors related to each time more frequent episodes of toxic blooms.

During the last decades, there is an increasing interest on the assessment of the occurrence and to study the nature of these biotoxins that can affect not only the natural environments but also the human health through food and water consumption [14].

In order to face the potential risk for human health caused by natural toxins together with the water scarcity problems in some areas, different regulations have been implemented. In Europe, the main regulations concerning the occurrence of biotoxins in the aquatic environment are the Water Framework Directive, the Drinking water directive (Council Directive 98/83/EC) [15] which is now under revision [16], and the EU Bathing Water Directive 2006/7/EC [17]. Several states have adopted their own regulation on drinking water safety although many directives were revised in response to the amendment to the Guidelines for Drinking Water Quality of the World Health Organization (WHO). In Table 1, the main national and international regulations are summarised.

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Table 1
Main international regulations and directives applied in different countries for surface water and drinking water's quality parameters.

Country	Committee	Regulation	Reference
Europe	European Council	Water Framework Directive; EU Bathing Water Directive	
USA	Environmental Protection Agency (EPA)	Title XIV of The Public Health Service Act: Safety of Public Water Systems (Safe Drinking Water Act)	[129]
Canada	Federal-Provincial-Territorial Committee on Drinking Water	Guidelines for Canadian Drinking Water Quality	[130]
Brasil	Ministry of Health (Ministerio do Saudade)	PORTARIA N° 2.914, DE 12 DE DEZEMBRO DE 2011 - 1.0 μg/L for equivalents MCs, 3.0 μg/L for STX equivalents	[131]
Australia	National Resource Management Ministerial Council	Australian Drinking Water Guidelines 6, 2011 - 1.3 μg/L expressed as toxicity equivalents of MC-LR	[132]
Italy	Ministry of Health (Ministero della Salute)	Decreto legislativo 31/2001 0.8 μg/L for MC-LR	[133]
South Africa	Department of Water Affairs and Forestry, Department of Health and Water Research Commission guides on the Quality of Domestic Water Supplies	Water Services Act, 1997 - 0.8/μg L for MC-LR.	[134]
China	Ministry of Health of China	Standards for Drinking Water Quality - MC-LR: 1 μg/L	[135]
Spain	Ministry of Health, social services and equality	Real Decreto 140/2003, February, 7th. Quality criteria of water intended for human consumption. MC-LR: 1 μg/L	[136]
Japan	Ministry of Health, Labour and Welfare	Waterworks Act - MC-LR: 1 μg/L	[137]

European Union (EU); microcystins (MCs); microcystin LR (MC-LR); saxitoxin (STX).

Considering the main toxins affecting the aquatic environment and drinking water reservoirs the following groups can be distinguished:

Plant toxins (Phytotoxins) are naturally produced as secondary metabolites, which the central role is to protect the organism against natural threats. The main groups of plant toxins are alkaloids, terpenes, glycosides, proteinaceous compounds, organic acids and resinoid compounds [18]. As shown in Table 2, each group is classified into different subgroups according to their structure. Crozier et al. [19], have characterised a wide number of phytotoxic compounds, and complete lists were also proposed by Quattrocchi [20]. In spite of the high number of plant toxins that potentially would end up in natural aquatic environments (according to their octanol/water partition constants distributed between sediments and water), the regulation concerning plant toxins in surface and drinking water has not been established yet. Moreover, the environmental fate and behaviour of phytotoxins has been investigated only for a limited number of compounds [21] such as glycoalkaloids produced from potato (*Solanum tuberosum*) [22], the ptaquiloside from bracken (*Pteridium aquilinum*) [23], or some isoflavones [24–26], showing high relevance in terms of their capacity to produce biological effects such as toxicity, carcinogenicity or estrogenicity. These toxins have been studied in food and feed, but the data about their environmental occurrence, fate and behaviour for most of them are still needed [21]. Due to the estrogenic activity isoflavones, and their use in pharmacy, during the last decade several analytical methods have been developed for their determination in aquatic environments and wastewaters. These analytical approaches have been mainly based on gas chromatography coupled to mass spectrometry (GC-MS) [27], tandem mass spectrometry (GC-MS/MS) [28], liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [29] and high resolution mass spectrometry (LC-HRMS) [30] to achieve enough sensitivity as it will be discussed in the following sections.

Table 2
Main groups of plant toxins according to their chemical structures.

Groups	Subgroups	Example
Alkaloids	Tropane/atropine like alkaloids	Atropine
	Pyrrolizidine alkaloids	Retronecine
	Pyridine/piperidine alkaloids	Coniine
	Pyrrolidine-pyridine alkaloids	Nicotine
	Purine alkaloids	Caffeine
	Quinoline alkaloids	Quinine
	Isoquinoline alkaloids	Morphine
	Indole/Indolizidine alkaloids	Strichnine
	Quinolizidine alkaloids	Anagyrine
	Steroidal glycoalkaloids	Solanidine
	Diterpenoid alkaloids	Aconitine
	Steroidal alkaloids	Jervine
	Phenylamine alkaloids	Ephedrine
Terpenes	Monoterpenes	Andromedotoxin
	Sesquiterpenes	Geigerin
	Triterpenes	Circubitacins
Glycosides	Cyanogenic glycosides	Amygdalin
	Cardionilides	Digitoxin
	Sapogenic glycosides	Argostemme
	Coumarin glycosides	Esculin
	Anthraquinone glycosides	
Proteinaceous compounds	Toxalbumins	
	Polypeptides	
	Amines	
Resin and resinoids		Tetrahydrocannabinol

Mycotoxins are produced by fungi of the genera *Fusarium*, *Aspergillus*, and *Penicillium* in particular conditions of temperature and humidity. They can enter in the human food chain either directly from contaminated drinking water, plant-based food components or by indirect contamination from the growth of toxigenic fungi on food or feed.

Mycotoxins can accumulate in cereals, corn, peanuts, soybeans and spices, among others during the maturation processes, the storage, and during transportation. Consumption of mycotoxin-contaminated food or feed can cause acute or chronic toxicity in human and animals. Showing genotoxic, carcinogenic and mutagenic effects, and some of them have immunosuppression activity [31]. For these reasons, the Regulation (EC) 1881/2006 [32] establishes the maximum levels of mycotoxins in food and the Commission Directive 2003/100/EC [33] which amended the Directive 2002/32/EC [34] modifying the maximum levels of mycotoxins in feed, but no limits are currently applied to surface water.

In addition, most of the current analytical approaches are optimised for their determination in food, being aflatoxins (AFLs) and ochratoxin A (OTA-A) the most studied ones. Regarding the most common analytical approaches, despite much interest in immunochemical methods [35–37] because of the rapid responses and potential of high-throughput cost-effective analysis, and the development of rapid methods based on biosensors [38,39], liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and high-resolution mass spectrometry (LC-HRMS) are the techniques of choice thanks to their sensitivity and selectivity [40–44]. It should be pointed out that the current analytical methods are generally focused on few analytes such as deoxynivalenol (DON), zearalenone (ZEA), ochratoxin (OTA), patulin (PAT), and trichothecenes [45–50]. On the other hand, fungi can produce their secondary metabolites in water [51] and it has been shown that can be a route of human exposure to mycotoxins [52], but only few studies have reported their presence. E.g., Phytoestrogens and mycotoxins were investigated in agricultural stream basins in Iowa. Mycotoxins were less spatially widespread than other phytoestrogens, and the detections of deoxynivalenol (6/56 measurements) suggested a more variable source due to the required combination of proper host and proper temperature and moisture conditions necessary to promote *Fusarium* spp. infections [53]. The occurrence of fungal metabolites, fumonisins were detected at the ng/L level in aqueous environmental samples [54]. Recently, mycotoxins have been detected in surface waters and drinking water sources [55] at concentrations up to 35 ng/L, and different fungi and yeast species have been identified in biofilms of drinking water networks [56]. In another interesting work the presence of mycotoxins was reported in bottled waters in Portugal, in which the aflatoxin B2 (AFL B2) was the most frequently detected with a maximum concentration of 0.48 ng/L followed by aflatoxin B1 (AFL B1), aflatoxin G1 (AFL G1), and OTA-A [57]. In spite of that, the presence of fungi in drinking water networks can be associated to the production of tastes and odours, a problem not very studied till now, and most of the works regarding this topic were focused on the determination of filamentous fungi [58].

Algal and autotrophic bacterial toxins Bacteria are heterotrophic microorganisms in various aquatic ecosystems, playing critical roles in biogeochemical cycles and being fundamental components of the aquatic food web. However, they can also be considered a source of toxins and diseases through drinking water. One of the most significant microbial risk associated with drinking water is related to faecal contamination by wastewater discharges in fresh waters and coastal seawaters, and some of the more relevant diseases that can be transmitted are cholera by *Vibrio cholerae*, gastroenteritis caused by different vibrios as *Vibrio parahaemolyticus* or typhoid fever and salmonellosis produced by different subspecies of *Salmonella enterica*. However, in this review, the main focus are toxins produced by bacteria of aquatic origin. Autotrophic bacteria are primary producers in aquatic systems as are the algae. For this reason, autotrophic bacteria (predominantly cyanobacteria) are often categorised as algae, though they are not related organisms. Under certain conditions, some strains of cyanobacteria can produce cyanotoxins, which are one the most important groups of natural toxins produced in freshwater environments. Cyanotoxins are represented by four different chemical groups like *cyclic peptides* such as microcystins, *alkaloids* such as senecionine or retronecine, *lipopolysaccharides polyketides* and *amino acids* such as β-aminomethyl-L-alanine. It should be mentioned that harmful cyanobacterial blooms have been increasing in frequency worldwide during the last decades and pose a threat to drinking and recreational water. The factor promoting favourable conditions for harmful blooms are the excess of nutrients, mainly phosphorus and nitrogen, temperatures around 20 °C, light, and calm waters [14]. Therefore, human activities such as runoff from agriculture, urban and sewer overflows that are leading to eutrophication and contamination processes also promotes the conditions for cyanobacterial blooms. Other factors such as the abundance or presence of other algal species and grazers in the aquatic ecosystem may also influence the dominance of certain cyanobacterial species associated with toxins production. While the factors that lead to the blooms of cyanobacteria are known, there is much less information that characterise when the toxic strains are dominant and in which conditions therefore they can produce toxins.

In the following sections, this review article presents the recent advances in the detection of natural toxins in freshwater environments with high throughput of molecular, biochemical and chemical methods. In this last section, particular attention will be given to quantitative methods based on LC-MS.

2. Sample collection, preservation and handling

The study of toxins in aquatic ecosystems includes different strategies for on-site sensing of blooms, e. g, to determine the horizontal distribution of cyanobacteria [59–61] or spectrofluorometric probes to investigate the vertical distribution of these cyanobacteria and algal blooms in the water column [61]. However, these methods are not proper methods in all cases, and they are expensive approaches. Additionally, errors in the estimation of bloom abundance might be caused by the spatial and temporal differences in the sampling site [61]. For laboratory analysis, surface water samples are commonly collected manually using amber glass containers to avoid potential adsorptions into plastic bottles and to minimise exposure to sunlight. Surface water samples are in general collected between 0.3 and 1 m depth [62], while the biomass samples are usually taken from the surface using phytoplankton nets. The samples are filtered after to separate the biomass containing intracellular toxins.

In situ passive solid-phase adsorption of biotoxins by solid-phase adsorption toxin tracking (SPATT) have been as well very much used over the past few years [63]. This method provides reliable, sensitive, time-integrated sampling to monitor the occurrence of toxic blooms. SPATT has several significant advantages over current phytoplankton and shellfish monitoring methods such as simplicity and low cost, and matrices are relatively clean which simplifies the extraction and provides toxin dynamics information. The best results till now have been obtained for lipophilic compounds, but new materials are being produced to selective retain the more polar water-soluble compounds.

After sampling, samples should be refrigerated in the dark to prevent toxins degradation, but it is essential a maximum storage

time minor of 24 h. Where prolonged storage is required, samples can be frozen at −40 to −80 °C. However, in the case of cyanotoxin analysis, samples freezing can lead to a release of toxins from the damaged cells and bring to the determination of the total amount of natural toxins in the sample. Prior filtration can be applied before the freezing step. Dissolved and intracellular toxins can be determined with a prior filtration of the dispersed cells and proceeding with a separate extraction.

3. Methods to detect natural toxins in freshwater environments

Nowadays, the most frequent approaches to identify natural toxins or their precursors are the chemical, biochemical, and molecular approaches.

3.1. Molecular methods

Molecular methods have been widely used since the 1990s to assess the presence of cyanobacteria and cyanotoxins in aquatic systems. They are based on the detection of genes present in cyanobacteria and those related to the synthesis of their toxins. These methods include polymerase chain reaction (PCR) techniques such as the conventional PCR, multiplex PCR, terminal restriction fragment length polymorphism (T-RFLP), random amplified polymorphic DNA (RAPD), denaturing gradient gel electrophoresis (DGGE), real-time PCR and non-PCR-based techniques such as fluorescence in situ hybridization (FISH) and DNA microarrays. The description of these techniques is beyond the scope of this review and can be found elsewhere [64].

The PCR analysis is the most frequently used, consisting of the *in vitro* amplification of a DNA sequence by specific primers that target the DNA specific sequence. There are several sequences used in the investigation of cyanobacteria such as the amplification of the 16S ribosomal RNA (rRNA) gene, phycocyanin operon, internal transcribed spacer (ITS) region, and the RNA polymerase β subunit gene (rpoB) using taxon-specific primers [65,66]. In the same way, biotoxins of the main groups of cyanotoxins such as microcystins (MCs) and their congeners, nodularin (NOD), saxitoxin (STX), anatoxin-a (ANA-a), and cylindrospermopsin (CYN) can be detected by molecular methods through the detection of the genes present in the gene clusters encoding these biosynthetic enzymes [67,68]. For example, toxin genes such as cyrJ, sxtA, mcyE, ndaF correlate to the production of a toxin or toxin group as CYN, paralytic shellfish toxins, cricystis and NOD [69], respectively. Different multiplexed PCR assays have been as well developed for different groups of cyanobacteria such as MCs [70], and the single-plex PCR assays for STX [67] and ANA-a [68]. On the other hand, the real-time PCR technique allows the quantification of both cyanobacteria and cyanotoxins with high sensitivity in environmental samples [71].

3.2. Biochemical methods

The main groups of techniques under this category are enzyme inhibition-based methods and the immunochemical approaches.

3.2.1. Methods based on enzyme inhibition

Based on the enzyme inhibition properties of specific groups of cyanotoxins, different methods to detect toxins and toxicity of a sample have been developed.

The cyanotoxin groups that can be determined by enzyme inhibition approaches are MCs, NOD, and ANA. Both MCs and NODs are phosphatase (PP) inhibitors, while the ANA-a group is an acetylcholinesterase (AChE) inhibitor. The first approaches were established by An and Carmichael [72] by using a colourimetric protein phosphatase assay to detect MC and NOD, but different variants succeeded. For example, MCs and NOD contained in drinking water were determined with the phosphate release from a substrate of phosphorylated protein [72]. The lowest detection level was achieved using an enzyme bioassay based on the quantitation of the ^{32}P radiolabelled phosphate [73,74]. Colorimetric and fluorimetric reactions were applied for several cyanotoxins detection with the measure of phosvitin. The colorimetric reaction is generally well combined with LC that achieves high levels of sensitivity and specificity given by the coupled techniques [75]. Posteriorly, the enzyme inhibition has been employed to develop different biosensors. For example, an electrochemical biosensor for the detection of MC based on the inhibition of the protein phosphatase 2A (PP2A) was developed by Campàs et al. [76]. In this system, the enzyme was immobilised by entrapment using a poly(vinyl alcohol) azide-unit pendant water-soluble photopolymer (PVA-AWP). Catechyl monophosphate (CMP), α-naphthyl phosphate (α-NP) and 4-methylumbelliferyl phosphate (4-MUP) were used as phosphorylated substrates to monitor the protein phosphatase activity by amperometry, the former providing the highest chronoamperometric currents at appropriate working potentials (+450 mV versus Ag/AgCl), and a limit of detection of 37 μg/L was achieved. In another example, another electrochemical biosensor to detect microcystin-LR (MC-LR) was developed based on the inhibition of recombinant protein phosphate type 1 (PP1α). In this case, phosphoparacetamol was shown to be an excellent synthetic substrate. The biosensor was constructed by entrapment of the enzyme in Polyvinyl Alcohol (azid unit) on Cobalt-Phtalocyanine (CoPC) modified screen-printed electrode. Electrocatalytic mediator demonstrated a significant improvement in the electrochemical detection of the dephosphorylated substrate. The standard inhibition curve has provided a limit of detection at 0.93 μg/L, demonstrating the improved analytical performance. In the case of ANA, some approaches have been developed based on biosensors. For example, a biosensor based on the amperometric detection of the activity of electric eel acetylcholinesterase was developed by Villate et al. [77] The system displayed a limit of detection of 1 μg/L ANA-a(s) in natural environmental sample, and the oxime reactivation was used to discriminate between the toxin and potential insecticides present in the sample. Using engineered acetylcholinesterase, Devic et al. [78], developed another biosensor with improved sensitivity, the limit of detection was brought to below the nanomole-per-litre level. However, the test is nonspecific for all the potential insecticides acetylcholinesterase inhibitors present in the same sample. For this reason, the authors used a four-mutant set of acetylcholinesterase variants, two mutants that are sensitive to ANA-a(s) and the other two which are sensitive to the insecticides, in this manner, it has been allowed specific detection of the cyanobacterial neurotoxin.

The main advantage of the methods based on enzyme inhibition is the rapid response without sample preparation. However, the main general limitations are the lack of specificity and versatility of these assays.

3.2.2. Immunochemical methods

The affinity properties between antibodies and antigens have been extensively explored to develop detection methods for natural toxins in the water. One of the first polyclonal rabbit antibodies against MCs was reported by Brooks and Codd in 1987 [79] after this early development different polyclonal and monoclonal antibodies were raised resulting in a variety of immunoassays. Among them, enzyme-linked immunosorbent assays (ELISA) have been very much employed and different commercial kits are available. Some of these antibodies could present broad specificity in front a range of cyanotoxins, which is an advantage for rapid screening but could be a

limitation in terms of specificity between congeners or structurally related compounds. For example, Sheng et al. [80], raised a polyclonal antibody for MCs group and developed a direct competitive to detect the MCs in waters, which showed a good cross-reactivity with MC-LR, microcystin-RR (MC-RR), microcystin-YR (MC-YR), microcystin-LF (MC-LF), microcystin-LW (MC-LW) and NOD, and have a LOD for MC-LR of 0.12 μg/L. Yang et al. [81], produced a monoclonal antibodies able to detect NOD and eight MCs with limits of detection (LOD) between 0.16 and 0.10 μg/L, and recoveries of 62–86%. While in some cases high selectivity can also be achieved. The group of Sheng at al [82]. Produced monoclonal antibody (Clone MC8C10) with high specificity against the most frequent and most toxic variant of MCs, MC-LR. An indirect competitive ELISA was established with a limit of detection for MC-LR of 0.1 μg/L and a limit of quantitation in the range from 0.3 to 10 μg/L. Some of these approaches present a high level of sensitivity. Lidner et al. [83], reported an immunoassay technique which allows LODs below 4 ng/L. Evolutions of this method, consisting in automated array biosensors, were able to reach LODs of about 500 pg/L [84].

A variety of immunosensors have been recently developed for rapid screening of cyanotoxins in water taken advantage of new nanomaterials to improve the assay in terms of sensitivity and robustness. Among these recent approaches, Zhang et al. [85], developed an electro-chemiluminescent immunosensor based on CdS quantum dots for ultrasensitive detection of MC-LR. In this case, a sandwich-type assay is proposed reaching a limit of detection of 0.0028 μg/L. Using photoelectrochemical detection an immunosensor incorporating graphene quantum dots and highly oriented silicon nanowires for the determination of MC-LR in water samples was presented by Tian et al., [86]. In this system, the specific recognition of MC-LR affected the optoelectronic properties of the electrode cluster antibody/graphene-quantum-dot/silicon-nanowires, leading to the photocurrent decrease. The optimal assay showed a limit of detection of 0.055 μg/L. The use of biocompatible nanomaterials has been as well explored. For example, a three-dimensional villiform-like carbon nanotube/cobalt silicate core-shell nanocomposites were synthesised to be used as the substrate to immobilise the antigen of MC-LR by Gan et al. [87]. In this immunosensor, Fe_3O_4 nanoclusters/polydopamine/gold nanoparticles core-shell magnetic nanocomposites were prepared as the label carrier to conjugate the second antibody and horseradish peroxidase. Due to the biocompatibility of the nanocomposite, the immunosensor can immobilise more antigens by the large surface area of the three-dimensional villiform-like structure, providing high electrochemical signals. This immunoassay showed a linear response to MC-LR in the range from 0.005 μg/L to 50 μg/L with a LOD of 0.004 μg/L. Recently, photo-electrochemistry has been used in several approaches. A photoelectrochemical immunosensor was also developed to detect MC-LR by using the Au nanoclusters as the substrate and silica-functionalized DNAzyme concatemers as the label carrier [88]. Modified branched TiO_2 nanorods decorated with CdS nanoparticles were used as photoelectrode, while the bioelectrode was prepared by in-situ electrodepositing Au nanoclusters on dopamine-modified glassy carbon electrode to immobilise the antigen. Silica nanospheres were used to conjugate the secondary antibody and G-quadruplex/hemin, which can accelerate the oxidation of 4-chloro-1-naphthol with H_2O_2 to yield the biocatalytic precipitation onto the electrode. By taking the advantages of the surface effect of Au nanoclusters, DNA amplification and high photoelectrocatalytic activity, the immunosensor detected MC-LR in a wide range of concentrations (0.001–100 μg/L) with, in addition, a very low limit of detection (0.7 ng/L). Besides the different strategies to obtain labelled assays with high sensitivity, several works explored the use of labelled free assays. Hu et al. [89], constructed a label-free electrochemical immunosensor for ultrasensitive detection of MC-LR based on multi-functionalized graphene oxide. The large surface area of graphene oxide facilitates immobilisation of the antibody. Moreover, the introduction of the Au nanoparticles and 1-butyl-3-methylimidazolium hexafluorophosphate improved electrical conductivity. This electrochemical immunosensor was prepared in an only one-step process, and differential pulse voltammetry was employed to detect the toxin showing a limit of detection of 0.1 μg/L.

In general terms, the main advantages of immunoassays are high-throughput analysis, therefore reduced sample preparation needs or even sample preparation is not required, and fast responses. In contrast, the main limitations are the cross-reactivity between structurally related compounds that rebound in low specificity. Matrix effects can also have a strong impact on the sensitivity of the assays. Finally, another drawback is the difficulty of raising antibodies against very toxic substances limiting their availability.

3.3. Chemical analysis

The most widely used analytical techniques to determine natural toxins in water samples are based on separative techniques such as LC or GC coupled to different detectors such as MS, fluorescence (FL), and ultraviolet (UV), and spectroscopy. However, LC–MS is nowadays the technique of choice because of their sensitivity and specificity. However, preconcentration and clean-up strategies are requested previous the analysis.

3.3.1. Extraction and clean-up strategies

Include liquid-liquid extraction [90], solid phase microextraction (SPME) [91], freeze-drying [92], and solid phase extraction (SPE) [93] among the more commonly used. However, among the different techniques, SPE is widely used due to their flexibility, the high number of different stationary phases available, the potential of automatization and lower solvents consumption. In some cases, to differentiate intracellular and extracellular concentrations of toxins, prior a filtration step is necessary to separate the cells from the water and then the two fractions are processed in parallel. If the total toxins content is required, the sample is subjected to ultrasonication, lyophilisation, and freeze-thawing to break the cells and obtain the toxins released.

In Table 3, the main sample preparation approaches for natural toxins in water samples are summarised. As can be seen in Table 3, only a limited number of methods have been reported for the analysis pf mycotoxins and phytotoxins in surface and drinking water. In general, the selection of the extraction solvents is carried out according to the nature of the toxins and also the nature of the sample. For example, in the case of cyanobacterial blooms that are rich in proteins, the extracts can result in complex mixtures. The use of pure water has been shown to lead dirtiest extracts with proteins content three times higher than using water acidified with acetic acid, whereas methanol extraction suppressed water-soluble proteins [94,95]. In another example, the use of methanol–water (50:50, *v:v*) was effective for extracting six MCs from biomass obtaining recoveries up to 90% [96]. Lawton et al. [97], reported the use of pure methanol as the versatile solvent for the extraction of different MCs (MC-LR, MC-LY, MC-LW, and MC-LF). Another relevant factor regarding the extraction of complex samples is the pH. However, the results reported so far are controversial between authors. Van der Westhuizen and Eloff [98] reported that the best recovery of MC-RR was achieved at pH 10, whereas other authors [99] showed that the solubility of MCs increased in methanol acidified with 1% trifluoroacetic acid (TFA). In any case, the posterior analysis should be as well considered and, e.g. TFA is not recommended during the extraction if the analytical method involves the use of MS.

Table 3
Summary of sample preparation methods and their recoveries for the analysis of natural toxins in water samples.

Mycotoxins						
Mycotoxins	Matrix	Sample volume	Sample preparation method	Elution solvents	Recoveries	Reference
DON; ZEA	Surface water	1 L	SPE Oasis HLB cartridge	Ethyl acetate	DON 95–108%; ZEA 70–102%	[46]
ZEA	Surface water, ground water; WWTP	1 L	SPE Zearala Test cartridges	MeOH	HPLC water, 86%; groundwater, 85%; surface water, 81-76%; WWTP, 74%.	[45]
3-Acetyl-DON, 15-DON, AFL (B1, B2, G1, G2, M1), citrinin, DON, fumonisin (B1, B2), patulin, HT-2 and T-2 toxins	Milli-Q, drainage, river water and WWTP effluent.	1 L	SPE Oasis HLB cartridge	MeOH	Milli-Q water, 20–109%; Drainage water, 62; River water, 56%; WWTP effluent, 57%	[47]
AFLs	Tank water	1,8 L	IAC-SPE cartridge for aflatoxins G_1, B_1, B_2, G, and M_1	MeOH	ND	[138]
AFLs, fumonisins, ochratoxin A	Natural bottled mineral waters and spring waters		Oasis HLB cartridges	CH_3CN and MeOH	AFLs 69–93% fumonisins 98–105% ochratoxin A 94%	[57]
Bacterial toxins						
Toxins	Matrix	Sample volume	Sample preparation method	Elution solvents	Recoveries	Reference
CYN	Brackish lake water	20 mL	SPE Oasis HLB cartridge	MeOH	CYN, 76%	[139]
MCs-NOD	HPLC water		SPE BondElut C_{18} cartridge	MeOH	ND	[140]
ANA-a	Freshwater samples	2 mL	SPME (PDMS) 100 μm		ND	[141]
ANA-a	Storage tank water	1.6 ml	SPME (PDMS, 100 μm) (PDMS–DVB, 60 μm) (PA, 85 μm) (CW–TPR, 50 μm)	MeOH–water (60:40 v/v)	ND	[108]
1 MCs (-RR –LR, -LY, -LW, -LF), NOD	Treated and raw water	2.5 L	SPE C_{18} cartridge	MeOH (0.1% v/v TFA)	Raw water: MCs 52-29%, NOD, 100–118%. Treated water: MCs 33–118%, NOD, 105–147%.	[97]
12 MCs (-RR, –LR, -LY, -LW, -LF, -LA, -HilR, -WR, [D-Asp3]-LR, [D-Asp3]-RR) CYN, ANA, NOD, OA and DA.	Lake water	0.4 L	SPE Oasis HLB and HyperSep Hypercarb PGC cartridges	DCM:MeOH (40:60, v/v)	44–113%	[100]
PTA	Stream water	0.1 L	SPE Oasis MAX cartridge	0.5 mL, 80% MeOH	57–106%	[115]
MCs (-LA, -LR, -LF, -LW, -YR, -RR)	Drinking water	0.5 L	SPE Oasis HLB and ImmunoSep (silica-based IAC) cartridges	IAC; 4% acetic acid in MeOH/H2O (8:2 v:v); MeOH	ImmunoSep, from 70 to 86%; Oasis HLB, from 70 to 94%	[104]
DABA; l-BMAA; AABA; β-ABA; GABA; BOAA	Lake water	0.5 L	SPE Oasis MCX cartridge	Ammonium hydroxide in MeOH, 5%)	ND	[142]
ANA	Aquaculture water samples		SPE LC-WCX cartridge	MeOH (0.2% TFA)	84–94%	[143]
MCs (-LA, -LR, -LF, -LW, -YR, -RR)	Tap water and lake water		SPE Sep-Pak C_{18} Plus Light cartridge	CH_3CN: H2O (90: 10, v/v) 0.1% FA	97–100%	[144]
ANA	Lake water	0.2 L	SPE Hypersep PGC cartridge	MeOH (0.1% TFA)	84%	[145]
Phytotoxins						
Toxins	Matrix	Sample volume	Sample preparation method	Elution solvents	Recoveries	Reference
PTA and PTB	Surface, untreated and stream water	20 mL	SPE Oasis MAX cartridge SPE Oasis HLB cartridge	MeOH:H_2O (80:20 v/v)	PTA, 85%; PTB, 86%	[103]
PTA and PTB	Ground water	20 mL	SPE Oasis MAX cartridge	MeOH:H_2O (80:20 v/v)	PTB; 84–89%, PTA, 52–66%.	[114]
Daidzein, genistein, formononetin, biochanin A and equol	Wastewater, surface water		SPE Oasis HLB cartridge	CH_2Cl_2/MeOH (50:50 v/v)	ND	[118]

3-methoxy-2-methyl-4-phenylbutyric acid (MMPB); β-aminobutyric acid (β-ABA), β-N-methylamino-l-alanine hydrochloride (l-BMAA); b- N-oxalylamino-L-alanine (BOAA); 2,4-diaminobutyric acid (DABA); Acetonitrile (CH_3CN); aflatoxin B2 (AFL B2); aflatoxin B1 (AFL B1); aflatoxin G1 (AFL G1); anatoxin-a (ANA-a); Anionic exchange cartridge (MAX); Carbowax fibre (CW); Cationic exchange cartridge (MCX); cylindrospermopsin (CYN); deoxynivalenol (DON); dichloromethane (DCM); dl-2-Aminobutyric acid (AABA); domoic acid (DA); formic acid (FA); Gamma-aminobutyric acid (GABA); hexasilane (C_6); high performances liquid chromatography grade (HPLC); immunoaffinity column (IAC); loganin (LOG); microcystin-YR (MC-YR); microcystin-LF (MC-LF); microcystin-LW (MC-LW); ochratoxin A (OTA-A); octadecylsilane(C_{18}); methanol (MeOH); microcystin-LR (MC-LR); microcystin-RR (MC-RR); not declared (ND); patulin (PAT); Poly-dimethyl-siloxane (PDMS); Poly-dimethyl-siloxane-divinyl benzene fibre (PDMS–DVB); Polyamide fibre (PA); polygraphityzed carbon (PGC); pterosin A (PTA) pterosin B (PTB); ptaquiloside (PTD); saxitoxin (STX); solid phase extraction cartridges (SPE); solid phase microextraction (SPME); trifluoracetic acid (TFA); waste water treatment plant (WWTP); zearalenone (ZEA).

As it has been mentioned before SPE is the more versatile and commonly used extraction technique, being the octadecyl silica (C_{18}) and methanol the most frequent combination, showing recoveries higher than 85% for some cyanotoxins such as MCs [94,95]. Rivasseau et al. [98], reported recoveries of cyanotoxins from drinking and river water between 75 and 80% and cleaner extracts using neutral pH. The use of polymeric sorbents is as well widespread. For example, the use of the copolymer hydrophilic–lipophilic-balanced Oasis HLB cartridges (Waters, Milford, CA) has been successfully validated for the analysis of different

mycotoxins in bottled water [57]. The same approach was previously proposed by Schenzel et al. [47]. These authors explored the suitability of the HLB cartridges in the analysis of neutral, basic and acidic mycotoxins in water obtaining average recoveries of 90%. Similar HLB cartridge, coupled with graphitised carbon materials such as HyperSep Hypercarb (ThermoFisher, Waltham, MA) has been used to obtain the best retention performances for various groups of natural toxins. Zervou et al. [100], used a combination of polar and less polar cartridges in tandem to extract cyanotoxins from different groups dissolved in water. In this work, the authors used pH > 10.5 to neutralise the charge of the polar toxins such as CYL, ANA-a, and domoic acid (DA).

In the case of some plant toxins and mycotoxins, the pH should be carefully controlled since some of them are not stable under strongly acidic or basic conditions. For instance, the rapid chemical hydrolysis occurs at pH < 4 and pH > 7 for ptaquiloside (PTD) [101] while for some mycotoxins such as OTA at pH > 7.3 the ring is opened (OP-OTA). OTA possesses two pK_a values, $pK_{a1} = 4.4$ (dissociation of carboxyl group); $pK_{a2} = 7.3$ (phenol hydroxyl group dissociation), so at pH higher than his pK_{a2} OTA converts to OP-OTA causing underestimation of determination, recoveries, and quantification [102]. In 2008, Bucheli et al. [46], reported for the first time the use of HLB cartridges prior LC-MS/MS analysis of DON and ZEA in water. In this case, labelled $^{13}C_{15}$-DON and D_6-ZEA have been used as internal standards to allow accurate quantification of the target compounds. In another example, PTD, loganin (LOG) and pterosin B (PTB) have been investigated by Clauson-Kaas et al. [103], which assessed the recoveries obtained with Oasis MAX and HLB SPE (Waters, Milford, CA). In both cases, toxins were successfully retained, but a lower amount of eluent (0.5 mL) was needed with Oasis MAX in comparison with the 2 mL necessary for the HLB column [103].

In addition to conventional stationary phases, recently, immunosorbents have improved the selectivity of the extraction of different groups of natural toxins. Aranda-Rodriguez et al. [104], compared the extraction and clean-up of six MCs between two different immunosorbents containing anti-MC-LR polyclonal antibodies and HLB cartridges. While the recoveries were similar for immunosorbents (>85%) and HLB (>90%) the main advantage with immunoaffinity columns was the higher clean-up. Interesting applications as well were reported by Wilcox et al. [105], for the analysis of mycotoxins. Immunoaffinity columns OCHRAPREP®, DZT MS-PREP®, AOF MS-PREP® and AFLAOCHRA PREP® (R-Biopharm AG, Darmstadt, GE) were used in tandem as a preconcentration step to analyse DON, ZEN, T-1, HT-2 toxins, fumonisins, and OTA-A in food samples reaching very high purity extracts free of any interference, and low detection limits.

Another interesting technique for the extraction and clean-up of contaminants from water samples is solid phase microextraction (SPME). This technique offers reusable fibres employing a variety of materials as carboxen, polydimethylsiloxane (PDMS), divinylbenzene (DVB), polystyrene (PS), carbopack-z, and polyacrylate (PA). However, SPME is an emerging technique which has not been very much employed for the analysis of natural toxins. Only a few examples of application have been reported for the extraction of MCs [106], NOD [107] and ANA-a [108] in waters.

3.3.2. Gas chromatography coupled to mass spectrometry

Due to the high selectivity of mass spectrometry, in this review GC will be considered only coupled to MS analysers. However, due to the low volatility and high polarity of most of the biotoxins that can be found in aquatic systems and their potential of degradation by temperature, few studies reported the use of this separative technique. In these cases, a derivatisation step prior to the separation by GC is necessary. Sano et al., developed a routine method for the analysis of ANA in blue-green algae [109]. In this work, they reported a method based on a quantitative analysis of 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) as an oxidation product of MCs by GC coupled to a single quadrupole MS (Q-MS). In this approach, MCs are oxidised first, derivatised and determined as their methyl esters [109]. Later, Harada et al. [110], proposed the use of ozonolysis to obtain the oxidation product of MCs, (3-methoxy-2-methyl-4-phenylbutyric acid). Following this approach, the reaction time was drastically reduced, including the sample preparation which was eliminated. The LODs reached with this approach were at picomole levels, providing not only the identification but also the quantitation of MCs. A recent application of GC-MS was proposed by Rocha et al. (2016) [111] for the analysis of endocrine disruptor compounds (EDC), including natural oestrogens and phytoestrogens such as formononetin, biochanin A, daidzein and genistein. In this work has been presented the use of an ion trap mass spectrometer (ITQ) coupled to a GC equipped with a programmable temperature vaporiser (PTV) for the analysis of EDC after a SPE clean-up and preconcentration step of river water samples. With this method, the method limits of detection (MLOD) were between 5.5 and 0.9 ng/L. A similar study was carried out in Portugal. Ribeiro et al. [112] confirmed the presence of natural toxins using a GC coupled to an ion trap mass spectrometer for the accurate mass identification of phytoestrogens and other natural compounds is estuarine waters at ng/L levels.

However, in general, the use of GC-MS methods for the analysis of natural toxins in freshwater have been minimal in comparison with the methods based on the separation by LC.

3.3.3. Liquid chromatography coupled to mass spectrometry

Toxins present in the aqueous phase are compounds with medium to high polarity. Therefore, LC is a more convenient approach offering the separation without derivatisation. LC coupled with detectors such as UV/VIS has been as well employed, but the identification of the toxins cannot be confirmed. For this reason, LC-MS methods are the most commonly used for enable the simultaneous identification and quantification [113]. In general, these approaches are based on the use of LC coupled to tandem mass spectrometry (MS/MS), but recently it has been well reported that some approaches taking the advantages of the high-resolution MS (HRMS). In Table 4, different methods and applications for the analysis of natural toxins in water are briefly reported. As it has been mentioned before, the analysis of natural plant toxins in waters including surface and groundwater, has not been intensively studied so far [48]. In spite of that, some good results for their trace analysis have been reported.

Phytotoxins: A sensitive analytical method based on SPE-LC-MS/MS for the analysis of the carcinogenic toxins produced by bracken fern (Pteridium aquilinum L), pterosin A (PTA) and their transformation product pterosin B (PTB), in groundwater was reported by Jensen et al. [114]. The method has LODs of 0.19 μg/L for PTA and 0.15 μg/L for PTB, which are 300–650 times better than the ones previously reported by LC-UV methods. The LC-MS/MS approach enables the quantification of these toxins at environmentally relevant concentrations with reliable identification [114]. More recently, an improved approach was presented by Clauson-Kaas et al. [103]. In this work, the authors developed a method based on SPE using Oasis MAX cartridges followed with Ultra-High-Performance LC (UHPLC) coupled to tandem mass spectrometry (MS/MS) using an electrospray (ESI) interface. The use of smaller column particles (sub 2 μm) helps to improve the speed, sensitivity and resolution. Therefore, thanks to the superior resolving performance of UHPLC, in only 5 min the authors achieved a good separation with method limits of detection of 8 and 4 ng/L for PTA and PTB, respectively. In addition, the pH adjustment of the samples to ~5.5 with ammonium acetate was shown to decisively increase the

Table 4
Analytical approaches for the analysis of biotoxins in drinking and freshwater.

Mycotoxin					
Toxins	Matrix	Instrumental approach	MLOD (ng/L)	MLOQ (ng/L)	Reference
DON; ZEA	Surface water	LC-APCI-MS/MS; C_{18} column	DON 1.4; ZEA 1.5	ND	[46]
ZEA	Surface Water; Groundwater; WWTP	HPLC-DAD and HPLC-FD; C_{18} column	HPLC water, 0.3; Groundwater, 0.3; Surface water, 0.4; WWTP, 0.5	ND	[45]
DON (3-Acetyl-,15-) AFL (B_1, B_2, G_1, G_2, M_1) citrinin, toxin (HT-2, T-2), fumonisin (B_1 and B_2) patulin	Milli-Q, drainage, river, and WWTP effluent water	HPLC-ESI-MS/MS; C_{18} column	Milli-Q water, 0.2–5.2; Drainage water, 0.3–44.9; River water, 0.3–29; WWTP, 0.4–47.7	ND	[47]
AFLs	Tank water	HPLC-FLD; C18 column	ND	ND	[138]
Bacterial toxins					
Toxin name	Matrix	Analytical equipment	MDL (ng/L)	MQL (ng/L)	Reference
MC-LR	River water	Electrochemical biosensor (PP2A)	35% inhibition LOD, 37000	ND	[76]
Anatoxin-a(s)	Lake freshwater	Acetylcholinesterase amperometric biosensor	ND	ND	[78]
MCs (-LR, -LF, -LW, -RR, -YR)	Effluent and drinking water	Direct competitive MC-LR ELISA immunoassay.	-LR, 120; -LF, 130; -LW, 140; -RR, 110; -YR, 160	ND	[80]
MC-LR	Raw drinking water	Phosphatase inhibition bioassay	2000	ND	[72]
CYN	Freshwater	LC/ESI-MS/MS, PFP column	40	100	[139]
MCs-NOD	HPLC water	HPLC- MS C_{18} column	0.2	0.7	[140]
ANA-a	Freshwater samples	GC-MS; HP 5MS fused-silica capillary column	2000	2500	[141]
ANA-a	Storage tank water	SPME–HPLC-FLD; C_{18} column	20000	ND	[108]
MCs (-RR, -LR, -LY, -LW, -LF); NOD	Treated and raw water	HPLC-PDA; C_{18} column	Treated water: 34; Raw water: 170.	ND	[97]
12 MCs (-RR, –LR, -LY, -LW, -LF, -LA, -HilR, -WR, [D-Asp_3]-LR, [D-Asp_3]-RR) CYN, ANA, NOD, OA and DA.	Lake water	LC–ESI-TSQMS; Atlantis T3 column	CYN, ANA, -RR, 1; DA, [D-Asp3], -RR, NOD, 2; -LA, 3; -YR, [D-Asp3]-LR, -LR, -LW, 4; -LF, 5; -HilR, -WR, -LY, 6; -HtyR, 7; OA, 10	ND	[100]
MC (-LA, -LR, -LF, -LW, -YR, -RR)	Drinking water	HPLC-PDA, C_{18} column	100	ND	[106]
DABA; I-BMAA; AABA; β-ABA; GABA; BOAA; ANA-a	Lake water	LC-ESI-ITMS/MS, C_{18} column	BMAA: 800. ANA-a: 3200	ND	[146]
ANA-a	Artificial bloom	LDTD-APCI-TQMS/MS	1000	3000	[128]
ANA-a	Aquaculture water	HPLC-FLD; C_{18} column	170 μg/L	580 μg/L	[127]
MC(-LA, -LF) and NOD	Freshwater	HPLC-ESI-TQMS/MS; C_{18} column	MC (-LA, -LF): 1; NOD: 9.	ND	[140]
MCs (-RR, -YR, -LR, -LY, -LW, -LF), ANA, CYN, NOD.	HPLC water	Online SPE-LC/HESI-MS/MS. Online C_{18} solid phase. Chromatography with a C_{18} column.	ANA and -LW, 10; CYN, -RR, -YR, -LR, -LY and -LF, 60;	ANA, 30; CYN, -LR, -RR, -LF, 50; -LW, 40; -LY 60; -YR, 70	[147]
MCs (-RR, -YR, -LR, -LA, -LW, -LF)	Tap and lake water	HPLC-ESI-MS/MS; C_8 column	-LA, -LW, -LF, 0.2; -RR, -LR and -YR, 0.16	-LA, -LW, -LF, 1; -RR, -LR, -YR, 0.6	[144]
ANA-a	Lake water	HPLC-ESI- MS/MS. C_{18} and an XDB columns	0.65	1.96	[145]
MC-LR, -YR, -RR, -LA, -LY and -LF, NOD, CYN, ANA-a, DA	Fresh and brackish water	UPLC–ESI-TQMS/MS; T3 column	ANA 5.6; DA, 1.2; CYN, 0.5; NOD, 1.4; -RR, 0.8; -LA, 0.7; -LR and -LY, 0.4; -YR 0.3	ANA, 18.5; DA, 3.9; CYN, 1.8; NOD, 4.6; -RR, 2.6; -LA, 2.4; -LR, 1.2; -LY, 1.3; -YR 0.8	[148]
Phytotoxins					
Toxin name	Matrix	Analytical equipment	MDL (ng/L)	MQL (ng/L)	Reference
PTA and PTB	Surface, and untreated stream water	UHPLC-MS/MS; C_{18} column	PTA, 130; PTB, 75	PTA, 440; PTB, 250	[103]
PTA and PTB	Groundwater	LC-ESI-TQMS	PTA, 190; PTB, 150 for LC-MS/MS	ND	[114]
PTD	Stream water	UPLC-ESI-TQMS; C_6-phenyl column	LC-MS/MS; PTA, 190; PTB, 150.	ND	[115]

3-methoxy-2-methyl-4-phenylbutyric acid (MMPB); β-aminobutyric acid (β-ABA), β-N-methylamino-l-alanine hydrochloride (l-BMAA); b- N-oxalylamino-ι-alanine (BOAA); 2,4-diaminobutyric acid (DABA); Acetonitrile (CH_3CN); aflatoxin B2 (AFL B2); aflatoxin B1 (AFL B1); aflatoxin G1 (AFL G1); anatoxin-a (ANA-a); Anionic exchange cartridge (MAX); Carbowax fibre (CW); Cationic exchange cartridge (MCX); cylindrospermopsin (CYN); deoxynivalenol (DON); dichloromethane (DCM); dl-2-Aminobutyric acid (AABA); domoic acid (DA); formic acid (FA); Gamma-aminobutyric acid (GABA); hexasilane (C_6); high performances liquid chromatography grade (HPLC); immunoaffinity column (IAC); loganin (LOG); microcystin-YR (MC-YR); microcystin-LF (MC-LF); microcystin-LW (MC-LW); ochratoxin A (OTA-A); octadecylsilane(C_{18}); methanol (MeOH); microcystin-LR (MC-LR); microcystin-RR (MC-RR); not declared (ND); patulin (PAT); Poly-dimethyl-siloxane (PDMS); Poly-dimethyl-siloxane-divinyl benzene fibre (PDMS–DVB); Polyamide fibre (PA); polygraphityzed carbon (PGC); pterosin A (PTA) pterosin B (PTB); ptaquiloside (PTD); saxitoxin (STX); solid phase extraction cartridges (SPE); solid phase microextraction (SPME); trifluoracetic acid (TFA); waste water treatment plant (WWTP); zearalenone (ZEA).

sample integrity during transportation and storing prior to extraction. On the other hand, the use of loganin as the internal standard was proposed to improve the repeatability of the analytical method, though it could not be employed for sample preparation. The optimised method was applied to the analysis of groundwater samples collected at the shallow water table below a Danish bracken stand, and PTD concentrations of 3.8 ± 0.24 μg/L (±sd, n = 3) were found, levels much higher than previously reported. The same group of authors has used a similar approach to investigate the occurrence of PTD in surrounding waters of Irish bracken ferns [115], in this last case using different solid phase extraction materials. And also, in another study, to assess the presence of PTD from bracken in stream water at base flow and during storm events [116]. During the last decade, several studies explore the presence of phytoestrogen residues in wastewater and natural waters. Cahill et al. [117], described a method using a linear triple quadrupole (LTQ) Orbitrap to determine with high mass resolution nine isoflavones (genistein, genistin, glycitein, daidzein, daidzin, (R,S)-equol, biochanin A, formononetin and coumestrol) in water. The samples were analysed in full scan, and several phytotoxins were tentatively identified in the samples at trace levels in a ranging from 0.0014 to 0.017 μg/L. In another study, Farré et al. [30], compared the potential of LC with triple quadrupole mass spectrometry (LC-QqQ-MS) compared with the UPLC-(Q-ToF)-MS for the analysis of biologically active compounds including phytoestrogens. The hybrid Q–ToF instrument offered the advantage of unequivocal identification of target compounds based on accurate mass measurement of the precursor ions and their products and their quantification. Accurate mass measurements of at least one production (two if available) provided qualitative information which was used for the identification of analytes in the real samples. However, high sensitivity was obtained with the QqQ instrument operated in multiple-reaction monitoring (MRM) mode with LOD from 1 to 50 ng/L for the water samples. In another study, the estrogenic activity of water with high cyanobacterial bloom was also assessed using a similar HPLC-QqQMS method in positive mode. Different phytoestrogens such as coumestrol, naringenin, daidzein biochanin A, apigenin, formononetin, equol and genistein were found [118]. This method exhibited limits of quantification (LOQs) in the range of 0.003 and 3 ng/L in water. Similar results were previously reported by Hoerger et al. (2009) which used a complementary LC-MS/MS method for the analysis of phytotoxins in river water with LOQs between 0.5 and 2.8 ng/L [48].

Mycotoxins: little attention has been paid to assess the occurrence of mycotoxins in natural waters. A first study to develop and apply a method for the quantification of DON and ZEA in natural aqueous samples at nanograms per litre concentration was reported by Bucheli et al. [46]. LC-MS/MS was coupled with APCI source in negative mode. MDL were 1.4 and 1.5 ng/L for DON and ZEA respectively. As reported by Wettstein et al. [119], DON is the most produced mytocoxin by the fungi of the genera *Fusarium*. An LC-MS/MS method was developed to evaluate the DON presence in three Swiss wastewater treatment plants. This application resulted in comparable LODs previously reported by the same authors in a different work [46].

As can be seen in Table 3, most of these approaches are focused on the analysis of the aflatoxins group and related compounds, such as OTA. As can be seen, LC-ESI-MS/MS is the technique of choice with methods LOD in the low ng/L range. Schenzel et al. [47], have developed and validated a multiresidue analytical method based on SPE using Oasis HLB cartridges followed by LC-ESI-MS/MS to assess 33 mycotoxins in waters. The method was operated under both positive and negative ionisation conditions to enlarge the range of the multiresidue approach, but higher matrix effects were observed when it was operated under negative conditions. The analytical approach showed excellent recovery percentages for most of the compounds with limits of detection below 10 ng/L for 27 of the 33 selected compounds. This method was applied to assess mycotoxins in natural waters and wastewater, and beauvericin and nivalenol were quantified in drainage and river waters, with mean concentrations of 6.7, 4.3 ng/L, 6.1 and 5.9 ng/L, respectively, for the first time [47]. A similar approach was used by Mata et al. [57], to investigate mycotoxins residues in bottled water with limits of quantification of 0.2 ng/L for aflatoxins and OTA-A. In this study, aflatoxin B2 (AFL B2) was the most frequently detected toxin with a maximum concentration of 0.48 ng/L followed by aflatoxin B1 (AFL B1), aflatoxin G1 (AFL G1), and OTA-A. Escrivá et al. [119], explored different extraction procedures for the analysis by LC-MS/MS of 11 mycotoxins (AFL B1, AFL B2, AFL G1, AFL G2, OTA, ZEA, beauvericine (BEA), enniatin A (EN A), enniatin B (EN B), enniatin A1 (EN A1) and enniatin B1(EN B1) in waters. The optimised method offered high sensitivity and unequivocal identification of the target compounds with LOD in the range of 0.1–15 μg/L [119]. A similar approach was used by Serrano et al. [120], for the analysis of several *Fusarium* mycotoxins. LC-MS/MS was used for the development of rapid analysis of emerging mycotoxins in water with LODs ranging between 0.06 and 0.17 μg/L, and LOQs in a range of 0.20–0.58 μg/L. Despite that, the presence of fungi in drinking water networks can be associated with the production of tastes and odours, and most of the works regarding this topic have been focussed on the determination of filamentous fungi [58]. In addition, recently it has been demonstrated that fungi can produce mycotoxins in water matrices in a non-negligible quantity and, as such, attention must be given to the presence of fungi in water [55].

Bacterial toxins: the most studied group of natural toxins in freshwater ecosystems are cyanotoxins. Most common approaches are as well based in SPE followed by LC or UHPLC-ESI-MS/MS methods. Oehrle et al. [121], developed a multi-residue method able to separate different MCs (RR, RY, LR, LA, LY, LW, and LF), NOD, Enkephalin, Cyclo (RADfV), ANA-a and CYN in less than 8 min. In another more recent example, the quantitative sensitive determination of MCs (LR, LY, LA, YR, RR, LF, LW) and NOD was achieved by HPLC-ESI-MS/MS [122]. The limit of detection for selected compounds was ranging from 0.1 to 0.9 ng/L.

Although chemical ionisation is not as widely used as ESI for the analysis of contaminants in water, interesting applications have been reported using laser diode thermal desorption-atmospheric pressure chemical ionisation interface coupled to tandem mass spectrometry (LDTD-APCI-MS/MS). For example, this technique was applied to the quantitative analysis of total MCs. The method consists in the cleavage of the Adda fragment contained in the MCs and the subsequent quantification of the 2-methyl-3-methoxy-4-phenyl butyric acid (MMPB) as an oxidation product obtained by MCs ozonolysis [123]. In this case, potassium permanganate was used to achieve the total oxidation of MCs contained in water samples in order to allow a faster determination [90].

Matrix-Assisted Laser Desorption/Ionisation with Time-of-Flight mass spectrometry (MALDI-TOF-MS) is another relevant technique that has been applied to the analysis of natural toxins in waters. One of the main advantages that offers MALDI-TOF-MS is the support for the tentative identification of biotoxins congeners even without standards, thanks to the high mass resolutions and the accurate mass measurements. For example, in the case of MCs there is a special Adda fragment which is contained in each variant. Moreover, this ion can be further fragmented in other ions (m/z 213 and 375) which are representative of each compound [124]. Generally, the aim of the works published using this detection technique is to characterise microbial strains or groups of MCs [124–126]. However, this technique is normally coupled to a second system such as HPLC, since quantification remains difficult.

Among the techniques based on HRMS, LC-HRMS using Orbitrap instruments provide excellent selectivity, specificity, sensitivity, and quantitation thanks to the high linear dynamic range. For example, the performance of MALDI-TOF/TOF-MS and LC-ESI-Orbitrap-MS was compared by Flores and Caixach [126] to determine intracellular and dissolved in water MCs. Both positive and negative ionisation modes were applied to obtain an extended amount of data used for the identification and confirmation of MCs. Nevertheless, high-resolution mass spectrometry can also lead to some errors, in fact, matrix effects can lead to a suppression of the signal for many matrices, surface water included. It was reported an ion suppression coming from the matrix for MC-LW, MC-YR, and MC-LW, an effect that can be reduced to assess more reliable results by using matrix-matched calibration, which can be done by preparing the calibration standards in matrix [122].

Suspected and non-target screening using HRMS techniques have been introduced to assess the presence of non-target toxins or the degradation products of natural toxins in the environment. In these cases, in general, standards are not available, and the possible structures are not clear, and only HRMS can support their tentative identification [127]. TOF-MS and Q-exactive Orbitrap are among the HRMS instruments able to carry out a full scan with a relative high acquisition frequency. Secondary metabolites, Na^+ and K^+ adducts and other unknown compounds can be included in a non-target analysis. Multiple reaction monitoring (MRM) analysis can be carried out by high-resolution detectors which are able to detect the total ion chromatogram of the fragment ions produced in a linear ion trap with high resolution and accurate mass measurements, also providing a very high sensitivity required to confirm the identity of the non-target analytes. In these cases, data processing and interpretation using specific software is required. Many online spectral libraries of natural toxins are available to be used by the search engine of the identification software. These approaches can identify those components without statistically significant differences between samples using the mass spectra reported in the libraries, helping to reach a higher confirmation level of the unknown compounds. For example, the screening of MC and ANA-a have been carried out by on-line-SPE-LC-QTOF-HRMS. In this case, MCs were previously analysed to obtain the full scan spectra and using the information for the non-target analysis of MCs variants generally not found in water. Up to 30 different most intense precursor ions were chosen from the full scan spectrum, a subsequent fragmentation by collision-induced dissociation in MRM mode was carried out. This approach is normally used for the natural toxins in water since is possible to recognize different MC compounds in algal blooms [128].

4. Future trends and conclusions

In terms of group objectives, there is a crucial gap of information about the presence of plant toxins and mycotoxins occurrence in natural waters and drinking water reservoirs. In addition, it continues to be necessary the study of natural toxins degradation products in aquatic environments and to assess their potential effects. In this regard, non-target screening and suspected screening are required approaches using techniques based on HRMS. However, the efficient application of these approaches still requires the development of specifically designed libraries.

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In general, the challenge for the analysis of this family of compounds is to develop the best testing method to face against natural toxins contamination as a preventive measure to avoid human contact. During recent years, few analytical methods for the analysis of natural toxins in water have been proposed. Most of them, are focused on the determination of single cyanobacterial toxins such as hepatotoxic microcystins and neurotoxic anatoxin. However, few works are focused on the determination of phytotoxins and mycotoxins in the aquatic environment. This could be connected to the important measures that are normally applied during the production of drinking water, that allows for obtaining safe water. However, several drinking water contamination episodes have been reported lately, which means that more efforts must be made to develop new and effective methods. Nevertheless, to date, the reported methods for the determination of natural toxins in water are generally specific for a class of similar analytes or a single compound [120,131-139].

Comparing what is reported in Table 3, most of the analytical methods were mainly focused on a group of toxins with similar characteristics or a single compound. The methods were then optimised, while taking into account only the chemical physical parameters of the selected compounds.

The first parameter is the recovery, which is influenced by the choice of a single compound to be determined. The higher the number of compounds, the lower is the recovery achieved for each. When working with only MCs, the average recovery is approximately between 45 to 90 %, while mixing MCs with alkaloids in water reduces the recovery to circa 53%. The differences in the chemical parameters and the polarity may be the most important factors that influence the retention capacity, with a competitive and preferential bonding with the polar moieties of the sorbent phase.

Moreover, most of the reported methods employ one sorbent phase (mostly HLB) which is the most used in environmental applications. The detection limits are also variable, leading to minimum LOD of 0.002 µg L^{-1} for MCs, 4 ng L^{-1} for mycotoxins and 0.4 ng L^{-1} for plant toxins.

However, the need for a comprehensive method to determine a wide range of structures, using the least possible time, is real. There are thousands of compounds that are not included in any screening protocol due to the low presence in water reported in the literature. However, the concomitant presence with other natural toxins can potentially constitute a threat for the surface water environment and for human health. For this reason, it is necessary to develop and optimise comprehensive methods that can determine a wide range of toxins in a single method, while taking into account the lack of standards for the confirmation step.

1.8 The environmental occurrence of natural toxins in surface water

Cyanotoxins

The study of the occurrence of cyanobacteria had its starting point due to the reports of livestock poisoning. In most cases, the animals had previously consumed water from water bodies where there was a clear contamination of scum formed by cyanobacteria on the lake surface [140]. Nowadays, it is known that cyanobacteria are ubiquitous organisms that can be found in different type of habitats, from the extreme regions, such as the arctic or deserts, to the temperate and tropical climate regions. Hepatotoxic cyanobacteria are common in Europe, Australia, and America, while CYL with other cyanotoxins, produced concomitantly, have been reported in Israel, Japan, Hungary, and Australia [141].

This may be assumed to have in a ubiquitous presence around the world (**Table 5**)

Among natural toxins, Microcystins are the most reported cyanotoxins worldwide (63 %; 699 out of 1118), followed by CYL (10 %; 107 out of 1118), and ANA (9 %; 100 out of 1118), while nodularins were the least reported cyanotoxins (2 %; 19 out of 1118) [142].

Table 5: Global occurrence of cyanotoxins [142]

Cyanotoxins	Europe (%)	North and Central America (%)	Asia (%)	Australia and New Zealand (%)	South America (%)	Africa (%)	Globe (%)
MCs	58	57	79	55	63	77	63
CYNs	18	4	10	8	3	2	10
ATXs	10	15	3	5	7	9	9
STXs	10	3	3	21	13	1	8
NODs	1	0	1	4	0	3	2
Others	1	22	4	7	14	8	9
Total samples	341	238	168	166	105	100	1118

Recently, Namsaraev et al.[143] described the occurrence of cyano-HAB in different climates in the Russian Federation. The geographical distribution of cyano-HAB events showed that contamination of MCs could affect most of the climate zones, including water environments from the polar regions to the arid regions.

Other groups of cyanotoxins such as Nodularin, produced by Nodularia spumigena, Aphanizomenon spp., and Anabaena spp., have been found less extensively in, for example, pelagic waters of the Baltic region. Nodularin occured especially during summer and covering huge surfaces of lakes and rivers. Nodularia spp. was detected in estuarine waters of the Baltic Sea. Further, MC-LR, MC-RR, MC-LY, MC-YR were detected at concentrations between 0.1 and 134.2 µg L^{-1} with one demethylated MC having a concentration of 7.5 µg L^{-1} [144,145].

CYL has been reported in different countries all over Europe. Germany, Hungary, Italy, Spain, Finland, Czech Republic, and France reported different bloom episodes with a production of dangerous *Cylindrospermopsis* spp. CYN that was reported to be present in different lakes in Germany between 1990 and 2000, while in 2004 a concentration of 0.34 - 1.80 µg L^{-1} was found in Melangsee and Lnager See,

respectively (Germany). CYN contamination in European waters has been enforced with the report of high concentrations in western France with concentrations higher than 1.95 µg L^{-1} due to the presence of *Aphanizomenon flos-aquae* bloom. Later, Italy experienced in 2004 a blooming onset in two lakes (Trasimeno and Albano) with concentrations ranging from 0.41 to 126 µg L^{-1}. Spain, Hungary, and the Czech Republic have reported CYN contaminations of their waters that are used for the production of drinking water or bathing activities with levels ranging from 0.16 to 9.4 µg L^{-1} [146].

Moreover, the occurrence of microcystins in rivers has been related with the seasonal climate variations and the chemical-physical parameters of the rivers around the globe. Northern America has been highly reported as the second highest occurrence zone for cyanobacterial blooms. Here, a report from Svirčev et al. [142] highlighted the presence of cyanobacterial species along different rivers in eastern, western, and mid-America. The most common genera identified in North and Central America were *Microcystis* spp., *Anabaena* spp., *Aphanizomenon* spp., *Lyngbya* spp., and *Cylindrospermopsis* spp, respectively. A slight dependence with the respect to the presence of phosphor in water can be observed (**Table 6**). Toxigenic genes increased as the phosphor levels raised in water. However, this parameter was not correlated with the production of microcystins. Only two sampling points reported the presence of MCs that were over the detection limits.

Table 6: Northern America survey for the occurrence of cyanobacteria genes and cyanotoxins.

	Total nitrogen (mg L^{-1})	***Total phosphorus (mg L^{-1})***	***DOM (mg L^{-1}***	***Cyanotoxins (µg L^{-1})***	***mcy Genes***
Connecticut River	0.541	0.035	6.7		0
Sacramento River	0.208	0.037	21.9		3
Willamette River	0.55	0.054	6.3		3
Delaware River	1.142	0.071	6		3
Susquehanna River	1.371	0.071	31.2		4
Mississippi River	4.596	0.126	52.7	0.1	14
Ohio River	1.74	0.159	63		8
Chattahoochee River	2.893	0.166	163		1
Missouri River	2.555	0.348	260.3		18
Kansas River	2.29	0.536	411.8	0.18	9
Trinity River	6.096	0.783	107.3		9

Similar behaviour was also reported in a survey [147] in Chinese lakes when cyanobacterial blooming occurred. Here, the relationship between the *Microcystis* biomass and the temperature was reported. Liu et al., [147] showed that the most prominent phytoplankton groups were positively correlated with pH, DOM and temperature. The blooming season was between May and November, which also followed the decrease of the N/P ratio < 30.

The Atlantic region and especially the islands in the Atlantic sea were also studied, reporting a great diversity on the cyanobacteria species, depending on the habitat.

Freshwater presents the highest number of taxa, with respect to the other studies in the literature, with the occurrence of 122 and 129 different species of cyanobacteria in Azores islands and Curaçao islands, respectively. Almost 75 % and 90 % of the territory is represented by a freshwater environment, however, brackish, thermal, marine, and terrestrial habitats increase the number of reported taxa to 187 and 127 taxa, respectively. Cyanobacteria richness is uneven among islands and is habitat dependent. The Azores and Cuba presented the highest cyanobacterial diversity, while Puerto Rico and the United States Virgin Islands registered the lowest number of species (1 and 3 respectively) [148].

Thirty-two (32) different species of *Nostocales, Oscillatoria*, and *Microcystis* were reported by Sant'Anna et al. [149]. Here, Brazilian lakes have been reviewed, reporting 14 species in the tropical zone and 27 species in the subtropical zone. Billings Reservoir in Brazil, also reported a high biomass composed of *Anabaena spiroides*, *Aphanocapsa sp, Woronichinia naegeliana, Cylindrospermopsis philippinensis,* and *C. raciborskii, Merismopedia tenuissima, Microcystis aeruginosa, M. panniformis, Microcrocis sp, Planktothrix agardhii, Pseudanabaena sp, and Galeata* [150].

Table 7 reports the maximum worldwide levels of cyanotoxins found in lakes and rivers. Three *Microcystis aeruginosa* strains were isolated from Moroccan lakes, Aguelmam, Azizgza, and Dayet Afourgah, respectively, in October 2005.

Here, four MCs congeners (MC-WR, MC-DM-WR, MC-YR, MC-RR) were identified [151]. Comparing the literature from 6 continents (Africa, Europe, South and North America, Oceania, and Asia) it is possible to state the widespread nature of cyanotoxins all over the world; a complete work was published by Díez-Quijada et al. [152].

When comparing the presented data in these works, it is possible to observe that there is no prevalence of a *Mycrocystis* genera with respect to the others. Europe has the highest variability in MCs congeners while the lowest is Oceania with only 7 congeners reported, among them anatoxins and cylindrospermopsins (**Figure 12**). Further, until now, Oceania is one of the regions that is the least affected by Cyanobacteria, while Europe is the most affected with many cases of cyano-HAB in different countries. This could also be due to the very different climatic regions that Europe has, ranging from the Mediterranean, continental, central European, and Maritime climates [153]. America experienced several algal bloom in different places, Argentina (Los padres Lake) with MC-LAG and MC-YR that ranged between 0.01 to 2.92 μg L^{-1}, while higher concentrations were found in Guatemala (MC-LR 6.48 μg L^{-1}), west EEUU (MC-LA, 54 μg L^{-1}) and Canada (MC-LA, 203 μg L^{-1}) [152].

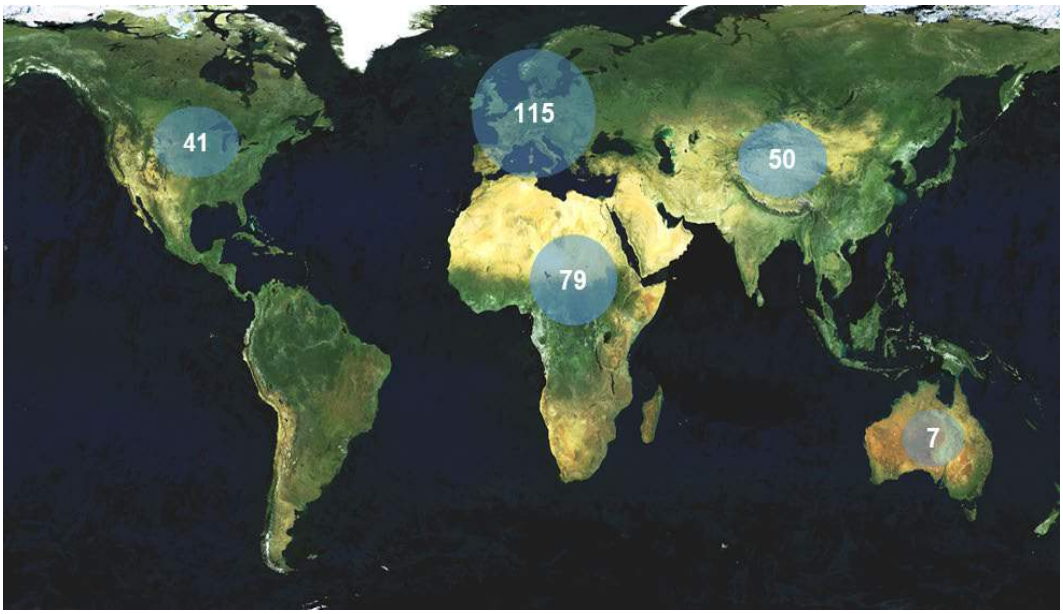

Figure 12: Number of cyanotoxins variants reported worldwide

In mixed field populations of cyanobacteria, MC variant occurrence depends on the interactions between the variable composition and the predominance of cyanobacterial strains in freshwater, which are related to its trophic status, and environmental factors such as light, nutrients, temperature, hydrological parameters, etc., although these relationships are complex. The occurrence of individual MC variants is very scarce, whereas a mixture of distinct MC variants is present in nearly all water reservoirs. Globally, among minority MC congeners, MC-YR and MC-LA are the variants which are most frequently detected in water samples, and in some cases they are predominant globally, although while it is recognised that the variability of MC profiles produced by cyanobacteria is highly strain-dependent, further studies are needed to clarify the role of climatic and environmental factors on the variability in the production of MC variants, particularly those other than MC-LR, in natural waters.

Table 7: Latest occurrence of cyanotoxins in water around the world

Country	Sampling point	Sampling period	Toxins	Max levels or ranges (µg/L)	Reference
Australia	Murray River	2016–2017	ATX	0.15 - 4	[113]
Argentina	Río de la Plata Estuary		MC-LR	8.6	[154]
	Salado River		STX	105.33	[155]
	Uruguay River		MC	0.6	
			STX	0.31	
			ANTX	0.055	
	Paraná river		MC-LR	1.9	[156]
			MC-RR	1.23	
			[D-Leu1] MC-LR	37.7	
	Paso de las Piedras		MC	0.17	[157]
	San Roque		MC-LR, RR, YR	920	
			ANTX	0.0066	[158]
	Piedras Moras		MC	0.23	[159]
	Salto Grande		MC-LR	48.6	[160]
	De los Padres		MC-LR	0.32	[136]
			MC-RR	12.3	
			MC-LA	2.14	[161]
			MC-YR	0.13	[162]
Colombia	Abreo Malpaso reservoir	May 2015- Oct 2016	MC-LR	6.715	[113]
			MC-YR	11.4	
			MC-RR	ND	
			[D-Asp3,(E)-Dhb7]-MC-RR	20.3	
			MC-LF	0.21	
			MC-LW	0.13	
			NOD	3.51	

			CYL	ND	
	Peñol reservoir		MC-LR	72.1	
			MC-YR	24.5	
			MC-RR	0.23	
			[D-Asp3,(E)-Dhb7]-MC-RR	10.9	
			MC-LF	6	
			MC-LW	4.6	
			NOD	1.5	
			CYL	1.3	
	Playas reservoir		MC-LR	15.2	
			MC-YR	19.2	
			MC-RR	ND	
			[D-Asp3,(E)-Dhb7]-MC-RR	21.3	
			MC-LF	1.7	
			MC-LW	0.35	
			NOD	1.7	
Czech Republic	94 water reservoirs	July – September 2004	MCs	37	[138]
France	Lake Aydat	September – October, 2011 – 2013	MCs,	0.077	[163]
	Reservoir Pen Mur	May 2016 – Apr 2018	MCs	60	[164]
Germany	Lakes Langer See and Melangsee	Jun - Sept 2004 Apr - Oct 2005	CYN	1.8	[165]
	Lakes Klostersee,	May - Oct 2015 (Klostersee)	MCs	6.7	[166]
Greece	Kastoria	Sep-2007	MC-RR	17.3	[167]
		Sep-2014	MC-LR	45.8	
		Oct-2014	dmMC-RR, MC-RR, MC-YR, MC-HtyR, dm3MC-LR, MC-LR, MC-HilR,MC-WR, MC-LA, MC-LY, MC-LW, MC-LF	9.6 - 354	

		Sep-2015	dmMC-RR, MC-RR, MC-YR, MC-HtyR, dm3MC-LR, MC-LR, MC-HilR,MC-WR, MC-LA, MC-LY, MC-LW, MC-LF	0.007 - 0.373	
		Oct-2015	dmMC-RR, MC-RR, MC-YR, MC-HtyR, dm3MC-LR, MC-LR, MC-HilR,MC-WR, MC-LA, MC-LY, MC-LW, MC-LF	0.1 -63	
		Sep-2016	dmMC-RR, MC-RR, MC-YR, MC-HtyR, dm3MC-LR, MC-LR, MC-HilR,MC-WR, MC-LA, MC-LY, MC-LW, MC-LF	0.05 - 36.5	
	Pamvotis	Sep-2014	CYN, MC-RR	2.8	
		Oct-2014	MC-RR, MC-YR, MC-LR	21.1 - 67	
	Zazari	Jun-2014	MC-RR, MC-LR	17 - 25	
	Vegoritis	Jul-2014	dm3MC-LR, MC-RR, MC-LR, MC-YR	11 - 109	
	Mikri Prespa	Nov-2014	MC-RR, MC-YR, MC-LR	36 - 41.2	
Italia	Garda	2016	[D-Asp3]-RR	70-100	[139]
			[D-Asp3]-LR	0-20	
			[D-Asp3]-HtyR	0-5	
			MC-LR	0-2	
			MC-RR	0-2	
	Iseo	2016	[D-Asp3]-RR	70-97	
			[D-Asp3]-LR	0-12	
			[D-Asp3]-HtyR	0-45	
			MC-LR	0-10	
			MC-RR	0-5	
	Como	2016	[D-Asp3]-RR	60-100	
			[D-Asp3]-LR	0-10	
			[D-Asp3]-HtyR	0-30	
			MC-LR	0-60	
			MC-RR	0-1	
	Lugano	2016	[D-Asp3]-RR	75-92	

			[D-Asp3]-LR	0-10	
			[D-Asp3]-HtyR	1-4	
			MC-LR	0-4	
			MC-RR	0-2	
	Lake Vico	Feb 2009 – Dec 2010	MCs	1.4	[168]
	Lake Alto Flumendosa	Oct 2011 – May 2013	MCs	100	[169]
	Lake Occhito	Apr 2009 – Dec 2012	MCs	7.5	[170]
	Pusiano lake	Apr 2009 – Dec 2012	MCs	4.6	
	Ledro Lake	Apr 2009 – Dec 2012	MCs	1.15	
	Garda Lake	Apr 2009 – Dec 2012	MCs	0.26	
	Garda Lake	Feb 2014 – Oct 2015	ANA	2.2	[171]
	Garda Lake	Sept 2008 – Sept 2013	MCs	0.23	[172]
Mexico	SMO crater lake, Nayarit	Sept 2014 - Apr 2016	MC-WR, MC-LR, MC-LA, MC-HilR, MC-LF, MC-YR, and MC-LY.	ND	[173]
Morocco	Aguelmam Azizgza	October 2005	MC-WR, MC-RR, MC-DM-WR, and MC-YR	859.6 (µg MC-LR eq./g biomass)	[151]
	Dayet Afourgah			688.4 (µg MC-LR eq./g biomass)	
Netherlands	Lakes Nuldernauw, Wolderwijd, Zoetermeerse Plas, De Put, De Grote Plas	Aug - 2013	MCs	0.31	[174]
Poland	Mytycze	May – Sept 2010 and 2011	MCs	30.68	[175]
	Tomaszne		MCs	23.62	
	Zemborzycki Dam	May – Sept 2005 –2006	MCs	22.2	[176]
			ANA	14.4	

	Lake Lubosinskie	Jul 2006 – Mar 2008	MCs	71.2	[177]
Portugal	Alvito lake	May – Dec 2005 Apr – Jul 2006	MCs	2.58	[178]
	Enxoé reservoir	May – Dec 2005 Apr – Jul 2006	MCs	0.63	
	Odivelas reservoir	May – Dec 2005 Apr – Jul 2006	MCs	0.5	
	Roxo reservoir	May – Dec 2005 Apr – Jul 2006	MCs	7.2	
	Reservoirs Alqueva and Beliche	Feb - Nov 2011	MCs	0.776	[179]
Russia	Lakes Suzdal	Jun – Oct 2010 Jun – Sept 2011	MCs,	41.37	[180]
	Sestroretskij Razliv	May – Sept 2012	ANA	0.54	[181]
	Volga river	Jun-2016	MC-LY, MC-LF, MC-LW, MC-LR, YR, [D-Asp3]MC-RR, [D-Asp3, Dha7]MC-RR, [D-Asp3]MC-LR	0.01 − 0.03	
		Aug-2016	MC-LY, MC-LF, MC-LW, MC-LR, YR, [D-Asp3]MC-RR, [D-Asp3, Dha7]MC-RR, [D-Asp3]MC-LR	7.2 − 16.4	
		Aug-2018	MC-LY, MC-LF, MC-LW, MC-LR, YR, [D-Asp3]MC-RR, [D-Asp3, Dha7]MC-RR, [D-Asp3]MC-LR	0.1 – 1.8	
	Kama reservoirs	Aug-2016	MC-LY, MC-LF, MC-LW, MC-LR, YR, [D-Asp3]MC-RR, [D-Asp3, Dha7]MC-RR, [D-Asp3]MC-LR	2.7 − 9.0	
	Tsimlyansk Reservoir	Aug-2018	MC-LY, MC-LF, MC-LW, MC-LR, YR, [D-Asp3]MC-RR, [D-Asp3, Dha7]MC-RR, [D-Asp3]MC-LR	2.2 – 3.6	

	Kazanka River	Aug-2016	MCs	1.4–12.1	[143]
		Aug-2016	ANA	0.057– 0.294	
	Olyutorskiy Bay	Jul-2017	STX	330	
Spain	Reservoir Ojos	Oct 2000 – Sept 2001	MCs	0.17	[48]
	Reservoir Cenajo		MCs	0.085	
	Reservoir Rosarito	Jun – Oct 2013	MCs	18.6	[182]
			ANA	2.1	
			STXs	0.12	
Turkey	Lake Egirdir	Apr – Dec 2013	MCs	20.5	[183]
	Lake Sapanca	Sept 2012 – Oct 2013	MCs	1.522	[184]

Mycotoxins

There is scant information available on the growth and the spreading of fungal species, in water environments. Data comparison is an additional issue due to the lack of standardised methodologies for their analysis in water samples. Also, up to now, the time-consuming colony-forming units (CFU) are employed universally to give summary information on their presence in water, and the data can be misinterpreted. Their occurrence in water can be linked to the fact that fungal species, plants, and bacteria can leach into water streams, thus leading to the contamination of surface water that is used for drinking water production [185]. Mycotoxins produced in streams tend to decay in the treatment plants during the potabilisation process. However, due to their tolerance and resistance to different environments, some species of fungi can colonise drinking water distribution systems, that are typically low in nutrients. They may enter drinking water distribution systems through several contamination pathways, including treatment deficiencies, water storage, or environmental conditions, among others. Once introduced, fungi can establish a layer into pipes thus producing biofilms within distribution systems, and consequently producing toxic secondary metabolites.

There is evidence on the presence of fungi and moulds in the drinking water supply systems. **Table 8** summarizes some examples of mycotoxins and fungi that have been reported to be present in different water environments, including surface, drinking, storage, and bottled water.

Table 8: List of mycotoxins and fungi reported in the literature found in water

Analyte	Genera	Origin/Matrix	Country	Results	Ref.
Bacteria	*Aspergillus spp.*	Surface water	Norway	1- 16 cfu mL^{-1}	[186]
	Aureobasidium spp.	Groundwater		1-3 cfu mL^{-1}	
	Beauveria spp.	Groundwater		1-3 cfu mL^{-1}	
	Cladosporium spp.	Surface water		1-14 cfu mL^{-1}	
	Fusarium spp.	Surface water		1-100 cfu mL^{-1}	
	Penicillium spp.	Groundwater		1-13 cfu mL^{-1}	
	Trichoderma sp.	Surface water		1-12 cfu mL^{-1}	
	Verticillium spp.	Surface water		1-2 cfu mL^{-1}	
Bacteria	*Phialophora, Acremonium, Exophiala, and Penicillium*	Drinking water, groundwater, tap water, and store tank	Germany	1-41 cfu mL^{-1}	[187]
Bacteria	*Penicillium, Acremonium sp, Phialophora, Cladosporium, Rhizopus, Alternaria, Aspergillus, and Chaetomium*	tap water	Portugal	1- 60 cfu L^{-1}	[131]

Bacteria	*Flagellospora curvula, Tetrachaetum and Alatospora*	Surface water	Africa		[188]
Fungi	*Allomyces spp., Fusarium, Penicillium, Trichoderma and verticillium, Aspergillus, Stachybotrys*	Surface water	Egipt	nd	[189]
aflatoxin B_2	*Aspergillus spp.*	Tap water	England	0.2 - 1.7 ug L^{-1} (B_2); 0.1 ug L^{-1} (G_2)	[28]
aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, fumonisin B_1,	*Cladosporium, Fusarium and Penicillium*	Bottled water	Portugal	0.48 ± 0.05 ng L^{-1} (B_2); 0.70 ng L^{-1} (B_1); 0.60 ng L^{-1} (G_1); 0.26 ng L^{-1} (OTA)	[190]
Fungi	*Aspergillus sp.*	Surface water, Groundwater	Portugal	Aspergillus sp. (100 – 1000 CFU 100 mL cfu mL^{-1}); Cladosporium (100-1000 CFU 100 mL^{-1}); Fusarium 1000 CFU 100 mL^{-1}); Penicillum (100 - 1000 CFU 100 mL^{-1})	[191]
zearalenone and 2 ZEA metabolites		Surface water	Italy	0.1 ug mL^{-1}	[192]
zearalenone		Surface water; groundwater	Poland	0.5 to 43.7 ng L^{-1}	[132]
citrinin; alternariol	*Alternaria; Penicillum; Cladosporium*	Mineral bottled water	Argentina	0.517 µg mL^{-1} (citrinin)	[193]
zearalenone	*Fusarium graminearum*	Drainage water from crop field	Switzerland	30 ng L^{-1}	[133]
zearalenone		Drinking water		15.0 ng L^{-1}	[135]

As can be seen, aflatoxins, zearalenone, and ochratoxin, together with their related fungi, were detected in different water samples. Aflatoxin B_1 and its congeners were reported in tap waters and bottled water. It is also recognised that fungal contamination is generally absent or minimal in domestic tap water, however, more knowledge on fungi and their toxins is still needed.

In the United Kingdom (UK), an average of 32 different fungal species were isolated from the surface and drinking waters. Results were compared showing the main presence of particular species of *Aspergillus, Cladosporium, Epicoccum, Penicillium*, and *Trichoderma*, which seem to be very common [78]. Similarly, bottled water can also present fungal contamination, as reported by Mata et al., [190]. In their study,

55 % of the samples were positive for aflatoxins and ochratoxin. AFLB_1 and B_2 were the most detected mycotoxins with concentrations of 0.70 and 0.48 ng L^{-1}, respectively, followed by AFL G_1(0.60 ng L^{-1}) and ochratoxin A (0.26 ng L^{-1}) [79]. Eighty-nine (89) genera of moulds occurred unexpectedly in water samples coming from unfavourable environments such as Norwegian surface and drinking water [186].

Plant toxins

Plant toxins are substances that are produced as secondary metabolites in poisonous plants that are able to grow in most of the different climates around the world. Their toxic effects can affect nearly all living creatures, from insects to humans. The major economic loss is due to livestock poisoning as a result of the ingestion of indolizidine alkaloid lupines, locoweeds, larkspurs, and other poisonous plants. There are many plants, some of which are in agricultural settings, and cultivated at high volumes, which can produce harmful phytotoxins. **Table 9** summarises the most reported phytotoxins and the producing plant that can generate them in the environment.

Table 9: Phytotoxins classes, plants producers (with toxins examples) and adverse effects

Toxicants	Plant family	Toxins	Toxicology
Alkenyl benzenes	*Myristicaceae, Labiatae, Lauraceae, Piperaceae*	Estragole, elemicin, apiole myristicin, safrole	Genotoxic and carcinogenic
Anthraquinones	*Labiatae, Lauraceae, Piperaceae, Verbenaceae, Rhamnaceae*	Aloin	Acute renal failure
Capsaicinoids	*Solanaceae*	Capsaicin, dihydrocapsaicin	Stomach ulcers, neurogenic inflammation, vasodilatation, analgesic properties
Coumarins	*Leguminosae, Rubiaceae, Umbelliferae*	Imperatorin, coumarin	Anti-inflammatory, anti-cancer, antibacterial
Cucurbitacins	*Cucurbitaceae*	Cucurbitacin -A, -B, -C	purgative, hepatoprotective, antifungal, anti-inflammatory, cytotoxic, and antineoplastic
Cyanogenic glycosides	*Leguminosae, Gramineneae, Rosaceae*	Prussic acid; amygdalin	acute cyanide poisoning; vomiting

Furocoumarins	*Umbelliferae, Rutaceae,*	Psoralen, imperatorin, xanthotoxin	Kidney and liver toxicity
Glucosinolates	*Cruciferae; Brassicaceae; capparaceae*	Sinigrin	Antimicrobial, insecticide, antifungal
Glycoalkaloids	*Solanaceae*	Tomatidine, solanidine, spirosolane	Insecticide, antifungal, proteinase inhibitor
Glycyrrhizinic acid	*Leguminosae, Sapindaceae*		Inhibitory effects on hepatocyte apoptosis and liver fibrosis; anti-inflammatory and antioxidant activities
Proteinase inhibitors	*Leguminosae*	Jasmonic acid; jasmonate	Digestive system toxicity
Isoflavonoids	*Leguminosae, Rosaceae, Vitaceae*	Daidzein and genistein	Antioxidant, antimutagenic, anticarcinogenic, antiproliferative activities
Lectins	*Leguminosae*	Coumestan and coumestrol; formononetin, biochanin-A; daidzein; genistein	Chronic renal diseases
Oligosaccharides	*Leguminosae; Cucurbitaceae, Oleaceae*	Asparagine	Cytokines inhibition; antiinflammatory
Pyrrolizidine alkaloids	*Asteracea, Boraginaceae, Leguminosae, Sapindacea*	Lasiocarpine; retronecine, heliotridine; senecionine	Hepatotoxic; chronic heart damages; necrosis
Quinolizidine alkaloids	*Berberidaceae, Chenopodiaceae, Leguminosae, Solanaceae; Asteraceae, Fabaceae*	Pyridone, anagryine; lupine; lupanine; sparteine	
Saponins	*Leguminosae; Chenopodiaceae*	Diosgenin	Surfactants; hyperlipidemic and hypolipidemic action
Sesquiterpene lactones	*Asteraceae, Convolvulaceae, Rutaceae, Umbelliferae*	Syringin, janerin, cynaropicrin; artemisinin; limonene, pinene	Antifeedant

Toxic amino acids	*Leguminosae*		Antimetabolites
Toxic fatty acid	*Cruciferae*		Cardiac toxicity
Xanthin alkaloids	*Buttneriaceae, Rubiaceae, Theaceae*	Caffeine; theobromine; theophylline	Ocular irritations, carcinogenicity, oral toxicity, genotoxicity

Most of these have been reported in edible fruits and vegetables. However, the potential presence of natural toxins in water is a reality that is connected to their chemical properties, which allow a rapid transfer from plants to soil and water (leaching) and their extremely extended presence all over the world. Leaching is defined as the removal of substances from plants by the action of aqueous solutions, such as rain, dew, mist, and fog. Hydrophilic properties of phytotoxins allow a rapid transfer into water bodies. One of the most important examples has been reported by Clauson Kaas, who provided some evidence of the presence of carcinogenic ptaquiloside in surface water [194] and groundwater [195]. The same behaviour of bracken ferns can also explain the similar leaching potential of other hydrophilic phytotoxins in water. **Figure 13** illustrates an example of the most probable contamination pathways of phytotoxins produced during agricultural activities and from the natural vegetation that is present in a given area.

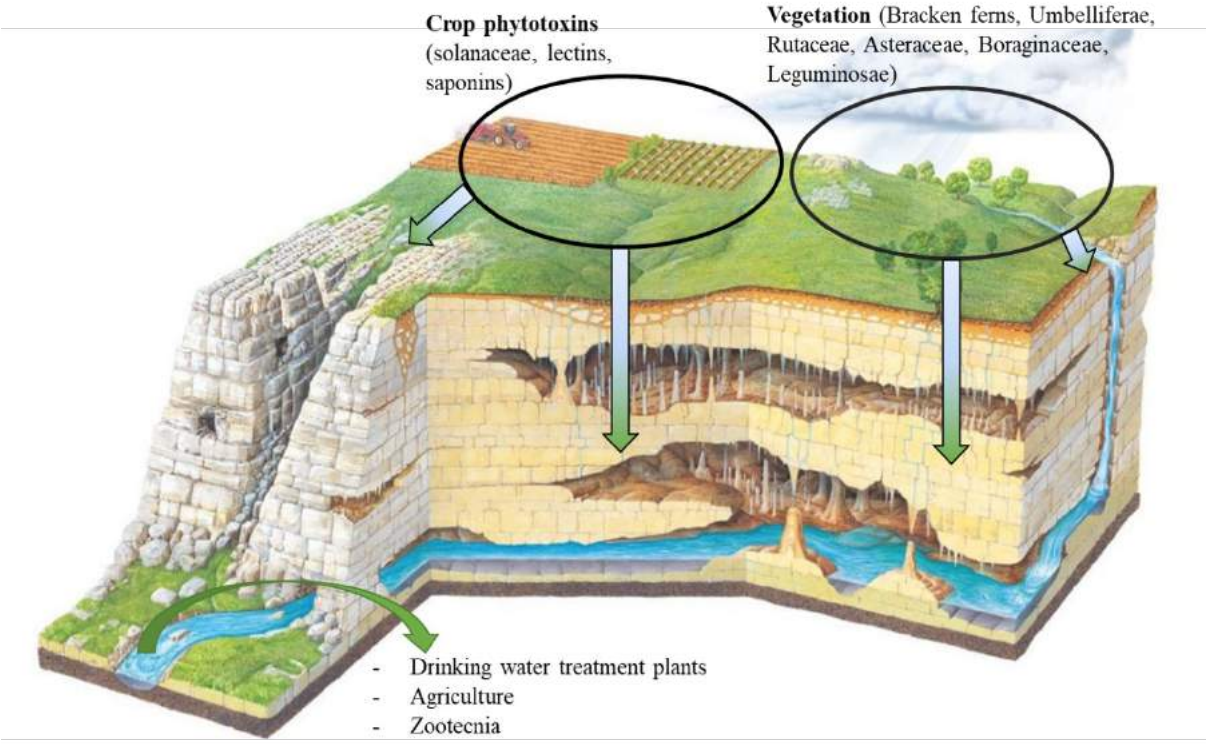

Figure 13: Example of sources of phytotoxins from agriculture and vegetation into groundwater and surface water.

However, the occurrence of water contamination by phytotoxins is poorly studied. Ptaquiloside was mostly reported in water and groundwater, while the first screening of plant toxins in Swiss and Spanish waters has been recently addressed by Günthardt et al. [196] and Picardo et al. [197], respectively.

Their effects on humans vary from causing individual poisoning to mass outbreaks, and from having common minor to rare fatal consequences. Very young children are at the highest risk from plant toxins, as they regularly eat or drink almost anything of suitable size. Further, they are more at risk due to their less effective immune systems, hence they form the most important risk group. For instance, water hemlock (*Cicuta sp.*), poison hemlock (*Conium maculatum*), and hemlock water dropwort (*Oenanthe crocata*) contain highly dangerous toxins. Toxins can be present in some or all parts of plants, including the roots, leaves, fruits, and seeds; and the toxin can be effective when taken internally or through contact with the skin.

2 . OBJECTIVES

2.1 Objectives

Natural toxins are diverse groups of toxic compounds produced by living organisms. Generally, they are not considered as environmental contaminants concerning water quality. Besides, their studies regarding the occurrence, distribution, and degradation processes in the environment. Due to the lack of identification and quantitation methods for natural toxins, the studies of the risk assessment are difficult and the toxicity degree generated by the presence of natural toxins remains unknown. Besides, during the last years, many scientific efforts are being spent to develop and optimize methods to screen, identify, and finally quantify known and unknown natural toxins.

Considering the very huge amount and variety of natural toxins that are possible to encounter in the water environment, the needs of analytical methods for their determination are of primary importance. However, most of the analytical methods published until now are mainly focused on the analysis of a single compound or a group of compounds with the same physic-chemical characteristics. Besides, the availability of certified standards remains a problem which makes the confirmation of natural toxins an issue requiring additional effort to confirm the suspect compounds. Considering this, it is necessary to develop and optimize methods that can isolate and screen a wide range of natural toxins from water.

Under this context the overall objectives of the present doctoral thesis are:

1. The development of a suspect screening analytical method for the tentative identification of natural toxins in surface water. Different groups of mycotoxins, phytotoxins, and cyanotoxins have been selected due to the extensively reported presence in rivers and lakes. Therefore, they are a recognized risk for human health due to the potential contamination of water resources used for drinking water production. From the analytical point of view, HRMS techniques were aimed to develop a suspect screening method for the tentative identification of suspect natural toxins in the water.

2. Study the occurrence of these groups of natural toxins in the Ter River (Barcelona, Spain) considering the different seasons and the possible variations that can occur depending on the blooming period.

3. Additional samples coming from Italy and Czech Republic have been analyzed in the frame of the development of a suspect screening approach using the All Ion fragmentation (AIF) acquisition mode.

The specific objectives were:

1. Develop and optimize a clean-up and concentration step for a wide range of natural toxins using offline SPE with different sorbents.

2. Develop and optimize an LC-HRMS/MS method for the analysis of a wide range of natural toxins in surface water aimed to carry out a suspect screening procedure to tentatively identify suspect compounds.
3. Build a suspect list of natural toxins mostly found in surface water to use it as a search list in the suspect screening procedure.
4. Apply the screening method to tentatively identify suspect natural toxins in different sampling points along the Ter River (Barcelona, Spain), one of them used as drinking water supply, taking into account the different sampling seasons and the botanical diversity

3. TARGET AND SUSPECT SCREENING OF NATURAL TOXINS IN SURFACE WATER

3.1 Introduction

The latest advances in analytical techniques using High-Resolution Mass Spectrometry (HRMS), permit an improvement in the confidence levels for the prioritisation of new contaminants in the aquatic environment. Natural harmful pollutants endangering wildlife and humans are still relatively unknown. Phytoestrogens, phytotoxins, mycotoxins, and microcystin (MC) variants have been previously detected in surface water. Once they are released into the environment, degradation and oxidation reactions can occur, generating transformation products that may be more persistent or more toxic [198]. Many of these metabolites are still unknown, hence their structural characterisation is of primary importance.

The main HRMS analysers, such as Quadrupole-Time of Flight (QTOF), Orbitrap, and Fourier Transform Ion Cyclotron Resonance (FTICR) have been used for suspect screening approaches [199]. However, several protocols which are aimed at the best fit of the data in order to attain the highest level of confidence in the structure identification, have been proposed by several authors [200-202] and, in a certain way, also by the European Community (EC) [203].

In the following sections, the latest criteria to be used as confidence levels in expressing the reliability of suspect compounds are reported. Under this frame, databases are also a crucial factor to be considered when applying a suspect screening method. Then, a chapter with a review of the literature has been included, in order to discuss the most used databases and lists which have been developed for the analysis of natural toxins.

3.2 Past and present analytical criteria for identification and confirmation purposes

The analytical criteria which are aimed at identifying and confirming suspect structures have changed over the years. In 1996, the European Community (EC) published the first Council Directive 96/23/EC of 29 April 1996 [204] on measures to monitor certain substances and residues. According to Council Directive 96/23/EC, two main groups (A and B) of substances should have been monitored to guarantee a high level of human health protection. Group A comprises the prohibited substances for which no maximum residue limits could be established, while group B encompasses all registered drugs and other contaminants in food and feed. Later, the document was revised thus setting out the basic regulations for the performance of the analytical method, including identification guidelines, performance assessment, and validation procedures [203]. The previous criteria that were used for identification purposes were absolute and relative retention times, UV, and spectra from MS. The confirmation was possible only when four ions with a given ratio were present. Then, the Commission Decision 2002/657/EC of 1 August 2002 introduced a system of identification points and given tolerances for the relative ion intensities [203].

In brief, full-scan (FS) and single ion monitoring (SIM) were considered. When working in FS, a minimum of four ions must be present with a relative intensity of ≥ 10 % with respect to the base peak. The molecular ion must be included in the

reference spectrum with a relative intensity of ≥ 10 %. Notwithstanding, the library search can be used and, in this case, the comparison between the experimental and theoretical spectra needs to reach a given match value, which will be determined experimentally for each analyte.
Then, when acquiring information in SIM with further precursor fragmentation, an identification points (IPs) method is used for confirming the data whereby, at least 4 IPs are required for a particular compound. A minimum of 3 IPs are required for the confirmation of group B [203]. In **Table 10,** the identification points and the different acquisition methods are reported.

Table 10: Mass fragments and identification points earned [adapted from OJ L 221, 17.8.2002, p. 8–36]

Mass Spectrometry	**ID points**
Low resolution mass spectrometry (LR)	1,0
LR-MS^n precursor ion	1,0
LR-MS^n transition products	1,5
HRMS	2,0
HR- MS^n precursor ion	2,0
HR-MS^n transition products	2,5

However, the Commission Decision 2002/657/EC was not purposed to identify environmental contaminants, and the recent advances in the analytical instrumentation were not included. New analytical methods with higher levels of accuracy for the identification and quantitation of environmental contaminants were required. Due to this reason, the use of FS-HRMS/MS has increased during recent years. HRMS/MS is instrumental in the identification of an extremely high number of compounds. Further, their resolutions support the reconstruction of highly selective retrieval of accurate mass spectra of target analytes in complex matrices.
Non-targeted evaluation of acquired data is an additional point that leads to detect non-a priori selected analytes [205]. In particular, the use of Ultra-High-Performance Liquid Chromatography (UHPLC) and HRMS allows us to acquire m/z values up to four decimal places or 0.001 Da of mass accuracy [206], employing both FS and SIM acquisition modes (TOF, FTICR, Orbitrap). Further, the Commission Decision 2002/657/EC gave basic knowledge on how to perform an identification of known compounds and introduced the new concepts of suspect and non-target screening.

Under this frame there was, therefore, great interest in the development of new criteria for the identification of known and unknown compounds in environmental matrices. Most of them were based on the identification point or identification levels, depending on the quality of the information obtained from the instruments. The concepts of "*known-knowns*", "*known-unknowns*", and "*unknown-unknowns*" were previously defined by Little et al. [207]. In brief, an analyte which is expected to be found in a sample, whose identity can be later confirmed by the MS/MS analysis of

the corresponding standard, can be named as “known-known.” On the contrary, when the compound is not expected to be present in the sample, but it is cited in databases, the body of literature, or MS references, it is recognised as “known-unknown”. Finally, if no previous information is available, compounds can be named “unknown-unknown” [207].

The approaches to characterising these components are diverse. “*Unknown-unknowns*” are the most difficult to be structurally identified. Many works report the development of Non-Target Screening using HRMS approaches. However, suspect and non-target are often confused as their characterisation needs multiple techniques, such as Infrared Spectroscopy (IR) and Nuclear Magnetic Resonance (NMR). However, HRMS coupled with in silico data treatment is generally considered sufficient for the identification of “known-unknown” and “known” compounds (**Table 11**).

Table 11: Relations and definitions of compounds and their approaches

Compound class and relative approach	*Definition*	*Minimum characteristics required*
Known-Known (Target screening)	A compound in which previous literature and information are available. The structure is defined and the presence of the analyte is expected in the sample.	• Flame ionization, UV detector. • Low-resolution MS (LRMS); QqQ, single quadrupole, Ion trap • Certified analytical standards
Known-Unknown (Target; Suspect screening)	The compound is cited in the literature and/or databases. Structural information is provided but it was not expected to be present in the sample. Post-acquisition data treatment is necessary.	• LRMS; QqQ • HRMS; QTOF, QTRAP, Orbitrap, FTICR. • Database, literature • Certified analytical standards
Unknown-Unknown (Non-target screening)	No previous information, structure unknown with no literature available no standards available.	• HRMS; QTOF, QTRAP, Orbitrap, FTICR • NMR and IR

Using HRMS, Bertrand Rochat [208] proposed a scale of confidence for the tentative identification of known-unknown compounds. The scale is based on the Commission Decision 2007/657/EC and the Metabolomics Standard Initiative (MSI) [209], and bridges metabolomics and screening information. This scale also depends on three criteria that involve chromatographic data, identification levels, and identification points. Four different identification levels have been proposed. Levels 4 and 3 are intended as unknown compounds while the level 2 corresponds to the match between a theoretical spectrum reported in the literature and the experimental spectrum.

Finally, the first level is defined as confirmation using standards. The chromatographic data involve a classification regarding the presence or absence of previous chromatographic examples in the body of literature. Finally, the act of attributing identification points (IP) is similar to the previously cited Commission Decision 2002/657/EC which differs on the sum of IP that can reach 30 IPs [208].
Even though it has been criticised [210], the IP strategy for the identification of molecules has been continuously optimised and adopted by different researchers/authors to finally become a "standard" in suspect screening approaches. The latest analytical techniques for the identification of *known-unknown* compounds using HRMS were considered in the identification confidence levels approach proposed by Schymanski et al. [201]. Considering the unavailability of many standards and the consequent lack of derived MS/MS data to confirm them, the structure elucidation of samples is of critical importance.
A methodology for unifying confidence levels between studies is proposed. Here, five identification levels are increasingly assigned from 5 to 1 where the fifth level is the lowest confidence and the first level is the confirmed structure. The approach does not aim at replacing the Commission Decision 2002/657/EC, but it does introduce a novel technique with the possibility of reporting identification confidence. As noted in **Figure 14**, several parameters are considered when reporting a particular level.

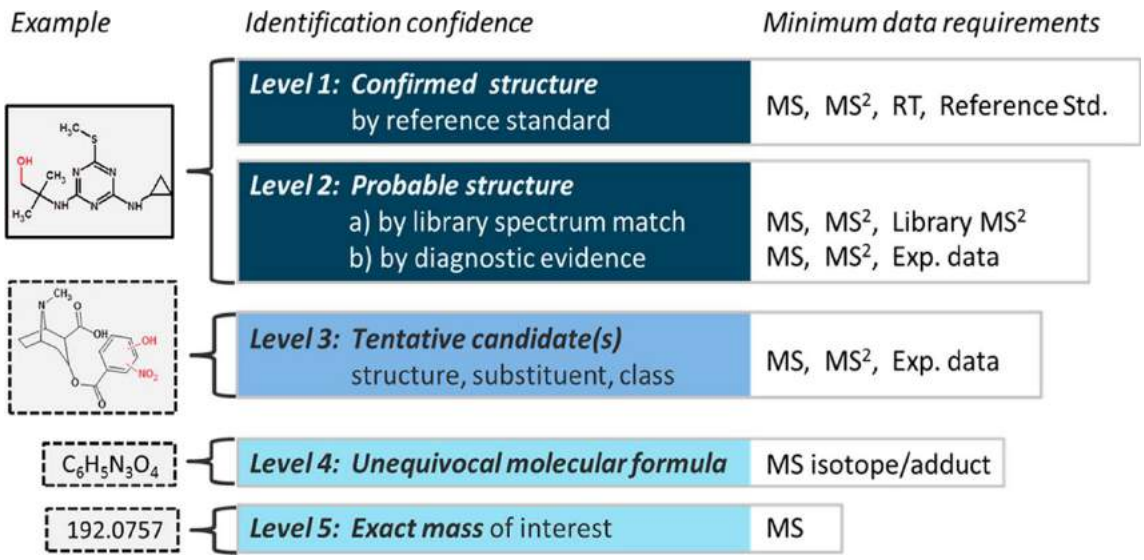

Figure 14: Identification levels proposed by Schymanski et al. [201] (Reused and adapted with permission from Environ. Sci. Technol. 2014, 48, 4, 2097-2098. Copyright (2020) American Chemical Society)

Level 5 is expressed as the presence of the exact mass. If no information about the exact mass is reported in the literature, the molecule can be reported as an unknown compound. Then, confidence increases as more data are added to the study. When adducts, isotopic masses, and fragmentation spectra are determined, it is possible to reach levels 4 and 3, respectively. Finally, a probable structure (level 2) is proposed through a comparison between theoretical and experimental MS/MS spectra, which

are contained in databases or the body of literature. Level 1 is reached with the comparison between the sample and the standard spectra.
To the best of my knowledge, this approach has become a “standard” that is widely reported as one of the best methods for reporting tentatively identified structures on a suspect screening approach. This approach has been applied in different research fields such as metabolomics [211], lipidomics [212], environmental chemistry [213], and medicine [214].

3.3 Online databases for natural toxins research

During recent decades, many research efforts have been effected to bridge the gap in the scant information regarding the occurrence and persistence of natural toxins and their degradation products in water.
The trend in publications regarding “Natural Toxins” is constantly increasing with a special peak in publications between 2000 and 2010, as a consequence of the reporting of human fatalities from cyanobacteria, due to contamination with microcystin-LR, -YR, and -AR in a dialysis centre [9,215]. After this, the risk perception of these natural chemicals and the demand for a safer environment were augmented, thus becoming an important factor in directing scientists towards focussing their research efforts in the direction of finding new ways to detect, identify, quantify, and avoid contact with these natural toxins in the environment
Currently, the greatest effort in the detection and identification of natural toxins is represented by the confirmation of the compounds, due to the lack of standards. Some natural toxins are available, but for most of them it is still not possible to retrieve a certified standard (i.e., ptaquiloside and other bracken fern metabolites such as pterosin A and B).
Under these premises, databases have a key role in natural toxins research.
Databases are the best option for collecting information regarding chemicals with chemical and physical parameters that are collected by other scientists. Today, there are hundreds of databases in chemistry research, many of which are privately owned and requiring payment for their use. However, the scientific world is continuously changing, and opting for Open Access publishing. To the best of my knowledge, there are at least sixty-five freely available chemistry databases compiling millions of compounds, substances, bioassays, bioactivities, proteomic, and other literature, within which natural toxins are reported. Thanks to this system, it is possible to easily find the compound of interest with a search query or search term. In **Table 12**, some of the main databases that can be found on Internet for prioritisation protocols, chemical data, occurrence, toxicology, and other research purposes and the links to the individual Websites, are summarised.

Table 12: List of the most used databases and chemical tools for research and prioritisation protocols

Databases	Website	Databases	Website
Chemical Identifier Resolver	https://cactus.nci.nih.gov/chemical/structure	**Kinase Knowledgebase**	eidogen-sertanty.com/kinasekb.php
ChemSpider Synthetic Pages	https://cssp.chemspider.com/	**LipidBank**	http://lipidbank.jp/
LipidMaps	www.lipidmaps.org	**LookChem**	https://www.lookchem.com/
AffinDB	http://pc1664.pharmazie.uni-marburg.de/affinity/	**Madison Metabolomics Consortium Database**	http://mmcd.nmrfam.wisc.edu/
BindingDB	www.bindingdb.org/bind/index.jsp	**MassBank**	https://massbank.eu/MassBank/
BRENDA	www.brenda-enzymes.org	**MassBank of North America**	https://mona.fiehnlab.ucdavis.edu/spectra/search
ChEBI (Chemical Entities of Biological Interest)	www.ebi.ac.uk/chebi/	**MatWeb**	http://www.matweb.com/
ChemBank	data.broadinstitute.org/chembank/assay/index.html	**MetaCyc**	https://metacyc.org/
ChemExper	www.chemexper.com	**METLIN**	https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage#
ChemID Plus	chem.nlm.nih.gov/chemidplus/	**MolPort**	https://www.molport.com/shop/index

ChemMine	https://chemminetools.ucr.edu/	**National Drug Code Registry**	https://www.accessdata.fda.gov/scripts/cder/ndc/index.cfm
ChemSpider	http://www.chemspider.com/	**MzCloud**	https://www.mzcloud.org/
ChemSynthesis	https://www.chemsynthesis.com/	**NIST Chemical Kinetics Database**	https://kinetics.nist.gov/kinetics/index.jsp
ChemWiki	http://chemwiki.wikidot.com/	**NIST Chemistry WebBook**	https://webbook.nist.gov/chemistry/
CoCoCo	https://omictools.com/cococo-tool	**NMRShiftDB**	https://nmrshiftdb.nmr.uni-koeln.de/
Common Chemistry	http://www.commonchemistry.org/	**NRG-CING**	https://omictools.com/nrg-cing-tool
Compendium of Common Pesticide Names	http://www.alanwood.net/pesticides/	**Organic Syntheses**	http://www.orgsyn.org/
Computational Chemistry Comparison and Benchmark DataBase	https://cccbdb.nist.gov/	**P450 Drug Interaction Table**	https://drug-interactions.medicine.iu.edu/MainTable.aspx
Crystallography Open Database	http://www.crystallography.net/cod/	**PheroBase**	https://www.pherobase.com/
DockBlaster	https://blaster.docking.org/	**Protein Data Bank**	https://www.rcsb.org/
Drug gene interaction Database	http://www.dgidb.org/	**Psychoactive Drug Screening Program Database**	https://pdspdb.unc.edu/pdspWeb/

DrugBank	https://www.drugbank.ca/	**PubChem**	https://pubchem.ncbi.nlm.nih.gov/
eMolecules	https://www.emolecules.com/info/plus/download-database	**RRuff**	http://rruff.info/
FDA Unique Ingredient Identifier	https://fdasis.nlm.nih.gov/srs/jsp/srs/uniiListDownload.jsp	**Side Effect Resource (SIDER)**	http://sideeffects.embl.de/
Heterocycles Web Edition	https://www.heterocycles.jp/newlibrary/libraries/prepress	**SCOPe (Structural Classification of Proteins — extended)**	https://scop.berkeley.edu/
Human Metabolome Database **IUPAC-NIST Solubility Database**	http://www.hmdb.ca/ https://srdata.nist.gov/solubility/	**Spectral Database for Organic Compounds**	https://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_list.cgi
KEGG	https://www.genome.jp/kegg/	**STITCH 2**	http://stitch.embl.de/

However, many of these databases are non-specific, reporting huge amounts of data of different classes of compounds (drugs, natural toxins, pharmaceuticals, pesticides, proteins, lipids, etc.) in a single list. In some cases, to retrieve complete information of a compound, it is necessary to search in more than one list to finally retrieve and assemble the required information. As can be seen in Table 9, no single database was dedicated solely to natural toxins. Only MassBank [23] contains an index with 2239 spectral record of 107 natural toxins and this will be discussed later.
In this context, where natural toxins are of growing interest for researchers as environmental pollutants, it is important to build dedicated databases for these compounds. To the best of my knowledge, there are at least 5 specific lists reporting information on natural toxins and their relative organisms, spectra, fragmentation patterns, structures, CAS registry number, occurrence, and toxicity information.
Additionally, the newest Open Access resource is of growing interest in order to spread information to the public, with no restrictions related to access fees or being reserved for specific groups. Academia and smaller organisations are particularly interested in Open Access to databases that can help to identify and predict chemical structures for prioritisation protocols.
PubChem is the best example, and this was launched in September 2004 by the US National Institute of Health [24] which reports circa 4800 literature sources for natural toxins with more than 50,000 compounds, among which are mycotoxins, plant toxins, and cyanotoxins. Contrary to what is reported by Baker, 2006 [216], Open Access initiatives are largely increasing during recent years, and millions of compounds are listed in Open Access databases. Within a period of 13 years, the number of compounds listed in PubChem increased from 23 million to 97 million compounds, plus circa 500 million compounds among which there are substances, bioactivities, and patents (December 2019).
Several organisations are focusing their efforts on continuously uploading and freely sharing their knowledge to help researchers in the detection and characterisation of natural toxins in the environment.
With these premises, below are reported specialised Open Access databases that focus on at least one or more groups of natural toxins. Also, included are repositories with filters or lists that help to discriminate natural toxins from other compounds.

Toxic Plants-Phytotoxins (TPPT) Database
The TPPT database was recently presented and described by Günthardt et al. [217] in 2018, and it is currently available from the Agroscope Website [27] in the Microsoft Excel Binary File (.xls) format or with extended function in SQLite format. This database includes 1586 examples, among which are alkaloids, terpenes, phenylpropanoids, steroids, and polyketides as potential natural aquatic pollutants. Further, 844 toxic plant species have been listed, thus developing a complete database that links the toxins connected to the relative plant species with toxicity, toxicological data, and persistence in freshwater.
The TPPT database has been conceived to easily assess the environmental risk and prioritise phytotoxins in the aquatic environment, depending on their toxicological

properties. An intense effort has been made to retrieve information on the botanical diversity in Switzerland, listing the most interesting Swiss plants that can produce phytotoxins and linking them with the compounds produced. Toxicological assessment of phytotoxins has also been described, reporting the acute toxicity in rodents, measured using prediction methods such as ProTox (Website for in silico prediction of oral toxicity in rodents). Further [218], the environmental behaviour of some highly toxic compounds, that are associated with their toxicological properties, helped those authors to complete a prioritisation list of the most dangerous compounds.

Database of Toxic Plants in the United States (DT PLUS)
Even if it is proposed as a veterinary list for equine health purposes, DT PLUS is a general database recording 337 plant species and their relative toxins. This list can be found on the Website of the University of Idaho (USA) [151]. It is very easy to browse since only 6 columns are reporting the common names, the scientific names, the biological injuries these toxins can provoke, plus the type, and some additional comments. It is easy to handle and extrapolate data from this Website, and it is considered to be a useful starting point for data collection and database build-up.

Planttox database (RIKILT, Wageningen University & Research, Netherlands)
Tropane alkaloids are secondary metabolites that occur in different plants. To date, hundreds of these compounds have been identified while reporting their risks for humans [219]. Wageningen Food Safety Research (RIKILT, Wageningen University & Research, Netherlands) developed their Planttox database in 2012, with features that are similar to those reported in the above. Briefly, more than 700 plant species have been reported, together with their typical toxins. The database is in .xls format and is extremely easy to use, with the interface being divided into two columns. The first column is to select the plant genus and, once selected, plant family name, phytotoxins, and the poisonous part of the plant will be displayed. In the second column, a compound family can be selected, thus retrieving the pertinent compounds. In this manner, it is possible to find the plant producer of a given toxin and vice versa. References are reported for all of the compounds that are collected within the database, which is extremely user friendly with no particular search skills required from the user. To the best of my knowledge, this database lacks structures and gives no other information regarding toxicological data or occurrence in the environment. However, it can be a useful tool as a starting point for further investigations, especially with previously known data regarding the botanical diversity in the area of interest and vice versa.

Toxin and Toxin Target Database (T3DB, Genome Canada, Genome Alberta, and Genome British Columbia)
Lim et al. [220] published a description of the T3DB database (or Toxic Exposome Database), which in 2010 provided a toxicology resource reporting a wide range of information about toxins and their metabolites. Links between biological targets and

toxins causing disease are reported, and human body interactions and toxicology effects with exposure data are also reported. The T3DB database currently provides 3678 (November 2019) structures between pesticides, natural toxins, food toxins, and medicines. This database is not intended as a specialised database solely for natural toxins, it has a filter tool that browses by category, thus providing a rapid retrieval service for natural toxins data.

The database structure is extremely easy to use, providing a complete dataset called ToxCard (with data fields or data types to be found in each ToxCard) [220]. Update and creation date followed by chemical data, such as structure, description, compound type biological, and physical properties, are listed. Further, biological information for toxicological assessments is included. The spectra row also includes links of predicted spectra that are stored in the internal servers, with different links to MoNA (MassBank of North America) that are reported for each compound at several collision energies, in positive and negative modes. However, as reported above, the only prediction of spectral fragments is reported, hence, in general further investigation is required. Furthermore, some of the MoNA links (URLs) are not functioning or are not included in the MoNa servers.

The category for natural toxins is included in the T3DB database. Filters need to be selected to retrieve the groups: *Bacterial toxins; Food toxins; Fungal toxins; Natural toxins, and Plant toxins*, and the attached information.

MassBank (NORMAN network)

The MassBank (NORMAN network of reference laboratories, research centres, and related organisations for monitoring of emerging environmental substances) database was previously reviewed by Horai et al. [221] hence, in this section, only its importance as a natural toxins repository will be discussed. Massbank can be accessed through MassBank Japan, and NORMAN MassBank, however, I will focus on the European NORMAN Website. MassBank is a high-resolution mass spectral database or chemical structural database (of small molecules). NORMAN MassBank has been created thanks to the contribution of different institutions under the NORMAN Association and MassBank Consortium. In **Figure 15**, a list of the contributors and the number of records that have been uploaded is reported. The first three organisations (RIKEN, Faculty of Engineering University of Tokyo, and Eawag) contributed with more than 10,000 records. Seven (7) contributors donated between 5,000 and 2,000 spectra of chemicals, while others uploaded between 900 and 4 mass spectra to the repository.

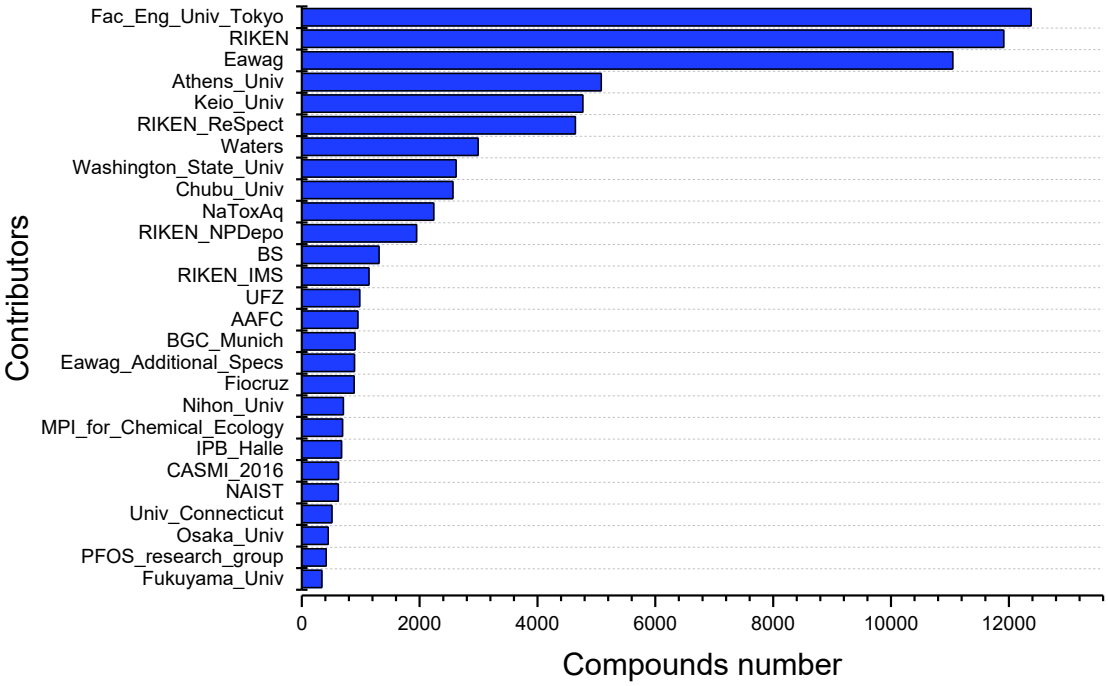

Figure 15: Contributors for MassBank (NORMAN) database and the number of compounds added

Figure 16 reports the most used acquisition methods and the mass spectrums that have been uploaded to MassBank. As one can see, soft ionisation techniques are prevailing over the hard ionisation approaches, such as Electron Impact (EI), which was the most used ionisation method until some years ago, when Gas Chromatography was introduced [222].

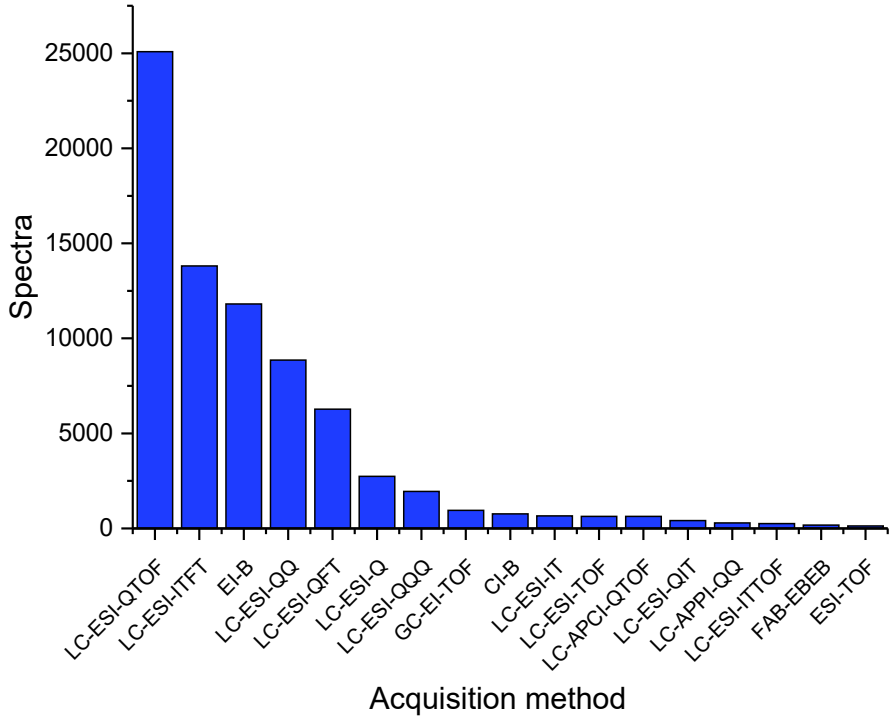

Figure 146: Spectrums uploaded on MassBank with the relative acquisition methods

LC with electrospray ion source (ESI) coupled with Quadrupole Time of Flight (QTOF) analyser is the most used acquisition method with 33 % of the total spectra being uploaded, followed by LC-ESI-Ion Trap Fourier Transform (ITFT), which reinforces the progressive increase in the use of soft ionisation techniques. Of the total compound number, 22,837 compounds have been acquired in negative mode while 53,099 were positive ions.
In addition to the huge amount of data in MassBank, there is a dedicated webpage (https://www.norman-network.com/?q=suspect-list-exchange) where are reported relevant suspect lists for the environmental monitoring of chemicals. Here, natural toxins from plants, bacteria, and fungi are well reported and well described. In particular, chemical lists S26 [223], S29 [224], and S40 [225] can be directly downloaded.
To the best of my knowledge, this database sums up the list of the most complete and useful identification repository and information search tools for natural toxins and other compounds. However, I did not manage to find a tool with which to group natural toxins by chemical and/or toxicological properties. However**,** this database is easy to use with complete MS and MS^n spectral information and it can be adopted as a search protocol for identification and screening purposes

CompTox Chemicals Dashboard [226]
CompTox Chemicals Dashboard was developed by the U.S. Environmental Protection Agency (EPA). It is a Web application that links to the EPA repository of chemicals and toxicological data that are relevant for environmental studies. Many data were collected from other Online sources, thus assembling a unique dataset within the EPA DSSTox project. Several resources came from public sources, such as the European Union (EU) Chemicals Agency (ECHA-CHEM) database [6], PubChem [24], PubMed [11], and other modelling tools such as Estimation Prediction Interface (EPI) from the EPI Suite (a screening-level tool) [7].
However, the primary database that is included in the dashboard is represented by the DSSTox [227] and other databases developed by the EPA National Center for Computational Toxicology (NCCT) including the Chemical and Product Categories database (CPC) [37], the ToxCast Dashboard [12], the Aggregated Computational Toxicology Online Resource (ACToR) [228], and the Endocrine Disruption Screening Program (EDSP) for the 21st Century (EDSP21) Dashboard [229].
These datasets have been used to evaluate potential adverse effects and risk to humans, reporting bioassay data, toxicological data, and predicting the physicochemical properties of thousands of compounds. Detailed information describing the CompTox Chemicals Dashboard has been extensively described by Williams et al. [226].
Natural toxins are well described in the CompTox Dashboard, and even if not all of the tabs are available and some data regarding toxicology are lacking, this is a complete repository for toxicology and risk assessment, also making available prediction data that are generally not reported in the literature, to the best of my knowledge. Further, a facility for easier recompilation of natural toxin lists is provided.

In the upper part of the main dashboard, between the six options that are available, “lists” is one of the most important for natural toxins, giving the option to add single toxins to a batch list to retrieve and download the data. It provides different lists of chemicals with the last update and a brief description. Using a query search with keyword “toxins”, five lists are provided:
ALGALTOX includes 54 marine algal species produced by bloom events which are referred to as: Harmful Algal Bloom (HAB) and red algae. The CYANOTOX list includes 7 congeners of microcystins (MCs) including MC-LR, MC-LA, MC-RR, MC-YR, MC-LW, MC-LF, and MC-LY. *MYCOTOXINS* and *MYCOTOX2* lists contain 88 and 328 mycotoxins, respectively. In the first case, 88 mycotoxins were extracted from MassBank, and providing the mass spectra.
Finally, the *PHYTOTOXINS* list contains the plant toxins collected from the TPPT database which is presented by Günthardt et al. [217].

Among the databases, there are many articles reporting lists of natural toxins that were studied or considered for an experiment. Thanks to this, it is possible to find lists of chemicals with many interesting examples of data to be used in an identification protocol. For this reason, manuscripts with lists of natural toxins that are generally considered for prioritisation protocols are reported in **Table 12**. Even if they are not intended as databases, these lists are helpful to retrieve possible natural toxins that are generally found in the environment.
In any application for identification and prioritisation protocols, databases have one of the main roles. However, retrieving data is not always easy and rapid, but requires a specific search for the compound of interest. Many times, natural toxins are merged in a list, and the same list can be used by different repositories. To date, the data in the literature and databases on natural toxins are scant, but they are continuously increasing thanks to the use of in silico tools for data prediction. This chapter makes clear the effort made towards developing and publishing databases in Open Access mode. Many organisations are involved in the research and sharing of results regarding natural toxins in the environment and many new tools will be developed to provide an easy and fast chemical online information retrieval.

Table 12: Manuscripts including lists of natural toxins encountered in literature.

Specie	Toxin type	Number of entries	Comments	Ref
Fungi	Mycotoxins	474 mycotoxins and fungal metabolites	List of metabolites, formula, Retention time, UV adsorption	[230]
Bacteria	Cyanotoxins	82 variants between microcystins, saxitoxins, gonyautoxins, and C-toxins	Guide to their public health consequences, monitoring, and management.	[19]
Bacteria	Cyanotoxins	369 Algal metabolites	List of cyanotoxins with raw formula, molecular weight, the retention time for target analytes, canonical smile notation, and fragment ions.	[231]
Bacteria	Botulinum toxins	133 peptides fragments	Peptide list of fragments	[232]
Bacteria	Toxins and antitoxins	120 molecules	Molecules, including TA complexes and free toxins/antitoxins.	[232]
Bacteria	Paralytic shellfish poisoning	25 toxins	Molecular formula, theoretical molecular mass of 25 PSP toxin and fragments ions.	[233]
Fungi	Mycotoxins	15 mycotoxins	List of mycotoxins and external links. Toxicological information, EU legislation, epidemiology, matrices and occurrence	[234]
Fungi	Mycotoxins	23 toxins and isomers	List of mycotoxin and analysis methods	[235]
Fungi	Mycotoxins	30 mycotoxins	Phytotoxic properties of mycotoxins and their effective doses on plants. Genus of the first isolation/description/main relevance is given.	[236]
Plant	Plant Toxins	38 plant toxins	Common names, phytotoxins, bioactivity, and reference are reported.	[237]
Plant	Alkaloids	14 alkaloids	List of ergot alkaloids identified, general chemical classification, and reference cited	[238]

3.4 Suspect screening of natural toxins in surface water reservoirs

During recent years, the interest of researchers and drinking water suppliers on the identification and confirmation of natural toxins in surface water has increased. However, an identification process of natural contaminants has not been well defined, but rather it leans towards the already well-known suspect and non-target screening approaches. Suspect screening is a good alternative when previous data are known for a given compound and, at the same time, the reference standards are not available. However, most of the analytical methods for the tentative identification of suspect natural toxins normally focus on a single toxin or a single group with the same chemical and physical parameters. In what follows is reported a published suspect screening approach for the tentative identification of natural toxins.

3.4.1 Scientific publication II

Suspect screening of natural toxins in surface and drinking water by high-performance liquid chromatography / high-resolution mass spectrometry

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Suspect screening of natural toxins in surface and drinking water by high performance liquid chromatography and high-resolution mass spectrometry

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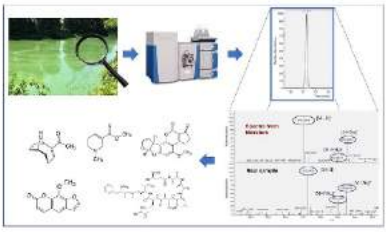
HIGHLIGHTS

- Single workflow for a comprehensive assessment of natural toxins in waters is presented.
- The procedure includes a suspect screening of natural toxins and target screening of a selection.
- The approach tentatively identified 23 natural toxins in the Ter River (Spain).

GRAPHICAL ABSTRACT



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ABSTRACT

Besides anthropogenic contamination, freshwater environments can also be affected by the presence of natural toxins. Mycotoxins, plant toxins and cyanotoxins are the most relevant groups that can be found in the aquatic system. However, until now, only cyanotoxins have been more carefully studied. In the present work, single workflow for the assessment of natural toxins in waters, based on suspect screening and target screening of a selected group of toxins is presented. The approach is based on a triple-stage solid-phase extraction (SPE) able to isolate a wide range of natural toxins of different polarities, followed by liquid chromatography coupled to high-resolution mass spectrometry (HPLC-ddHRMS^2) using a Q-Exactive Orbitrap analyser. The acquisition was performed in full-scan (FS) and data-dependant acquisition (ddMS^2) mode, working under positive and negative mode. For the tentative identification, different on-line databases such as ChemSpider and MzCloud and an in-house natural toxins list with 2384 structures, that includes cyanotoxins, plant toxins and mycotoxins, were used. Also, thanks to the MS^2 data, it was possible to achieve a high level of tentative identification confidence, but confirmation was only possible comparing the standards of the suspected compounds. For those, the analytical parameters of the developed method were also validated, and the quantification was possible by external calibration. Validation showed recoveries in the range between 53 and 95%, and method limits of detection (MDL) between 0.02 and 1.22 μg/L. This approach was applied to study natural toxins in 4 sampling sites along the Ter River in Catalonia (NE Spain). In this preliminary study 23 natural toxins were tentatively identified, and 9 of them confirmed (aflatoxin B1, anatoxin-a, nodularin, microcystin-LR, baicalein, kojic acid, cinchonine, B-asarone and atropine). The results of the quantification of these compounds showed concentrations below 1 μg/L in all cases, that is considered safe according to the

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actual legislation. This suspect screening approach allows a more comprehensive assessment of natural toxins in natural waters.

1. Introduction

The occurrence of natural toxins in the surface and drinking water reservoirs is a problem of increasing concern for water producers and environmental scientists. Natural toxins are secondary metabolites produced by a multitude of organisms and especially by fungi (fungal toxins or mycotoxins), plants (phytotoxins), bacteria and algae. Natural toxins are naturally synthesized by living organisms and it has been hypothesised that defence or predation mechanisms can be some of the factors ruling their production (Clucas et al., 2010). The release of natural toxins into the environment, and in particular their occurrence in surface waters, can constitute a hazard for human health due of possible ingestion and/or recreational and bathing activities (Hodgson and Hodgson, 2012; Picardo et al., 2019). Moreover, the agriculture, intensive animal husbandry and urban wastewater, producing high concentration of nitrogen, phosphorus and other nutrients, together with climate change, enhance the eutrophication processes and provide the propitious conditions of harmful algal and cyanobacterial blooms – (HABs and cyano HABs), which have increased in intensity and frequency during the last years (Davis et al., 2009). Because of the chemical diversity of natural toxins, they may produce different toxic effects. The most common adverse effects include hepatotoxicity (e.g. microcystins) (Testai et al., 2016), neurotoxicity (e.g. anatoxin) (dos Santos et al., 2019), dermal toxicity (Funari and Testai, 2008) (e.g. T-2 toxins) and cytotoxicity (Froscio et al., 2009) (e.g. cylindrospermopsin) to the general inhibition of protein synthesis. The toxicity assessment and the hazard caused by natural toxins can be understood thanks to their physical and chemical properties, fate and occurrence in the environment.

Several legislations have been implemented around the world. In Europe, the principal regulations are the Water Framework Directive, the Drinking water directive (Council Directive 98/83/EC, currently under revision), and the EU Bathing Water Directive 2006/7/EC (Council directive 98/83/E, 1998; National Center for Biotechnology Information, 2020). However, these regulations are based on the analysis of target compounds, while other groups of biotoxins, such as plant toxins, are insufficiently studied and not yet regulated. Besides that, due to climate change, the occurrence of certain groups of toxins not expected in some regions will increase. As reported by Manning et al. (Manning and Nobles, 2017) human pollution and the consequent eutrophication of water bodies, will cause an unbalanced competition between cyanobacteria and other bacteria. This will allow longer HABs events with higher levels of cyanotoxins (Manning and Nobles, 2017). For these reasons, the characterisation of the complete composition of natural toxins in surface waters, including secondary metabolites and degradation products, is needed.

During the last decade, a significant number of analytical methods have been developed for the target quantification of natural toxins in freshwater environments, in particular, based on liquid chromatography coupled to mass spectrometry (LC-MS). In most cases, the analytical approaches have been developed to quantify targeted toxins or a selected group of toxins. For example, for cyanotoxins, showing in general limits of detection ranging between 0.16 ng/L and 37 μg/L (Turner et al., 2018; Gambaro et al., 2012; Yen et al., 2011; Aguete et al., 2003; Roy-Lachapelle et al., 2019).

The availability of high-resolution mass spectrometry techniques (HRMS) opened a new window for the detection of low molecular weight compounds, such as natural toxins. These techniques allow the research of unknown compounds and their tentative identification in different degrees of confidence, as previously reported by Schymansky et al. (Schymanski et al., 2014). Besides, suspect and non-target screening approaches are of particular interest in the research of emerging contaminants and those lacking analytical standards, as it is the case for a significant number of natural toxins. It should be highlighted that, in spite of the great variety of natural toxins currently identified, the information of their presence in the environment, such as in surface waters and water reservoirs, is still quite limited.

Moreover, different analytical approaches for the detection of target compounds or groups with similar chemical characteristics, have been developed. However, there is still a lack of analytical methods able to identify a wide range of natural toxins part of the total natural charge in the aquatic environment. Regarding the research of natural toxins, the lack of analytical standards is an additional issue for their identification.

This work aimed to develop a suspect screening method to establish the natural toxins profiles in natural waters. Therefore, the specific goals of this study were: (i) develop a homemade triple-stage solid-phase extraction (SPE) procedure to isolate a wide range of natural toxins from different groups and physico-chemical characteristics. The good performances of the extraction procedure were tested with a selected group of natural toxins part of different classes and polarities including cyanotoxins Anatoxin (Ana-a), cylindrospermopsin (Cyn), microcystins (MCs) and their congeners -LR, -LY and -YR, nodularins (Nods); fungal toxins aflatoxin B_1 ($AflB_1$), kojic acid (Kja) and ochratoxin-A (Ot-A); and plant toxins baicalein (Bai), scopolamine (Sco), atropine (Atr), cinchonine (Chn), and B-asarone (B-as); (ii) to collect an in-house natural-toxins list, including cyanotoxins, plant toxins, and mycotoxins that are susceptible to be found in natural waters, to facilitate the screening process; (iii) to develop and validate a suspect screening approach based on high-performance liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) using a Q-Exactive Orbitrap mass analyser; (iv) to validate the method for the target analysis of selected compounds; and (v) to assess the performance of the new analytical combined approach in real samples collected in the Ter River near the Barcelona water catchment area.

Despite the significant work carried out during the recent years in suspect screening approaches, to the authors' knowledge, the screening method proposed is the first one specifically designed for the comprehensive tentative identification of a wide range of natural toxins in surface waters.

2. Experimental

2.1. Chemicals and reagents

Ana-a 1 mg (99%) standard was supplied from Santa Cruz Biotechnology (Dallas, TX, USA). Cyn, 25 μg (99%) was purchased from BOCSci (BOC Sciences, Ramsey Road Shirley, NY, USA). MC-LR,

10 μg/mL; MC-YR, 10 μg/mL, MC-LY, 10 μg/mL and Nod 10 μg/mL; were >99% purity from Cyano (Cyanobiotech GmbH, Berlin, Germany). $AflB_1$, 25 μg/mL (99%) were bought from Merck (Darmstadt, Germany). Baicalein (Bai), 100 mg (98%); Scopolamine (Sco)1 mg (98%); (+)-cinchonine; (Chn) 25 g (99%); Atr, 1 g (99%); Kja 100 mg (98%); B-asa, 1 g (98%) and Ota-a 1 mg (99%) were purchased from Merck (Darmstadt, Germany) (Table S1). Methanol and acetone HPLC grade were supplied from Merck (Darmstadt, Germany) while water HPLC grade was obtained from Baker (Madrid, Spain).

2.2. Samples and sampling sites

Freshwater samples were collected in the Ter River, in central Catalonia (NE Spain), in one of the drinking water catchment areas of Barcelona Metropolitan Area to assess the performance of the proposed analytical approach. A total number of 16 samples were collected in four sampling sites Point 1 (M1) 41.986133; 2.603488, Point 2 (M2) 41.982191; 2.585539, Point 3 (M3) 41.991090; 2.570144, Point 4 (M4) 41.975693; 2.395398. Sampling was carried out twice per month during May and July 2018. May is just before the expected algal bloom and July because it is one of the months more affected in general by cyanotoxins in Spain (Carrasco et al., 2006). Besides, June, July and August are mostly dry seasons were no precipitations can cause leaching of toxins in the soil while September is considered the start of the wet season expecting highest levels of plant toxins in the water. Mycotoxins are instead produced along the entire year depending on the climate conditions. But, during summer near water bodies, the higher relative humidity, increase the production of mycotoxins (Al-Gabr et al., 2014).

The samples were collected in amber glass bottles, and before the sampling, the pH, temperature, pO_2 and conductivity were measured on-site. Samples were transported at 4 °C and then were frozen at −40 °C till the initiation of the analytical process.

2.3. Sample pre-treatment

Each sample was processed in an ultrasonic bath for 20 min at a power of 200 W and a frequency of 60 Hz to disrupt cells and release the intracellular toxins. After sonication, the samples were filtered through a glass microfiber filter GF/B grade (Sigma Aldrich, Steinheim, Germany) and toxins were isolated by off-line solid-phase extraction (SPE). A hand-made cartridge was prepared and coupled to a HLB plus cartridge to achieve the maximum recovery of toxins with different ranges of polarity. The custom SPE cartridge was prepared as follows: a 3 mL volume glass cartridge was manually filled with a double layer of 200 mg (top side) of porous graphitized carbon (PGC) 120 mesh (Sigma Aldrich, Steinheim, Germany) and 200 mg of Bond-Elut PPL (PPL) 120 mesh (Agilent, Santa Clara, CA, USA) separated by a Teflon frit. The cartridge was connected to an Oasis HLB plus (225 mg sorbent/6 mL) cartridge (Waters Corporations, Mil-ford, MA). Water samples (100 mL) were loaded through the cartridges at a flow rate of 2 mL/min previously conditioned with 10 mL of methanol and 10 mL of water, both acidified with 0.5% of formic acid (FA). After loading, the cartridges were dried under vacuum and were switched to elute the analytes in backflush. To achieve this, the PGC/PPL cartridge was reversed, while the HLB cartridge remained in the same position connected to the bottom side with the same flow direction. The toxins were eluted with 15 mL of water/methanol 2:8 (*v/v*), followed by 15 mL of methanol and 15 mL of acetone/methanol 50:50 (*v/v*). All the solvents were previously warmed at 45 °C before each elution. The eluate was evaporated almost to dryness and re-dissolved in 1 mL of mobile phase.

2.4. Liquid chromatography

The chromatograph was an Acquity high-performance liquid chromatograph system (Waters Corp, Milford, MA, USA). The chromatographic separation was achieved using a C_{18} reversed-phase column Lichrosphere, (125 mm × 2 mm i.d., 5 μm) (Merck, Barcelona, ES). A binary mobile phase was composed of water (solvent A) and acetonitrile (solvent B) both acidified with 0.1% of FA. The elution gradient was programmed as follows: From 0 to 3 min, 10% B; from 3 to 13 min, B was linearly increased to 90%; 13−15 min, stabilized at 90% B; 15−16 min B decreased linearly to 10%; 16−20 min, column stabilization with a 10% of solvent B. Extracts were kept at 8 °C in the autosampler. An aliquot of 20 μL was injected into the LC-HRMS system with a flow rate of 0.25 mL/min.

2.5. Orbitrap Q-Exactive mass spectrometry

A Thermo Scientific Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with heated electrospray ionisation (HESI) probe was used in positive and negative mode. The optimal source parameters were as follows: spray voltage of 3.75 kV (+) and −3.25 kV (−), sheath flow gas of 20 a.u., auxiliary gas of 20 a.u., and sweep gas of 5 a.u. Heater and capillary temperature were set at 300 °C with an S-lens RF level at 60%. The acquisition was performed in full-scan-data dependant acquisition (FS-$ddMS^2$) mode with an inclusion list of 100 most probable suspect compounds. In Table 1, the acquisition parameters are summarised.

2.6. Data processing: suspect screening of natural biotoxins

Data processing was performed using the Xcalibur Qual Browser software (Thermo Fisher Scientific, San Jose, CA, USA), and Compound Discoverer software version 2.1 v. x86 (Thermo Fisher Scientific, San Jose, CA, USA), respectively.

For the suspect screening approach, first a home-made list of natural toxins was assembled collecting data from literature and other databases containing the exact mass of 2380 compounds (Table S2 of the supporting information). Besides, the information contained in two online databases, Chemspider for structural information and MzCloud, as mass spectra database was used. The workflow (Fig. 1) and the tentative identification criteria were based on those described by Krauss (Krauss et al., 2010) and Schymanski (Schymanski et al., 2014). Other filters applied were mass tolerance of 5 ppm, the retention time (tolerance ± 2.5% min) if available, isotopic fit (>90%), fragmentation and the previously mentioned parameters in addition to the mass peak resolution and

Table 1
Acquisition parameters of the reported MS methods.

	Acquisition parameters
Method duration (min)	20
Acquisition mode	Full MS/$ddMS^2$
Polarity	(+); (−)
Resolution (FWHD)	35.000 FS 17.500 $ddMS^2$
AGC target	1.00E+05
Max IT	50
Scan Range (*m/z*)	75−1100
Spectrum data type	Centroid
Loop count	3
Top-N	3
Isolation window (*m/z*)	2
Collision Energy (CE)	35 (All toxins) 70 (Aflatoxins)

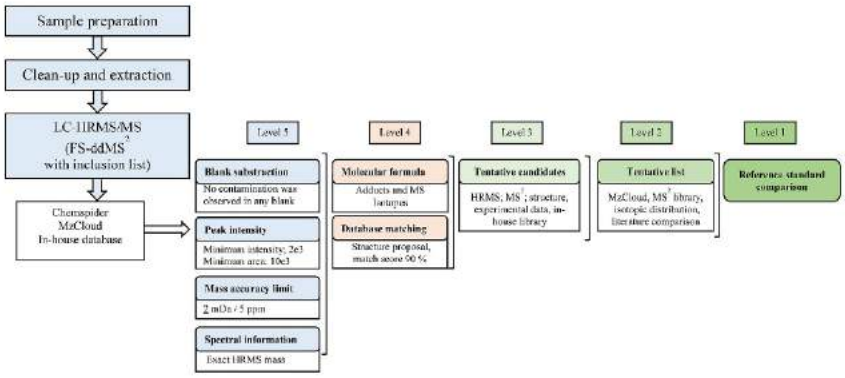

Fig. 1. Tentative identification workflow inspired by Schymansky et al. (Schymanski et al., 2014).

response. The suspect screening was performed as follows; the FS-ddMS^2 spectra were processed with Compound Discoverer that helped to obtain a list of candidates between the ones contained in the inclusion list. These suspect natural toxins were finally checked and confirmed to level 2 matching the experimental fragmentation spectra and comparing them with theoretical patterns encountered in the literature. Finally, coincident compounds with the available standards were confirmed, identification at level 1.

2.7. Quality assurance

The proposed suspect screening was tested using spiked samples with 14 representative natural toxins pertaining to different groups and showing different physicochemical properties; Plant toxins (Bai, Sco, Atr, Chn, B-asa); Cyanotoxins (Ana-a, Cyn, MC-LR, MC-LY, Nod and MC-YR); Fungal toxins (Ota-a, Kja, AflB$_1$). First, the extraction performance was assessed with the spiked samples. Then, the samples were processed to evaluate the suspect screening approach. Finally, the analytical parameters of the entire method for target analysis of selected toxins were assessed, and the recoveries, method limits of detection (MDL), quantification (MQL), selectivity, linearity, and precision were determined.

2.7.1. Spiking experiments

Fortified samples were prepared in ultra-pure water and artificial freshwater (AFW). AFW was prepared according to the description of Lipschitz and Michel (2002), the organic matter was simulated by adding 10 mg/L of humic acid of technical grade from Sigma-Aldrich (reference 53,680), and the pH was adjusted to 6.5 with formic acid 1.0 M.

In both matrices, the spiked samples were prepared by fortification at seven different concentrations between 0.5 and 100 μg/L, using the 14 representative toxins. The spiked samples were left for 1 h at room temperature to ensure proper distribution in the matrix. Besides, three pristine natural samples, free of the representative selected toxins, were additionally spiked at the same concentrations following the same procedure. Samples were processed as reported in section 2.3 working in triplicates adding three procedural blanks that showed no contamination for the entire method. However, procedural blanks were performed during each analysis and the signals in the blanks were quantified and subtracted from the signals of samples. To assess the good extraction performance, the spiked samples were compared with the corresponding calibration curve prepared in the initial composition of the mobile phase according to the elution gradient of the chromatography. In the Supporting Information section, the SPE optimization results are presented.

2.7.2. Validation of the suspect screening approach

First, the extraction procedure was evaluated in front of the selected natural toxins, and the recoveries for the representative compounds were calculated. Then, the suspect screening approach was tested using the Compound Discoverer and the data bases using the same approach as reported in 2.6.

2.7.3. Evaluation of the analytical approach for target analysis of the selected natural toxins

The linearity range, intra-assay precision, accuracy, matrix effects, the limit of detection (LOD) and limits of quantification (LOQ) for the 14 selected toxins was carried out according to the EURACHEM guidelines (Magnusson and Örnemark, 2014). The instrumental limits of detection (iLOD) were calculated by progressive dilution to the lowest concentration where each compound could be detected. Instrumental linearity and sensitivity were estimated as the squared Pearson index (R^2) and the slopes of the calibration curves, respectively. Instrumental reproducibility (inter-day precision) was calculated as the average percentage of the relative standard deviation (RSD %) of standard solutions (six replicates) at seven concentration levels on three consecutive days.

The MDL and MQL were based on matrix-matched calibration curve points. MDL of each analyte was defined as the lowest concentration for which the peak area was, at least, three times the signal-to-noise, while the MQLs were established as the lowest concentrations which fulfilled the criteria: signal-to-noise ratio, at least, 10; relative standard deviation of three replicates, below 19%; Gaussian peak shapes; less than 5 ppm of exact mass error; and isotopic pattern similarity.

Recoveries and precision were assessed by repeating six times the procedure at a concentration of 0.5 μg/L during three different days. Precision expressed as intraday and inter-day repeatability was calculated repeating the procedure in the same day and during a period of 3 months (2 times per week), respectively.

3. Results and discussion

3.1. Sample preparation for a wide range of natural toxins in water

An SPE procedure was developed to extract wide polarity range of natural toxins from water samples. Based on previously reported results four different sorbents were tested: Oasis HLB Plus (Oasis-

HLB), from Waters (Waters Corp, Milford, MA, USA), ISOLUTE®C_{18} (C_{18}) from Biotage (Biotage AB, Uppsala, Sweden), EnviCarb polygraphitized carbon (PGC) from Sigma Aldrich (Steinheim, Germany), and Bond-Elut PPL from Agilent (Santa Clara, CA, USA).

Oasis HLB is a polymeric reversed-phase sorbent composed by a copolymer of divinylbenzene and vinyl pyrrolidinone that has been efficiently used for the enrichment of natural toxins from environmental samples (Schenzel et al., 2010). Good recoveries were obtained for polar to moderately polar toxins as expected such as aflatoxins, nodularin and microcystins in general, except for MC-LF (recoveries < 10%) because it is a non-polar compound. C_{18} is a hydrophobic silica bonded sorbent efficient to isolate non-polar and moderately polar compounds from aqueous matrices. Some studies reported the high recovery of natural toxins when it was combined with other phases such as PGC (Liu and Scott, 2011). Here, C_{18} showed good recoveries for polar to moderately polar compounds as well such as microcystins but with lower recoveries than Oasis HLB. Kja, Sco, Atr, Ana-a, Chn, Cyn, were well retained with the PGC (24–58%). The Bond Elut PPL is a styrene-divinylbenzene (SDVB) polymer that has been modified with a non-polar surface and is efficient in extracting dissolved organic matter (Dąbrowska et al., 2003; Raeke et al., 2016; Goss et al., 2017). As expected, this sorbent alone was not suitable for the retention of natural toxins but combined with other phases it improved the recoveries thanks to the lower content in organic matter into the final sample and to the diminished matrix effects.

With these results, a homemade triple-stage SPE method was developed and optimised, consisting of PGC-PPL-HLB in series. This combination showed the best recoveries, as can be shown in Fig. S1 of the Supporting Information. The correspondent K_{ow} have been also reported as supplementary information in Table S1.

Elution solvents were as well optimised. In Fig. 2, the recoveries obtained with different solvents or mixtures in a single step are summarised. The best elution was achieved with water: methanol 8:2 (v/v) which eluted the most of the natural toxins especially MC-LR, Kja, Sco, Ana-a, Atr, Bai and Cyl with recoveries ranging between 24 and 79%. The final volume was set at 15 mL to ensure a better recovery of toxins. Then, different mixtures were tested to improve the recovery from different sorbents. A mix of methanol/acetone 1:1 (v/v) was selected to ensure a better contact with the most polar phase (PGC). As can be seen in Fig. S1A, the recoveries were between 2.4 and 53%.

Finally, the best elution sequence was shown to be 15 mL of water: methanol 8:2 (v/v) followed by 15 mL of methanol/acetone 1:1 (v/v) and 15 mL more of methanol 100%.

The influence of the pH of elution solvents was studied as well. pH 2 showed in general, low recovery rates, basic pH (pH 11) gave satisfactory rates for Ana-a, MC-LR and MC-YR, but pH 7 yield the best compromise between loading efficiency and compound neutrality (**Fig. S1B**). Elution temperature (T°) was studied. The strongest elution was obtained working with warm solvents at 40 °C. The optimal approach gave recovery rates between 53 and 98% for the selected group of natural toxins of different polarities.

3.2. Suspect screening

Tentative structural assignments were based on the criteria previously described by Schymanski et al. (2015). From the suspect list, one hundred compounds were selected for the inclusion list considering the botanical diversity of the area, the most probable species present and the climatic conditions that can determine the production of certain natural toxins. This limit was decided as a compromise of the analytical time and the scan rate of the QExactive. A first attempt with the entire suspect list (2377 precursor ions) resulted in a very poor fragmentation spectrum with less than one scan for each peak which are not enough for the identification purposes of this step. QExactive data scan speed at a resolution of 17,500 can reach 12.5 Hz with an injection time of 50 ms in a range of 75–1100 *m/z*, but it required a higher analytical time to process the whole number of entries. Finally, a lower number of entries

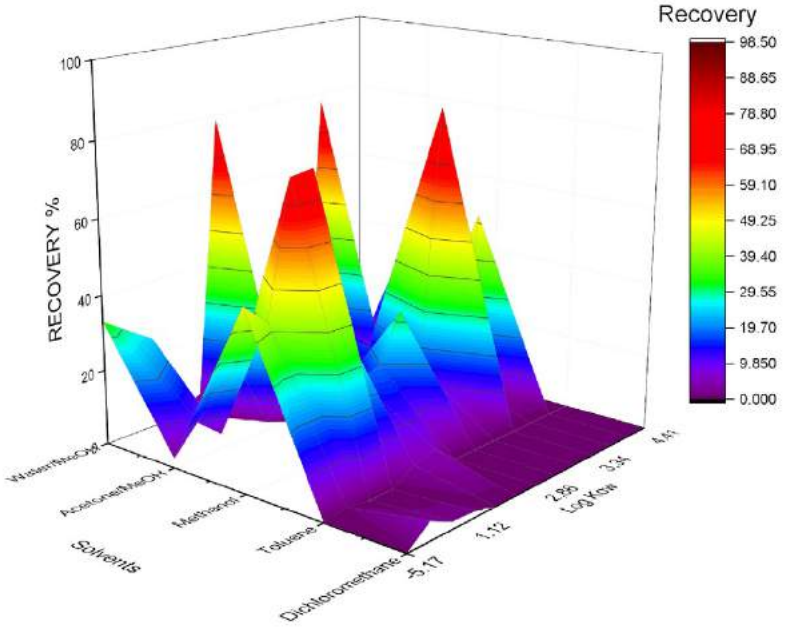

Fig. 2. Recoveries obtained using different solvents during the elution step of the SPE.

provided a more precise mass spectrum and at least three scans per peak, enough for identification purposes and it has then been used for the entire experiments. After pre-identification using Compound Discoverer, the fragmentation of the tentatively selected compounds was compared with known fragmentation patterns collected in on-line libraries such as MzCloud and MassBank.

The lowest levels of identification confidence (level 5, 4 and 3) comes from the similarity between experimental and the exact mass typical fragments and adducts proposed by the online databases. (<5 ppm). The probable structures were established matching the MS^2 spectrums obtained with the FS-ddMS^2 mode and the spectrums reported in the literature, considering the same transitions and the same ratio of the product-ions (level 2). This is a high level of confidence but cannot be confirmed since it can lead to false positives. Finally, only those compared with standards and with coincident retention time compared at a tolerance of ±2.5% were confirmed to level 1 (Fig. 1).

These criteria were applied to tentatively identify natural toxins in 16 samples collected from four different sampling sites along the Ter River near the water catchment area of Barcelona city. In Fig. 3, an example of tentative identification in a real sample is presented. Following this procedure, a range between 14 and 25 structures have been tentatively identified in each point. In the case of MCs and Nod, the typical ADDA fragment (135.0505 *m/z*) was used to establish as tentative candidate. The ADDA fragment is a highly stable product ion originated by the typical moiety corresponding to the [phenyl-$CH_2CH(OCH_3)^+$ ion contained in MCs and Nod, that has been considered in various analytical methods for the identification of MCs and Nod using LC-*ESI*-MS/MS (Mbukwa et al., 2012; Hummert et al., 2001).

Finally, 23 natural toxins candidates were tentatively identified with a confidence level 2 (Table 2).

Most of them were frequently reported toxins except two of them aspionene, and laudanosine, which have been few times reported (Lehner et al., 2011; Fodale and Santamaria, 2002). It should be highlighted that a wide variety of toxins tentatively identified were phytotoxins (12), followed by mycotoxins (8).

The 3-acetoxytropane and hygrine are tropane alkaloids found in Datura stramonium of the Solanaceae family. Their alkaloids produce psychoactive effects and eventually, death (Boopathi, 2019). On the other hands, phytoestrogens such as coumestans exhibit estrogenic activity with reproductive effects on cattle. The presence of both groups of compounds tentatively identified in the Ter river was not surprising. Datura stramonium is extensively present along the entire region of Catalonia (Ministerio para la transición ecológica y el reto demografico, 2020), and its presence can be related to the leaching from the aerial parts of the plants into the water. Allantoin, geranylacetone and 20-deoxyingenol are as well plant secondary metabolites that were tentatively identified in some of the samples, and their production increases in the response of stress periods enhancing seedling tolerance (Takagi et al., 2016). Another compound that was tentatively identified in the samples was, xanthotoxol, which is the most abundant toxin from *Cnidium monnieri*. The abundance of *Cnidium monnieri* in Catalonia can be related to the presence of suspect xanthotoxol in Ter River. Moreover, there are different plants endemic of this area that can produce this metabolite. On the other hand, the chemical properties of xanthotoxol allow a quick transfer from plants to soils

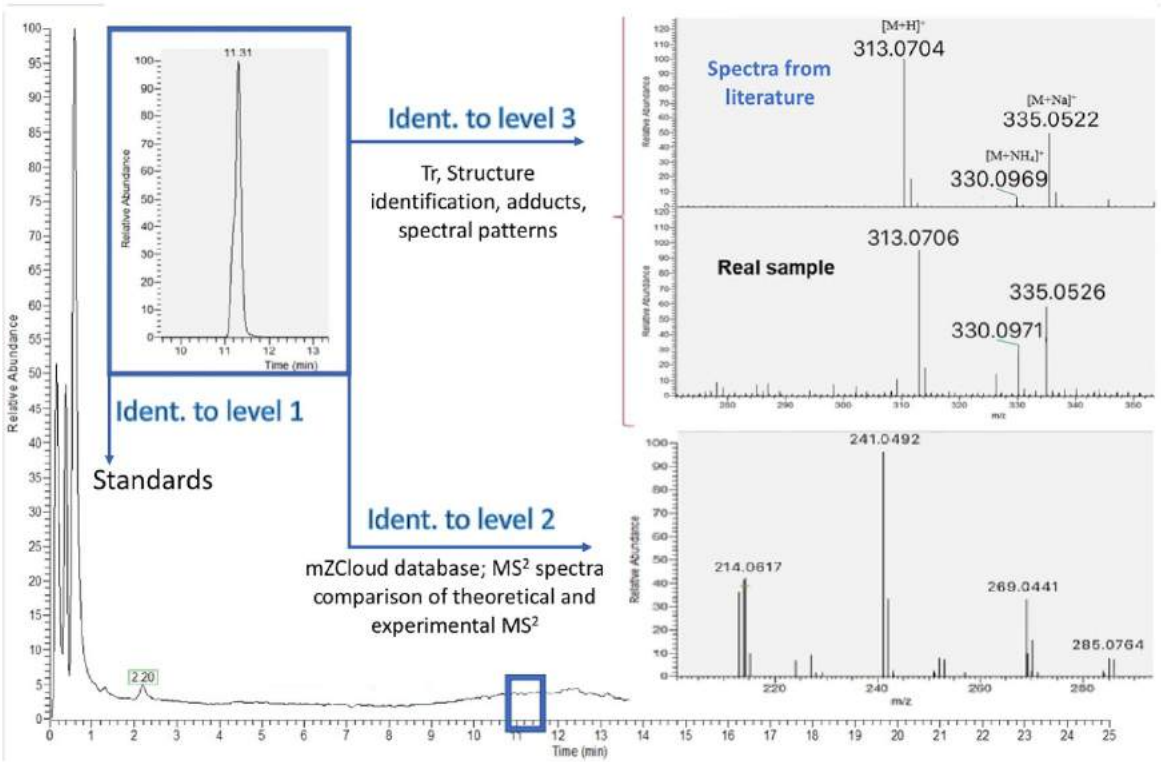

Fig. 3. Example of tentative identification of aflatoxin B_1

Table 2
Structures tentatively identified, chemical and features for their identification.

Compound Name	Chemical formula	Ret. Time [min]	Most int. ion species [m/z]	Ion specie	Theoretical [M+H]+	Observed [M+H]+	Rel. mass dev. [ppm]	m/z found	Ion specie	MS2	Ion specie
3-Acetoxytropane	$C_{10}H_{17}NO_2$	9.1	206.1151	$[M+Na]^+$	184.1334	184.1332	−1.1	184.1332	$[M-H]^-$	125.12062 146.09452	$[M+H-CH3COOH]$ $[M+Na-CH3COOH]^-$
20-Deoxyingenol	$C_{20}H_{28}O_4$	13.2	301.1396	$[M-H-CH_2OH]^-$	333.2060	333.2046	−4.3	355.1879	$[M-Na]^+$	315.1953	$[M+H-H_2O]^-$
Aflatoxin B_2	$C_{17}H_{14}O_6$	11.2	315.0863	$[M+H]^+$	315.0863	315.0863	0.0	337.0682	$[M-Na]^+$	297.2076 271.1543	$[M+H-H_2O]^-$ $[M+H-CO_2]^-$
Aflatoxin G_1	$C_{17}H_{12}O_7$	11.0	329.0655	$[M+H]^+$	329.0656	329.0655	−0.2	367.1270	$[M-K]^+$	311.1077 312.1309 285.0643	$[M+H-H2O]^+$ $[M+H-OH]^-$ $[M+H-CO_2]^+$
Aflatoxin G_2	$C_{17}H_{14}O_7$	10.7	331.0812	$[M+H]^+$	331.0812	331.0812	0.0	353.0631	$[M-Na]^+$	313.0898 287.1463 286.1130	$[M+H-H_2O]^+$ $[M+H-CO_2]^+$ $[M+H-COOH]^-$
Aflatoxin B_1	$C_{17}H_{12}O_6$	11.3	313.0707	$[M+H]^+$	313.0707	313.0707	0.1	335.0526 351.0266	$[M-Na]^+$ $[M-K]^+$	253.0495 269.08138 241.0477	$[M+H-CH_3COOH]^+$ $[M+H-CO_2]^+$ $[M+H-C_2H_5COCH_2]^+$
Allantoin	$C_4H_6N_4O_3$	0.4	159.0506	$[M+H]^+$	159.0513	159.0506	−4.2	197.0158 181.0063	$[M-K]^+$ $[M-Na]^+$	144.0050 111.0235	$[M+H-NH_3]^+$ $[M+H-CHOHNH_2]^+$
Anatoxin-a	$C_{10}H_{15}NO$	0.7	166.1226	$[M+H]^+$	166.1223	166.1226	1.8	204.1032 188.0488	$[M-K]^+$ $[M-Na]^+$	123.1048 151.0997	$[M+H-CH_3CO]^+$ $[M+H-CH3]^+$
Arecoline	$C_8H_{13}NO_2$	1.9	156.1019	$[M+H]^+$	156.1019	156.1019	0.0	194.0582 178.0838	$[M-K]^+$ $[M-Na]^+$	141.0789 96.08132	$[M+H-CH_3]^+$ $[M+H-CH_3OOH]^+$
(−)-Aspidospermine	$C_{22}H_{30}N_2O_2$	12.5	355.2380	$[M+H]^+$	355.2380	355.2380	0.0	377.2199 393.1940	$[M-Na]_+$ $[M-K]_+$	337.1187 322.1774	$[M+H-H_2O]^+$ $[M+H-CH_4OH]^-$
Aspionene	$C_9H_{16}O_4$	11.0	187.0976	$[M-H]^-$	187.0976	187.0976	0.0	209.0751 231.9252	$[M-H+CH_3OH]^-$ $[M-H+HCOOH]^-$	142.0953 168.9833	$[M-H-COOH]^-$ $[M-H-H_2O]^-$
Azelaic Acid	$C_9H_{16}O_4$	11.0	189.1122	$[M+H]^+$	189.1121	189.1122	0.1	227.0680 211.0941	$[M-K]_+$ $[M-Na]_+$	172.10994 144.11503	$[M+H-OH]$ $[M+H-COOH]^-$
Coumestan	$C_{15}H_8O_3$	0.2	237.0546	$[M+H]^+$	237.0546	237.0546	0.1	259.0471 274.8998 254.0404 268.8908 281.0709	$[M-Na]_+$ $[M-K]_+$ $[M-NH_4]_+$ $[M-CH_3OH]^-$ $[M-FA]^+$	219.0446 220.9143	$[M+H-H_2O]^+$ $[M+H-OH]$
Cryptolepine	$C_{16}H_{12}N_2$	0.4	243.1064	$[M+K]^+$	233.0700	233.0701	0.5	246.1253 263.1173 255.0740	$[M-CH_3CN+H]^+$ $[M-CH_3COOH-H]^+$ $[M-Na]^-$	218.0938	$[M+H-CH_4]^+$
Embelin	$C_{17}H_{26}O_4$	12.5	295.1904	$[M+H]^+$	295.1904	295.1904	0.0	317.1723 333.1459	$[M-Na]^+$ $[M-K]^+$	278.1174	$[M+H-OH]$
Geranylacetone	$C_{12}H_{22}O$	0.1	217.0174	$[M+Na]^+$	195.1743	195.1743	0.0	233.1541	$[M-K]^+$	177.1643 178.0937	$[M+H-H_2O]^+$ $[M+H-OH]^-$
Hygrine	$C_8H_{15}NO$	2.18	142.1222	$[M+H]^+$	142.1226	142.1222	−2.8	142.1222 180.1015	$[M-H]^-$ $[M-K]^+$	126.0909 111.0387 84.0805	$[M+H-CH_3]^+$ $[M+H-CH_2OH]$ $[M+H-C_3H_6O]^-$
Lantadene D	$C_{34}H_{52}O_5$	12.7	579.3507	$[M+K]^+$	541.3888	541.3879	−1.7	563.3225	$[M-Na]^+$	468.3190 439.091 343.2533	$[M+H-C_4H_8O_2]^+$ $[M+H-C_{10}H_{14}O_2N]^+$

(continued on next page)

Table 2 (*continued*)

Compound Name	Chemical formula	Ret. Time [min]	Most int. ion species [m/z]	Ion specie	Theoretical [M+H]+	Observed [M+H]+	Rel. mass dev. [ppm]	m/z found	Ion specie	MS2	Ion specie
Laudanosine	$C_{21}H_{27}NO_4$	13.2	325.2201	$[M+H-CH_3OH]^-$	358.2013	358.2010	−0.8	390.2423	$[M+H-CH_3OH]$	206.1175 189.091 151.0754	$[M+H-C_{13}H_{24}O]^+$ $[M+H-C_9H_{12}O_2]^+$ $[M+H-C_{10}H_{14}O_2N]^+$
Microcystin-LR	$C_{49}H_{74}N_{10}O_{12}$	12.0	995.5560	$[M+H]^-$	995.5560	995.5560	0.0	498.7860	$[M+H]^{2+}$	135.0440 931.4331	$[M+H-C_{11}H_{14}O_2N]^+$ [M+H-ADDA] $[M+H-CN_3H_4]^-$
Nodularin	$C_{41}H_{60}N_8O_{10}$	11.7	825.4505	[M+H]	825.4505	825.4505	0.0	848.6317 466.2278	$[M+Na]^+$ $[M+H]^{2+}$	807.4016 764.3502	$[M+H-H_2O]^+$ $[M+H-CN_3H_4]^-$
Stipitatic acid	$C_8H_6O_5$	0.4	183.0269	$[M+H]^-$	183.0288	183.0289	0.5	205.0369 221.0000	$[M+Na]^+$ $[M+K]^+$	137.9976 166.9685 204.0627	[M+H-COOH] $[M+H-OH]^+$ $[M+Na-OH]^+$
Xanthotoxol	$C_{11}H_6O_4$	0.4	225.0103	$[M+Na]^+$	203.0379	203.0399	9.9	240.9447 261.0761	$[M+K]^+$ $[M+CH_3COOH-H]^+$	184.8984 157.0714	$[M+H-H_2O]^-$ $[M+H-CHOOH]^-$

and water (Real et al., 2019; Moreno-Pedraza et al., 2019). However, no quantification was possible due to the lack of standards, and levels are only relatives to the intensities.

In Fig. 4, the frequency of the different groups of toxins tentatively identified in each point is reported. Also, in Table 3, the potential structures in each point and sampling month are summarised. M1 corresponds to the location near the Barcelona water catchment area which is downstream respect to the other sampling sites. Due to their characteristics of temperature, sun exposition, low water flow, and vegetation, it presented the major variety of toxins, as expected. In the same manner, M4 is located upstream and was the point with a lower occurrence of natural

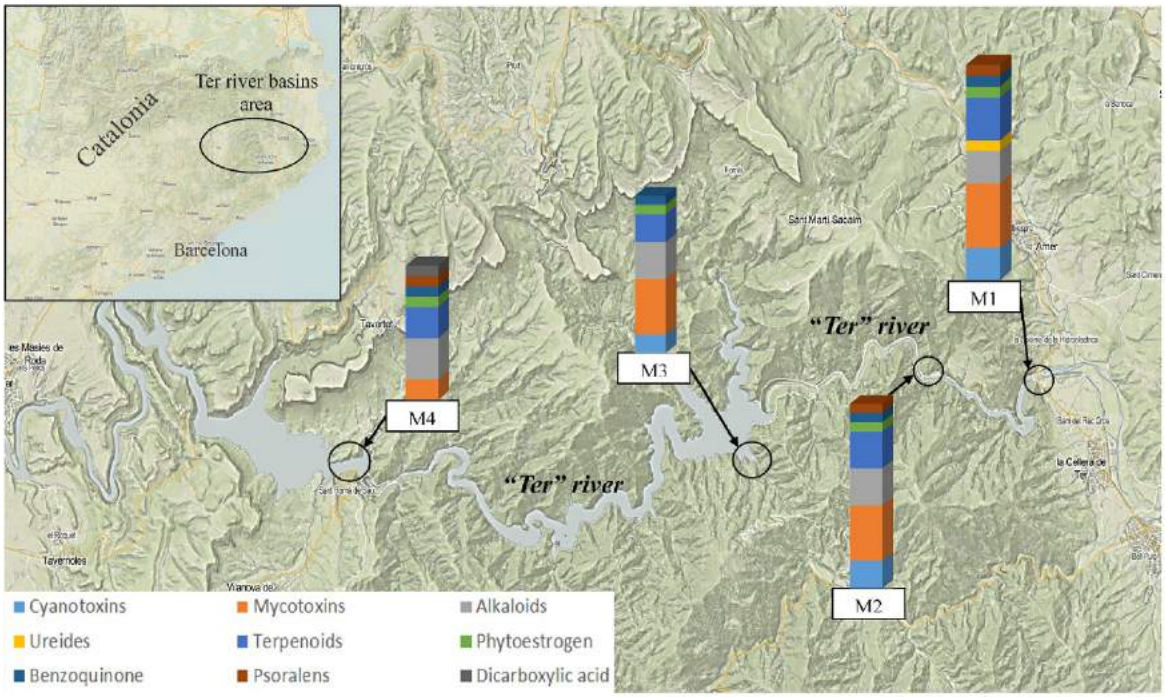

Fig. 4. Natural toxins screening results along the four points of the Ter river in Catalonia, Spain (Sampling points M1, M2, M3, M4).

Table 3
List of natural toxins tentatively identified in four sampling points during May and July; areas encountered between 10^4 and 10^6 a.u., quantification was not possible due of the lack of standards.

Sampling points	MAY				JULY			
	M1	M2	M3	M4	M1	M2	M3	M4
Natural toxins	***Mycotoxins***							
Aflatoxin B_1	X			X	X	X	X	
Aflatoxin B_2					X			
Aflatoxin G_1					X			
Aflatoxin G_2					X			
Allantoin					X	X	X	X
Aspionene							X	
Stiphatic acid			X	X	X			
Kojic acid	X	X	X	X	X		X	X
Azelaic Acid				X	X			
	Phytotoxins							
Embelin								X
Buddledin B	X		X					X
Xanthotoxol	X	X	X	X	X	X	X	X
Arecoline						X	X	X
(−)-Aspidospermine	X	X	X	X	X	X	X	X
20-Deoxyingenol		X	X		X		X	
3-Acetoxytropane	X	X	X			X		X
Coumestal/ coumestrol	X	X			X	X	X	
Geranylacetone					X	X	X	
Lantadene D			X		X			
Cryptolepine				X				X
Laudanosine	X		X	X		X	X	X
B-Asarone	X	X	X	X	X	X	X	X
Hygrine			X			X		
	Cyanotoxins							
Anatoxin-a	X				X	X		
Microcystin-LR	X				X	X		
Nodularin	X				X	X	X	

toxins tentatively identified.

Downstream, an increasing presence of natural toxins can be observed especially in July.

Comparing the results of May and July, mycotoxins and cyanotoxins presented a significant increase on the number of structures tentatively identified, while in the case of phytotoxins the number of compounds in July was only slightly higher than during May. Especially in the case of cyanotoxins, a significant increase was observed in July in the case of MC-LR and Nod.

Very few information is available on the growth of fungal species, especially in water environments.

Fungal species that naturally grow in streams tend to decay and to be adsorbed into plants. From the plants, bacteria and mycotoxins can leach into water streams leading to the contamination of surface water used for drinking water production. Moreover, mycotoxins can grow in surface water, groundwater and into the drinking water supply system (Al-Gabr et al., 2014; Hageskal et al., 2006; Magwaza et al., 2017).

During July and May, Catalonian's climate becomes warmer and humid, with more sun exposition with a consequent increase of water temperatures. During May and July 2017, the average temperature of inland Catalonia was above the 26 °C. with low precipitations (Servei Meteorològic de Catalunya). These factors aimed at the onset of the cyanobacterial algal bloom and also created the optimal conditions to the growth of moulds and fungi. The production of cyanotoxins and mycotoxins is independent, and to the author's knowledge, no article reported a dependence or correlation with the production of mycotoxins and cyanotoxins.

Finally, $AflB_1$, Ana-a, Atr, Bai, B-Asa, Chn, Kja, Nod and MC-LR were confirmed by standards and quantified.

3.3. Target analysis and quantification

The analytical procedure based on SPE-HPLC-HRMS employed for the suspect screening of natural toxins was validated for a selected group of toxins that were as well used for the spiking experiments. The recoveries obtained were in a range between 53 and 96%. MDLs ranges were between 0.07 and 1.22 μg/L while MQLs were between 0.02 and 4 μg/L. In Table S1 of the supporting information, the analytical parameters are summarised. The results of the 16 analysed samples reported 26 natural toxins tentatively identified in these natural waters (Table 3). Nine of them were confirmed and quantified (Table 4). As reported in Table 4, only Ana-a MC-LR and Nod were above the MQL in the sampling points M1, M2 and M3. MC-LR is the only toxin which has been regulated (Council directive 98/83/E, 1998) with a limit of 1 μg/L, and as can be seen in none of the samples, this limit was exceeded.

4. Conclusions

HRMS showed versatility and potential to carry out a suspect screening of natural toxins in water reservoirs and recreational waters. Suspect screening could be used as a routine tool to perform a comprehensive assessment of natural toxins in water reservoirs and recreational waters. The clean-up approach using a homemade triple-stage SPE was shown efficient for a wide range of polarities with recovery rated between 53 and 96% and showed good MQLs, between 0.02 and 4 μg/L, for the selected toxins using external calibration.

The suspect screening approach was carried out in different sampling sites close to one of the Barcelona catchment sites. The results showed a good performance of the new approach. One of the main contributions of this study is that 23 natural toxins were tentatively identified, indicating the presence of plant toxins and mycotoxins that in general are not monitored in surface waters but they can contribute to the final toxicity. Finally, 9 toxins ($AflaB_1$,

Table 4
Concentrations of the natural toxins confirmed with standards.

	MAY 1st Sampling				MAY 2nd Sampling			
	M1	M2	M3	M4	M1	M2	M3	M4
Toxin name	μg/L				μg/L			
$AflaB_1$	<MDL	<MDL	<MDL	<MDL	0.36	<MDL	<MDL	0.47
Ana-a	<MDL	<MDL	<MDL	<MDL	0.21	<MDL	<MDL	<MDL
Atr	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Bai	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
B-Asa	3.1	2.2	0.8	0.6	2.8	1.5	1.2	0.2
Chn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Kja	2.3	0.3	4.1	3.2	2.5	<MDL	3.8	2.6
Nod	<MDL	<MDL	<MDL	<MDL	0.25	<MDL	<MDL	<MDL
MC-LR	<MDL	<MDL	<MDL	<MDL	0.50	<MDL	<MDL	<MDL
	JULY 1st Sampling				**JULY 2nd Sampling**			
	M1	**M2**	**M3**	**M4**	**M1**	**M2**	**M3**	**M4**
Toxin name	μg/L				μg/L			
$AflaB_1$	<MDL	0.27	0.36	<MDL	0.26	0.27	0.39	<MDL
Ana-a	0.73	0.55	<MDL	<MDL	0.60	<MDL	<MDL	<MDL
Atr	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Bai	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
B-Asa	2.3	1.3	<MDL	4.3	1.2	1.0	0.4	3.8
Chn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Kja	0.9	1.5	0.6	0.7	<MDL	1.2	0.8	1.2
Nod	0.91	0.49	0.4	<MDL	0.85	0.45	0.2	<MDL
MC-LR	0.24	<MDL	<MDL	<MDL	0.23	0.24	<MDL	<MDL

$AflaB_1$: aflatoxin B_1, *Ana-a*: anatoxin-a, *Atr*: atropine, *Bai*: baicalein, *B-Asa*: b-asarone, *Chn*: cinchonine, *Kja*: kojic acid, *MC-LR*: Microcystin-LR, *Nod*: nodularin.

Ana-a, Atr, Bai, B-Asa, Chn, Kja, Nod, MC-LR) were confirmed. It should be highlighted that MC-LR, the only natural toxin under regulation in natural waters, was present in 2 of the 4 sampling sites here reported, but cyanotoxins concentration did not exceed the maximum level of 1 μg/L set by the EU legislation.

Credit author statement

Massimo Picardo: Investigation, Formal analysis and writing-Original draft. Josep Sanchís: Methodology and Writing - Review & Editing. Oscar Núñez:Supervision and Writing - Review & Editing. Marinella Farré: Conceptualization,Methodology, Supervision and Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127888.

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Supporting Information

Suspect screening of natural toxins in surface and drinking water by high performance liquid chromatography and high-resolution mass spectrometry

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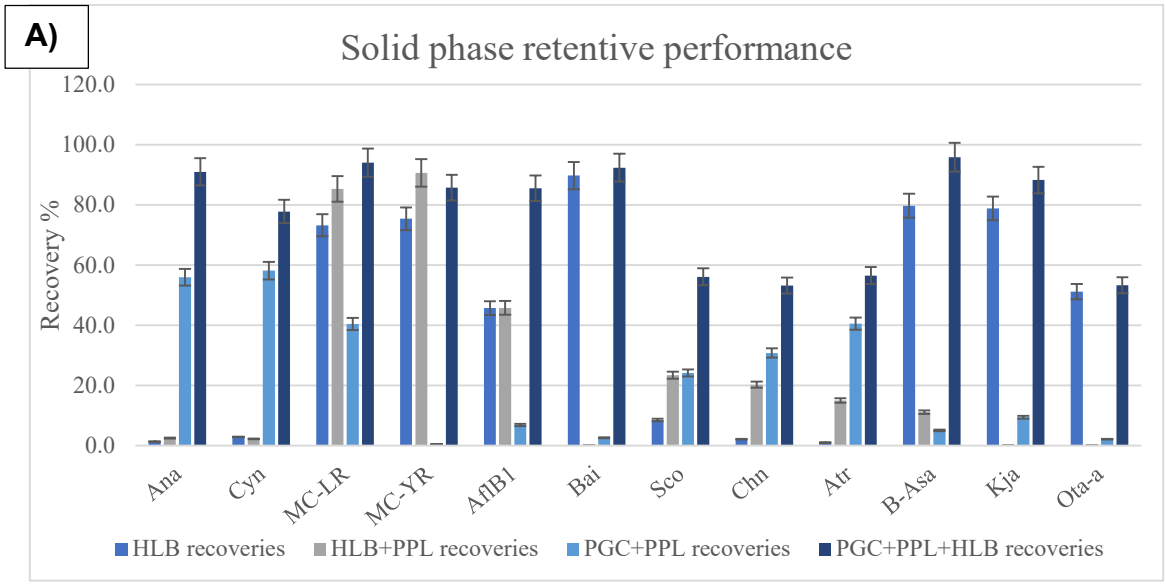
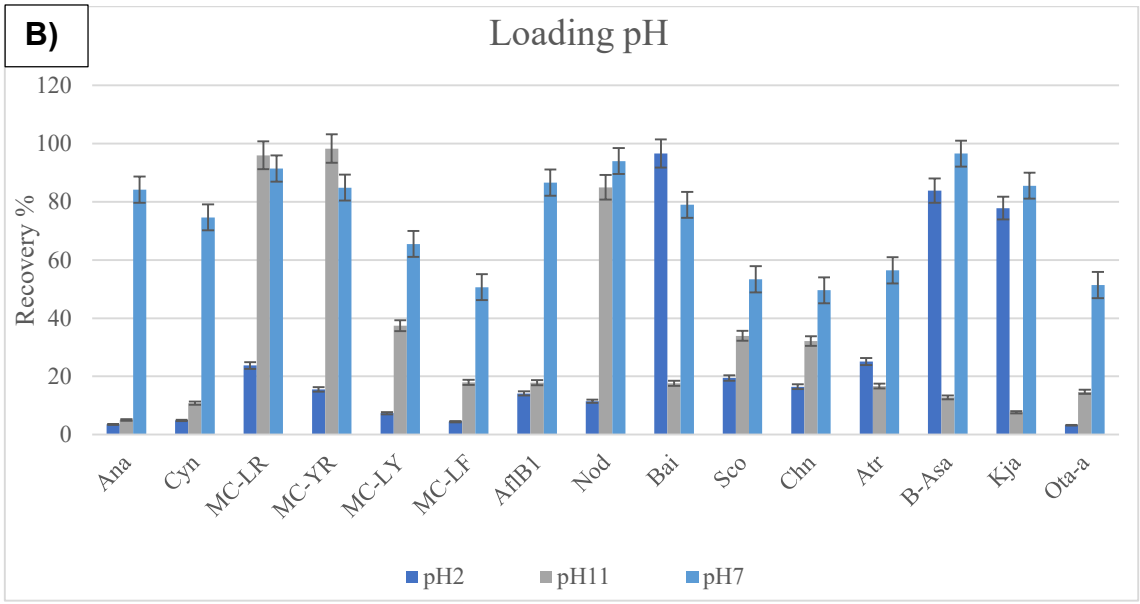
Figure S1

A) Recoveries obtained by different sorbent materials HBL, PGC and the combination of PGC+PPL and PGC sorbents for the selected natural toxins

B) Recoveries using loading at different pH





$AflB_1$: Aflatoxin B_1; Ana: anatoxin-a; Atr: atropine; Bai: baicalein; B-asa: beta-asarone; Chn: cinchonine; Cyn: cylindrospermopsins; HLB: hydrophilic lipophilic balance; Kja: kojic acid; MC-LR: microcystin –LR; MC-YR: microcystin-YR; Nod: nodularin; Ota-a: ochratoxin-a; PGC: polygraphytized carbon: PPL: polymeric sorbent phase;

Table S1: Analytical parameters of the target approach

Toxins	Molecular formula	Exact mass	HR m/z [M+H]+	Recovery %	RSD%	MDL ug/L	MQL ug/L	Ion suppression %	R^2	Kow	Reference
Ana-a	$C_{10}H_{15}NO$	165.1154	166.1234	84	8.0	0.2	0.5	10.4	0.989	0.8	506
Cyn	$C_{15}H_{21}N_5O_7S$	415.1162	313.2878	74	13.0	1.2	4.1	2.7	0.994	-2.6	506
AflB1	$C_{17}H_{12}O_6$	312.2798	416.1242	86	9.9	0.2	0.7	25.5	0.999	1.6	507
MC-LR	$C_{49}H_{74}N_{10}O_{12}$	994.5488	995.5568	78	3.3	0.2	0.5	5.1	0.995	-1.2	508
MC-LY	$C_{52}H_{72}N_{10}O_{13}$	1001.5110	1002.5190	65	5.8	0.1	0.2	7.8	0.981	-0.65	508
Nod	$C_{41}H_{60}N_8O_{10}$	824.4432	825.4512	94	16.2	0.2	0.8	4.2	0.992	1.7	509
MC-YR	$C_{54}H_{72}N_8O_{12}$	1044.5281	1045.5361	84	16.9	0.4	1.5	12.1	0.943	3.9	505
Bai	$C_{15}H_{10}O_5$	271.0600	270.0522	96	5.2	0.09	0.30	1.5	0.9984	2.71	510
Sco	$C_{17}H_{21}NO_4$	304.1543	303.1465	53	1.8	0.08	0.27	0.8	0.9883	0.9	511
Chn	$C_{19}H_{22}N_2O$	295.1804	294.1726	50	0.6	0.09	0.29	1.9	0.9900	3.16	512
Atr	$C_{17}H_{23}NO_3$	290.1750	289.1672	56	1.1	0.04	0.13	0.8	0.9909	1.83	513
Kja	$C_{12}H_{16}O_3$	209.1172	208.1093	85	6.4	0.02	0.08	1.9	0.9907	-0.9	507
Ota-a	$C_6H_6O_4$	143.0338	142.0260	89	3.7	0.16	0.53	0.9	0.99	4.7	507
B-asa	$C_{20}H_{18}ClNO_6$	404.0895	403.0817	51	5.4	0.08	0.28	1.0	0.9971	3.03	514

$AflB_1$: Aflatoxin B_1; Ana: anatoxin-a; Atr: atropine; Bai: baicalein; B-asa: beta-asarone; Chn: cinchonine; Cyn: cylindrospermopsin; HR m/z: High resolution m/z; Kja: kojic acid; MC-LR: microcystin –LR; MC-YR: microcystin-YR; MDL: Method detection limits; MQL Method quantification limits; Nod: nodularin; Ota-a: ochratoxin-a; RSD: relative standard deviation (precision)

Table 2A only reports the first 33 compounds in the in house suspect list that have been included as informative purpose. However, the list counts with 2384 compounds, and can be found in the online supporting information if required.

Table 2A: Natural toxins list used for the suspect screening method

Toxins name	Molecular weight	Molecular formula	CASRN	Group	References
Nostocarboline	217.0527	C12H10ClN2	874440-44-5		18
Falcarinolone	258.1614315	C17 H22 O2	18089-23-1	Polyacetylene	39
Durantoside I	552.1837424	C26H32O13	53526-67-3	Terpene, Monoterpene, Iridoid glycoside	43
Durantoside II	582.194307	C27H34O14	533526-66-2	Terpene, Monoterpene, Iridoid glycoside	43
Isocupressic acid	320.2345964	C20H32O3	1909-91-7	Terpene, Diterpene, Diterpene acid	43
Urushiol I (C15:0)	320.2709819	C21H36O2	492-89-7	Polyketide, Alkylphenol	47
Urushiol III (C15:2)	316.2396818	C21H32O2	492-91-1	Polyketide, Alkylphenol	47
Urushiol IV (C15:3)	314.2240317	C21H30O2	83532-40-5	Polyketide, Alkylphenol	47
Ailantinol B	394.1622192	C20H26O8	177794-39-7	Terpene, Triterpene, Triterpene lactone, Quassinoid	53
Canthin-6-one	220.0631144	C14H8N2O	479-43-6	Alkaloid, Indole alkaloid, beta-Carboline alkaloid	66
Cypripedin	284.067925	C16H12O5	8031-72-9	Quinone	66
Harmaline	214.1100646	C13H14N2O	304-21-2	Alkaloid, Indole alkaloid, beta-Carboline alkaloid	66
Harmine	212.0944146	C13H12N2O	442-51-3	Alkaloid, Indole alkaloid, beta-Carboline alkaloid	66
Hydroxycanthin-6-one	236.058029	C14H8N2O2	80787-59-3	Alkaloid, Indole alkaloid, beta-Carboline alkaloid	66
Osthole	244.1093959	C15H16O3	484-12-8	Phenylpropanoid, Furanocoumarin	66
Saniculoside N	1100.576175	C55H88O22	196955-52-9	Saponin, Triterpenoid saponin	66

Saniculoside R-1				Saponin, Triterpenoid saponin	66
Tetrahydroharmine	216.1257147	C13H16N2O	7759-46-8	Alkaloid, Indole alkaloid, beta-Carboline alkaloid	66
Enniatin B3	611.37818	C31H53N3O9			67
Enniatin D	653.42513	C34H59N3O9			67
Enniatin J1	611.37818	C31H53N3O9			67
Enniatin K1	625.39383	C32H55N3O9			67
Echihumiline	397.2095037	C20H31NO7		Alkaloid, Pyrrolizidine alkaloid	71
Echivulgarine	479.2513685	C25H37NO8		Alkaloid, Pyrrolizidine alkaloid	71
Leptanthine	315.1676389	C15H25NO6		Alkaloid, Pyrrolizidine alkaloid	71
Uplandicine	357.1782036	C17H27NO7	74202-10-1	Alkaloid, Pyrrolizidine alkaloid	71
[(6Z)-Adda5]MC-RR	1037.565222	C49H75N13O12		Mycrocystin; Cyanotoxins	105
[6(Z)-Adda3]NOD	824.4432217	C41H60N8O10		Mycrocystin; Cyanotoxins	105
[6Z-Adda5]MC-LR	994.5482217	C49H74N10O12		Mycrocystin; Cyanotoxins	105
[9-AcO-Adda5]MC-RR [ADMAdda5]MC-RR	1065.560122	C50H75N13O13		Mycrocystin; Cyanotoxins	105
[ADMAdda5, Dha7]MC-LR	1008.527422	C49H72N10O13		Mycrocystin; Cyanotoxins	105
[ADMAdda5, MeSer7]MC-LR	1040.553622	C50H76N10O14		Mycrocystin; Cyanotoxins	105
[ADMAdda5]MC-(H4)YHar	1090.569322	C54H78N10O14		Mycrocystin; Cyanotoxins	105

3.4.2 Publication III

Tentative identification of suspect natural toxins. A case study for their seasonal occurrence in Ter River (Catalonia, Spain).

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Toxins, 12, Accepted
DOI in Process

toxins

MDPI

Article

Suspect and Target Screening of Natural Toxins in the Ter River Catchment Area in NE Spain and Prioritisation by Their Toxicity

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Abstract: This study presents the application of a suspect screening approach to screen a wide range of natural toxins, including mycotoxins, bacterial toxins, and plant toxins, in surface waters. The method is based on a generic solid-phase extraction procedure, using three sorbent phases in two cartridges that are connected in series, hence covering a wide range of polarities, followed by liquid chromatography coupled to high-resolution mass spectrometry. The acquisition was performed in the full-scan and data-dependent modes while working under positive and negative ionisation conditions. This method was applied in order to assess the natural toxins in the Ter River water reservoirs, which are used to produce drinking water for Barcelona city (Spain). The study was carried out during a period of seven months, covering the expected prior, during, and post-peak blooming periods of the natural toxins. Fifty-three (53) compounds were tentatively identified, and nine of these were confirmed and quantified. Phytotoxins were identified as the most frequent group of natural toxins in the water, particularly the alkaloids group. Finally, the toxins identified to levels 2 and 1 were prioritised according to their bioaccumulation factor, biodegradability, frequency of detection, and toxicity. This screening and prioritisation approach resulted in different natural toxins that should be further assessed for their ecotoxicological effects and considered in future studies.

Keywords: natural toxins; cyanotoxins; phytotoxins; mycotoxins; suspected screening; HRMS

Key Contribution: A suspect screening approach has been applied to assess natural toxins in one of the water reservoirs of Barcelona city, NE Spain. The toxins that were tentatively identified were prioritised.

1. Introduction

Natural toxins in the aquatic ecosystem can be produced by different organisms, including bacteria, plants and fungi, thus grouping together a wide variety of structures and physicochemical properties and effects [1]. The risk of water contamination by natural toxins generates environmental and public health issues. In some cases, natural toxins can be accumulated in aquatic organisms and transferred throughout the aquatic food chain to humans [2].

However, if we consider freshwater environments, the primary route of human exposure includes the consumption of contaminated water, dermal exposure, and inhalation during recreational activities. Intoxication can include different symptoms, such as a severe headache, a

fever, and respiratory paralysis, as well as a variety of possible effects that include hepatotoxicity, neurotoxicity, carcinogenicity, and dermal toxicity. Due to their diversity, toxicological assessment is still challenging and there is also an information gap concerning their occurrence, due to the lack of analytical methods and certified standards. Therefore, the concentration of natural toxins in drinking water for most of these groups is not yet well regulated, and this is also of concern for countries in the European Union (EU).

Among the natural toxins, the cyanotoxins group is one of the most studied groups in freshwater ecosystems. Cyanotoxins can be released by cyanobacterial blooms, which is a frequent natural phenomenon that is characterised by an algal biomass accumulation in surface water. These secondary metabolites include hepatotoxins (microcystins and nodularins), neurotoxins (such as anatoxins, saxitoxins, and β-methylamino-l-alanine), cytotoxins (such as cylindrospermopsin), and dermatotoxins (lipopolysaccharide, lyngbyatoxins, and aplysiatoxin). Among them, microcystins (MCs), produced by freshwater cyanobacteria genera such as *Microcystis*, *Aphanizomenon*, *Planktothrix*, *Dolichospermum*, etc. [3], are the most diverse group and the best described in the literature [4]. However, only one congener is regulated. The World Health Organization (WHO) has issued a guideline value of 1 μg/L in drinking water for microcystin-LR (MC-LR), which is one of the most toxic and widespread toxins in water supplies [5].

Another relevant group is represented by mycotoxins, which are secondary metabolites produced by fungi. Due to their diverse chemical structures, mycotoxins can present a wide range of toxicity, such as hepatotoxicity, nephrotoxicity, neurotoxicity, and immunotoxicity, and some of them have been recognised as being teratogenic, mutagenic, and carcinogenic [3]. Their biological effects have been extensively reported and regulated in food and feed [6,7] but not in water. However, many environmental species (particularly of the genus *Aspergillus*) show resistance to the commonly used water disinfection procedures, allowing them to enter water distribution/reticulation systems [8,9]. Moreover, those species can form mixed biofilm communities with bacteria, algae, and protozoa. These biofilms increase the ability to survive heat treatments and chlorination procedures. Therefore, fungal presence in tap water distribution systems also leads to an increase in the presence of temperature-tolerant fungi, which are the target of many studies that note this as a serious health risk [10].

The phytotoxins group includes secondary metabolites that are produced by plants as a defence mechanism against herbivores, insects, or other plant species [11]. They can include different chemical structures, including peptides, terpenoids, flavones, glycosides, and phenolic compounds (<3500 Da) [12]. Phytotoxins can be grouped into three major chemical structures: alkaloids, terpenes, and phenols. Among them, furanocoumarins, lectins, glycoalkaloids, and pyrrolizidine alkaloids are the most studied [1,13,14]. These compounds can end up in water bodies due to leaching from leaves and soil, and some of them can present high toxicity, such as the case of the carcinogenic ptaquiloside, which is produced by bracken fern [15]. However, in general, few studies have explored their presence in surface waters [16], despite their potentially high toxicity alone or in combination with other anthropogenic contaminants.

During the recent decades, the contamination and over-enrichment of nutrients (eutrophication) of surface waters have increased the number of harmful algal bloom events. Moreover, the increasing temperatures and light intensity promote the algal bloom events and consequently the production of natural toxins [17]. Their chemical diversity, the variety of their structures with structural features that are comparable to common anthropogenic contaminants, and their low concentrations can lead to harmful effects, making their determination in surface waters a great challenge. For these reasons, it is of primary importance to investigate the occurrence of natural toxins in the aquatic environment.

The most common approaches using multi-residue analysis include a limited number of compounds [18,19]. Most approaches cannot determine a wide range of polarities, in that they are mostly applied for one particular compound or a group of compounds with similar characteristics. The suspect screening methods that are based on high-resolution mass spectrometry (HRMS) opened a new window for the comprehensive study of natural toxins in surface waters.

In this regard, the main goal of the present study was to apply a recently developed method [20], based on a generic three-step solid-phase extraction (SPE) procedure followed by liquid chromatography (LC) coupled to high-resolution mass spectrometry (HRMS), with full-scan (FS) and data-dependent MS^2 (DDA) acquisition using a Q-Exactive Orbitrap analyser, to study the natural toxins in different water reservoirs that are used to produce tap water in Barcelona city (Catalonia, NE Spain).

Here, we present the data that was originated by the analysis of a complete set of samples that were collected during a sampling campaign in the period of March to September 2018. The data reported in the previous work have been omitted in the present one. In this sampling campaign, the 48 samples were collected at 4 sites along the Ter River. Sample collection was carried out twice a month from March to September 2018. In our previous study, the 16 samples that came from the Ter River were collected using a different sampling campaign, specifically designed to assess the good performance of the newly developed approach, and was carried out in May and July, and thus needless to say at different days from the samples presented here. Moreover, a prioritisation protocol, including a scoring system, is reported now, designed to elucidate the most significant natural toxins of concern in the drinking water reservoirs.

The suspect screening was carried out using a suspect list containing 2384 items of natural toxin data that were collected from the literature and online databases (mzCloud and ChemSpider). The confidence levels for the identification of suspect natural toxins were based on the approach that was previously reported by Sckymansky et al. [21], consisting of mass accuracy, isotopic fit, fragmentation, and final confirmation, using standards and retention times. Finally, the suspect natural toxins were prioritised according to their toxicity, frequency of detection, biodegradability, and bioaccumulation factors. The results of this screening and prioritisation protocol present a set of natural toxins that could be assessed for their toxicological effects and should also be considered in future water monitoring studies. To the best of our knowledge, this is the first study providing the prioritisation of natural toxins in a water reservoir in Spain.

2. Results and Discussion

2.1. Tentatively Identified Compounds

In this study, after removal of the background and the very small signals under the minimum intensity threshold, 4404 suspect masses were detected in the 48 water samples by using Compound Discoverer 3.1 software. Among them, 381 compounds (8.6%) were assessed as suspect natural toxins that were included in the in-house database and finally selected for further screening. It is noteworthy that the compounds of the study were natural toxins pertaining to three major groups in water, phytotoxins, mycotoxins, and cyanotoxins. Other compounds, such as pesticides, were discarded in this study. Among these 381 structures, after filtering by way of the isotopic patterns, ionisation efficiency, and fragmentation patterns, the number of suspected identified compounds diminished to 191 structures (50.1% of the initial potential for natural toxins). Finally, the comparison with in-silico MS^2 patterns gave 50 structures that were tentatively identified at level 2 (25.7% of the initial potential for natural toxins) (Table 1 and Figure 1). Finally, nine natural toxins were confirmed and quantified by injections of the standard.

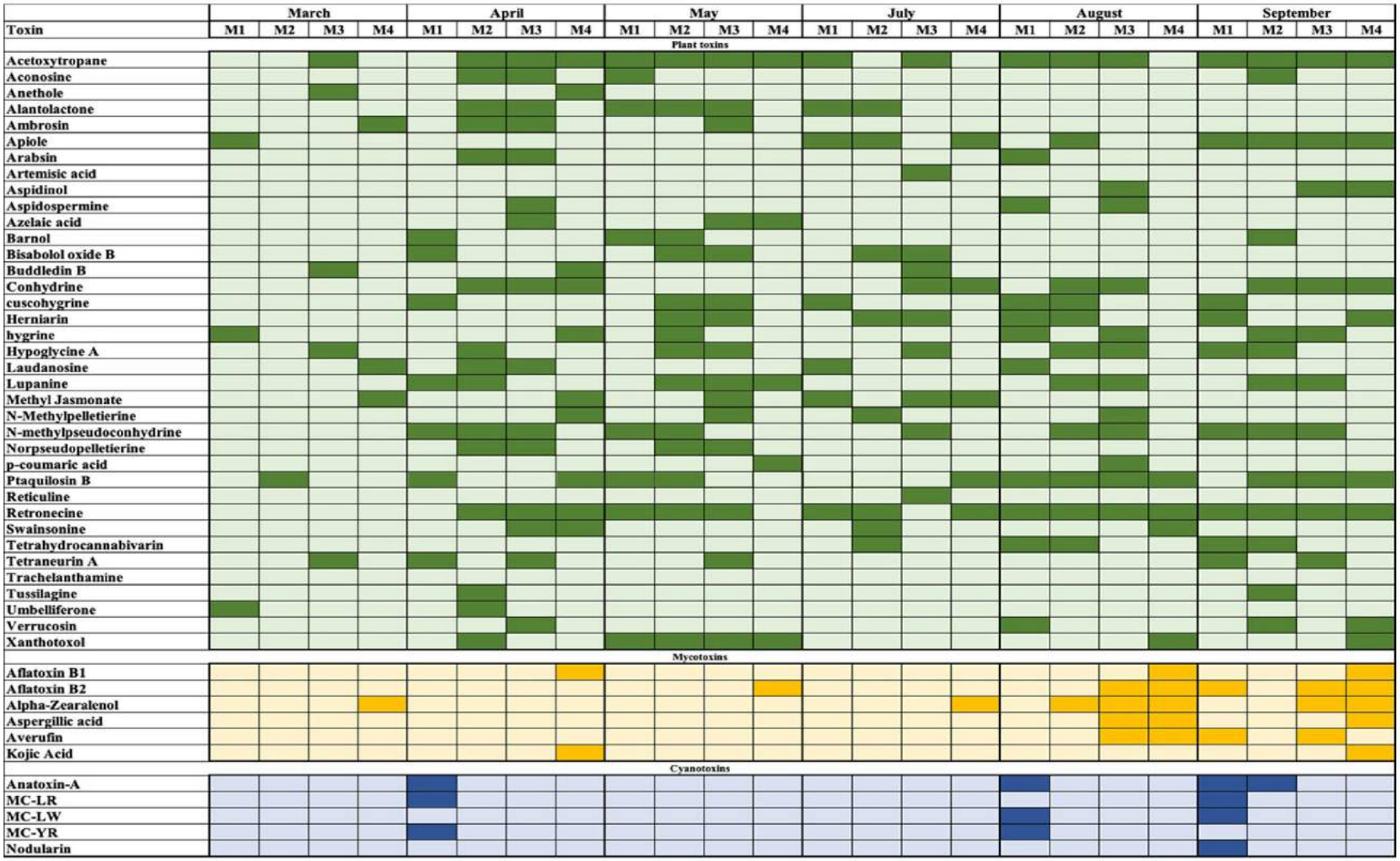

Figure 1. Hits diagram. A dark colour indicates a positive hit.

Table 1. List of suspect compounds (level 2) after tentative identification in the four sampling sites along water reservoirs in the Ter River.

Toxins	Formula	[M + H]	Rt	MS^2 (1)	$[M-e]^+$	MS^2 (2)	$[M-e]^+$	MS^2 (3)	$[M-e]^+$	MS^2 (4)	$[M-e]^+$
Plant Toxins											
Acetoxytropane	$C_{10}H_{17}NO_2$	184.1332	9.1	123.0805	$C_8H_{11}O$	142.0864	$C_7H_{12}NO_2$	125.0599	$C_7H_9O_2$	165.0913	$C_{10}H_{13}O_2$
Aconosine	$C_{22}H_{35}NO_4$	378.2639	11.3	283.1701	$C_{19}H_{23}O_2$	269.1539	$C_{18}H_{21}O_2$	235.1324	$C_{14}H_{19}O_3$	137.0599	$C_8H_9O_2$
Anethole	$C_{10}H_{12}O$	149.0961	9.8	115.0544	C_9H_7	103.0543	C_8H_7	145.065	$C_{10}H_9O$	121.0649	C_8H_9O
Ambrosin	$C_{15}H_{18}O_3$	247.1332	8.5	229.1227	$C_{15}H_{17}O_2$	201.1267	$C_{13}H_{13}O_2$	119.0857	C_9H_{11}		
Apiol	$C_{12}H_{14}O_4$	223.0965	11.9	105.07	C_8H_9	119.0857	C_9H_{11}	163.0755	$C_{10}H_{11}O_2$	149.0963	$C_{10}H_{13}O$
Arabsin	$C_{15}H_{22}O_4$	266.1521	10.8	249.1488	$C_{15}H_{21}O_3$	231.1384	$C_{15}H_{19}O_2$	221.1539	$C_{14}H_{21}O_2$		
Artemisic acid	$C_{15}H_{22}O_2$	235.1702	14	179.1069	$C_{11}H_{15}O_2$	165.0901	$C_{10}H_{13}O_2$	119.0853	C_9H_{11}		
Aspidinol	$C_{12}H_{16}O_4$	225.1121	9.5	107.0492	C_7H_7O	137.0599	$C_8H_9O_2$	123.0441	$C_7H_7O_2$	109.0649	C_7H_7O
Aspidospermine	$C_{22}H_{30}N_2O_2$	355.2380	12.5	107.0492	C_7H_7O	136.0759	$C_8H_{10}NO$	174.0915	$C_{11}H_{12}NO$	148.0759	$C_9H_{10}NO$
Azelaic acid	$C_9H_{16}O_4$	189.1121	11.0	107.0854	C_8H_{11}	155.0704	$C_8H_{11}O_3$	111.0806	$C_7H_{11}O$	115.0391	$C_5H_7O_3$
Barnol	$C_{10}H_{14}O_3$	183.1016	10.8	119.0857	C_9H_{11}	135.0806	$C_9H_{11}O$	163.0755	$C_{10}H_{11}O_2$	181.086	$C_{10}H_{13}O_3$
Bisabolol oxide	$C_{15}H_{26}O_2$	239.2006	12.4	133.1013	$C_{10}H_{13}$	121.1013	C_9H_{13}	149.1326	$C_{11}H_{17}$	187.1483	$C_{14}H_{19}$
Buddledin B	$C_{15}H_{22}O_2$	235.1693	12.9	113.0598	$C_6H_9O_2$	179.0106	$C_{11}H_{15}O_2$	193.1225	$C_{12}H_{17}O_2$	155.1067	$C_9H_{15}O_2$
Conhydrine	$C_8H_{17}NO$	144.1383	11.6	107.0856	C_8H_{11}	125.0962	$C_8H_{11}O$	138.0915	$C_8H_{12}NO$		
Cuscohygrine	$C_{13}H_{24}N_2O$	225.1961	12.3	123.0805	$C_8H_{11}O$	109.0649	C_7H_9O	163.1118	$C_{11}H_{15}O$	150.0914	$C_9H_{12}NO$
Curassavine	$C_{16}H_{29}NO_4$	300.2169	12.6	155.0703	$C_8H_{11}O_3$	107.0856	C_8H_{11}	123.0805	$C_8H_{11}O$	173.081	$C_8H_{13}O_4$
Herniarin	$C_{10}H_8O_3$	176.0477	11.8	121.0649	$C_7H_5O_2$	133.0653	C_9H_9O				
Hydroxyarbusculin A	$C_{15}H_{22}O_4$	267.1585	13.3	159.1169	$C_{12}H_{15}$	123.0805	$C_8H_{11}O$				
Hydroxycoumarin	$C_9H_6O_3$	163.0390	15.1	121.0284	$C_7H_5O_2$	149.0233	$C_8H_5O_3$	163.0389	$C_9H_7O_3$	105.0335	C_7H_5O
Hygrine	$C_8H_{15}NO$	142.1226	10.9	109.065	C_7H_9O	124.0758	$C_7H_{10}NO$	111.0804	$C_7H_{11}O$	140.1069	$C_8H_{14}NO$
Hypoglycine A	$C_7H_{11}NO_2$	142.0862	2.34	97.0287	$C_5H_5O_2$	120.0444	C_7H_6NO	124.0757	$C_7H_{10}NO$		
Laudanosine	$C_{21}H_{27}NO_4$	358.2013	13.2	121.0285	$C_7H_5O_2$	115.0543	C_9H_7	159.088	$C_{11}H_{11}O$	147.0805	$C_{10}H_{11}O$
Lupanine	$C_{15}H_{24}N_2O$	249.1961	5.3	110.0965	$C_7H_{12}N$	120.0808	$C_8H_{10}N$	122.0966	$C_8H_{12}N$	138.0915	$C_8H_{12}NO$
Methyl Jasmonate	$C_{13}H_{20}O_3$	225.1485	0.1	107.0855	C_8H_{11}	121.1012	C_8H_{13}	175.112	$C_{12}H_{15}O$	165.1275	$C_{11}H_{17}O$
Methylpelletierine	$C_9H_{17}NO$	156.1386	2.2	107.0705	C_8H_{11}	140.105	$C_8H_{14}No$				
Methylpseudoconhydrine	$C_9H_{19}NO$	158.1539	11.9	107.0856	C_8H_{11}	114.0914	$C_6H_{12}NO$	123.0805	$C_8H_{11}O$	109.0649	C_7H_9O
Norpseudopelletierine	$C_8H_{13}NO$	140.1070	9.1	109.0649	C_7H_9O	121.0649	C_8H_9O	138.0917	$C_8H_{12}NO$	123.0806	$C_8H_{11}O$

p-Coumaric acid	$C_9H_8O_3$	165.0546	12.5	105.07	C_8H_9	123.0441	$C_7H_7O_2$	133.0649	C_9H_9O	125.0598	$C_7H_9O_2$
Ptaquilosin B	$C_{14}H_{20}O_3$	237.1485	11.2	119.0857	C_9H_{11}	159.0807	$C_{11}H_{11}O$	145.1013	$C_{11}H_{13}$	111.0442	$C_6H_7O_2$
Reticuline	$C_{19}H_{23}NO_4$	330.1700	13.2	115.0543	C_9H_7	125.0597	$C_7H_9O_2$	145.0646	$C_{10}H_9O$	135.0441	$C_8H_7O_2$
Retronecine	$C_8H_{13}NO_2$	156.1019	1.9	152.0709	$C_8H_{10}NO_2$	118.0652	C_8H_8N	114.0916	$C_6H_{12}NO$	124.0758	$C_7H_{10}NO$
Swainsonine	$C_8H_{15}NO_3$	174.1125	8.1	140.0682	$C_7H_{10}NO_2$	114.0914	$C_6H_{12}NO$	125.0598	$C_7H_9O_2$	118.0652	C_8H_8N
Tetrahydrocannabivarin	$C_{19}H_{26}O_2$	287.2006	12.9	105.07	C_8H_9	163.1118	$C_{11}H_{15}O$	175.0755	$C_{11}H_{11}O_2$	217.0123	$C_{14}H_{17}O_2$
Tetraneurin A	$C_{17}H_{22}O_6$	323.1489	12.6	281.0996	$C_{14}H_{17}O_6$	199.0968	$C_{19}H_{15}O_4$	155.0704	$C_8H_{11}O_3$	213.112	$C_{11}H_{17}O_4$
Trachelanthamine	$C_{15}H_{27}NO_4$	286.2013	12.5	155.0704	$C_8H_{11}O_3$	107.085	C_8H_{11}	159.0655	$C_7H_{11}O_4$	215.1269	$C_{11}H_{19}O_4$
Tussilagine	$C_{10}H_{17}NO_3$	200.1281	10.6	180.1021	$C_{10}H_{14}NO_2$	165.0912	$C_{10}H_{13}O_2$	151.0756	$C_9H_{11}O_2$	134.0967	$C_9H_{12}N$
Umbelliferone	$C_9H_6O_3$	163.0390	11.1	147.0441	$C_9H_7O_2$	135.0442	$C_8H_7O_2$	111.0441	$C_6H_7O_2$	123.0441	$C_7H_7O_2$
Verrucosin	$C_{20}H_{24}O_5$	345.1697	13.0	301.143	$C_{18}H_{21}O_4$	121.0286	$C_7H_5O_2$	141.0548	$C_7H_9O_3$	247.1332	$C_{15}H_{19}O_3$
Xanthotoxol	$C_{11}H_6O_4$	203.0348	1.3	147.1173	$C_9H_{10}O_2$	177.0188	$C_9H_5O_4$	173.0239	$C_{10}H_5O_3$		
Mycotoxins											
Aflatoxin B_1	$C_{17}H_{12}O_6$	313.0707	11.2	213.0547	$C_{13}H_9O_3$	269.0444	$C_{15}H_9O_5$	285.0761	$C_{16}H_{13}O_5$	217.0497	$C_{12}H_9O_4$
Aflatoxin B_2	$C_{17}H_{14}O_6$	315.0863	11.6	273.0761	$C_{15}H_{13}O_5$	255.0654	$C_{15}H_{11}04$	68.9979	C_3HO_2		
Alpha-Zearalenol	$C_{18}H_{24}O_5$	321.1674	14.8	149.133	$C_{11}H_{17}$	121.1016	C_9H_{13}	139.1123	$C_9H_{15}O$		
Aspergillic acid	$C_{12}H_{20}N_2O_2$	225.1598	9.4	114.0915	$C_6H_{12}NO$	144.0889	$C_6H_{12}N_2O_2$	150.0915	$C_9H_{12}NO$	128.07	$C_6H_{10}NO_2$
Averufin	$C_{20}H_{16}O_7$	369.0969	10.6	327.0853	$C_{18}H_{15}O_6$	299.0555	$C_{16}H_{11}O_6$	137.0236	$C_7H_5O_3$		
Kojic Acid	$C_6H_6O_4$	143.0344	1.38	125.0239	$C_6H_5O_3$	97.02844	$C_5H_5O_2$	69.0335	C_4H_5O		
Cyanotoxins											
ANA-a	$C_{10}H_{15}NO$	166.1226	0.5	149.1	$C_{10}H_{13}O$	131.0859	$C_{10}H_{11}$	107.0858	C_8H_{11}		
MC-LR	$C_{49}H_{74}N_{10}O_{12}$	995.556	9	135.0807	$C_9H_{11}O$	213.087	$C_9H_{13}N_2O_4$	375.1914	$C_{20}H_{27}N_2O_5$		
MC-LW	$C_{54}H_{72}N_8O_{12}$	1025.5343	12	135.0807	$C_9H_{11}O$	376.1926	$C_{19}H_{21}N_{10}$	288.1354	$C_{17}H_{20}O_4$		
MC-YR	$C_{52}H_{72}N_{10}O_{13}$	1045.5317	8.9	135.0807	$C_9H_{11}O$	375.1935	$C_{19}H_{21}N_9$	213.0874	$C_9H_{13}N_2O_4$		
NOD	$C_{41}H_{60}N_8O_{10}$	824.4446	8.6	135.0807	$C_9H_{11}O$	389.2079	$C_{21}H_{29}NO_5$	691.3795	$C_{34}H_{53}O_{10}N_5$		

Plant toxins were the most prominent group in the studied samples (73% of the tentatively identified compounds), with a prevalence of the alkaloids group. The most frequently identified phytotoxins were acetoxytropane, retronecine, and N-methyl pseudo conhydrine in 71%, 70%, and 46% of the samples, respectively. These results are in agreement with the diversity of endemic plants of the area [22], due to the different climatic zones of the occidental Pyrenees and the variation in dry and wet periods. The occurrence of some of these toxins was at a maximum in April, May, August, and September. These two peaks of natural toxins can be related to the leaching into the water immediately after the flowering period in the Mediterranean area, corresponding to April and May, and posteriorly the release of toxins from the dead plant with the consequent rain-washing effect into the river in August and September. For example, in Figure 2, the intensity of the signals of three alkaloids, acetoxytropane, anethole, and retronecine, which can be attributed to the *Symphytum officinale*, *Pimpinella anisum* [23], and *Apiaceae* families, are displayed. As can be seen, the maximum intensities of the toxins were between May and September. In addition to the alkaloids, some terpenes were also tentatively identified. A common species in this area and in the general region of the Iberian Peninsula is bracken (*Pteridium aquilinum*) [24], which produces ptaquiloside [15]. Ptaquiloside is a carcinogen norsesquiterpene glucoside that is responsible for haemorrhagic disease and bright blindness in livestock and can produce gastric cancer in humans [25]. As can be seen in Figure 1, in this study the degradation product of ptaquiloside, ptaquilosin B (PTB) [26], was identified in 33% of the samples, while ptaquiloside was not detected. The degradation of ptaquiloside in soils and the start of the rainy season explains the leaching of PTB into the water, which is coincident with the maximum intensities of the signals in the samples that were collected in August and September (Figure 3). Another relevant group of phytotoxins, the phenolic group, was less represented in the samples that were identified, and the representatives of this group were present in a minor number of samples. An example was p-coumaric acid, which was found in only 8% of the samples.

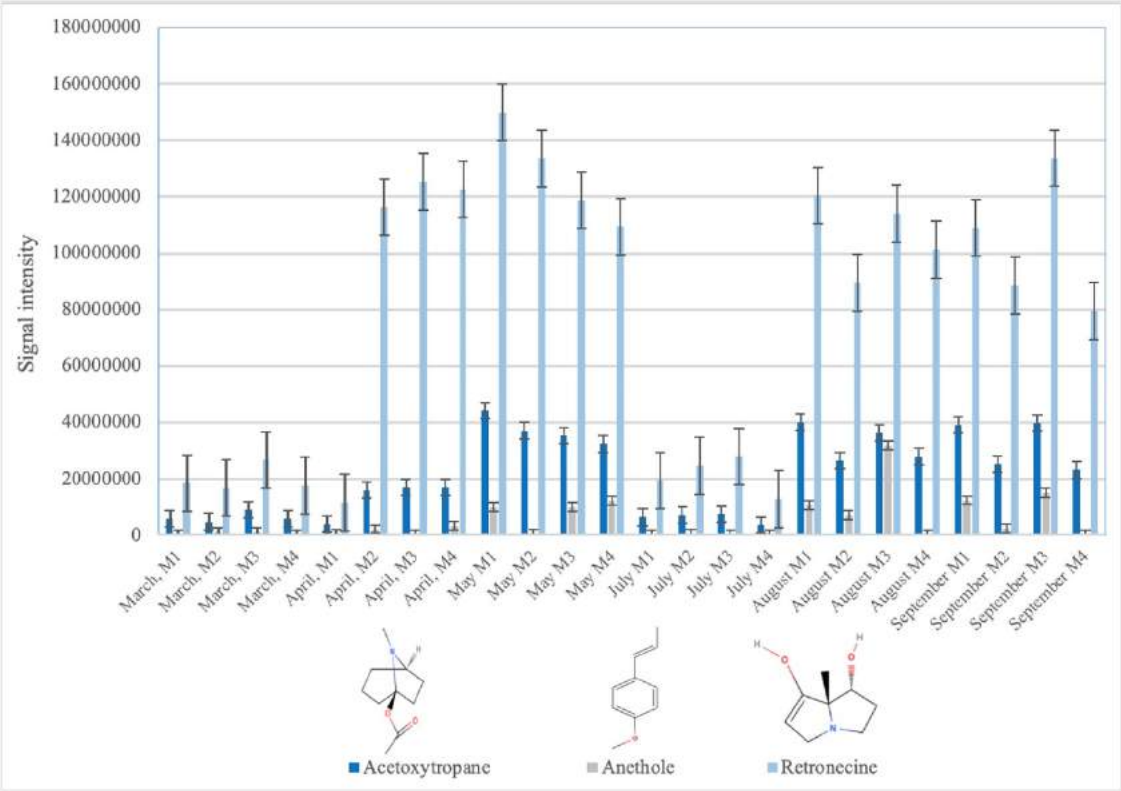

Figure 2. Signal intensities of three alkaloids: acetoxytropane, anethole, and retronecine.

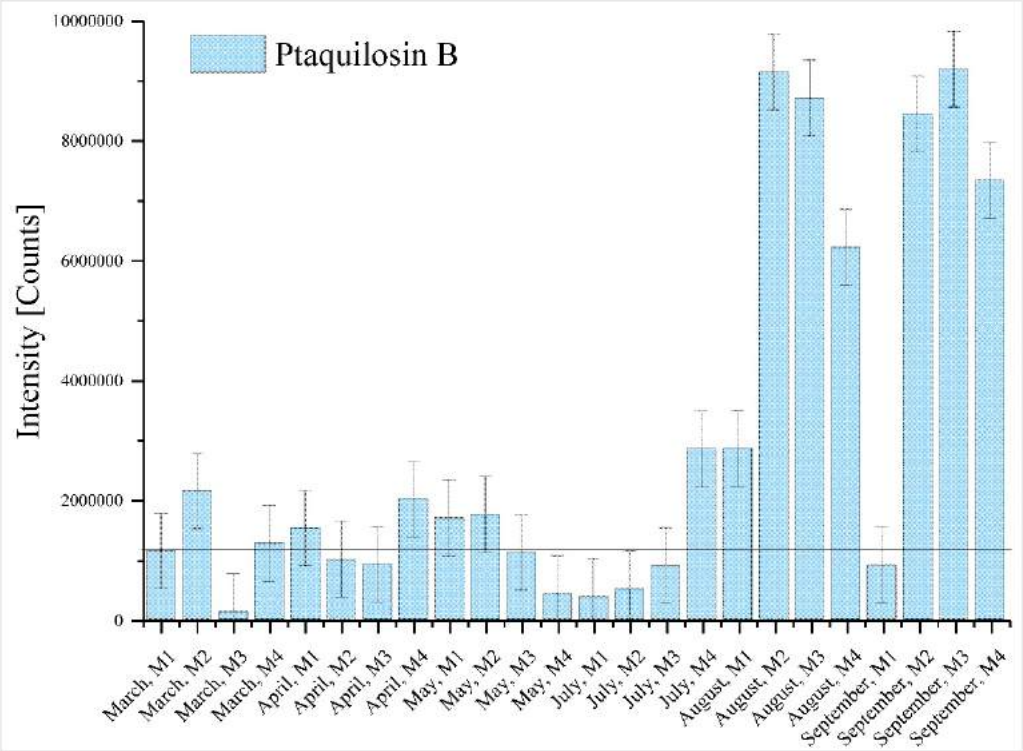

Figure 3. Ptaquilosin B intensity signals along the sampling period.

Mycotoxins were marginally detectable in the samples, and 58% of the studied water samples did not present detectable concentrations. Alpha-zearalenol was the most prevalent suspect mycotoxin with an occurrence of 29%, followed by aflatoxin B_2 (25%), aflatoxin B_1 (12%), and averufin, which is an anthraquinoid precursor of aflatoxins [27,28]. Regarding the distribution during the study period, mycotoxins were almost exclusively detected in August and September when the rainy season started, indicating that their presence in water could be due to the washing effect of plants infected with *Aspergillus flavus* and *Aspergillus parasiticus* in the case of aflatoxins and *Fusarium* mycotoxins in the case of alpha-zearalenol. As can be seen in Figure 2, and on the principal component analysis (PCA) presented in Figure 4, the occurrence of natural toxins in natural waters is influenced by seasonality, and the months with a higher charge of natural toxins were in this case April, August, and September, while a very low presence of natural toxins was found at the end of winter and during the driest months. Contrary to what can be expected, the samples from May and July were almost free of cyanotoxins. Only in M1 and M2 during April, August, and September was the occurrence of cyanotoxins detected, in agreement with the two peaking algal blooms in the Mediterranean region. This site (M1) corresponded to the area of Pasteral dam, which is the reservoir that is located downstream of the other reservoirs and presenting slightly higher levels of eutrophication in comparison with the other three areas. The more frequently found cyanotoxins were anatoxin-a, which was present in four samples, followed by microcystin LR, LW, and YR.

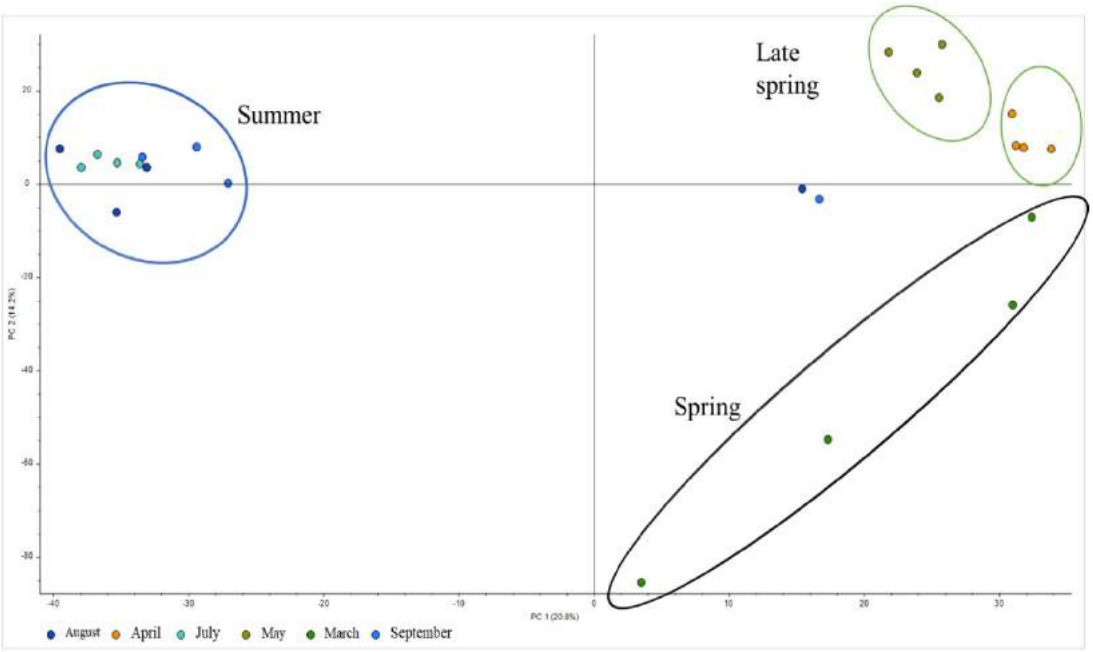

Figure 4. PCA of the results during the sampling period.

The concomitant presence of three MCs, both with anatoxin-a, at the sampling point M1, suggests this area is of a higher risk in terms of the occurrence of MCs, and therefore of MC producers. This is in line with the previous studies reporting benthonic species in the NE of Catalonia. Thirty-two different species have been identified as endemic in this area [29]. Toxins producing genera of freshwater cyanobacteria include *Phormidium* spp., *Oscillatoria* spp., *Nostoc* spp., and *Pseudanabaena* spp. [27]. These were considered to be the main producers of MC-LR, MC-YR, and –LW found in the M1 point in May and July. The occurrence of cyanotoxins can be related to increments in temperature and eutrophication, as was confirmed by the Catalan Water Agency [28] and CARIMED 2018 [30] for this area during the period studied. On the other hand, M1 is the downstream point of the studied area, which receives nutrients from areas in the upper river, with nitrate levels between 0.67 and 10 mg $N\text{-}NO_3^-$/L.

2.2. Target Analysis

A target analysis of 27 natural toxins was carried out using certified standards that are summarised in Table A1 of Appendix A. Matrix-matched calibration curves were used for the quantification of eight natural toxins. The limits of detection (LODs) were between 0.002 to 0.4 µg/L while the limits of quantification (LOQs) were between 0.07 and 1.5 µg/L. The analytical parameters are summarised in Table A3. Nine toxins were confirmed (Ana, AflB1, MC-LR, MC-LW, Nod, MC-YR, Kja, 7-methoxycoumarin, and umbelliferone). Concentrations were under the limit of 1 µg/L as proposed by the World Health Organisation [24] and they were used as an arbitrary reference limit in this work. MC-LR was confirmed in only two sampling points (April M1 and September M1), where the precursor ion $[M+H]^+$ 995.5560 *m/z* was detected for both with the fragment 135.0806 *m/z*, which is typically generated by the ADDA structure. Finally, MC-LR was confirmed with standards in these two samples. MC-LW and MC-YR were detected at the M1 point in September, August, and, surprisingly, in April, which correspond to the same months where the MC-LR was detected. Anatoxin-a was further detected in the same periods. 7-methoxycoumarin and umbelliferone were confirmed by certified standards. The concentrations of the detected natural toxins are reported in Table 2, showing their presence at relatively low levels in water.

Table 2. Quantification of the confirmed compounds detected in the Ter River.

Toxin	Month	Sampling Point	Concentration (μg L^{-1})
Ana-a	April	M1	0.12
	August	M1	0.03
	September	M1	0.06
Afla B_1	September	M2	0.28
	September	M4	0.9
Kja	April	M4	0.7
Nod	September	M1	0.1
MC-YR	April	M1	0.1
	August	M1	0.2
MC-LW	August	M1	0.4
MC-LR	September	M1	0.1
	April	M1	0.2
	September	M1	0.7
Umbelliferone	May	M3	<LOD
	July	M2	<LOD
		M3	0.1
	August	M2	<LOD
		M3	<LOD
7-methoxycoumarin	May	M2	0.17
		M3	0.008
	July	M2	0.08
		M3	0.18
	August	M2	0.06
		M3	0.03
	September	M1	0.04

Abbreviations: Afla B_1: aflatoxin B_1; Ana-a: anatoxin-a; Kja: Kojic acid; Nod: nodularin; MC-YR: microcystin-YR; MC-LW: microcystin-LW; MC-LR: microcystin-LR.

2.3. *Prioritisation*

In this study, a scoring system was designed to highlight the most significant natural toxins of concern in drinking water reservoirs. The scoring system was in accordance with the previous protocol that was published by Choi et al. [31], which is based on the risk-relevant parameters such as the detection frequency in percentage, biodegradability, log BAF, and the toxicity values based on the 50% lethal dose (LD50) laboratory tests in mice. A score in the range of 0 to 100 for each parameter was used, and 100 points were additionally added if carcinogenicity or neurotoxicity was already reported for the substance as what happens, for example, with $AflB_1$ and $AflB_2$. Thus, the maximum total for a given toxin can be 500. In Table 3, detailed information on the parameterisation and scoring is provided, and in Table 4, the parameters used for each tentatively identified substance are shown. It is noteworthy that the biodegradability and the bioaccumulation factor (BAF), used as log BAF, were calculated using EPI Suite™ software (United States Environmental Protection Agency, U.S. EPA).

Table 3. Scoring system for prioritisation of the quantified substances with the risk relevant parameters (detection frequency, biodegradability, bioaccumulation factor (BAF), and toxicity value).

Detection Frequency	Biodegradability *	Log BAF *	EC50 (mg/kg)	Score
<5%	Days	<2	>1000	0
5~30%	Weeks	2~3	100~1000	25
30~55%	Weeks–Months	3~4	10~100	50
55~80%	Months	4~5	1~10	75
>80%	Recalcitrant	>5	<1	100

* Biodegradability and BAF were estimated using EPI Suite software (United States Environmental Protection Agency, US EPA).

Table 4. Parameters used for the prioritisation of the tentatively identified compounds.

Toxin	CAS No.	Frequency %	Log Kow	Biodegradation Frame *	Log BAF *	LD50 (Mouse) mg/Kg	Effects	Ref.	Smileys
Phytotoxins									
Acetoxytropane	3423-26-5	71	1.5	Week–Months	1	1830	Diarrhoea and hypoactivity after administration of 50 and 200 mg/kg	[32]	CC(=O)OC12CCCC(N1C)CC2
Aconosine	38839-95-1	17	1.2	Months	0.5	0.27		[33]	CCN1CC2CCC(C34C2CC(C31)C5(CC(C6CC4C5C6O)OC)O)OC
Anethole	104-46-1	13	2.7	Weeks	2.31	2090	Lethal oral toxicity in rats at 2 g/kg	[34]	CC=CC1=CC=C(C=C1)OC
Alantolactone	546-43-0	29	3.47	Week–Months	2.06	1200	Carcinogenic/anticarcinogenic potential; Cytotoxic in vitro	[35]	CC1CCCC2(C1=CC3C(C2)OC(=O)C3=C)C
Ambrosin	509-93-3	17	1,03	Week–Months	0.21		NF-κβ inhibitor	[36,37]	CC1CCC2C(C3(C1C=CC3=O)C)OC(=O)C2=C
Apiole	523-80-8	38	2.7	Week–Months	2.21	4200	Acute oral LD50 in rats 3.96 g/kg, in mice 1.52 g/kg;	[38]	COC1=C2C(=C(C(=C1)CC=C)OC)OCO2

							acute dermal LD50 in rabbits > 5 g/kg		
Arabsin	38412-44-1	13	0.76	Weeks	−0.02			[39]	CC1C2CCC3(C(CC(=O)C(C3C2OC1=O)C)O)C
Artemisic acid	80286-58-4	4	3.8	Week–Months	4.39	50	Cytotoxicity	[40]	CC1CCC(C2C1CCC(=C2)C)C(=C)C(=O)O
Aspidinol	519-40-4	13	2.6	Week–Months	1.01	50	anti-MRSA activity, with antibacterial effect. Inhibition of the formation of the ribosome	[41]	CCCC(=O)C1=C(C(=C(C=C1O)OC)C)O
Aspidospermine	466-49-9	13	3.78	Recalcitrant	1.76	46.3	Cytotoxicity against mouse NIH3T3 cells	[42]	CCC12CCCN3C1C4(CC3)C(CC2)N(C5=C4C=CC=C5OC)C(=O)C
Bisabolol oxide B	26184-88-3	21	2.5	Months	2.63	633	Skin reaction; hepatic toxicity	[43]	CC1=CCC(CC1)C2(CCC(O2)C(C)(C)O)C
Buddledin B	62346-21-8	13	2.9	Week–Months	2.97		Piscicidal activity	[44]	CC1=CCCC(=C)C2CC(C2C(C1=O)O)(C)C
Conhydrine	495-20-5	50	1.21	Months	0.39	11	Activation and then blocking of nicotinic acetylcholine receptors	[45]	CN1CCC23C4C1CC5=C2C(=C(C=C5)OC)OC3C(CC4)O
Cuscohygrine	454-14-8	29	1	Months	0	111	Autonomic nervous system blockade	[46]	CN1CCC[C@@H]1CC(=O)C[C@@H]2CCCN2C
Herniarin	531-59-9	29	1.74	Weeks	0.72	4300	Inhibition of human carbonic anhydrase with a concentration of 2.4 μM	[47]	COC1=CC2=C(C=C1)C=CC(=O)O2
Hygrine	496-49-1	29	0.5	Week–Months	−0.02	91		[48]	CC(=O)C[C@H]1CCCN1C
Hypoglycine A	156-56-9	33	-2.5	Day–Weeks	−0.05	98	Jamaican vomiting sickness; hypoglycaemia and death; encephalopathy	[49]	C=C1CC1CC(C(=O)O)N
Laudanosine	2688-77-9	25	3.7	Months	1.59	410	GABA receptors interaction glycine receptors, involved	[50]	CN1CCC2=CC(=C(C=C2C1CC3=CC(=C(C=C3)OC)OC)OC)OC

							in epilepsy and other types of seizures		
Lupanine	550-90-3	38	1.6	Week–Months	0.65	410	Tremor, Muscle contraction and dyspnoea within mouse	[51]	C1CCN2CC3CC(C2C1)CN4C3CCCC4=O
Methyl-Jasmonate	1211-29-6	25	2.76	Weeks	1.25	5000	Anti-inflammatory activity in LPS-stimulation within mouse	[52]	CCC=CCC1C(CCC1=O)CC(=O)OC
Methylpelletierine	40199-45-9	17	0.8	Week–Months	0.05	40	Taenicide	[53,54]	CC(=O)CC1CCCCN1C
Methylpseudoconhydrine	140-55-6	46	1.5	Week–Months	0.33	250	Antinociceptive	[55]	CC(C(C1=CC=CC=C1)O)N(C)C
Norpseudopelletierine	4390-39-0	17	0.2	Weeks	0.15		Causes severe skin burns and eye damage; genotoxic in vitro + in vivo	[56]	C1CC2CC(=O)CC(C1)N2
p-Coumaric acid	7400-08-0	8	1.46	Day–Weeks	1.81	1.2	Reproductive toxicity	[57]	C1=CC(=CC=C1C=CC(=O)O)O
Ptaquilosin B	87625-62-5	33	ND	Months	0.42		Generation of carcinogenic ADN adducts	[35]	CC1CC2(C=C(C3(CC3)C(C2C1=O)(C)O)C)O
Reticuline	485-19-8	0	3	Months	0.61	56	Ptosis, somnolence, convulsions.	[36]	CN1CCC2=CC(=C(C=C2C1CC3=CC(=C(C=C3)OC)O)O)OC
Retronecine	480-85-3	71	-0.56	Weeks	−0.04	634	Carcinogenic, pulmonary oedema, blood lymphoma, convulsions	[38]	C1CN2CC=C(C2C1O)CO
Swainsonine	72741-87-8	17	-1.3	Weeks	−0.05	0.35	Locoweed intoxication; It is a potent inhibitor of Golgi alpha-mannosidase II	[58]	C1CC(C2C(C(CN2C1)O)O)O
Tetrahydrocannabivarin	31262-37-0	21	5.76	Months	3.06	3	Neurotoxicity	[59]	CCCC1=CC(=C2C3C=C(CCC3C(OC2=C1)(C)C)C)O
Tetraneurin A	22621-72-3	29	0.6	Week–Months	−0.04	42	Antiviral activity; Ear thickness in rats; dermatitis	[60]	CC(=O)OCC1CCC2C(C3(C1(CCC3=O)O)C)OC(=O)C2=C
Trachelanthamine	14140-18-2	0	1.4	Week–Months	0.69	1500	Somnolence, tremor, muscle weakness	[61]	CC(C)C(C(C)O)(C(=O)OCC1CCN2C1CCC2)O

Tussilagine	80151-77-5	8	0.6	Week–Months	−0.04	28.8	Carcinogenic in vivo	[43,62]	CC1(CN2CCCC2C1C(=O)OC)O
Umbelliferone	93-35-6	21	1,58	Weeks	0.4	10000	Inhibition of human carbonic anhydrase 9 catalytic domain	[63]	C1=CC(=CC2=C1C=CC(=O)O2)O
Xanthotoxol	2009-24-7	29	1.16	Weeks	0.22	480	Inhibitors of Secretory Acid Sphingomyelinase (S-ASM);	[64]	C1=CC(=O)OC2=C(C3=C(C=CO3)C=C21)O
Mycotoxins									
Aflatoxin B_1	1162-65-8	13	1.45	Week–Months	0.1	3.2	Carcinogenic, terathogenic	[65]	COC1=C2C3=C(C(=O)CC3)C(=O)OC2=C4C5C=COC5OC4=C1
Aflatoxin B_2	7220-81-7	25	0.855	Week–Months	0.18	100	Carcinogenic, terathogenic; hepatotoxic	[66]	COC1=C2C3=C(C(=O)CC3)C(=O)OC2=C4C5CCOC5OC4=C1
Alpha-Zearalenol	36455-72-8	29	4	Weeks	1.41	0.010	Chronic toxicity and carcinogenic	[67]	CC1CCCC(CCCC=CC2=C(C(=CC(=C2)O)O)C(=O)O1)O
Aspergillic acid	2152-59-2	13	1.7	Week–Months	0.8	100	Antibiotic substance; animal toxicity	[49,68]	CCC(C)C1=CN=C(C(=O)N1O)CC(C)C
Averufin	14016-29-6	17	3	Months	1.09	20.64	Inhibition of deaminase	[69]	CC12CCCC(O1)C3=C(O2)C=C4C(=C3O)C(=O)C5=C(C4=O)C=C(C=C5O)O
Kojic Acid	501-30-4	8	-0,64	Weeks	−0.05	23.8	Inhibition of human recombinant DAAO	[70]	C1=C(OC=C(C1=O)O)CO
Azelaic acid	19619-43-3	13	1.55	Day–Weeks	0.64	5	Irritant	[71]	C(CCCC(=O)O)CCCC(=O)O
Barnol	2151-18-0	0	2.26	Week–Months	0.79			[56,62]	CCC1=C(C(=C(C(=C1C)O)O)O)C
Cyanotoxins									
Anatoxin-a	64285-06-9	17	0.8	Weeks	0.36	420	Neurotoxicity; muscular fasciculation, respiratory paralysis.	[72]	CC(=O)C1=CCCC2CCC1N2
MC-LR	101043-37-2	8	-1.2	Recalcitrant	−0.01	5	Hepatotoxicity; visual disturbance, respiratory	[73]	CC1C(NC(=O)C(NC(=O)C(C(NC(=O)C(NC(=O)C(NC(=O)C(=C)N(C(=O)CCC(NC1=O)

							irritation; vomiting, and muscle weakness		C(=O)O)C)C)CC(C)C)C(=O)O)C)CCCN=C(N)N)C=CC(=CC(C)C(CC2=CC=CC=C2)OC)C
MC-LW	157622-02-1	8	5.2	Recalcitrant	0.81	0.25-0.33	Hepatotoxicity; visual disturbance, respiratory irritation; vomiting, and muscle weakness	[74]	CC1C(NC(=O)C(NC(=O)C(C(NC(=O)C(NC(=O)C(NC(=O)C(=C)N(C(=O)CCC(NC1=O)C(=O)O)C)C)CC(C)C)C(=O)O)C)CC2=CNC3=CC=CC=C32)C=CC(=CC(C)C(CC4=CC=CC=C4)OC)C
MC-YR	101064-48-6	8	-0.2	Recalcitrant	−0.02	40	Hepatotoxicity; visual disturbance, respiratory irritation; vomiting, and muscle weakness	[75]	CC1C(NC(=O)C(NC(=O)C(C(NC(=O)C(NC(=O)C(NC(=O)C(=C)N(C(=O)CCC(NC1=O)C(=O)O)C)C)CC2=CC=C(C=C2)O)C(=O)O)C)CCCN=C(N)N)C=CC(=CC(C)C(CC3=CC=CC=C3)OC)C
Nodularin	118399-22-7	4	1.7	Months	−0.04	0.060	Hepatotoxicity; visual disturbance, respiratory irritation; vomiting, and muscle weakness	[76]	CC=C1C(=O)NC(C(C(=O)NC(C(=O)NC(C(C(=O)NC(CCC(=O)N1C)C(=O)O)C)C=CC(=CC(C)C(CC2=CC=CC=C2)OC)C)CCCN=C(N)N)C)C(=O)O

In Table 5, the ranking of the tentatively identified substances is presented. Four substances, namely, tetrahydrocannabivarin, MC-LW, aconosine, and MC-LR, were ranked with more than 300 points, and 13 toxins were ranked with more than 200 points. In this case, it was considered to be the frequency during the sampling period, which includes seasons with a lower incidence of the substances in water.

Table 5. Prioritisation for ranking the substances detected in the Ter River.

Ranking	Tentatively Identified Substance
325	Tetrahydrocannabivarin
325	MC-LW
300	Aconosine
300	MC-LR
275	MC-YR
275	Nodularin
250	Aflatoxin B1
250	Alpha-Zearalenol
225	Ptaquilosin B
225	Retronecine
225	Tussilagine
225	Aflatoxin B2
200	Aspidospermine
175	Artemisic acid
175	Conhydrine
175	Anatoxin-a
150	Bisabolol oxide B
150	Swainsonine
150	Averufin
125	Acetoxytropane
125	Apiole
125	Aspidinol
125	Cuscohygrine
125	Hygrine
125	Laudanosine
125	Lupanine
125	Methylpelletierine
125	Methylpseudoconhydrine
125	Reticuline
125	Tetraneurin A
125	Aspergillic acid
100	Alantolactone
100	Buddledin B
100	Hypoglycine A
100	p-Coumaric acid
100	Kojic Acid
100	Azelaic acid
75	Anethole
75	Ambrosin
75	Xanthotoxol
50	Arabsin
50	Herniarin
50	Methyl-Jasmonate
50	Norpseudopelletierine

50	Trachelanthamine
50	Umbelliferone
50	Barnol

However, following a month-by-month inspection, for certain substances the frequency was higher; hence, this ranking then varies a little and a higher number of toxins reaches 300 points.

For this reason, in spite of the low concentrations of the substances that are quantified as the top 12 toxins to be tentatively identified, Barcelona city water reservoirs should be monitored at least from May to September, which were the months with higher occurrences of natural toxins.

3. Conclusions

The method described in this article is a good alternative for tentatively identifying suspect natural toxins in surface water. We have shown that the presence of organic matter near the river can potentially cause the leaching of mycotoxins. Moreover, in this study, plant toxins were mostly spread across different points in relation to the presence of different endemic plants. Notwithstanding, the botanical diversity influences the presence of natural toxins as equally as the precipitation and dry periods. The concentrations of natural toxins were not determined due to the lack of certified standards; however, a correlation between the rain and the leaching in water was described and assessed.

Thanks to these results, we report on the importance of the suspect screening for the identification of natural toxins and their final inclusion in prioritisation lists in order to control their presence in water environments, in particular in drinking water reservoirs. It is also important to increase the amount of data, to help scientists identify environmental compounds when no standards are available, or where they are excessively expensive. Many MC congeners are still not included in databases such as MzCloud and Chemspider. Hence, the retrieval of MS^2 spectrums for the MC congeners is an issue that is being solved with the efforts of the scientific community via the constant updating of data in dedicated databases for environmental research. For comparison purposes, future works should apply this method of analysing natural toxins across different climates worldwide.

4. Materials and Methods

4.1. Chemicals and Reagents

Twenty-seven (27) natural toxin standards with a maximum purity between 95 and 99% were selected for the targeted analysis. In Table A1 of Appendix A, the list of standards, their main chemical parameters, and providers are listed. Methanol (MeOH), acetone, and acetonitrile (ACN) of HPLC grade were from Merck (Darmstadt, Germany). HPLC water grade was from Baker (Madrid, Spain).

4.2. Samples and Sampling Sites

Forty-eight surface water samples were collected from the Ter River (Catalonia, NE Spain) at four sampling sites: (M1) 41.986133, 2.603488; Point 2 (M2) 41.982191, 2.585539; Point 3 (M3) 41.991090, 2.570144; and Point 4 (M4) 41.975693, 2.395398, in the area of Pasteral, Susqueda, and Sau dams, which are the freshwater reservoirs for Barcelona city tap water.

The sampling was carried out from March to September 2018, except for June, twice per month, in order to study the prior, during, and after blooming periods, when higher concentrations of natural toxins are expected [77]. In each sampling site, the pH, conductivity, and pO_2 were measured. Water samples were collected in amber glass bottles that had previously been rinsed, transported at 4 °C, and maintained frozen at -40 °C until the start of the analytical process.

4.3. Sample Pre-Treatment

Sample pre-treatment was based on the generic methodology to isolate natural toxins from water, as recently developed by Picardo et al. [20]. Briefly, each sample was processed in an ultrasonic bath for 20 min to disrupt the microbial cells and to release the intracellular toxins. Then, the sonicated samples were filtered through a glass microfibre filter of GF/B grade (Sigma Aldrich, Steinheim, Germany). Natural toxins were isolated from the filtrate via a three-step solid-phase extraction (SPE) method, using a hand-made cartridge that had been prepared with 200 mg of a porous graphitised carbon (PGC) 120 mesh (Sigma Aldrich, Steinheim, Germany) and 200 mg of a Bond-Elut PPL (PPL) 120 mesh (Agilent, Santa Clara, CA, USA), coupled to an HLB plus cartridge (225 mg sorbent) (Waters Corporations, Milford, MA, USA).

Then, water samples, each of 100 mL, were loaded into the cartridges at a flow rate of 2 mL/min, previously conditioned with 10 mL of MeOH and 10 mL of water, and both solvents were acidified with 0.5% of formic acid (FA). After loading, the cartridges were dried and switched to elute the analytes in the backflush mode. The PGC/PPL cartridge was reversed, while the HLB cartridge maintained the same position. The toxins were eluted with 15 mL of water/MeOH 2:8 (v/v), followed by 15 mL of MeOH and 15 mL of acetone/MeOH 50:50 (v/v). All the solvents were previously warmed at 45 °C before each elution. The eluate was evaporated almost to dryness and re-dissolved in 1 mL of the mobile phase.

4.4. Liquid Chromatography Coupled with High-Resolution Mass Spectrometry

According to the method described by Picardo et al., 2020 [20], the chromatographic separation was carried out using a C18 reversed-phase Lichrosphere (125 mm × 2 mm i.d., 5 µm) column (Merck, Barcelona, ES) connected to an Acquity high-performance liquid chromatography system (Waters Corp, Milford, MA, USA). The binary mobile phase was composed of water (solvent A) and acetonitrile (solvent B) and both had been acidified with 0.1% of FA. The elution gradient was as follows: from 0–3 min, 10% B; from 3–13 min, B was linearly increased to 90%; 13–15 min, stabilised at 90% B; 15–16 min B decreased linearly to 10%; 16–20 min, column stabilisation with 10% of solvent B. A 20 µL injection volume was used with a mobile phase flow rate of 0.25 mL/min.

The HPLC system was coupled to a Thermo Scientific Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionisation source (HESI), and used in the positive and negative ionisation modes. The acquisition was performed using a full-scan and data-dependent analysis (FS-DDA) from m/z = 75 to m/z = 1100, with a resolution of 35,000 full widths at half maximum (FWHM) for the FS and 17,500 FWHM for the DDA There was a spray voltage of 3.75 kV (+) and −3.25 kV (−), a sheath flow gas of 20 a.u., an auxiliary gas of 20 a.u., and a sweep gas of 5 a.u. Heater and capillary temperatures were set at 300 °C with an S-lens RF level at 60%. An inclusion list of the 100 most probable suspect compounds was used (Appendix A Table A2).

4.5. Data Processing: Suspect Screening of Natural Toxins

The suspect screening procedure that was previously described by Picardo et al. [20] was employed with minor changes. Briefly, the FS chromatograms that were obtained with the acquisition software Xcalibur Qual Browser (Thermo Fisher Scientific) were processed, using an automated screening with Compound Discoverer software version 3.1 v. x86 (Thermo Fisher Scientific, San Jose, CA, USA). The first screening steps included peak picking, RT alignment, and grouping of isotopes and adducts (to form compounds), as well as the grouping of compounds across samples. Suspect compounds were marked as background if their peak area in the samples was less than three times larger than the maximum peak area in the blanks. Suspects were tentatively identified using the exact mass with a mass error of 5 ppm. This created a first list of suspect compounds that were further filtered by comparison with a homemade database containing the exact mass of more than 2384 natural toxins. Further filtering steps consisted of the comparison of isotopic patterns, ionisation efficiency, and fragmentation patterns. In Figure 5, the general workflow is summarised, which is

similar to the workflows of Krauss [78] and Schymanski [21]. Finally, the MS/MS spectrum was compared with the spectrum of a standard or the predicted fragmentation pattern using the ChemSpider and MzCloud online databases. Unequivocal confirmation was only possible when a reference standard was available (identification at level 1).

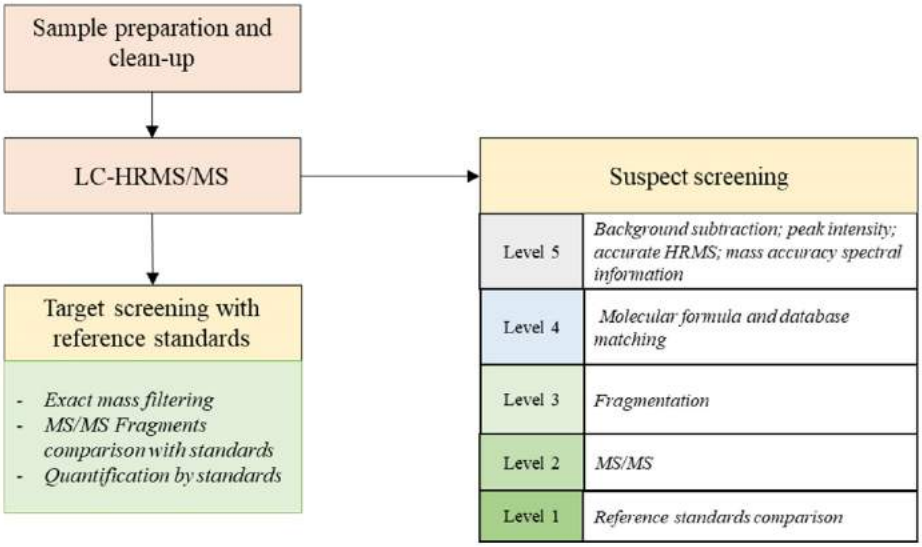

Figure 5. General workflow for suspect screening as proposed by Schymansky et al. [21].

4.6. Accuracy, Precision, Limits of Detection, and Quantification

Quantification was achieved through calibration curves that were prepared in an artificial freshwater matrix (AFW). The AFW was prepared using the same ingredients that were reported by Lipschitz and Michel [79]. Briefly, the organic matter was simulated with 10 mg/L of technical grade humic acid (Sigma-Aldrich, reference 53,680), and the pH was adjusted to 6.5 with 1.0 M formic acid. Matrix-matched calibration curves were produced using spiked samples from 0.5 to 100 µg/L. Intra-assay precision, accuracy, LOD, and LOQ for the confirmed toxins were calculated according to the EURACHEM guidelines [80]. The instrumental limits of detection (iLOD) were obtained by progressive dilution to the lowest concentration, whereby each compound could be detected. Instrumental reproducibility (inter-day precision) was calculated as the average percentage of the relative standard deviation (RSD%) of the standard solutions (six replicates) at seven concentration levels on three consecutive days.

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Appendix A

Table A1. List of the natural toxin standards employed for the confirmation.

Toxin	Toxic Group	Chemical Formula	Exact Mass	Purity (%)	Supplied by
Microcystin LA	Cyanotoxin	C46H67N7O12	909.4847	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Microcystin LF	Cyanotoxin	C52H71N7O12	985.5160	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Microcystin LR	Cyanotoxin	C49H74N10O12	994.5488	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Microcystin LY	Cyanotoxin	C52H71N7O13	1001.5109	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Microcystin LW	Cyanotoxin	C54H72N8O12	1024.5269	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Microcystin YR	Cyanotoxin	C52H72N10O13	1044.5353	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Nodularin	Cyanotoxin	C41H60N8O10	824.4432	>95	Cyano (Cyanobiotech GmbH, Berlin, Germany)
Anatoxin-a	Cyanotoxin	C10H15NO	165.2320	>98	Santa Cruz Biotechnology (Dallas, TX, USA)
Cylindrospermopsin	Cyanotoxin	C15H21N5O7S	399.1219	99	BOCSci (BOC Sciences, Ramsey Road Shirley, NY, USA)
Aflatoxin B1	Mycotoxin	C17H12O6	312.0632	>98	Merck (Darmstadt, Germany)
Ochratoxin-A	Mycotoxin	C20H18ClNO6	403.0823	>98	Merck (Darmstadt, Germany)
Baicalein	Phytotoxin	C15H10O5	270.0528	98	Merck (Darmstadt, Germany)
Genistein	Phytotoxin	C15H10O5	270.0528	>98	Merck (Darmstadt, Germany)
Amygdalin	Phytotoxin	C20H27NO11	457.158	>99	Merck (Darmstadt, Germany)
Scopolamine	Phytotoxin	C17H21NO4	303.147	>98	Merck (Darmstadt, Germany)
Cinchonine	Phytotoxin	C19H22N2O	294.1732	>98	Merck (Darmstadt, Germany)
Atropine	Phytotoxin	C17H23NO3	289.1682	>99	Merck (Darmstadt, Germany)
Kojic Acid	Mycotoxin	C6H6O4	142.0274	>98	Merck (Darmstadt, Germany)
b-Asarone	Phytotoxin	C12H16O3	208.1099	70	Merck (Darmstadt, Germany)
p-Coumaric acid	Phytotoxin	C9H8O3	164.0471	>98	Merck (Darmstadt, Germany)
Abietic acid	Phytotoxin	C20H30O2	302.2256	>95	Merck (Darmstadt, Germany)
7-Ethoxyoumarin	Phytotoxin	C11H10O3	190.0634	≥97%	Merck (Darmstadt, Germany)
7-Metoxycoumarin	Phytotoxin	C10H8O3	176.0479	>98	Merck (Darmstadt, Germany)
Arbutin	Phytotoxin	C12H16O7	272.0986	>98	Merck (Darmstadt, Germany)
Umbelliferone	Phytotoxin	C9H6O3	162.0327	>99	Merck (Darmstadt, Germany)
Thujone	Phytotoxin	C10H16O	152.1235	>99	Merck (Darmstadt, Germany)
Cotinine	Phytotoxin	C10H12N2O	176.0956	>99	Merck (Darmstadt, Germany)

Table A2. Inclusion list of the 100 most probable suspect compounds.

Mass [M + H]+	Formula [M]	CE	Toxin and Possible Isomers
239.1542	C16H18N2	35	(−)-Agroclavine
180.1019	C10H13NO2	35	(−)-Salsolinol, Fusaric acid
398.0961	C18H24BrNO4	35	(−)-Scopolamin bromide
128.1433	C8H17N	35	(+)-Coniine
142.1226	C8H15NO	35	(+)-Hygrine
249.1961	C15H24N2O	35	(+)-Lupanine
333.2060	C20 H28 O4	35	20-Deoxyingenol
184.1332	C10 H17 N O2	35	3-Acetoxytropane
197.1536	C12H20O2	35	3-Thujyl acetate
646.3221	C34H47NO11	35	Aconitine
313.0706	C17 H12 O6	70	Aflatoxin B_1
315.0863	C17 H14 O6	35	Aflatoxin B_2
329.065	C17 H12 O7	35	Aflatoxin G_1
331.0812	C17H14O7	35	Aflatoxin G_2
502.2951	C32H39NO4	35	Aflatrem
159.0513	C4 H6 N4 O3	35	Allantoin
924.4951	C47H73NO17	35	Amphotericin Bh
458.1656	C20H27NO11	60	Amygdalin
456.1511	C20H27NO11	35	Amygdalin negative
166.1226	C10 H15 N O	45	Anatoxin-A
187.03897	C11H6O3	35	Angelicin (Isopsoralen)
504.343	C28H45N3O5	35	Antillatoxin
624.3755	C34H49N5O6	35	Apicidin
271.0601	C15H10O5	35	Apigenin
283.1540	C15H22O5	35	Artemisinin
189.1121	C9 H16 O4	35	Aspionene
290.1751	C17H23NO3	50	Atropine
369.0968	C20H16O7	35	Averufin
321.1696	C18H24O5	35	a-Zearalenol
261.1597	C15H20N2O2	35	Baptifoline
784.4167	C45H57N3O9	35	Beauvericin
641.2891	C34H44N2O8S	35	Belladonnine
209.1172	C12H16O3	50	beta-Asarone
285.0757	C16H12O5	35	Biochanin A (BIO)
438.2638	C27H35NO4	35	b-Paxitriol
281.1747	C16 H24 O4	35	Brefeldin A
235.1692	C15 H22 O2	35	Buddledin B
317.2111	C20H28O3	35	Cafestol
195.0876	C8H10N4O2	35	Caffeine
153.1273	C10H16O	35	Carveol
261.1849	C17H24O2	35	Cicudiol
259.1692	C17 H22 O2	35	Cicutoxin
1111.5836	C60H86O19	35	Ciguatoxin
295.1804	C19H22N2O	35	Cinchonine
279.0863	C14H14O6	35	Citreoisocoumarin
403.2115	C23H30O6	35	Citreoviridin
400.1754	C22H25NO6	35	Colchicine

144.1382	C8H17NO	35	Conhydrine
127.0389	C6H6O3	35	Coumarin
300.2169	C16 H29 N O4	35	Curassavine
225.1961	C13H24N2O	35	Cuscohygrine
416.1234	C15H21N5O7S	45	Cylindrospermopsin
255.0651	C15H10O4	35	Daidzein (DAI)
417.1180	C21H20O9	35	Daidzin
589.1915	C29H32O13	35	Dalbin
427.1387	C23H22O8	35	Dalbinol
249.1485	C15H20O3	35	Damsin
291.1227	C16H18O5	35	Dehydrocurvularin
355.1176	C20H18O6	35	Deoxynivalenol
411.1074	C22H18O8	35	Desertorin A
367.1751	C19H26O7	35	Diacetoxyscirpenol
765.4419	C41H64O13	35	Digitoxin
415.3206	C27H42O3	35	Diosgenin
295.1903	C17 H26 O4	50	Embelin
271.0601	C15H10O5	35	Emodin
1095.5662	C60H74N10O10	35	Ergoclavin
350.1598	C18H23NO6	35	Erucifoline
269.0808	C16H12O4	35	Formononetin (FOR)
209.0444	C10H8O5	35	Fraxetin
271.0601	C15H10O5	50	Genistein or baicalein
155.1430	C10H18O	35	Geraniol
781.4368	C41H64O14	35	Gitoxin
156.1019	C8 H13 N O2	35	Heliotridine
304.1543	C17H21NO4	35	Hyoscine
143.0338	C6H6O4	35	Kojic acid
541.3887	C34 H52 O5	35	Lantadene D
358.2012	C21 H27 N O4	35	Laudanosine
910.4920	C46H67N7O12	35	MC-LA
995.5560	C49H74N10O12	35	MC-LR
1025.5344	C54H72N8O12	35	MC-LW
1045.5353	C52H72N10O13	35	MC-YR
192.0781	C11H12O3	35	Myristicin
825.4505	C41 H60 N8 O10	35	Nodularin
128.1069	C7H13NO	35	Norhygrine
152.0566	C5H5N5O	35	Nostocine
404.0895	C20H18ClNO6	70	Ochratoxin-a
215.1277	C11H18O4	35	Pestalotin
165.0658	C8H8N2O2	35	Ricinine
194.1175	C11H15NO2	35	Salsoline
868.5053	C45H73NO15	35	Solanine
746.4837	C42H67NO10	35	Spirolide
183.0288	C8H6O5	35	Stipitatic acid
174.11247	C8H15NO3	35	Swainsonine
153.1273	C10H16O	35	Thujone
115.0389	C5H6O3	35	Tulipalin B
163.0389	C9H6O3	35	Umbelliferone
355.2380	C22 H30 N2 O2	35	Vincaminorein (Aspidospermine)

203.0338	C11 H6 O4	35	Xanthotoxol

Table A3. Calibration curve parameters for the quantification of the confirmed compounds.

Toxins	Molecular Formula	[M+H]+	Recovery %	RSD %	LOD µg/L	LOQ µg/L	R^2
Ana	$C_{10}H_{15}NO$	166.1234	84	8.0	0.2	0.5	0.989
AflB1	$C_{17}H_{12}O_6$	416.1242	86	9.9	0.2	0.7	0.999
MC-LR	$C_{49}H_{74}N_{10}O_{12}$	995.5568	78	3.3	0.2	0.5	0.995
MC-LW	$C_{54}H_{72}N_8O_{12}$	1025.5342	55	5.8	0.1	0.5	0.991
Nod	$C_{41}H_{60}N_8O_{10}$	825.4512	94	16.2	0.2	0.8	0.992
MC-YR	$C_{54}H_{72}N_8O_{12}$	1045.5361	84	16.9	0.4	1.5	0.943
Kja	$C_{12}H_{16}O_3$	208.1093	85	6.4	0.02	0.08	0.990
7-methoxycoumarin	$C_{10}H_8O_3$	177.0546	82	7	0.002	0.007	0.999
Umbelliferone	$C_9H_6O_3$	163.0389	79	11.2	0.009	0.03	0.998

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3.4.3 Publication IV

All Ion Fragmentation (AIF) analysis as a tentative identification tool for the suspect screening of natural toxins

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MethodsX

A Data Independent Acquisition All Ion Fragmentation mode tool for suspect screening of natural toxins in surface water .

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Abstract:	Among natural freshwater pollutants, cyanotoxins, mycotoxins, and phytotoxins are the most important and less studied. The identification is challenging. Most target methods focus one or a single group of compounds with similar characteristics. Here we present an AIF fast method for the tentative identification of natural toxins in water. Respect to the previous method [1], it offers higher performances for the acquisition of unknown compounds at low levels for higher number of analytes. The key aspects of the method are:The qualitative screening DIA-AIF workflow using QExactive orbitrap. Both targeted and suspect screening bases have been combined with online databases and suspect list to retrieve candidates as suspect natural toxins and their metabolites or degradation products. The in-silico analysis of mass spectrums allowed a fast structural characterization. The workflow has been finally applied to real samples coming from the Czech Republic, Italy, and Spain allowing the determination of 17 suspect natural toxins, 4 of them confirmed. None toxin passed the limit of 1 µg/L taken from the legislation applied for microcystin LR and arbitrarily extended to all toxins.
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Method Article – Title Page

Title	A Data Independent Acquisition All Ion Fragmentation mode tool for the suspect screening of natural toxins in surface water
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ABSTRACT

Among natural freshwater pollutants, cyanotoxins, mycotoxins, and phytotoxins are the most important and less studied. The identification is challenging. Most target methods focus one or a single group of compounds with similar characteristics. Here we present an **AIF** fast method for the **tentative identification** of natural toxins in water. Respect to the previous method [1], it offers higher performances for the acquisition of unknown compounds at low levels for higher number of analytes.

The key aspects of the method are:

- The qualitative screening DIA-AIF workflow using **QExactive orbitrap**. Both targeted and suspect screening bases have been combined with online databases and suspect list to retrieve candidates as suspect natural toxins and their metabolites or degradation products.

- The in-silico analysis of mass spectrums allowed a fast structural characterization.

- The workflow has been finally applied to real samples coming from the Czech Republic, Italy, and Spain allowing the determination of 17 suspect natural toxins, 4 of them confirmed. None toxin passed the limit of 1 µg/L taken from the legislation applied for microcystin LR and arbitrarily extended to all toxins.

SPECIFICATIONS TABLE

Subject Area	Chemistry
More specific subject area	*Environmental Analytical Chemistry*
Method name	Data Independent Acquisition All Ion Fragmentation mode
Name and reference of original method	*Picardo M., Sanchís, J., Nuñez O., Farré M. Suspect screening of natural toxins in surface and drinking water by high performance liquid chromatography and high-resolution mass spectrometry.* *Chemosphere Volume 261, December 2020, 127888*
Resource availability	*Compound Discoverer 3.1 (ThermoFisher); MzCloud; Metfrag; Xcalibur*

Method details

Common approaches for the analysis of natural toxins in surface waters rely on solid-phase extraction as a sample preparation protocol followed by target analysis with Data Dependent Acquisition methods for a limited number of compounds. Most methods are specifically designed for a group of toxins with similar parameters or a single compound, depending on its physic-chemical characteristics. However, the prioritization of natural toxins and their degradation products in the surface water environment is of increasing importance due to their different eco-toxicological properties [2].

The need for identification protocols is critical, especially considering the low availability of certified standards. Among them, targeted approaches are generally used to analyze known chemicals of interest while non-targeted approaches are more challenging. This is due to the need for identification and structure characterization protocols that require the use of multiple instruments (NMR and IR) which usually are not available or highly expensive.

Hig-Resolution Mass Spectrometry (HRMS) based on high-resolution instruments such as QExactive orbitrap, QTOF, and FTICR are helping to fulfill the need for reliable identification methods, providing sensitive fragmentation spectrums (MS/MS) for the identification of known and known-unknown compounds [3]. HRMS provides a high amount of information for characterization and identification purposes (molecular formula, isotopic patterns, double bond equivalents) comparing the experimental results with online or in-house databases of chemical compounds. Tandem mass spectrometry and the consequent fragmentation spectra are mandatory to achieve a tentative structural characterization. In these regards, the data-acquisition methodology used to acquire MS/MS spectrums is of critical consideration that influences the type of data generated, and the choice of which method to use is largely dependent on the aim of the approach. Among them, Data-Dependent Acquisition (DDA), Single Reaction Monitoring (SRM), and Data-Independent Acquisition (DIA) are the most used (Figure S1 of the Supporting Information).

This work aims to introduce the **A**ll Ions **F**ragmentation (**AIF**) acquisition approach as a suspect screening method for a wide range of natural toxins in surface water. The AIF acquisition for all theoretical fragment ions was used to acquire the entire MS/MS spectrum with no precursor preselection. Data processing and information extraction required the use of various bioinformatics tools to deconvolute complex mass spectra, using data from prior experiments in DDA mode to generate spectral libraries that were used in the interrogation of DIA data [4]. The objectives can be resumed in **i)** develop a robust workflow for the determination of natural toxins in surface water samples using the AIF mode; **ii)** provide a reliable workflow to describe how to process the acquired data, **iii)** demonstrate the advantages to use this approach as a tentative identification protocol for the screening of natural toxins using real samples.

A QExactive orbitrap was used to obtain the full scan and MS/MS spectrums with the AIF mode. Data mining was then carried out using Compound Discoverer using a published suspect list with 2384 entries [1] and the online databases Chemspider, Metfrag, and MassBank [5]. The "Fish score" option was used to structurally characterize the MS/MS patterns. Finally, 24 natural toxins have been tentatively identified from surface water samples coming from three sampling sites in Europe.

Standard solutions

Table S1 of the supporting information reports the standards used for method optimization. Compounds **1-5, 7-12, 14, 15, and 22-26** were supplied from Merck (Darmstadt, Germany). Compound **6** was supplied from Santa Cruz Biotechnology (Dallas, TX, USA). **16-21** were from Cyano (Cyanobiotech GmbH, Berlin, Germany). Methanol (MeOH), acetone, and acetonitrile (ACN) HPLC grade were from Merck (Darmstadt, Germany). HPLC water grade was from Baker (Madrid, Spain). Fortified samples with the 23 compounds (**Table S1**) at a concentration of 1 µg/L were prepared in both HPLC water and artificial freshwater (AFW) to simulate the presence of matrix interference. To prepare the AFW we followed the description of Lipschitz and Michel [6], the organic matter was simulated by adding 10 mg/L of humic acid of technical grade from Sigma-Aldrich (reference 53680), and the pH was adjusted to 7.5 with formic acid 1.0 M. The method optimization was carried out analyzing the standard solution in pure HPLC and artificial water, previously mixed for an hour at 25 ºC and processed as reported below.

Sample preparation

Sample preparation was previously reported by Picardo et al. [1]. Briefly, intracellular toxins were released by sonication for 20 minutes and further filtered with a GF/B microfiber filter grade (Sigma Aldrich, Steinheim, Germany). Solid-phase extraction (SPE) consisted of a 3 mL cartridge filled with 200 mg of porous graphitized carbon (PGC) (Sigma Aldrich, Steinheim, Germany) and 200 mg of Bond-Elut PPL (PPL) (Agilent, Santa Clara, CA, USA) separated by a Teflon frit. The third sorbent was the Oasis HLB plus, 225 mg (Waters Corporations, Mil-ford, MA) connected at the end of the cartridge. Conditioning required 10 mL of methanol followed by 10 mL of water acidified at 0.5% formic acid. 100 mL of sample was loaded at a constant flow rate (2 mL/min). After the procedure, analytes were eluted in backflush with 15 mL of water/methanol 20:80 (*v/v*), 15 mL of methanol, and 15 mL of acetone/methanol 1:1 (*v/v*). Solvents were warmed at 45 ºC before elution. The eluate was concentrated to 100 µL and re-dissolved to 1 mL of mobile phase. Finally, 20 µL of samples were injected

Liquid Chromatography-Mass Spectrometry

Chromatographic separation followed the same parameters reported by Picardo et al., [1]. Briefly, the separation was performed with an Acquity UPLC System (Waters, Milford, MA, USA) using a Lichrosphere column, 125 mm × 2 mm i.d., 5 µm (Merck, Barcelona, ES). 20 µL of samples were injected, the constant flow rate was 250 µL/min. Mobile phases were water (A) and acetonitrile (B) acidified with 0.1 % of FA. The gradient was 0-3 min, 10% of B; at 3-13 min B was increased to 90% and kept at a constant concentration from 13 to 15 min; 15-16 min B decreased to 10%; 16-20 min equilibration at 10% B. Total run time was 20 min. The analysis was performed using a Q Exactive™ Orbitrap mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA). Samples were acquired in Full Scan and AIF mode in positive (+) mode in a range from 75 to 1100 *m/z*. Collision Induced Dissociation (CID) was obtained using a collision energy of 35 eV. The mass spectrometer parameters are reported in **Table 1.**

Suspect screening workflow.

After the acquisition, spectral data were further processed to tentatively identify suspect natural toxins with the screening approach as reported below.

1) In silico processing

No inclusion list of precursors ions was used in the acquisition method however, the suspect list reported by Picardo et al., [1] was used in combination with the in silico tools for the data processing. Blanks of the entire procedure were processed to exclude background noise. Then, raw files from Orbitrap were uploaded to Compound Discoverer 3.1 (Thermo Fischer Scientific, San Jose, CA, USA) and processed with the Environmental Untargeted Metabolomics workflow. Here, peak alignment, unknown compound detection, compound grouping across all samples, elemental composition prediction, and chemical background hiding (using blank samples) were applied with a mass error of <5 ppm. Finally, a tentative list of various compounds is displayed. **Table 2** reports the parameters used for the compound identification with Compound Discoverer 3.1

2) Structural identification

The structural characterization of the compounds in the candidate list was based on accurate mass data. The molecular ions, potassium, sodium, and ammonium adducts and their transitions have been used as identification parameters. The spectrums have been submitted to MzCloud online search to obtain the

corresponding similarity score (SS). Compounds with SS lower than 70% were discarded. MzLogic and Fish scoring algorithms have been applied to compare the experimental and theoretical spectrums obtaining a similarity score (rejected if < 50%). Furthermore, Metfrag [7] was the last step for structural identification. Here candidates have been retrieved using the molecular formula, neutral mass with a mass error of 5 ppm using the KEGG database. Then, the MS/MS spectrums and the relative intensities have been uploaded. Only the first 10 candidates with a similarity score higher than 0.9/1.0 have been considered as valid candidates for the last step. Finally, each suspect natural toxin that fulfilled the requirements, was checked with Xcalibur (Thermo Fischer Scientific, San Jose, CA, USA) to control the elution profile and the retention times overlapping of the precursors and their transitions.

3) Confirmation

Confirmation was not possible for all the compounds due to the lack of standards. However, for the ones available the same procedure was carried out to obtain the AIF spectra from the standards to finally confirm the suspect compounds. (Level 1). Here identification levels from 1 to 5 were assigned to the suspect compounds, following what was previously reported by Schymanski et al. [8]. The lowest level 5 corresponded to the accurate mass, while level 4 was achieved using the spectral information to assign a molecular formula. Level 3 resulted at the end of the first identification step when the primary tentative candidate was proposed when existing some evidence to recognize a possible structure. Finally, levels 2 and 1 were achieved using databases reporting diagnostic evidence to assign an exact structure and using the standard respectively.

Application on real samples

The procedure was then applied to real samples coming from different sites in Europe. Briefly, 2 samples were from Piave River 46°10'12.6"N 12°15'58.2"E (Belluno, Italy), 3 from Sykovec (Tri Studne, Czech Republic), Brno Dam (49°13'58.1"N 16°31'03.3"E, Czech Republic) and Jedovnice (49°20'04.2"N 16°45'58.7"E, Czech Republic) respectively. 1 from Cardener River (41°40'48.2"N 1°50'39.1"E Barcelona, Spain). All samples were in triplicate. Sampling was carried out between July and August were the highest biological activity was expected in the cited areas.

Optimization and suspect screening using AFW standards solutions

Unlikely by what is generally applied with the $ddMS^2$, all the characteristic MS/MS transitions that can ensure a positive assignment were considered. **Figure S2** reports the mass spectrum of the umbelliferone standard $[M+H]^+$ m/z 163.0394 in artificial surface water after processing with Compound Discoverer 3.1. The picture reports the Full Scan and the AIF spectrum. As expected, the fragmentation spectra differ between the one obtained with DDA since also other transitions coming from interfering ions are displayed. As shown in **Figure 1,** the sensitivity increased when the spectrum is acquired in AIF mode providing higher intensities for the same fragments under the same experimental conditions (CE energy, concentration, MS parameters). A similar result was also highlighted by Sentandreu et al., [9] who reported that the breakdown pattern of previously isolated compounds and AIF patterns cannot be comparable. An over-breakdown is generally observed when AIF is applied retrieving a higher transition intensity rather than the molecular ion at the same collision energy. The tentative list obtained after the first analysis of AFW samples resulted in a list where appeared both compounds of interest and interferents. The noise was further hidden from the background using blanks. The first tentative structure was obtained using the "*mzLogic*" algorithm who matched the $HR[M+H]^+$ with the Chemspider and the in-house suspect list using the exact mass of the precursor ion 163.0195 m/z with a maximum error of 5 ppm. Here, umbelliferone had a full match (100%) from our suspect list and a partial match in Chemspider with a score of 86 %. Then, the "*FISh scoring"* algorithm was employed to match the MS/MS spectrum in a list of expected fragments reported in online databases. The most abundant fragment at 107.0492 *m/z* produced by the loss of –CO and –COH was observed followed in intensity by the 95.0492 and the 79.0180 *m/z*. However, during this experiment at least 5 positive fragments (green highlight in **Figure 2**) were necessary to consider the compound as a tentative candidate. As a result of this processing, is possible to observe that even if there were 80 unmatched transitions produced by interference, 16 were recognized as structural fragments**.** Spectra comparison depends on the Collision Energy applied. Here at 35 eV, umbelliferone structure $C_9H_6O_3$ (7-hydroxycoumarine) was confirmed with a match score of 87.7 % which is over the threshold required to accept a candidate to be further investigated. The MS/MS spectrum was also investigated using MetFrag [7] to assign a formula for each transition. Here 2 candidates were displayed (umbelliferone and 4-hydroxycoumarin). The mass spectrum obtained a final similarity score of 1.0 /1.0 for umbelliferone and 0.962/1.0 for the 4-hydroxycoumarin. A total of 29 fragments have been identified for the first compound and

27 for the second. **Table 3** reports the structures, the formula, and the exact masses of the fragments considered for the tentative identification to level 2 of umbelliferone.

The final step to reach identification level 2 as reported by Schymansky [8] was the manual check with XCalibur (Thermo Fischer Scientific, San Jose, CA, USA) to ensure the peak fitting under the same retention time of the precursor ion. **Figure 3** reports the MS^2 spectra with the fragments considered for the tentative identification of umbelliferone. The intensities are higher with respect to the noise originated by the interferences allowing a clear recognition of the peaks. The procedure resulted in the overlap of retention times with the same peak shape and intensities of the MS/MS spectrums confirming the good performances obtained in the identification of spectral patterns. Here three fragments were considered as qualitative ions, with a mass error under 5 ppm, briefly: $[C_8H_6O_2]^+$ 134.0368 m/z, 3.8 ppm; $[C_7H_7O]^+$ 107.0495 m/z, 3.8 ppm; $[C_6H_7O]^+$ 95.0495 m/z, 4 ppm; $[C_7H_7]^+$ 91.0546 m/z, 4.2 ppm; $[C_6H_7]^+$ 79.0546 m/z, 4.3 ppm. These steps were necessary to achieve the tentative identification level 2, however, the confirmation was only possible using standards.

Confirmation

The last step to confirm the suspect natural toxins to level 1, required the comparison with the standard. **Figure 4** shows the confirmation of the umbelliferone to the identification level 1. The standard solution at 1 µg/L in HPLC water was injected using the same acquisition method. As expected, the typical fragment ions reported above and used as qualifier ions at m/z 134.0368, 107.0495, 95.0495, and 79.0546 were at the same retention time but with higher intensity. The signal was more intense due to the absence of interferents in the solution. The separation performance was comparable with the standards dissolved in AFW. The measured results were within the required limits for the identification of natural toxins in surface water samples. The same procedure was repeated with all the standards available. In **Table 4** the results in AFW and HPLC water are reported. For each compound, more than 4 qualitative ions have been encountered in both AFW and HPLC water solutions. AFW samples presented as expected a lower signal suppressed by the most intense signals of the humic acids. However, the procedure allowed us to identify the standards and to validate the procedure for their determination. Quantitative validation was not included in this work since it is out of the aims.

Surface water samples analysis

Water samples coming from Italy, Spain, and Czech Republic were processed as described, performing the screening and the further identification and confirmation of different natural toxins. For the one in which standards were not available identification levels (ILs) system was applied [8]. This ILs method has been used by other authors to identify low molecular mass molecules when using data-independent acquisition [1, 10]. 138 compounds have been proposed as suspect candidates in the first identification step. However, only 27 were reported as suspect natural toxins, 3 (cotinine, abscisic acid, and ptaquilosin B) were false positives and 4 (methoxycoumarin, MC-LR, abietic acid, and umbelliferone) were confirmed comparing by standards (**Table 5**). For the compounds that had previous literature with mass spectra under similar conditions, the MS/MS interpretation was less time-consuming. For instance, the mass spectrum of azelaic acid matched with the one reported in MassBank [5]. Comparing the common fragments *m/z*= 83.08897, 97.10339, 103.05256, and 125.09818 were found in both spectrums and the tentative identification level 2 was assigned. Then, the presence of suspect ptaquilosin B was also investigated. Ptaquiloside, a carcinogenic bracken fern toxin, is converted to the aglycone ptaquilosin B (PTB) in aqueous solutions due to the liberation of D-glucose to be then converted to pterosin B [11]. Here PTB was detected in the first identification step. However, the conversion rate of PTB depends on the temperature and the pH >9. Here, since samples were frozen to -24 ºC and the initial pH was 7.8 further investigation was required. Since D-glucose is released when converting ptaquiloside, its molecular ion m/z 181.07066 was searched. No D-glucose was found besides, the total absence of its precursor ptaquiloside brought to discard this compound as a tentative candidate. Finally, fragment analysis of 61 peaks revealed a strong similarity (1.0/1.0) with the 4-Heptyloxybenzoic acid [12] a carboxylic acid used with different purposes with no environmental importance for this work. Finally, 24 compounds have been detected and tentatively identified as suspect natural toxins. However, the confirmation to level 1 through mass spectrums comparison was carried out for 4 compounds with standards available (MC-LR, abietic acid, methoxycoumarin, and umbelliferone). Samples coming from the Czech Republic were collected in a blooming area which was characterized by green algal slime. This was the first signal to further investigate the presence of algal toxins such as microcystins. Here the tentative candidate microcystin LR was detected with the typical molecular ion at m/z= 995.5545. The doubly charged ion at 498.2822 *m/z* was also encountered at T_r 9.12 min with the typical

higher intensity respect to the molecular ion [13]. Finally, the MS/MS spectra revealed the presence of the typical fragment at 135.0803 *m/z* which is the exact mass of the ADDA fragment part of all the microcystins structure. After manual analysis of the MS/MS spectra, the precursor and 3 common fragments were found to be consistent with the MC-LR structure (**Figure 5**). Finally, the MC-LR was confirmed to level 1 using the standard solution that revealed the presence of the qualitative fragment ions in both mass spectrums. The same confirmation procedure was applied for methoxycoumarin, abietic acid, and umbelliferone while 20 structures were proposed as suspect natural toxins with an identification level 2.

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Declaration of interests:

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

***References:**

[1] M. Picardo, J. Sanchís, O. Núñez, M. Farré, Suspect screening of natural toxins in surface and drinking water by high-performance liquid chromatography and high-resolution mass spectrometry, Chemosphere 261 (2020) 127888.
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Figures and tables

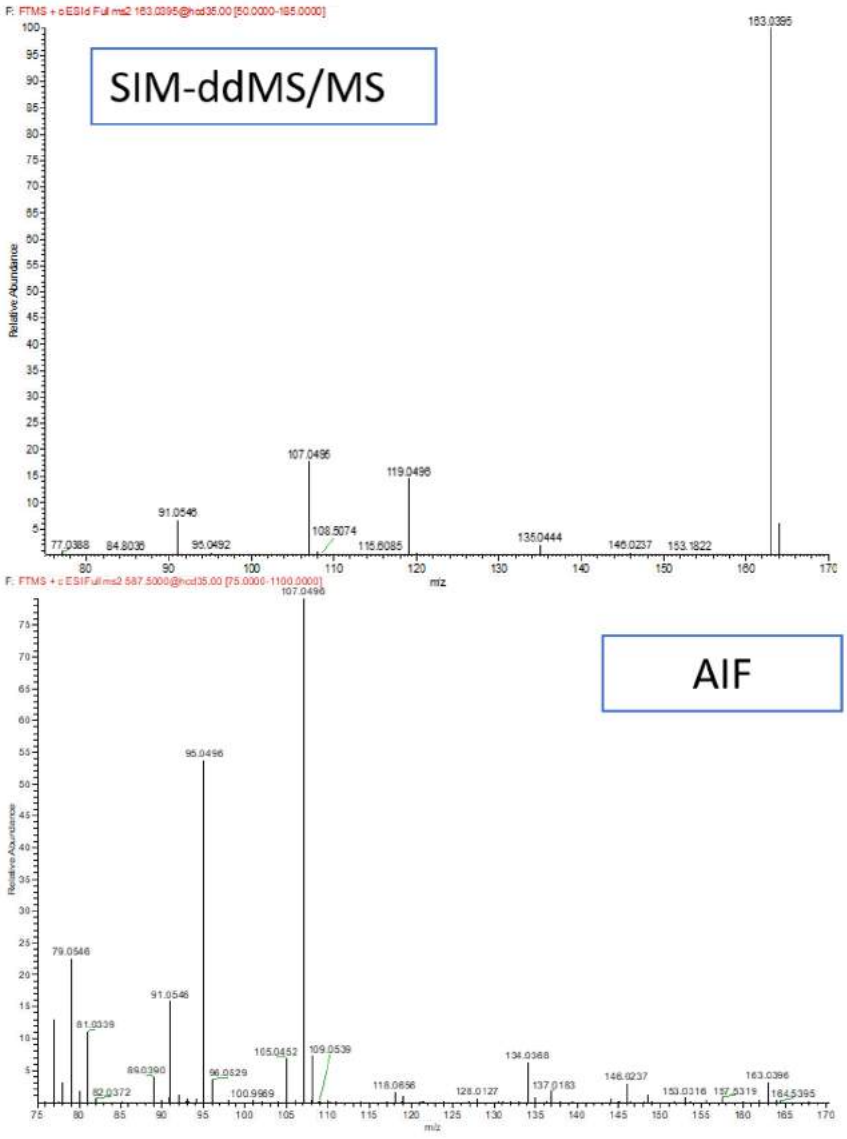
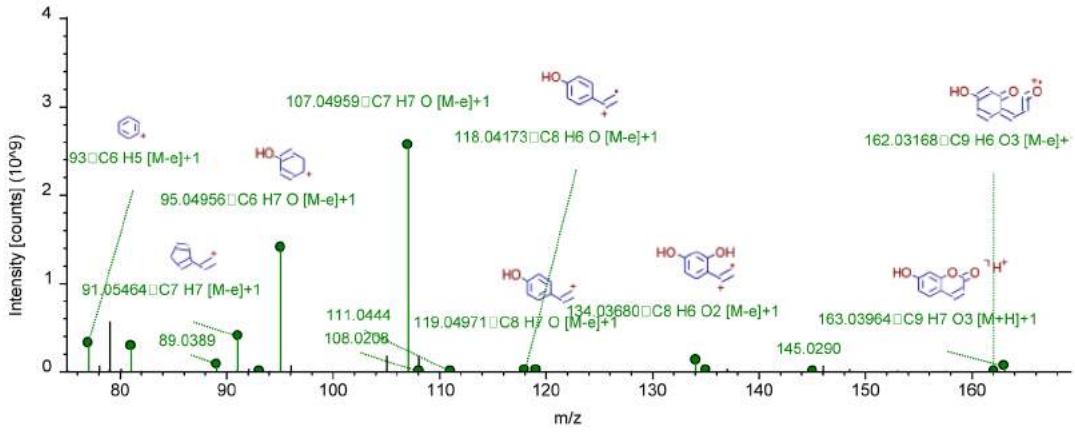


Figure 1: *Sensitivity comparison between SIM-ddMS/MS and AIF under the same experimental conditions*



306

307 **Figure 2:** *FISh coverage results for the analysis of umbelliferone*

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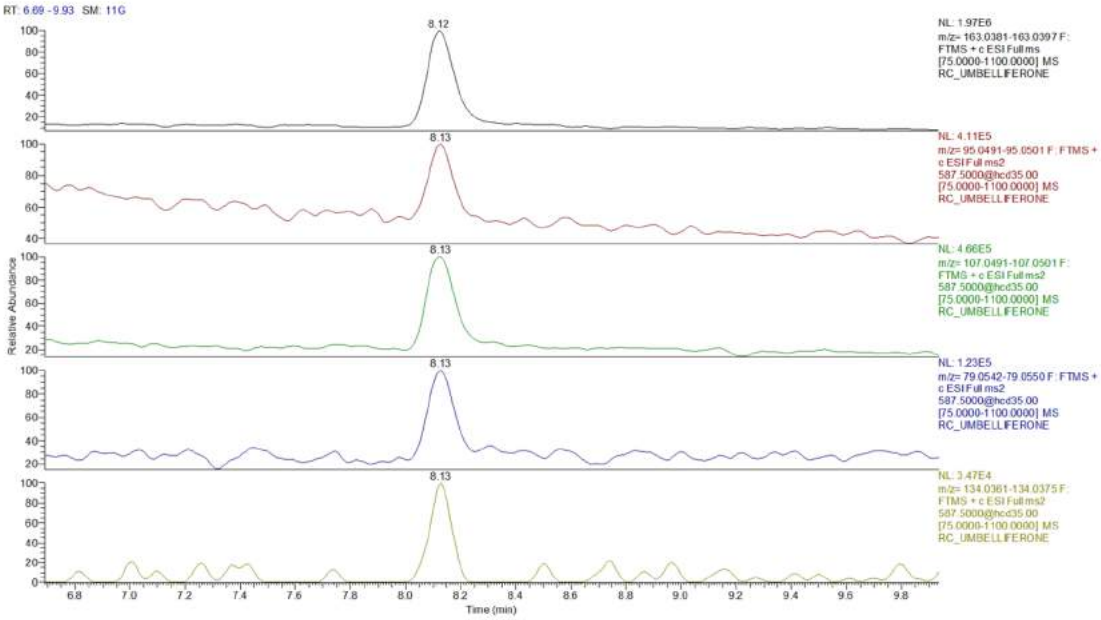

310

311 **Figure 3:** *Manual check with Xcalibur for peak shape and retention time fitting of precursor and*
312 *transitions*

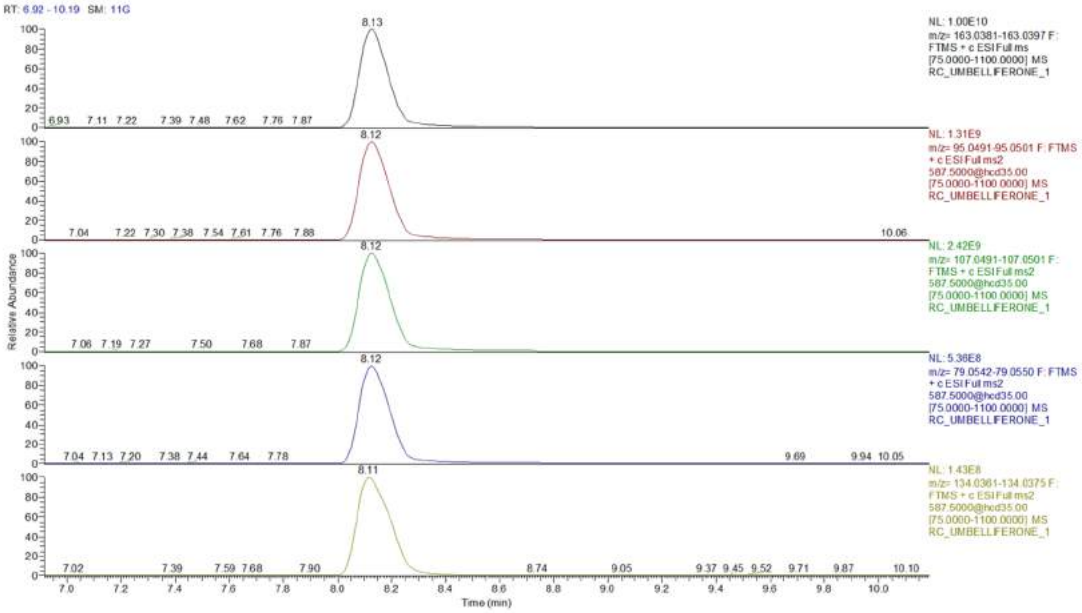

Figure 4: *Mass spectrums of the standard umbelliferone and its fragmentation products.*

Table 1: *Analyser acquisition parameters*

Tune Data	
Spray Voltage:	3250 V
Capillary Temperature (+ or +-):	300 ºC
Sheath Gas (+ or +-):	40
Aux Gas (+ or +-):	25
Spare Gas (+ or +-):	0
Max Spray Current (+):	100
Probe Heater Temp. (+ or +-):	300 ºC
S-Lens RF Level:	70
Ion Source:	HESI
Resolution FS	35000 FWHM
Resolution AIF	17500 FWHM
AGC target	$10e^5$
Max Inject time	100ms
Top N	3
Microscans	1

Table 2: *Compound Discoverer 3.1 parameters for the peak alignment and identification.*

Workflow node	Advanced Parameters	Parameter	Value
Select spectra	Spectrum filter	Min precursor Mass	50 Da
		Max precursor Mass	1200 Da
	Scan event filter	Polarity mode	Positive
		Min Collision Energy	0
		Max Collision Energy	70
		Scan type	Any
	Peak filter	S/N threshold	3
Align retention times	General settings	Adaptive alignment	
		Max shift	2 min
		Mass tolerance	5 ppm
Find expected compounds	General settings	Mass tolerance	5 ppm
		Intensity tolerance [%]	50
		Intensity threshold [%]	0.1
		S/N threshold	3
		Min peak Intensity	100000
Detect compounds	General settings	Mass tolerance	5 ppm
		Intensity tolerance [%]	40
		S/N threshold	3
		Min peak Intensity	100000
		Ions Checked	[M+ACN+H]+, [M+H]+, $[M+K]^+$, $[M+Na]^+$
		Min Elements count	C, H, O
Group Compounds	Compound Consolidation	Mass tolerance	5 ppm
		RT tolerance	1 min
	Fragment data selection	Preferred Precursor Ions	[M+ACN+H]+, [M+H]+, [M+K]+, [M+Na]+
Search ChemSpider	Search settings	Databases	MassBank, Toxin, Toxin-target database,
		Search Mode	By Formula and mass
		Mass tolerance	5 ppm
		Max results per compound	20
		Max predicted compounds	3
Search MzCloud	General settings	Compound classes	Natural toxins
		Library	Autoprocessed; Reference
	DDA Search	identity search	HighChem; HighRes
		Match Activation Type	True
		Match Activation energy	Match with tolerance
		Activation energy tolerance	35
		Apply intensity threshold	True
		Similarity search	Confidence forward
		Match factor Threshold	40
	DIA Search	Use DIA scans for search	True
		Max isolation width	500

		Match activation type	False
		Match Activation energy	Any
		Activation energy tolerance	100
		Apply intensity threshold	False
		Match factor Threshold	10
Search Mass list	General settings	Mass List	In house suspect list
		Use retention times	False
		RT tolerance	-
		Mass Tolerance	5 ppm

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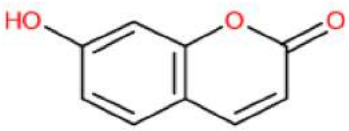
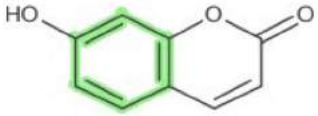
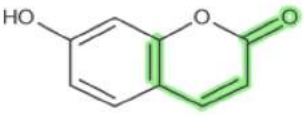
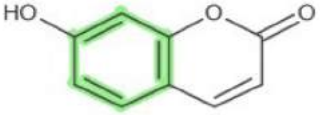
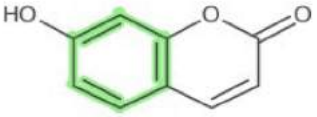
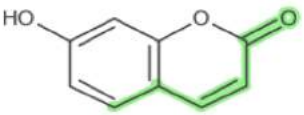
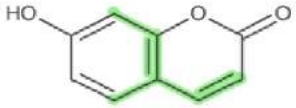
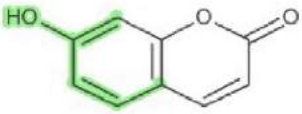
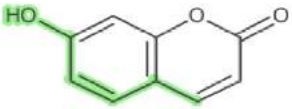
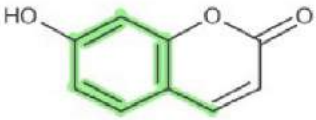
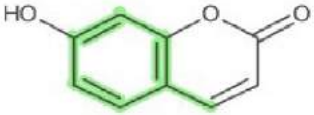
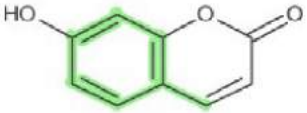
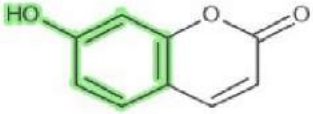
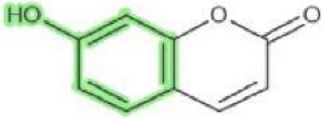
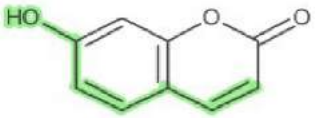
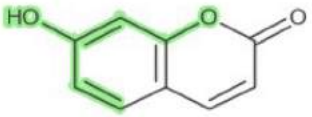
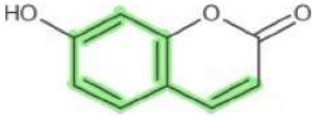
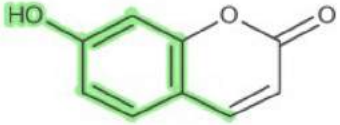
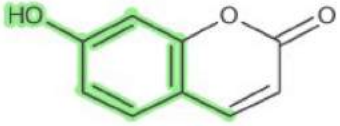
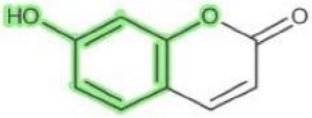
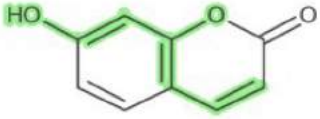
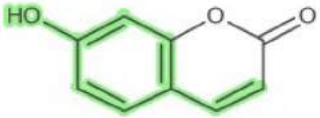
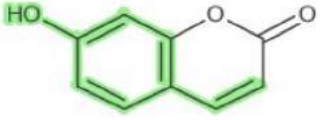
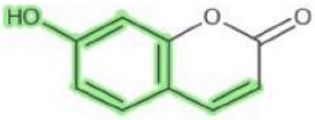
327 **Table 3**: *fragmentation patterns recognised by MetFrag*

Precursor		
	**CompoundName** **Molecualr formula** **Identifier**	Umbelliferone C9H6O3 C9H6O3
Fragments		
	**Formula** **Mass** **Peak m/z**	[C5H2O-H]+ 77.00219 77.00256
	**Formula** **Mass** **Peak m/z**	[C5H2O-H]+ 77.00219 77.00256
	**Formula** **Mass** **Peak m/z**	[C6H3+H]+H+ 77.0386 77.03893
	**Formula** **Mass** **Peak m/z**	[C6H3+2H]+H+ 78.04643 78.04679
	**Formula** **Mass** **Peak m/z**	[C5H3O]+ 79.01785 79.01823

	**Formula** **Mass** **Peak m/z**	[C6H4+2H]+H+ 79.05426 79.0546
	**Formula** **Mass** **Peak m/z**	[C5H4O]+H+ 81.03351 81.03388
	**Formula** **Mass** **Peak m/z**	[C5H4O+2H]+H+ 83.04917 83.04953
	**Formula** **Mass** **Peak m/z**	[C7H4]+H+ 89.0386 89.03898
	**Formula** **Mass** **Peak m/z**	[C7H4+H]+H+ 90.04643 90.0468
	**Formula** **Mass** **Peak m/z**	[C7H4+2H]+H+ 91.05426 91.05464
	**Formula** **Mass** **Peak m/z**	[C6H4O]+H+ 93.03351 93.03397
	**Formula** **Mass** **Peak m/z**	[C6H4O+H]+H+ 94.04134 94.04169
	**Formula** **Mass** **Peak m/z**	[C6H5O+H]+H+ 95.04917 95.04956

	**Formula** **Mass** **Peak m/z**	[C5H4O2+H]+H+ 98.03625 98.03667
	**Formula** **Mass** **Peak m/z**	[C8H5]+ 101.0386 101.03919
	**Formula** **Mass** **Peak m/z**	[C7H5O]+ 105.03351 105.03397
	**Formula** **Mass** **Peak m/z**	[C7H5O+H]+H+ 107.04917 107.04959
	**Formula** **Mass** **Peak m/z**	[C6H4O2]+ 108.02059 108.02084
	**Formula** **Mass** **Peak m/z**	[C6H4O2+2H]+H+ 111.04408 111.04446
	**Formula** **Mass** **Peak m/z**	[C8H6O-H]+ 117.03351 117.03391
	**Formula** **Mass** **Peak m/z**	[C8H6O]+ 118.04134 118.04173
	**Formula** **Mass** **Peak m/z**	[C8H6O]+H+ 119.04917 119.04971

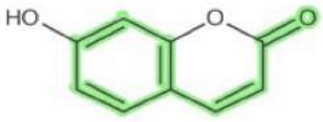
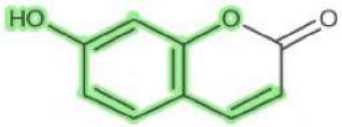
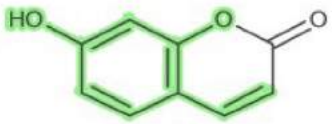
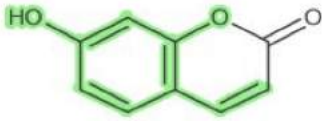
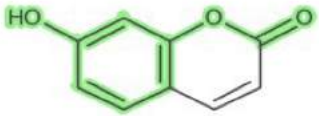
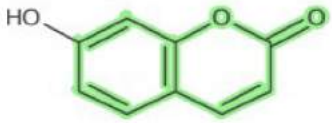
	**Formula** **Mass** **Peak m/z**	[C9H5O+H]+H+ 131.04917 131.04965
	**Formula** **Mass** **Peak m/z**	[C8H6O2-H]+ 133.02842 133.0289
	**Formula** **Mass** **Peak m/z**	[C8H6O2]+ 134.03625 134.0368
	**Formula** **Mass** **Peak m/z**	[C8H6O2]+H+ 135.04408 135.04466
	**Formula** **Mass** **Peak m/z**	[C7H4O3+H]+H+ 138.03116 138.03174
	**Formula** **Mass** **Peak m/z**	$[C9H5O2]^+$ 145.02842 145.02901

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Table 41: *Fragmentation patterns of the 23 natural toxins standards*

Compound	Rt	Precursor	Qi (1)	Structure	Qi (2)	Structure	Qi (3)	Structure	Qi (4)	Structure	Qi (5)	Structure
Ethoxycoumarin	11.2	191.0698	107.0492	C_7H_7O	95.0492	C_6H_7O	163.039	$C_9H_7O_3$	119.0492	C_8H_7O	91.0543	C_7H_7
Methoxycoumarin	10.1	177.0542	77.0386	C_6H_5	162.0310	$C_9H_6O_3$	106.0413	C_7H_6O	121.0647	C_8H_9O	134.0361	$C_8H_6O_2$
Abietic acid	1.5	303.2323	257.2269	$C_{19}H_{29}$	121.1014	C_9H_{13}	147.1171	$C_{11}H_{15}$	287.2010	$C_{19}H_{27}O_2$	241.1954	$C_{18}H_{25}$
Aflatoxin B_1	9.8	313.0696	285.0763	$C_{16}H_{12}O_5$	269.0449	$C_{15}H_{10}O_5$	241.0499	$C_{14}H_9O_4$	214.0627	$C_{13}H_{10}O_3$	201.0913	$C_{12}H_8O_3$
Amygdalin	6.4	480.1483 [M+Na]+	85.0285	$C_4H_5O_2$	107.0492	C_7H_7O	325.11325	$C_{12}H_{21}O_{10}$	163.0602	$C_8H_{11}O_5$	127.0391	$C_6H_7O_3$
Anatoxin-a	1.6	166.1228	149.0964	$C_{10}H_{13}O$	95.0493	C_6H_7O	105.0700	C_8H_9	91.0544	C_7H_7	79.0544	C_6H_7
Atropine	6.8	290.1747	124.1120	$C_8H_{14}N$	93.06989	C_7H_9	103.0542	C_8H_7	260.1644	$C_{16}H_{22}NO_2$	142.1226	$C_8H_{16}NO$
B-Asarone	12.2	209.1166	179.0705	$C_{10}H_{11}O_3$	151.0756	$C_9H_{11}O_2$	121.0649	C_8H_9O	91.05446	C_7H_7	107.0493	C_7H_7O
Cinchonine	6	294.1733	79.0544	C_6H_7	184.0759	$C_{12}H_{10}NO$	130.0654	C_9H_8N	154.0653	$C_{11}H_8N$	142.0654	$C_{10}H_8N$
Cotinine	1.4	177.1029	80.0499	C_5H_6N	98.0606	C_5H_8NO	146.0609	C_9H_8NO	106.0657	C_7H_8N		
Cylindrospermopsin	1.5	415.1166	336.1675	$C_{15}H_{22}N_5O_4$	194.1293	$C_{10}H_{16}N_3O$	274.0864	$C_{10}H_{16}N_3O_4S$	318.1570	$C_{15}H_{20}N5O_3$		
Kojic Acid	2.3	143.0336	113.0234	$C_5H_5O_3$	126.0313	$C_6H_6O_3$	97.02863	$C_5H_5O_2$	87.00786	$C_3H_3O_3$		
Microcystin LA	11.5	910.4882	135.0808	$C_9H_{11}O$	227.0224	$C_9H_9N_2O_5$	299.0621	$C_{11}H_{15}N_4O_6$	155.0689	$C_6H_8N_3O_2$	297.0829	$C_{11}H_{15}N_4O_6$
Microcystin LF	12.3	986.5225	135.0808	$C_9H_{11}O$	213.0871	$C_9H_{13}N_2O_4$	258.1855	$C_{17}H_{24}N\ O$	461.2398	$C_{23}H_{33}N_4O_6$	580.3016	$C_{32}H_{42}N_3O_7$
Microcystin LR	9.1	995.5575	135.0806	$C_9H_{11}O$	382.2089	$C_{17}H_{28}N_5O_5$	213.08728	$C_9H_{13}N_2O_4$	470.2729	$C_{20}H_{36}N_7O_6$	103.0544	$C_8\ H_7$
Microcystin YR	11.7	1045.5355	135.0806	$C_9H_{11}O$	213.1364	$C_9H_{16}N_4O_2$	265.1609	$C_{19}H_{23}O$	323.1800	$C_{14}H_{24}N_6O_3$	466.2589	$C_{26}H_{36}N_4O_4$
Microcystin LY	11.2	1002.5353	135.0806	$C_9H_{11}O$	375.1918	$C_{20}H_{27}N_2O_5$	494.2616	$C_{28}H_{36}N_3O_5$	213.08723	$C_9H_{12}N_2O_4$	243.1343	$C_{11}H_{18}N_2O_4$
Nodularin	8.8	825.4505	135.080	$C_9H_{11}O$	227.103	$C_{10}H_{15}O_4N_2$	389.2074	$C_{21}H_{29}O_5N_2$	691.3768	$C_{29}H_{53}O_{12}N_7$	285.1668	$C_{11}H_{21}O_3N_6$
Ochratoxin-A	11.8	404.0885	358.0835	$C_{19}H_{17}ClNO_4$	257.0211	$C_{11}H_{10}ClO_5$	239.0105	$C_{11}H_8ClO_4$	120.0808	$C_8H_{10}N$	211.0157	$C_{10}H_8ClO_3$
P-Coumaric acid	7.8	165.0544	91.0543	C_7H_7	81.0336	C_5H_5O	81.03363	C_5H_5O	119.0492	C_8H_7O	147.0441	$C_9H_7O_2$
Scopolamine	6.21	304.1538	138.0912	$C_8H_{12}NO$	103.0542	C_8H_7	110.09641	$C_7H_{12}N$	103.0542	C_8H_7	121.0647	C_8H_9O
Thujone	12	153.1269	139.1120	$C_9H_{15}O$	97.0650	C_6H_9O	121.10143	C_9H_{13}	109.0651	C_7H_9O	135.1171	$C_{10}H_{15}$
Umbelliferone	8.12	163.0386	107.0492	C_7H_7O	95.0492	C_6H_7O	91.0546	C_7H_7	119.0493	C_8H_7O	134.0363	$C_8H_6O_2$

Table 5: *results of the suspect screneing with AIF acquisition in water samples*

Comp Nº	Compound	Molecular formula	[M+H]+	Transition 1	Structure	Transition 2	Structure	Transition 3	Structure	Transition 4	Structure	Transition 5	Structure	Conf. Level
1	Aspidospermine	$C_{22}H_{30}N_2O_2$	355.2373	119.0491	C_8H_6O	107.0491	C_7H_6O	146.06	C_9H_9NO	228.1379	$C_{15}H_{18}NO$	152.1072	$C_9H_{15}NO$	2
2	O-Acetyltropine	$C_{10}H_{17}NO_2$	184.1329	108.0807	$C_7H_{11}N$	109.0648	$C_7H_{11}O$	127.0754	$C_7H_{11}O_2$	140.1068	$C_8H_{14}NO$	138.0913	$C_8H_{13}NO$	2
3	Microcystin LR	$C_{49}H_{74}N_{10}O_{12}$	995.5545	135.0805	$C_9H_{11}O$	382.2089	$C_{17}H_{28}N_5O_5$	213.0872	$C_9H_{13}N_2O_4$	265.1585	$C_{15}H_{21}O$	103.0544	C_8H_7	1
4	Heliotridine	$C_8H_{13}NO_2$	156.1018	120.0808	C8H11N	122.0965	$C_8H_{11}N$	124.0758	$C_7H_{10}N_O$	110.0601	C_6H_9NO	108.0808	C_7H_9N	2
5	4-Heptyloxybenzoic acid	$C_{14}H_{20}O_3$	237.1481	105.0699	C_8H_{11}	133.1012	$C_9H_{11}O$	147.0854	C10H13O	123.0848	C8H12O	161.0961	$C_{11}H_{15}O$	False Ptaquiloside B
6	Hypoglicine A	$C_7H_{11}NO_2$	142.0861	107.0492	C_7H_8O	126.0550	$C_6H_9NO_2$	111.0441	C6H7O2	125.0597	C7H9O2	108.0808	$C_7H_{10}N$	2
7	Salsolinol	$C_{10}H_{13}NO_2$	180.1017	105.0700	C_8H_{10}	107.0493	C_7H_5O	118.0652	$C_8H_{10}N$	144.0810	$C_{10}H_{11}N$	162.0916	$C_{10}H_{12}NO$	2
8	Fumigaclavine C	$C_{23}H_{30}N_2O_2$	367.2374	105.0699	C_8H_9	119.0855	C_9H_{12}	243.1375	$C_{16}H_{17}O_2$	130.0653	C_9H_8N	144.0808	$C_{10}H_{11}N$	2
9	4-hydroxymellein	$C_{10}H_{10}O_4$	195.065	149.0234	$C_8H_6O_3$	121.0285	$C_7H_6O_2$	181.0496	$C_8H_7O_4$	163.0756	$C_{10}H_6O_2$	141.0543	$C_7H_7O_3$	2
10	(R)-reticuline	$C_{19}H_{23}NO_4$	330.1695	121.0285	$C_7H_6O_2$	111.0441	$C_6H_4O_2$	135.0805	C_9H_9O	125.0598	$C_7H_7O_2$	138.0914	$C_8H_{10}NO$	2
11	Apiol	$C_{12}H_{14}O_4$	223.0961	109.0648	C_7H_9O	135.0440	$C_8H_7O_2$	151.0754	$C_9H_9O_2$	147.0805	$C_{10}H_9O$	163.0391	$C_9H_8O_3$	2
12	Conhydrine	$C_8H_{17}NO$	144.1382	107.0856	C_8H_{11}	123.0805	$C_8H_{11}O$	138.0915	$C_8H_{12}NO$	111.0805	$C_7H_{11}O$	100.112	$C_5H_{14}N$	2
13	5-(N-Methyl-4,5-dihydro-1H-pyrrol-2-yl)pyridin-2-ol	$C_{10}H_{12}NO_2$	177.1019	120.0327	$C_5H_4N_2O$	107.0494	C_7H_4O	91.05438	C_7H_6	89.03877	C_7H_5-H	80.0496	C_5H_4N	False cotinine
14	Ridentin	$C_{15}H_{20}O_4$	265.1434	163.0757	$C_{10}H_{11}O_2$	123.0805	$C_8H_{11}O$	191.0709	$C_{11}H_{11}O_3$	207.1388	$C_{13}H_{19}O_2$	163.0757	$C_{10}H_{11}O_2$	False abscissic acid
15	Abietic acid	$C_{20}H_{30}O_3$	303.2317	257.226	$C_{19}H_{29}$	121.10145	C_9H_{13}	147.1171	$C_{11}H_{15}$	173.1328	$C_{13}H_{17}$	95.0945	C_5H_7O	1
16	Jervine	$C_{27}H_{39}NO$·	426.2997	187.1120	$C_{13}H_{16}O$	191.1438	$C_{13}H_{19}O$	215.14362	$C_{15}H_{18}O$	219.1745	$C_{15}H_{22}O$	121.0650	$C_8H_{11}O$	2
17	Umbelliferone	$C_9H_6O_3$	163.0387	107.0492	C_7H_7O	119.0493	C_8H_7O	119.0493	C_8H_7O	135.0441	$C_8H_7O_2$	147.0441	$C_9H_7O_2$	1

18	Vincaminorein (Aspidospermine)	$C_{22}H_{30}N_2O_2$	355.2373	270.1859	$C_{18}H_{24}NO$	107.0491	C_7H_6O	119.0491	C_8H_6O	145.0652	$C_{10}H_{10}O$	98.0603	C_5H_8NO	2
19	Swainsonine	$C_8H_{15}NO_3$	174.1128	86.0603	C_4H_8NO	87.0443	$C_4H_7O_2$	124.0761	$C_7H_{12}NO$	140.0712	$C_7H_{12}NO_2$	138.0918	$C_8H_{13}NO$	2
20	Salsoline	$C_{11}H_{15}NO_2$	194.1173	91.0545	C_7H_8	179.1069	$C_{11}H_{14}O_2$	163.0759	$C_{10}H_{12}O_2$	107.0494	C_7H_9O	96.0809	C_6H_9N	2
21	Methoxycoumarin	$C_{10}H_8O_3$	177.0545	91.0546	C_7H_8	149.0239	$C_8H_5O_3$	163.0395	$C_8H_5O_3$	134.0361	$C_8H_6O_2$	121.0647	C_8H_9O	1
22	Azealic acid (Aspionene)	$C_9H_{16}O_4$	189.112	75.0439	$C_3H_5O_2$	83.0857	C_6H_{12}	97.0650	$C_6H_{10}O$	101.0599	$C_5H_9O_2$	143.1071	$C_9H_{15}O_2$	2
23	Aspergillic acid	$C_{12}H_{20}N_2O_2$	225.1602	98.0604	C_5H_8NO	124.0761	$C_7H_{10}NO$	209.1290	$C_{11}H_{17}N2O2$	152.0712	$C_8H_{11}NO_2$	86.0603	C_4H_5NO	2
24	Coniferyl acetate	$C_{12}H_{14}O_4$	223.0961	91.0546	C_7H_7	149.0238	$C_8H_7O_3$	121.0288	$C_7H_5O_2$	137.0602	$C_8H_8O_2$	177.0911	$C_{11}H_{10}O_2$	2

Supplementary material *and/or* Additional information:

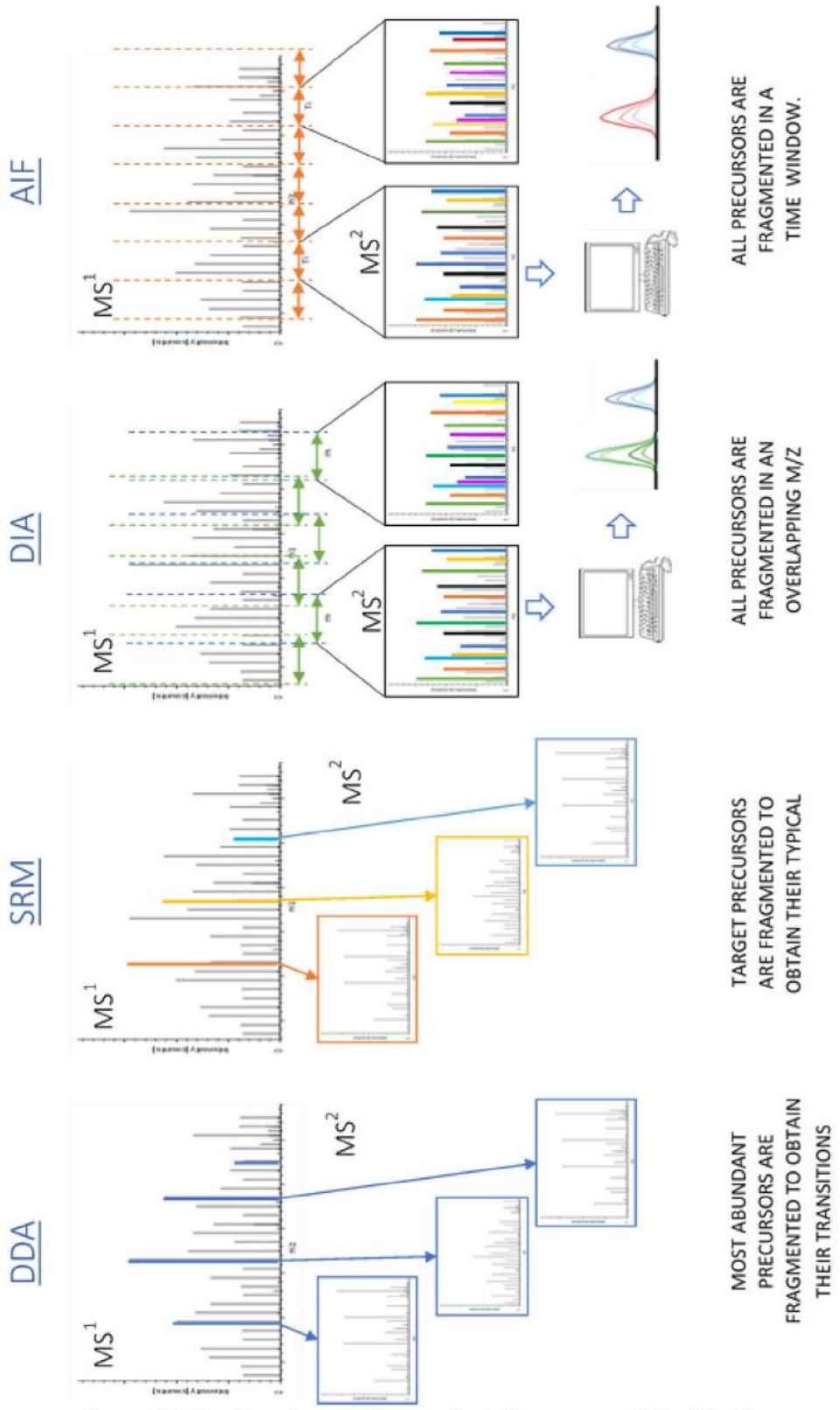


Figure S1: Most used acquisition methods for compound identification

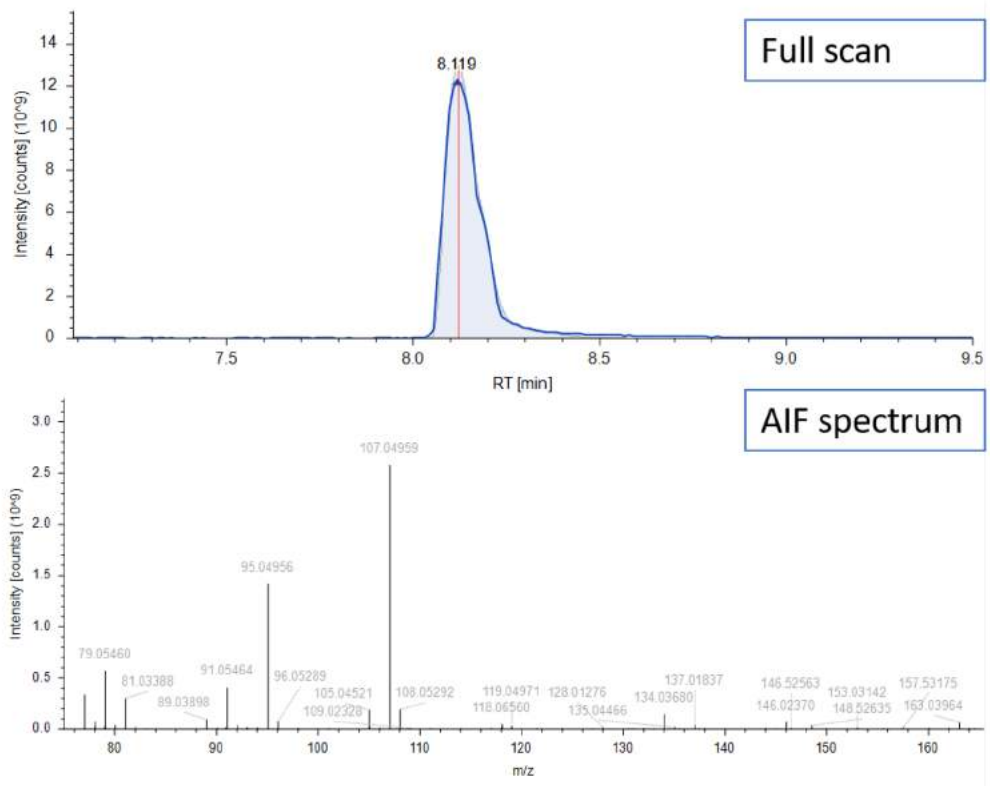
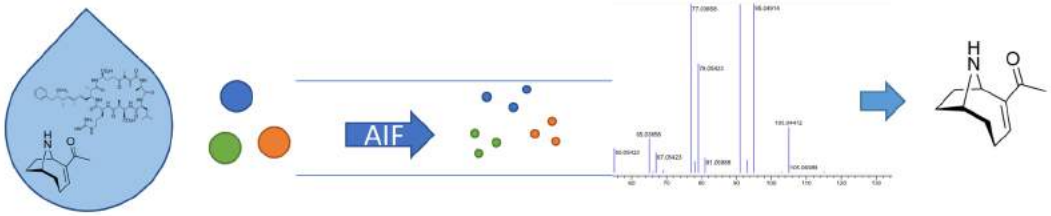


Figure S2: *Layout after the first step of the tentative identification of umbelliferone standard in AFW solution*

Table S2: *Standards list*

Nº	Toxin	Toxic group	Chemical formula	Exact mass	Purity %	Weight
1	*Ethoxycoumarin*	Phytotoxin	$C_{11}H_{10}O_3$	190.0634	>98	25 mg
2	*Metoxycoumarin*	Phytotoxin	$C_{10}H8O3$	176.0479	99	10 mg
3	*Abietic acid*	Phytotoxin	$C_{20}H_{30}O_2$	302.2256	>75	25 g
4	*Aflatoxin B_1*	Mycotoxin	$C_{17}H_{12}O_6$	312.0632	99	1mg
5	*Amygdalin*	Phytotoxin	$C_{20}H_{27}NO_{11}$	457.158	> 97	5 g
6	*Anatoxin-a*	Cyanotoxin	$C_{10}H_{15}NO$	165.2320	> 99	1 mg
7	*Atropine*	Phytotoxin	$C_{17}H_{23}NO_3$	289.1682	> 99	1 g
8	*B-Asarone*	Phytotoxin	$C_{12}H_{16}O_3$	208.1099	> 70	1 g
9	*Cinchonine*	Phytotoxin	$C_{19}H_{22}N_2O$	294.1732	> 99	50 mg
10	*Cotinine*	Phytotoxin	$C_{10}H_{12}N_2O$	176.0956	99	1 mg/mL
11	*Cylindrospermopsin*	Cyanotoxin	$C_{15}H_{21}N_5O_7S$	399.1219	99	10 µg/mL
12	*Kojic Acid*	Mycotoxin	$C_6H_6O_4$	142.0274	> 99	5 g
13	*Microcystin LA*	Cyanotoxin	$C_{46}H_{67}N_7O_{12}$	909.4847	> 95	10 µg/mL
14	*Microcystin LF*	Cyanotoxin	$C_{52}H_{71}N_7O_{12}$	985.5160	> 95	10 µg/mL
15	*Microcystin LR*	Cyanotoxin	$C_{49}H_{74}N_{10}O_{12}$	994.5488	> 95	10 µg/mL
16	*Microcystin YR*	Cyanotoxin	$C_{52}H_{72}N_{10}O_{13}$	1045.5355	> 95	10 µg/mL
17	*Microcystin LY*	Cyanotoxin	$C_{52}H_{71}N_7O_{13}$	1001.5109	> 95	7.5µg/mL
18	*Nodularin*	Cyanotoxin	$C_{41}H_{60}N_8O_{10}$	824.4432	99	7 µg/mL
19	*Ochratoxin-A*	Mycotoxin	$C_{20}H_{18}ClNO_6$	403.0823	99	10 µg/mL
20	*P-Coumaric acid*	Phytotoxin	C_9H8O_3	164.0471	> 98	1 g
21	*Scopolamine*	Phytotoxin	$C_{17}H_{21}NO_4$	303.147	> 98	1 g
22	*Thujone*	Phytotoxin	$C_{10}H_{16}O$	152.1235	99	1 mL
23	*Umbelliferone*	Phytotoxin	$C_9H_6O_3$	162.0327	99	10 g

Graphical abstract



4. DISCUSSION

4.1 Natural toxins load in Ter River

Cyanotoxins

The Mediterranean area is well known to be affected by algal bloom phenomena, which is also due to global warming (**Figure 17**) [239-242].

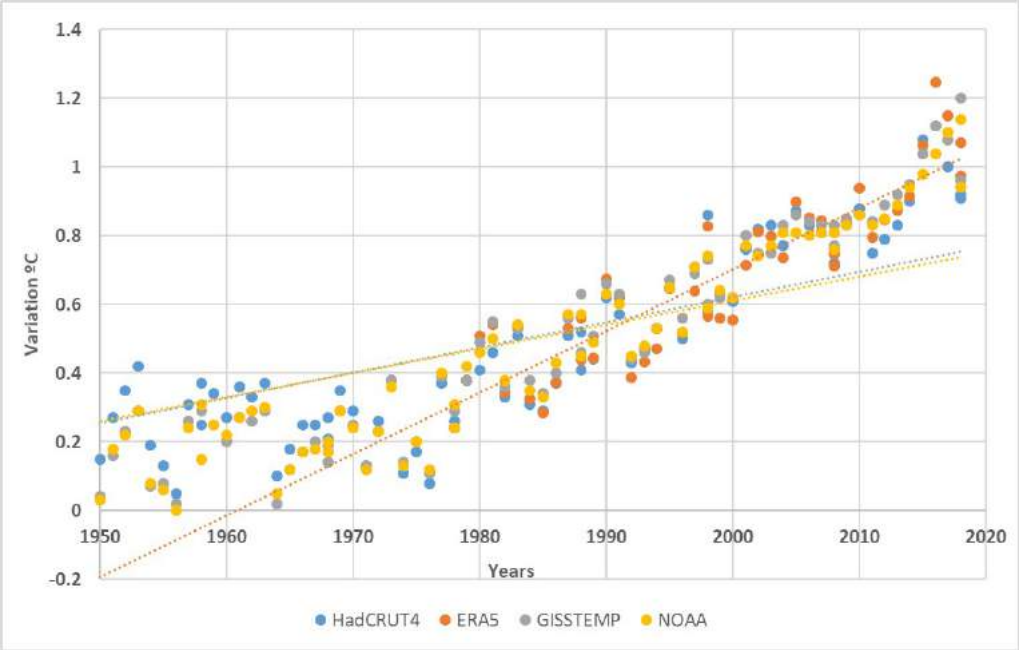


Figure 17: Global average temperatures from 1950 to 2020

The increasing temperature, which is projected to rise to 1.5 °C over the next 10 years is creating favourable conditions for algal bloom to express itself as an increasingly frequent phenomenon. This reproduction is also controlled by environmental factors such as nutrients. Using these premises, the physical-chemical characteristics of the Ter River waters (Catalonia, Spain) have been registered and compared with the previous data that were collected during the research of CARIMED 2018 [243], in order to report the ecological risk of eutrophication and to link the eventual presence of natural toxins.

Figure 18 shows the sampling points that were considered for CARIMED 2018 with the coloured spots referring to the different eutrophication risk levels, while the sampling points refer to publication III and publication I which are represented as arrows with the same names that are reported in the respective articles.

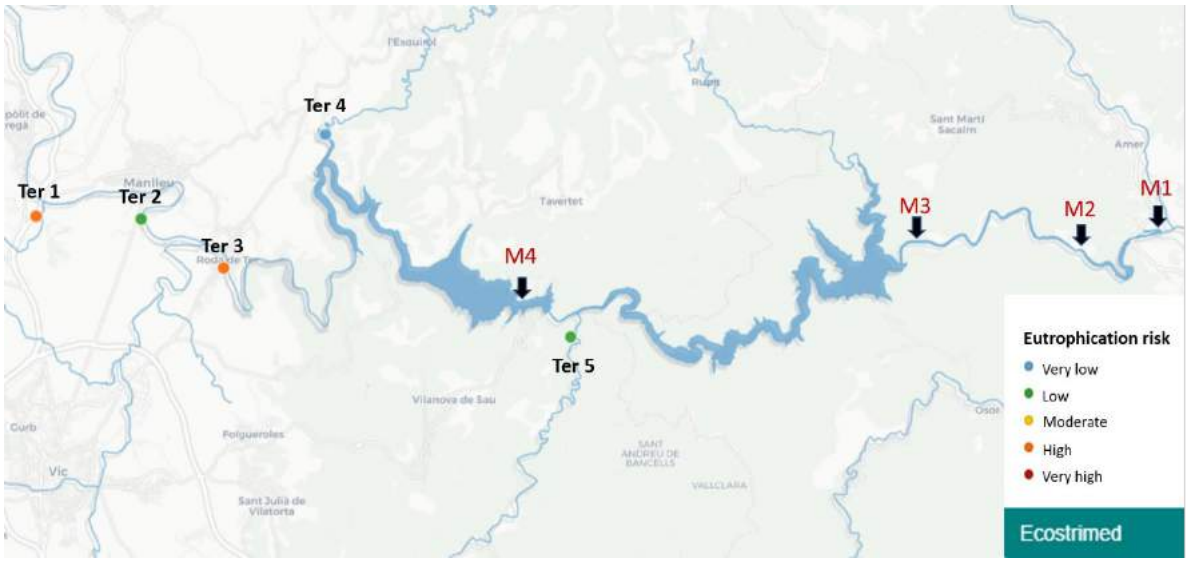


Figure 18: Ecological situation using the ECOSTRIMED index in the Ter River (Catalonia, Spain) (Figure adapted from [243])

In 2018, two sampling points were considered to be at high risk of eutrophication (Ter 1 and Ter 3) and they are located in an agricultural area. The comparison between the two sampling campaigns is been reported in **Table 13**. The chemical parameters are homogeneous among the entire part of the river that was considered for this work. Conductivity and pH vary depending on the season while the oxygen saturation falls to circa 60 % levels during summer. Temperature limits the oxygen saturation water can hold, in general, during summertime, and the water is less oxygenated with respect to the winter water. The dispersed oxygen in the water column is produced by photosynthesis, being transported by water circulation and mixing, diffusion, and biochemical consumption [244]. These processes modulate the oxygen concentration over different time scales such as diurnal, seasonal, annual variations [245,246]. These premises can aid to understand the further discovery of microcystins in the Ter River, as reported below. Here, as described in publication I and publication III, MC-LR, MC-LW, MC-YR, and Nod have been detected in the M1 and M2 points during July, August, and September 2018 (highlighted in green colour in **Table 13**).

Table 13: Chemical physical parameters of the sampling points for CARIMED and that reported in publications I and III

Sampling point	Date	Volume L/s	Temp ºC	Cond.	pH	Oxygen mg/l	Oxygen %
Ter 1	18/04/2018		13	915.00	8.8		
	28/05/2019	184.40	14.00	542.00	7.9	10.7	120.40
Ter 2	11/07/2018		20	367	8.2		
	28/05/2019	8323.2	17	409	8.1	7.3	71.8
Ter 3	30/05/2018	38519	15	360	7.3		
	28/05/2019	12401	17	338	7.9	7.2	64.3
Ter 4	30/05/2018		14	658	8.1		
	18/06/2019	137.9	17	568	8.2	8.4	81.1
Ter 5	01/06/2018	86	16.6	164	8.1	13.1	134.6
	15/05/2019	59	14.5	234	7.7	9.6	93.8
	05/09/2018	89	18.4	251	7.9	7.5	79.6
	07/08/2019	17	22.3	341.8	7.9	8.7	99.6
M1	28/03/2018	-	11.1	314.4	9.3	20.91	190.3
	13/04/2018		16	302.2	9.2	7.6	69.8
	25/05/2018		18	348.4	8.1	9.6	107.1
	17/07/2018		18.5	302	8.4	8.4	77.2
	24/08/2018		22.3	256	8.4	5.6	51.5
	20/09/2018		20.3	286	8.2	5.0	45.9
M2	28/03/2018	-	11.1	215	9.4	20.2	185.6
	13/04/2018		11.4	265.1	8.6	9.3	85.4
	25/05/2018		16	246.2	7.2	10.3	114.9
	17/07/2018		18.2	296	8.3	10.2	93.7
	24/08/2018		21.2	297.5	8.1	9.4	86.4
	20/09/2018		22	274	7.9	9.2	84.5
M3	28/03/2018	-	9	306.3	8.9	8.2	75.3
	13/04/2018		7.2	423	8.5	6.2	57.0
	25/05/2018		13.5	451	7.6	7	64.3
	17/07/2018		17.2	394	7.8	8.3	76.3
	24/08/2018		20.5	384.2	8.2	9.3	85.4
	20/09/2018		21.7	426	8.3	10.9	100.2
M4	28/03/2018		6.1	321.2	8.9	13.6	125.0
	13/04/2018		8.2	345.8	8.4	7.3	67.1
	25/05/2018		20.8	332.9	8.07	8.29	92.7
	17/07/2018		19.2	306.2	8.1	7.6	69.8
	24/08/2018		21.6	278.1	8.6	5.3	48.7
	20/09/2018		20.4	326	8.2	6.3	57.9

Sampling point	Ammonia mg N-NH_4/L	Nitrite mg NO_2/L	Nitrate mg NO_3/L	Phosphate mg P-PO_4/L	Sulfate mg/L	Chlorine mg/L
Ter 1	0.082	0.006	8.96	0.03	106.0	37.0
	0.577	0.021	4.56	0.16	61.0	25.0
Ter 2	0.08	0.006	1.49	0.029	38	11
	0.08	0.006	0.56	0.03	28	5
Ter 3	0.41	0.006	1.422	0.029	37	16
	0.16	0.024	1.44	0.065	32	18
Ter 4	0.08	0.024	2.28	0.029	44	20
	0.165	0.027	2.054	0.098	39	24
Ter 5	0.087	0.001	0.618	0.017	10.9	7.5
	0.041	0.005	0.411	0.005	11.2	14
	0.084	0.001	1.03	0.05	4.3	3.3
	0.041	0.012	0.14	0	11.4	19.7

Nitrogen expressed as nitrates (mg NO_3 L^{-1}) concentration can also affect the development of bloom-forming bacteria. **Figure 19** reports the latest control points from the Catalan Waters Agency (ACA) showing that the M1 point in 2019 was included among 4 areas (01, 07, 03, and 08) where the eutrophication risk is higher with respect to other areas with the some points at NO_3^- concentration < 50 mg NO_3 L^{-1} [247]. Also, agriculture activities near the M1 point can potentially be responsible for the presence of cyanobacteria.

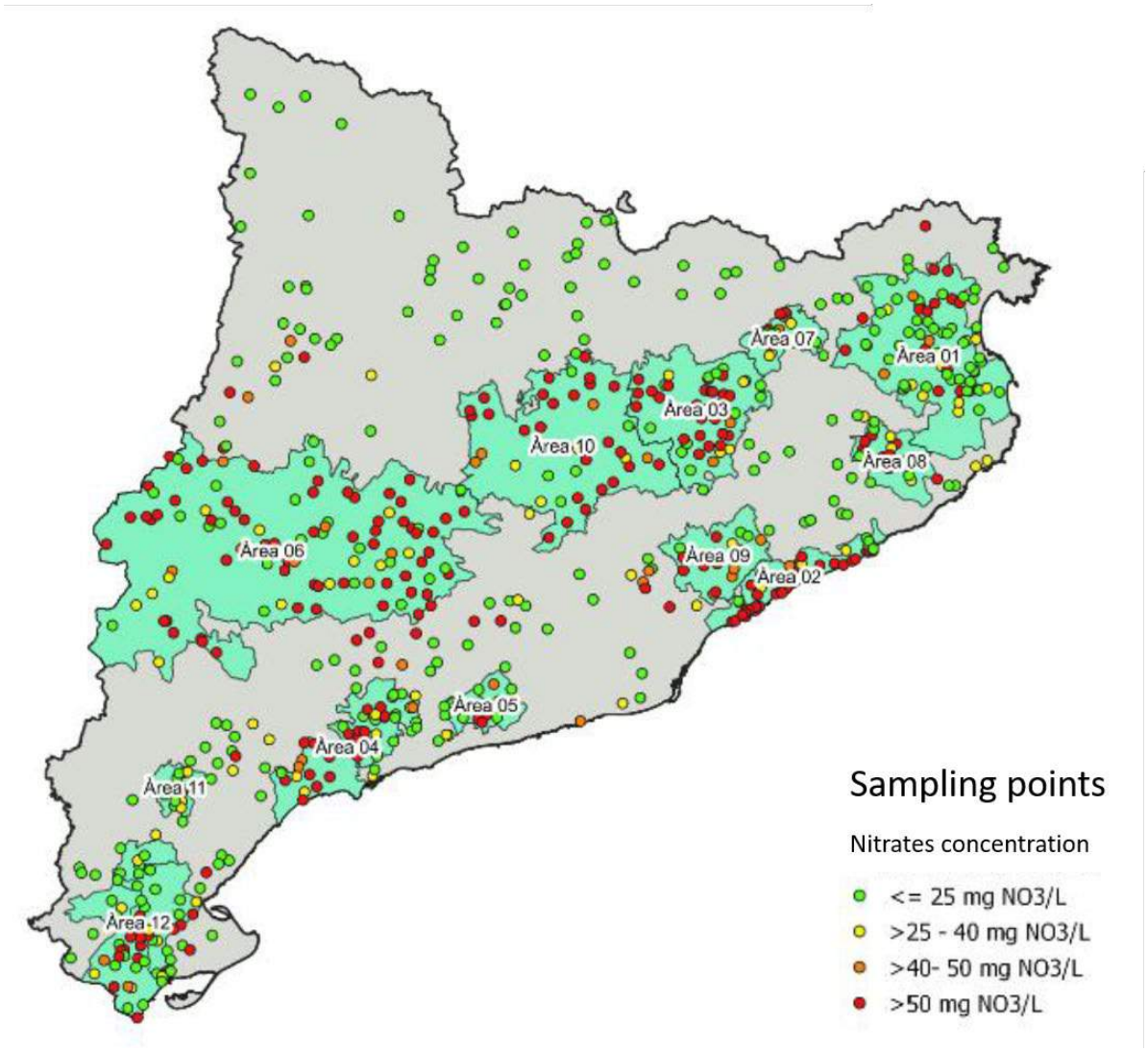


Figure 19: Picture of the sampling points considered for the NO3 control during the 2019 campaign (Figure adapted from Agencia Catalana De l'Aigua [247])

Thus can potentially explain the presence of cyanotoxins in the lower water body. MCs concentrations were not high and all were below the limit of 1 µg L^{-1}, and only during September did the concentration of MC-LR increase to 0.7 µg L^{-1}. That can be related to the high temperatures that were registered in that period with an average temperature of 24.1 °C [248], which created favourable conditions for their production in these spots; MC-YR and MC-LW remained always under 0.5 µg L^{-1}. Nodularins were also found, and in Ter River low concentrations are in line with the

other MCs levels. The presence is not surprising due to the recognised presence of *Nodularia spp.* common in brackish water bodies worldwide, suggesting a special adaptation at severe to moderate salinities [249]. Here, it has been shown that the positive combination of light exposure, nutrients, and temperature can potentially create a favourable environment for the growth of cyanobacteria and consequently, cyanotoxins.

Fungal toxins

Aflatoxins B and G groups, allantoin, aspionene, stipitatic acid, kojic and azelaic acids have also been encountered in different sampling sites. Only aflatoxin B_1 could be confirmed to level 1 using certified standards, while the other suspect toxins reached level 2. Due to the recognised ubiquitary occurrence, mycotoxins are generally expected in water environments, due also to the continuous water movement and the increment or decrement of the river flow during rainy or dry seasons, thus causing unexpected variations in concentrations [134].

Firstly, abiotic and anthropogenic factors influencing the water ecology were investigated to explain the presence of mycotoxins. Following what has been reported by Hageskal et al. [186,250], I consider that surface water can be highly affected by fungi, with respect to groundwater. Several authors have reported a fungal cell count in surface and spring water between 1,750 CFU 100 ml^{-1} and 1,025 CFU 100 ml^{-1} , respectively [191]. Nevertheless, the larger amount of organic matter provides nutrients and substrate for fungal growth.

Similar to cyanobacteria, the temperature may influence fungal growth. Ter River temperatures can vary on the upper and mid surface depending on the season and they tend to change suddenly in almost dry spots under solar irradiation, thus creating favourable conditions for their growth. However, the fungal biota can vary with predominant species at certain temperatures. Colony formation can occur at 15-25 °C in any case, and cold water can host several mould species [187] with respect to warm water that may host *Aspergillus* spp [251].

Fungi are also able to reuse the organic matter through extracellular enzymatic digestion [252] and different aquatic taxa have been recorded to be present in water streams and rivers: *Tetracladium marchalianum*, Lemonniera a*quatica*, *Anguillospora longissima, Articulospora tetracladia*, *Fusarium cavispermum*, *Tricladium angulatum*, *Clavariopsis aquatica, and Fusarium* sp. [253]. Considering this premise, the presence of *Aspergillus sp* can be assumed, which is the main causing agent of the presence of aflatoxins B_1 and B_2, of which B_1 was confirmed. Moreover, the sampling sites where most suspect mycotoxins were tentatively identified were characterised with abundant organic matter, such as leaves and wooden pieces in a relatively low flow spot. Here, low water recirculation, mild sunlight exposure, and warm temperature combination probably allowed to create favourable conditions to produce mycotoxins. May and September presented variable levels of aflatoxin B_1 and suspect aspionene, stipitatic acid, and azelaic acid while the rest of the year they were under the LOD. Concentrations above 1 mg L^{-1} near crops were reported in water streams near agricultural areas and snow-melting

effluents [254], that could be helpful to understand the occurrence of mycotoxins in Ter River surface waters. May is the period for melting snow which drags nutrients, while July is the start of the summer season which creates the perfect conditions for the growth of Aspergillus and the consequent aflatoxins.

Plant toxins

Among the variety of suspect natural toxins that have been found in Ter River, cyanotoxins and mycotoxins are negligible with respect to the relatively high number of phytotoxins that can be released by plants.

According to the Catalan Biodiversity Data Base [255], bracken ferns of the species *Pteridium aquilinum* are endemic in the Catalan region. **Figure 20** shows the geographical abundance of these brackens in the Catalan region.

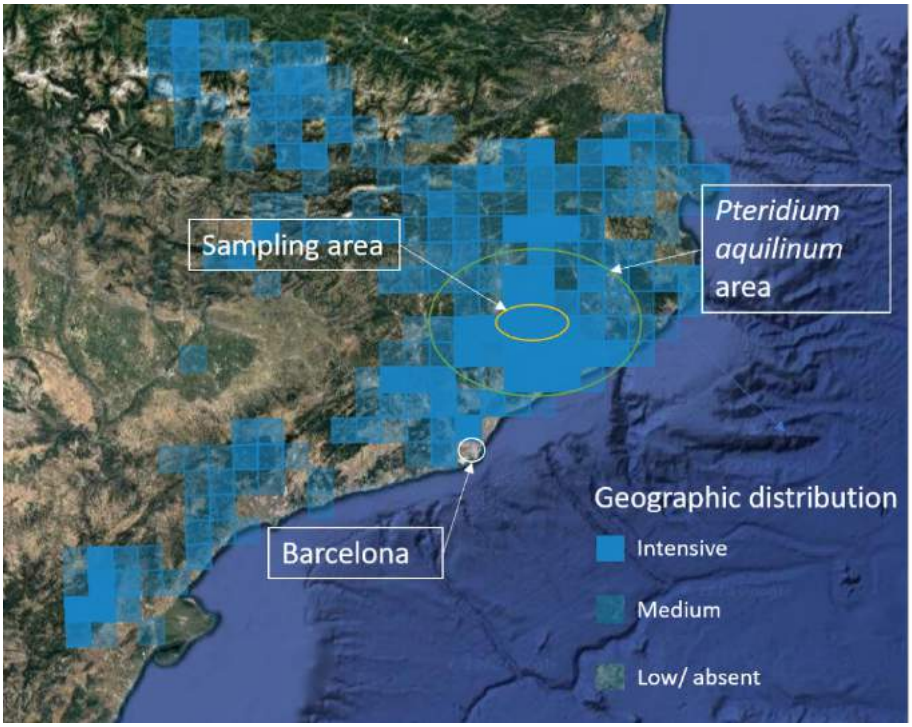


Figure 20: Distribution of Pteridium aquilinum in Catalonia

The sampling sites of the Ter River were in the centre of the endemic area. Most of the plants were situated at a higher level with respect to the water surface and they were not homogeneously distributed along the river. The sampling sites where bracken ferns were observed the most were M2 and M3. According to what is reported in publication III, these sites reported the highest levels of the suspect intermediate degradation product of ptaquiloside (PTA) and ptaquilosin (PTN). PTA is a toxin from bracken fern (*Pteridium sp.)* with genotoxic effects.

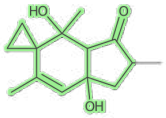
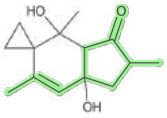
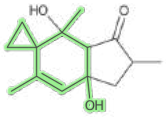
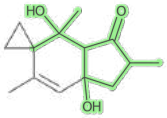
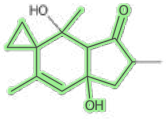
The acidic or basic conditions of the water environment at 25 °C allow the aromatisation of PTA with the loss of the D-glucoside to produce PTN [256]. Its hydrolysis in water leads to the production of the less toxic and more stable ptaquilosin B (PTB) [257].

Considering the Ter River water with pH > 8 and T > 20 °C during the summer season at the M2 and M3 sites (**Table 18**), it is possible to trace back to the high levels of PTN that were encountered in these locations. Lately, the presence of PTN was also linked to climate conditions. High temperatures and dry periods lead the aerial parts drying with a brown colour (**Figure 26**). Water sampling was undertaken 2 days after heavy rains, hence the highest presence of suspect PTN was expected in the M2 and M3 sites. The rain has a flushing effect which permits the accumulation of PTN in water.

Figure 2115: Dry bracken ferns at the M3 site.

Ptaquiloside in Bracken litter has been reported to range from 0.09–23.5 $\mu g \cdot g^{-1}$ [258],hence the consequent levels in the water of the degradation products PTN and PTB may vary. During identification, the PTN required to manually check each of the 92 MS/MS fragments due to the lack of HRMS/MS spectra is available Online. To the best of my knowledge, there are no HRMS/MS spectra of PTN available, however, the fragments which were used for the tentative identification that are reported in **Table 14** are partially in agreement with those reported by Kiyoshi et al. [259].
Further investigation was then focused on the potential presence of the final aromatisation product, the PTB, which is much more stable than PTA and it is usually indirectly quantified by converting the PTA to PTB [260]. At pH < 4 the PTA can degrade in a period ranging between hours to days, while at pH < 7 it can degrade within hours [261]. The presence of suspect PTN in water samples suggested the recent conversion onset of PTA to PTB. Then, suspect PTB was tentatively identified in the M3 point. Over 117 peaks were assumed to be structural fragments of PTB m/z 218.1301 with a match score of 1.0/1.0 on MetFrag [262], allowing to set the confidence levels to 2.

Table 34; Transitions used for the tentative identification of ptaquilosin in Ter River

Tentative structure	***HRMS m/z***	***Tentative molecular formula***
	221.1175	$C_{13}H_{17}O_3$
	135.08055	$C_9H_{11}O$
	149.09628	$C_{10}H_{13}O$
	167.07037	$C_9H_{12}O_3$
	205.1225	$C_{13}H_{16}O_2$

Bracken ferns are just one of the very long list of plants that can be present in a geographic zone. The Ter River zone presents a very broad biodiversity, as reported by the Catalan Institute of Plant Biodiversity [263]. Among the plants, many can potentially release natural toxins into the water. **Table 15** reports the genera of plants with toxins that can be potentially released

Table15: Biodiversity of the Ter River zone near the sampling points

Family name	Taxa	Toxins
Apocianaceae	*Vinca minor*	Vincaminoreine
Compostae	*Senecio inaequidens*	Senecionine
Boraginaceae	*Heliotropium europaeum L.*	Pyrrolizidine alkaloyds
Compostae	*Artemisia vulgaris L.*	Artemisin, thujone
Compostae	*Artemisia verlotiorum*	Artemisin, thujone
Compostae	*Artemisia absinthium L.*	Artemisin, thujone
Gutiferae	*Hypericum tetrapterum*	Hypericin and hyperforin
Gutiferae	*Hypericum montanum*	Hypericin and hyperforin
Umbelliferae	*Ammi majus L.*	8-methoxypsoralen (xantotoxin)
Umbelliferae	*Angelica sylvestris L.*	Antibacterial activity , furanocoumarins
Umbelliferae	*Anthriscus caucalis*	Furanocoumarins
Umbelliferae	*Anthriscus sylvestris*	Furanocoumarins
Umbelliferae	*Apium nodiflorum*	Furanocoumarins
Umbelliferae	*Bifora testiculata*	Furanocoumarins
Umbelliferae	*Conium maculatum L.*	Cicutoxin, conhydrine, coniine, N-Methylconiine
Umbelliferae	*Conopodium majus*	Furanocoumarins
Umbelliferae	*Laserpitium latifolium*	Furanocoumarins
Umbelliferae	*Pastinaca sativa L.*	Furanocoumarins
Umbelliferae	*Pimpinella saxifraga L.*	Furanocoumarins, psoralen
Umbelliferae	*Sanicula europaea L.*	Furanocoumarins, psoralen
Umbelliferae	*Oenanthe lachenalii*	Furanocoumarins, psoralen
Euphorbiaceae	*Euphorbia amygdaloides*	Bisabolol, cineole, geraniol, eugenol
Euphorbiaceae	*Euphorbia dulcis*	Bisabolol, cineole, geraniol, eugenol
Euphorbiaceae	*Euphorbia helioscopia*	Bisabolol, cineole, geraniol, eugenol
Euphorbiaceae	*Euphorbia maculata L.*	Bisabolol, cineole, geraniol, eugenol
Cucurbitaceae	*Bryonia cretica*	Cucurbitacins
Cucurbitaceae	*Ecballium elaterium*	Cucurbitacins
Solanaceae	*Atropa belladonna L.*	Hyoscyamine, Atropine, Tropane and Scopolamine
Solanaceae	*Datura stramonium L.*	Hyoscyamine, Atropine, Tropane and Scopolamine
Solanaceae	*Hyoscyamus niger*	Hyosciamine
Solanaceae	*Physalis alkekengi*	Solanine
Solanaceae	*Solanum dulcamara L.*	Solanine
Solanaceae	*Solanum lycopersicum L*	Solanine
Solanaceae	*Solanum nigrum L.*	Solanine
Solanaceae	*Solanum tuberosum L.*	Solanine

As shown, *Umbelliferae* species are well present in this area covering almost 80 % of the sampling point area. As a result, 3 hydroxycoumarin, N-methyl-pseudo conhydrine, and Conhydrine have been tentatively identified in Ter River while Umbelliferone, Coumarin, and Herniarin have been confirmed and quantified. Concentrations followed a climatic variation depending on the occurrence of dry and/or wet season (**Figure 22**).

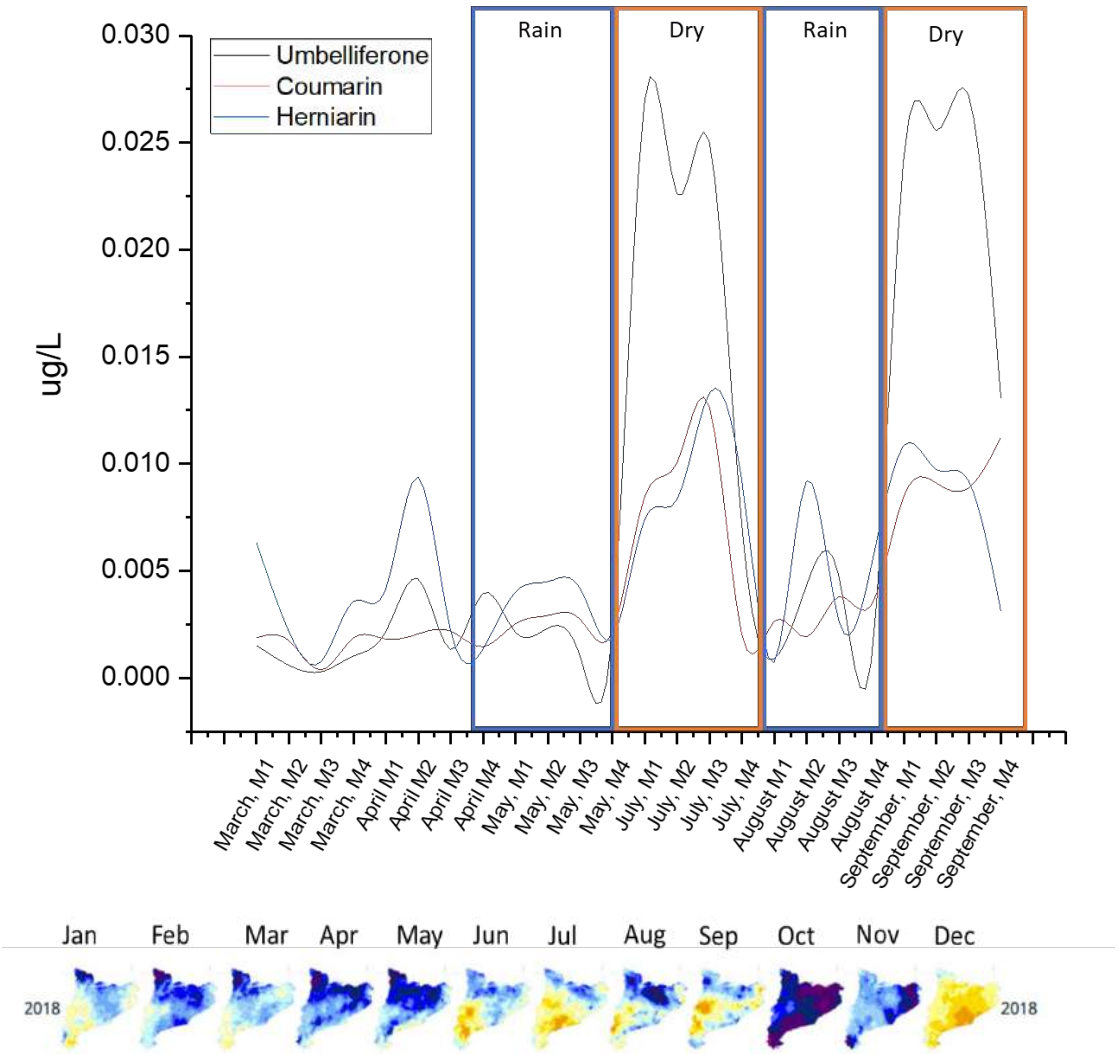


Figure 162: Variation of furanocoumarin concentration in Ter River during the wet and dry seasons.

4.2 Risks assessment of natural toxins in surface water

Phytotoxicity data for aquatic plants had a minor role in regulatory decisions concerning the environmental hazard of natural toxins.

The risk assessment of natural toxins in surface water remains difficult, due to the different uses of water, the human exposure pathways, and the lack of an occurrence overview in a given geographical area. Notwithstanding, the in vivo information regarding the effect or lethal concentration (EC50 or LC50) values are generally not available or they must be estimated using prediction tools. In what follows, a theoretical risk assessment is discussed using estimated parameters with EPI Suite™ Estimation Program Interface v4.11. Among these parameters, BIOWIN 3 and BIOWIN4 algorithms were used to estimate the biodegradation in the water environment and to understand possible risks connected to the presence of natural toxins in surface water. BIOWIN contains six models that are aimed at providing screening-level estimates of aerobic biodegradability for organic chemicals. Here, two models have been used. In brief, BIOWIN 3 is an expert survey ultimate biodegradation model while BIOWIN 4 is a primary biodegradation model. The purpose of both is to calculate the probability score that a substance under aerobic conditions with mixed cultures of microorganisms will biodegrade rapidly or slowly in the environment, according to expert studies in the body of literature [264,265].

The model results for any given chemical constitute a number from one to five that can be used either ‘as is’ or if desired it can be related to the words that are used in the expert survey (e.g., ‘days’, ‘weeks’).

The theoretical parameters of natural toxins are reported in **Table 16**. Pyrrolizidine alkaloids, cyanotoxins, and mycotoxins that were retrieved from the Ter River (Spain), the Piave River (Italy), and the Czech Republic show a Biodegradation Index between 1.62 and 3.90. In BIOWIN3, values > 2.75 and <= 3.25 are assigned the term “weeks”, while a value > 3.25 is assigned the term “month”.

Thus, the values estimated for the reported natural toxins show their persistence in the water, at least for longer than 1 week. According to what was reported by the ECHA [265], the term “week” means a quick degradation in the water environment.

To select the most persistent compounds, I selected an arbitrary threshold of 3.0 on the BIOWIN3scale because it suggests that the compound exceeds the monthly persistence in water. Only Coumarin, Herniarin, 3-Hydroxycoumarin, Hypoglicine A, Salsolinol, 4-hydroxymellein, and Conhydrine were estimated to be slowly biodegradable. The other compounds show relatively faster biodegradation.

Other parameters considered were the octanol/water (K_{ow}) and the organic carbon/water partition coefficient (K_{oc}). According to the classification proposed by McCall et al.[266], the partition coefficients can be used to understand the chemical’s behaviour in soil and water. Most of the natural toxins that are reported in this work present moderate to high affinity to water with Log K_{ow} lower than 4.5. Also, Log K_{oc} under 3 corresponds to good mobility in the soil, which may explain their presence in the water thanks also to the low retention of the organic carbon represented by the soil.

Table 16: EPISuite predicted persistence data for natural toxins

Estimated (EPISuite)	Log Kow	Log Koa	Log Koc	Water solubility (mg/L, EPISuite WSKowwin v1.43 Estimate)	Volatilization (half-life hrs)	Volatilization (half-life days)	Log BCF (L/kg wet-wt)	Half-life in fish (days)	BIOWIN 3 degradation
Ptaquilosin	1.33	9.60	1.03	2.08E+03	6.77E+06	2.82E+05	0.54	0.10	2.02
Pterosin B	3.19	10.96	2.47	2.13E+02	1.23E+07	5.11E+05	0.50	0.13	2.55
Coumarin	1.51	5.06	1.63	5.13E+03	7.14E+03	2.97E+02	0.58	0.12	3.01
Herniarin	1.59	6.50	1.87	1.88E+03	1.89E+03	7.90E+01	0.82	0.10	3.90
3-Hydroxycoumarin	0.63	4.08	0.79	1.95E+04	1.07E+03	4.44E+01	0.50	0.05	3.14
Acetoxytropane	2.54	6.78	2.19	3.68E+03	5.72E+02	2.38E+01	1.34	0.03	2.46
Heliotridine	-0.56	8.20	0.10	1.00E+06	1.80E+07	7.49E+05	0.50	0.01	2.93
Hypoglicine A	-1.29	5.84	1.16	8.94E+03	3.87E+05	1.61E+04	0.50	0.04	3.27
Salsolinol	1.2	13.60	3.20	2.01E+05	8.35E+10	3.48E+09	0.46	0.01	2.86
4-hydroxymellein	0.86	9.21	1.33	1.06E+05	7.63E+06	3.18E+05	0.50	0.00	3.12
Apiol	3.61	9.49	3.31	2.77E+01	2.75E+04	1.14E+03	2.05	0.61	2.40
Conhydrine	1.05	8.20	3.12	1.05E+05	4.77E+05	1.99E+04	0.36	0.17	3.06
Vincaminorein (Aspidospermine)	3.68	13.77	3.43	4.21E+00	5.57E+08	2.32E+07	2.09	0.15	1.62
Swainsonine	-1.31	8.92	0.29	7.60E-01	5.39E+08	2.25E+07	0.50	0.01	3.04
Coniferyl acetate	2.19	10.78	2.50	4.49E+02	3.11E+06	1.29E+05	1.11	0.02	2.84
Artemisin	0.24	10.34	1.46	4.09E+04	4.91E+08	2.05E+07	0.50	0.02	2.68
xantotoxin	2.14	7.78	2.68	7.06E+02	2.15E+04	8.97E+02	0.99	0.05	2.80
Methoxycoumarin	0.8	4.52	1.72	1.18E+04	1.71E+02	7.14E+00	0.50	0.09	2.94

Finally, human exposure to surface water chemicals can be intended as negligible regarding plant toxins which present very low levels, and, to the author's knowledge, no previous water ingestion intoxications have been reported.

Cyanotoxins and mycotoxins may be included as natural pollutants of concern while their presence in surface water has been well documented [23,132,137,186,215,250]. However, the presence of these natural toxins in Ter River (Spain), Brno Dam (Czech Republic), and Piave River (Italy) cannot be intended as dangerous due to the efficient potabilization methods used by the drinking water companies.

For example, Aigües de Barcelona (Barcelona Water Company) which provides drinking water using two treatment stations, uses Ter River water. Here the water is submitted to a potabilization process where activated carbon filters eliminate all the organic compounds (including phytotoxins, cyanotoxins, and mycotoxins) producing safe and drinkable water.

Possible exposure could be related to bathing activities and irrigation with contaminated water. However, the estimation of human exposure cannot be addressed with the current data available.

5. FUTURE PERSPECTIVES AND CONCLUSIONS

5.1 Future trends

Despite natural toxins control strategies, the presence of natural toxins in surface water is a documented reality. Among the toxins, cyanotoxins, mycotoxins, and plant toxins are the most widely spread in the environment, with increasing pollution events due to the climatic change and human activities. Most of the limits have not been established for natural toxins in surface water. Therefore, regulatory efforts are needed to provide reliable and standardised control methods for drinking water producers and environmental operators.

Future regional and national regulations need to be implemented in an environmental control plan for a wide range of natural toxins in surface water.

Among natural toxins, cyanotoxins especially MCs, have led the World Health Organization (WHO) to establish a tolerable daily intake (TDI) (0.04 μg kg^{-1}) and a provisional guideline value for MC-LR in drinking water (1 μg L^{-1}). However, mycotoxins and plant toxins have not been regulated having similar exposure ways of cyanotoxins: chronic and accidental ingestion of contaminated drinking water; inhalation and dermal contact with toxins during recreational activities such as swimming, or bathing; consumption of contaminated vegetables and fruits irrigated with water containing natural toxins.

In summary, Mycotoxins and plant toxins should be included in monitoring programmes to keep their levels under control, while taking into account the seasonality and the raining events that may increase the levels of undesired natural toxins in water.

In terms of assessing their occurrence, the sampling procedure has a central role. The lack of definitions, methodologies, and analytical approaches complicates their assessment in the environment. Faster analytical procedures for different classes of compounds will be needed to reach a rapid and inexpensive control of the waters. Firstly, an improvement in the availability of certified standards is required, taking into account the high cost of some compounds hence some extraction and isolation technologies could be experimented.

Regarding drinking water companies, strict control and reduction of natural toxins in water catchment areas can help to improve the strategies that are needed to obtain safe and clean water, while reducing the costs that are derived from the potabilisation steps.

5.2 Conclusions

According to the aims that I previously discussed in my thesis, it is possible to report the following conclusions:

- Natural toxins can be produced by different organisms. They can have different chemical parameters, thus making it difficult to separate them from the water matrix.

As reported in publication I, different methods use SPE as a cleanup step. However, almost all of them are applied to one compound or a group of compounds of the same class and chemical properties.
Here, an SPE that is used in the cleanup and concentration steps has been reported to be an effective method for the retention of a wide range of natural toxins with recoveries in the range of 71 to 95 %. Matrix interference was reported to be between 2.5 and 25 %, while detection limits were almost all below 1µg L^{-1}.

- Similarly to what is reported in the above, different analytical techniques have been reported to be able to identify natural toxins in surface water (HPLC and GC coupled with MALDI-TOF/MS, QExactive, Triple Quadrupole). However, most of them have not been optimised for the identification of a wide range of compounds with different chemical properties. These approaches, compared to the immunochemical and biochemical methods, allow us to identify the structures of new compounds through high-resolution mass spectrometry. The analytical method that is reported in publication II, permitted to tentatively identify 23 suspect natural toxins in the Ter River, 6 of which were then confirmed by using certified standards. Concentrations were always below the limit of 1 µg L^{-1}. The tentative identification was also made possible with the suspect list that was developed with this aim.

- A second application is proposed in this thesis, using the AIF approach to further tentatively identify natural toxins in surface water. The method was shown to be a reliable alternative to the most used ddMS/MS acquisition modes. A higher number of qualifying fragments was obtained to further tentatively identify suspect natural toxins. Samples coming from different sites in Italy, Spain, and the Czech Republic were analysed using this method. Further comparison with certified standards showed the reliability of this approach, confirmation (level 1) was possible for 4 compounds out of the 25 compounds at were tentatively identified to level 2.

- The occurrence of natural toxins in the Ter River was then studied with the application of the suspect screening method for the sampling campaign that was carried out during March and September 2018. Here, 53 suspect natural toxins have been tentatively identified, and the corresponding occurrences, depending on the biodiversity of the zone and the climatic variations, were assessed. Finally, 6 compounds were confirmed to level 1.

The suspect screening of natural toxins in surface water and the further optimisation of the quantification approaches may help to develop a complete risk assessment of the area. Also, the geographic biodiversity drives possible contamination of the hydric resources, depending on the climatic variations.

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