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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 Nº 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; l-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-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).

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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