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 MS² (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 Scymansky 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 MS² 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.
| Toxin                        | March | April | May | July | August | September |
|------------------------------|-------|-------|-----|------|--------|-----------|
| Acetoxytropane               |       |       |     |      |        |           |
| Aconosine                    |       |       |     |      |        |           |
| Anaethole                    |       |       |     |      |        |           |
| Analoacteone                 |       |       |     |      |        |           |
| Ambrosin                     |       |       |     |      |        |           |
| Apiole                       |       |       |     |      |        |           |
| Arabin                      |       |       |     |      |        |           |
| Artemistic acid              |       |       |     |      |        |           |
| Aspidinol                    |       |       |     |      |        |           |
| Aspidospermine               |       |       |     |      |        |           |
| Azelaic acid                 |       |       |     |      |        |           |
| Barnol                      |       |       |     |      |        |           |
| Bisabolol oxide B            |       |       |     |      |        |           |
| Buddleidin B                 |       |       |     |      |        |           |
| Conhydrine                   |       |       |     |      |        |           |
| Cuscohygrine                 |       |       |     |      |        |           |
| Herniarin                    |       |       |     |      |        |           |
| Hygrine                      |       |       |     |      |        |           |
| Hyophygrine A                |       |       |     |      |        |           |
| Laudanosine                  |       |       |     |      |        |           |
| Lepamine                     |       |       |     |      |        |           |
| Methyl Jasmonate             |       |       |     |      |        |           |
| N-Methylpelleterine          |       |       |     |      |        |           |
| N-methylpseudocoumarhydrine  |       |       |     |      |        |           |
| Norgaupelleterine            |       |       |     |      |        |           |
| P-coumaric acid              |       |       |     |      |        |           |
| Pterocillin                  |       |       |     |      |        |           |
| Reticoline                   |       |       |     |      |        |           |
| Retronecine                  |       |       |     |      |        |           |
| Swainsonine                  |       |       |     |      |        |           |
| Tetrahydropallavicin         |       |       |     |      |        |           |
| Tetranerin A                 |       |       |     |      |        |           |
| Trachelanthamine             |       |       |     |      |        |           |
| Tussilagine                  |       |       |     |      |        |           |
| Umbelliferone                |       |       |     |      |        |           |
| Verrucosin                   |       |       |     |      |        |           |
| Xanthotoxin                  |       |       |     |      |        |           |
| Aflatoxin B1                 |       |       |     |      |        |           |
| Aflatoxin B2                 |       |       |     |      |        |           |
| Alpha-Zearalenol             |       |       |     |      |        |           |
| Aspergillus acid             |       |       |     |      |        |           |
| Averufin                     |       |       |     |      |        |           |
| Kojic Acid                   |       |       |     |      |        |           |
| Anatoxin-A                   |       |       |     |      |        |           |
| MC-L-R                      |       |       |     |      |        |           |
| MC-LW                       |       |       |     |      |        |           |
| MC-YR                       |       |       |     |      |        |           |
| Nodularin                   |       |       |     |      |        |           |

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  | MS² (1) | [M-e⁺] | MS² (2) | [M-e⁺] | MS² (3) | [M-e⁺] | MS² (4) | [M-e⁺] |
|---------------------|------------------|-----------|-----|---------|--------|---------|--------|---------|--------|---------|--------|
| Acetoxytropane      | C₁₀H₁₇NO₂        | 184.1332  | 9.1 | 123.0805| 142.0864| 125.0599| 165.0913| C₁₀H₁₃O₂ |
| Aconosine           | C₂₂H₃₅NO₄        | 378.2639  | 11.3| 283.1701| 269.1539| 235.1324| 137.0599| C₁₄H₂₉O₂ |
| Anethole            | C₈H₁₁O           | 149.0961  | 9.8 | 115.0544| 103.0543| 145.065  | 121.0649| C₈H₂O    |
| Ambrosin            | C₁₅H₁₈O₃         | 247.1332  | 8.5 | 229.1227| 201.1267| 119.0857| C₁₁H₁₁  |
| Apiol               | C₁₂H₁₄O₄         | 223.0965  | 11.9| 105.07  | 119.0857| C₁₁H₁₁   | 163.0755| C₁₀H₂O   |
| Arabsin             | C₁₅H₂₂O₄         | 266.1521  | 10.8| 249.1488| 231.1384| 221.1539| C₁₄H₂O₂  |
| Artemisic acid      | C₁₅H₂₉O₄        | 235.1702  | 14  | 179.1069| 165.0901| C₁₁H₂O₂  | 119.0853| C₁₁H₁    |
| Aspidinol           | C₁₂H₁₄O₄        | 225.1101  | 9.5 | 107.0492| 137.0599| 123.0441| 109.0649| C₉H₂O    |
| Aspidospermine      | C₂₂H₃₀N₂O₂       | 355.2380  | 12.5| 107.0492| 136.0759| C₁₁H₂NO  | 148.0759| C₁₃H₂O₇   |
| Azelaic acid        | C₁₉H₃₄O₄        | 189.1121  | 11  | 107.0854| 155.0704| 111.0806| 115.0391| C₁₅H₂O₇  |
| Barnol              | C₁₈H₂₂O₄        | 183.1016  | 10.8| 119.0857| 135.0806| C₁₁H₂O₂  | 181.086  | C₁₀H₂O₅  |
| Bisabolol oxide     | C₁₅H₂₂O₂         | 239.2006  | 12.4| 133.1013| 121.1013| 149.1326| 187.1483| C₁₄H₁₉  |
| Buddledin B         | C₁₅H₂₂O₂         | 235.1693  | 12.9| 113.0598| 179.1016| 193.1225| 155.1067| C₁₄H₂O₂  |
| Conhydrine          | C₁₅H₂₁O₂         | 144.1383  | 11.6| 107.0856| 125.0962| 138.0915| C₁₅H₂O₇  |
| Cuscohygrine        | C₁₅H₂₉O₄        | 225.1961  | 12.3| 123.0805| 109.0649| 163.1118| 150.0914| C₁₅H₂O₇  |
| Curassavine         | C₁₅H₂₂O₄        | 300.2169  | 12.6| 155.0703| 107.0856| C₁₁H₂O₄  | 173.081  | C₁₄H₂O₄  |
| Herniarin           | C₁₅H₂₉O₄        | 176.0477  | 11.8| 121.0649| 133.0653| C₉H₂O    |
| Hydroxyarbusculin A | C₁₅H₂₈O₄        | 267.1585  | 13.3| 159.1169| 123.0805| C₁₁H₂O₄  |
| Hydroxyecoumarin    | C₁₅H₂₈O₄        | 163.0390  | 15.1| 121.0284| 149.0233| 163.0389| 105.0335| C₉H₂O    |
| Hygrine             | C₁₉H₃₄O₄        | 142.1226  | 10.9| 109.0655| 124.0758| 111.0804| 140.1069| C₁₃H₃NO  |
| Hypoglycine A       | C₁₉H₃₄O₄        | 142.0862  | 2.34| 97.0287 | 120.0444| C₁₃H₂NO  | 124.0757| C₁₃H₂NO  |
| Laudanosine         | C₁₅H₂₉N₂O₄      | 358.2013  | 13.2| 121.0285| 115.0543| C₁₁H₂O₂  | 159.088  | C₁₃H₂O₇  |
| Lupanine            | C₁₅H₂₈N₂O₄      | 249.1961  | 5.3 | 110.0965| 120.0808| C₁₂H₂N₂  | 138.0915| C₁₃H₂O₇  |
| Methyl Jasmonate    | C₁₃H₂₀O₃        | 225.1485  | 0.1 | 107.0855| 121.1012| 175.112  | 165.1275| C₁₃H₂O₂  |
| Methylpelletierine  | C₁₅H₂₁O₂        | 156.1386  | 2.2 | 107.0705| C₁₁H₁₁  | 140.105  | C₁₃H₄NO  |
| Methylpseudoconhyd- | C₁₃H₂₉NO        | 158.1539  | 11.9| 107.0856| 114.0914| C₁₂H₂NO  | 123.0805| 109.0649| C₉H₂O    |
| Norpseudoconhydrine | C₁₅H₂₉NO        | 140.1070  | 9.1 | 109.0649| 121.0649| 138.0917| 123.0806| C₁₃H₂O   |
| Substance                    | Molecular Formula | CAS Number | pKa | MW   |
|------------------------------|-------------------|------------|-----|------|
| p-Coumaric acid              | C_9H_8O_3         | 165.0546   | 12.5| 165.0594|
| Ptaquilosin B                | C_9H_9O_2         | 237.1485   | 11.2| 237.0857|
| Reticuline                   | C_9H_11O_2        | 330.1700   | 13.2| 330.0543|
| Retronecine                  | C_10H_13O_2       | 156.1019   | 1.9 | 152.0709|
| Swainsonine                  | C_9H_11O_2        | 174.1125   | 8.1 | 140.0682|
| Tetrahydrocannabinarin       | C_9H_12O_2        | 287.2006   | 12.9| 163.1118|
| Tetraneurin A                | C_10H_20O_6       | 323.1489   | 12.6| 281.0996|
| Trachelanthamine             | C_10H_17O_2       | 286.2013   | 12.5| 155.0704|
| Tussilagine                  | C_10H_17O_3       | 300.1281   | 10.6| 301.143|
| Umbelliferone                | C_9H_13O_2        | 163.0390   | 11.1| 147.0441|
| Verrucosin                   | C_10H_18O_4       | 345.1697   | 13.0| 301.143|
| Xanthotoxol                  | C_12H_20O_4       | 203.0348   | 1.3 | 147.1173|
| Aflatoxin B1                 | C_17H_12O_6       | 313.0707   | 11.2| 213.0547|
| Aflatoxin B2                 | C_17H_14O_6       | 315.0863   | 11.6| 273.0761|
| Alpha-Zearalenol             | C_18H_24O_5       | 321.1674   | 14.8| 149.133|
| Aspergillusic acid           | C_12H_18O_3       | 225.1598   | 9.4 | 114.0915|
| Averufin                     | C_10H_16O_7       | 369.0969   | 10.6| 327.0853|
| Kojic Acid                   | C_4H_6O_4         | 143.0344   | 1.38| 125.0239|
| ANA-a                        | C_10H_18O_3       | 166.1226   | 0.5 | 149.1   |
| MC-LR                        | C_8H_14N_1O_2     | 995.556    | 9   | 135.0807|
| MC-LW                        | C_8H_16N_3O_2     | 1025.5343  | 12  | 135.0807|
| MC-YR                        | C_9H_12N_1O_3     | 1045.5317  | 8.9 | 135.0807|
| NOD                          | C_10H_18N_3O_10   | 824.4446   | 8.6 | 135.0807|

**Mycotoxins**

| Substance                    | Molecular Formula | CAS Number | pKa | MW   |
|------------------------------|-------------------|------------|-----|------|
| Aflatoxin B1                 | C_17H_12O_5       | 313.0707   | 11.2| 269.0444|
| Aflatoxin B2                 | C_17H_14O_5       | 315.0863   | 11.6| 255.0654|
| Alpha-Zearalenol             | C_18H_24O_4       | 321.1674   | 14.8| 149.133|
| Aspergillusic acid           | C_12H_18O_3       | 225.1598   | 9.4 | 144.0889|
| Averufin                     | C_10H_16O_7       | 369.0969   | 10.6| 299.0555|
| Kojic Acid                   | C_4H_6O_4         | 143.0344   | 1.38| 97.02844|

**Cyanotoxins**

| Substance                    | Molecular Formula | CAS Number | pKa | MW   |
|------------------------------|-------------------|------------|-----|------|
| ANA-a                        | C_10H_18O_3       | 166.1226   | 0.5 | 149.1   |
| MC-LR                        | C_8H_14N_1O_2     | 995.556    | 9   | 213.087 |
| MC-LW                        | C_8H_16N_3O_2     | 1025.5343  | 12  | 375.1914|
| MC-YR                        | C_9H_12N_1O_3     | 1045.5317  | 8.9 | 375.1935|
| NOD                          | C_10H_18N_3O_10   | 824.4446   | 8.6 | 389.2079|

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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 B₁ (25%), aflatoxin B₂ (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 Pastoral 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.
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-NO₃/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                   |
|            | September | M2        | 0.28                   |
| Afla B₁    | September | M4        | 0.9                    |
| Kja        | April  | M4             | 0.7                    |
| Nod        | September | M1        | 0.1                    |
| MC-YR      | April  | M1             | 0.1                    |
| MC-LW      | August | M1             | 0.2                    |
|            | September | M1        | 0.4                    |
| MC-LR      | April  | M1             | 0.1                    |
|            | September | M1        | 0.7                    |
| Umbelliferone | May    | M3             | <LOD                   |
|            | July   | M3             | 0.1                    |
|            | August | M3             | <LOD                   |
| 7-methoxycoumarin | May | M3             | 0.008                  |
|            | July   | M3             | 0.08                   |
|            | August | M3             | 0.06                   |
|            | September | M1    | 0.04                   |

Abbreviations: Afla B₁: aflatoxin B₁; 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 AflB₁ and AflB₂. 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 |
|-----------|-----------|-------------|---------|------------------------|-----------|---------------------|--------------------------------------------------------------------------|------|---------|
| 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                | Carcinogenic/anticarcinogenic potential; Cytotoxic in vitro               | [33] | CC1CC2CCC(C34C2CC(C31)C5(CCC(C5CC6CCO)OC)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] | CC1CCC2C1=C3C3(C2)OC(=O)C3=C |
| Ambrosin  | 509-93-3  | 17          | 1.03    | Week–Months            | 0.21      |                    | NF-κβ inhibitor                                                           | [36,37] | CC1CCC2C(C3C1=CC3=O)C0C(=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=O)OCO2 |
|          | CAS    | NO | Type                        | Duration | ADME   | IC50 | 
|----------|--------|----|-----------------------------|----------|--------|------| 
| Arabsin  | 38412-44-1 | 13 | 0.76                        | Weeks    | −0.02  |      | acute dermal LD50 in rabbits > 5 g/kg |
| Artemisic acid | 80286-58-4  | 4  | 3.8                         | Week–Months | 4.39   | 50   | anti-MRSA activity, with antibacterial effect. Inhibition of the formation of the ribosome |
| Aspidinol | 519-40-4   | 13 | 2.6                         | Week–Months | 1.01   | 50   | Cytotoxicity against mouse NIH3T3 cells |
| Aspidospermine | 466-49-9  | 13 | 3.78                        | Recalcitrant | 1.76   | 46.3 | Skin reaction; hepatic toxicity |
| Bisabolol oxide B | 26184-88-3 | 21 | 2.5                         | Months    | 2.63   | 633  | Piscidical activity |
| Buddedrin B | 62346-21-8  | 13 | 2.9                         | Week–Months | 2.97   |      | Activation and then blocking of nicotinic acetylcholine receptors |
| Conhydrine | 495-20-5   | 50 | 1.21                        | Months    | 0.39   | 11   | Autonomic nervous system blockade |
| Cuscohygrine | 454-14-8   | 29 | 1                           | Months    | 0      | 111  | Inhibition of human carbonic anhydrase with a concentration of 2.4 µM |
| Herniarin | 531-59-9   | 29 | 1.74                        | Weeks     | 0.72   | 4300 | Jamaican vomiting sickness; hypoglycaemia and death; encephalopathy |
| Hygrine   | 496-49-1   | 29 | 0.5                         | Week–Months | −0.02 | 91   | GABA receptors interaction glycine receptors, involved |
| Hypoglycine A | 156-56-9  | 33 | −2.5                        | Day–Weeks | −0.05 | 98   | Jamaican vomiting sickness; hypoglycaemia and death; encephalopathy |
| Laudanosine | 2688-77-9   | 25 | 3.7                         | Months    | 1.59   | 410  | GABA receptors interaction glycine receptors, involved |

**Chemical Structures:**
- [39] NC1CCC(C=C=C)(C=O)OC
- [40] CC1CCC(C=C(C=C(C=C(O)(C)(C(O)O)C)O)OC)OC
- [41] CCC=C(C=C(C=CC(C=CC(C=CC(C=C(=O)(O)(O)O)O)O)O)O)O)
- [42] CCC12CCCN3C1C4(CC3C(=O)O)C
- [43] CCC1=CCC(CC1)C2(CCC(O2)C(C)(C)O)OC
- [44] CCC1=CCCC(=C)C2CC(C2(C1=O)OC)OC
- [45] C1=CCC23C4C1CC5=C2C(O=C=C5)OC(O)CC4O
- [46] CN1CC23C4CC(C1C5C=OC=C(C=C5)OC3CC(C4)OC)
- [47] COC1=CC2=C(C=C1)C=CC(=O)O2
- [48] CC(=O)C[CC@H]1CCC1NC
- [49] C=C1CC1CC(C=O)O)
- [50] CN1CC2=CC(C=C=C2CC1CC3=CC(C=C3)OC)OC)OC)OC
| Substance                  | CAS Number | EC Number | LD50 (mg/kg) | Route                  | Effect                                                                 | Reference |
|---------------------------|------------|-----------|--------------|------------------------|----------------------------------------------------------------------|-----------|
| Lupanine                  | 550-90-3   | 38        | 1.6          | Week–Months            | 0.65 410 Tremor, Muscle contraction and dyspnoea within mouse         | [51]      |
| Methyl-Jasmonate          | 1211-29-6  | 25        | 2.76         | Weeks                  | 1.25 5000 Anti-inflammatory activity in LPS-stimulation within mouse | [52]      |
| Methylpseudocominhydine   | 40199-45-9 | 17        | 0.8          | Week–Months            | 0.05 40 Taenicide                                                    | [53,54]   |
| Norpseudopelletine        | 4390-39-0  | 17        | 0.2          | Weeks                  | 0.15 Causes severe skin burns and eye damage; genotoxic in vitro + in vivo | [56]      |
| p-Coumaric acid           | 7400-08-0  | 8         | 1.46         | Day–Weeks              | 1.81 1.2 Reproductive toxicity                                      | [57]      |
| Ptaquilosin B             | 87625-62-5 | 33        | ND           | Months                 | 0.42 Generation of carcinogenic ADN adducts Ptosis, somnolence, convulsions. | [35]      |
| Reticuline                | 485-19-8   | 0         | 3            | Months                 | 0.61 56 Carcinogenic, pulmonary oedema, blood lymphoma, convulsions   | [36]      |
| Retronecine               | 480-85-3   | 71        | -0.56        | Weeks                  | -0.04 634 Locoweed intoxication; It is a potent inhibitor of Golgi alpha-mannosidase II | [38]      |
| 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]      |
| Tetrahydrocannabivarin    | 31262-37-0 | 21        | 5.76         | Months                 | 3.06 3 Neurotoxicity                                                | [59]      |
| Tetranenurin A            | 22621-72-3 | 29        | 0.6          | Week–Months            | -0.04 42 Antiviral activity; Ear thickness in rats; dermatitis        | [60]      |
| Trachelanthamine          | 14140-18-2 | 0         | 1.4          | Week–Months            | 0.69 1500 Somnolence, tremor, muscle weakness                      | [61]      |
| Compound                  | CAS Registry | T [Weeks] | K [μM] | IC50 [μM] | Effect                                    | Mechanism                                                                 |
|--------------------------|--------------|-----------|--------|-----------|------------------------------------------|--------------------------------------------------------------------------|
| Tussilagine              | 80151-77-5   | 8         | 0.6    | -0.04     | Carcinogenic in vivo                      | Inhibition of human carbonic anhydrase 9 catalytic domain                |
| Umbelliferone            | 93-35-6      | 21        | 1.58   | 0.4       | 10000                                     | Inhibitors of Secretory Acid Sphingomyelinase (S-ASM)                    |
| Xanthotoxol              | 2009-24-7    | 29        | 1.16   | 0.22      | 480                                       | Inhibitors of Secretory Acid Sphingomyelinase (S-ASM)                    |
| Mycotoxins               |              |           |        |           |                                          |                                                                          |
| Aflatoxin B1             | 1162-65-8    | 13        | 1.45   | 0.1       | Carcinogenic, terathogenic                | Carcinogenic, terathogenic                                              |
| Aflatoxin B2             | 7220-81-7    | 25        | 0.855  | 0.18      | Carcinogenic, terathogenic; hepatotoxic   | Carcinogenic, terathogenic; hepatotoxic                                  |
| Alpha-Zearalenol         | 36455-72-8   | 29        | 4      | 1.41      | Chronic toxicity and carcinogenic         | Chronic toxicity and carcinogenic                                         |
| Aspergilllic acid        | 2152-59-2    | 13        | 1.7    | 0.8       | Antibiotic substance; animal toxicity     | Antibiotic substance; animal toxicity                                   |
| Averufin                 | 14016-29-6   | 17        | 3      | 1.09      | Inhibition of deaminase                   | Inhibition of deaminase                                                 |
| Kojic Acid               | 501-30-4     | 8         | -0.64  | -0.05     | 23.8                                      | Inhibition of human recombinant DAAO                                     |
| Azelaic acid             | 19619-43-3   | 13        | 1.55   | 0.64      | 5                                         | Irritant                                                                 |
| Barnol                   | 2151-18-0    | 0         | 2.26   | 0.79      |                                          |                                                                          |
| Cyanotoxins              |              |           |        |           |                                          |                                                                          |
| Anatoxin-a               | 64285-06-9   | 17        | 0.8    | 0.36      | Neurotoxicity; muscular fasciculation, respiratory paralysis. | Neurotoxicity; muscular fasciculation, respiratory paralysis.            |
| MC-LR                    | 101043-37-2  | 8         | -1.2   | -0.01     | Hepatotoxicity; visual disturbance, respiratory | Hepatotoxicity; visual disturbance, respiratory                            |
| Toxin   | CAS Number   | Efficacy | Recalcitrant | LD | LD50 [mg/kg] |
|---------|--------------|----------|--------------|----|--------------|
| MC-LW   | 157622-02-1  | 8        | 5.2          | 0.81 | 0.25-0.33   |
| MC-YR   | 101064-48-6  | 8        | -0.2         | -0.02 | 40          |
| Nodularin | 118399-22-7 | 4        | 1.7 Months   | -0.04 | 0.060       |

Hepatotoxicity; visual disturbance, respiratory irritation; vomiting, and muscle weakness

| MC-LW   | [74] C(=O)O)C)C(C(C)C(=O)(=O)O)C C C N=N=C(N)=C(C(=O)O)C)C=CC=CC=CC(=CC(C)C=CC=CC=CC(=CC=C2)OC)C
|---------|-------------------------------------------------
| MC-YR   | [75] C(=O)O)C)C(C(C)C(=O)(=O)O)C C C N=N=C(N)=C(C(=O)O)C)C=CC=CC=CC(=CC=C2)OC)C
| Nodularin | [76] C(=O)O)C)C(C(C)C(=O)(=O)O)C C C N=N=C(N)=C(C(=O)O)C)C=CC=CC=CC(=CC=C2)OC)C

Irritation; vomiting, and muscle weakness
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     | Tetranueein 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      | Norpseudopelletierine                             |
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 MS² 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 pO₂ 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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**Conflicts of Interest:** The authors declare no conflict of interest.
Appendix A

| 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)       |
| Cylindrospermopsis     | Cyanotoxin    | C15H21N5O7S      | 399.1219   | 99         | BOCSci (BOC Sciences, Ramsey Road Shirley, NY, USA) |
| Afatoxin B1            | Mycotoxin     | C17H12O6         | 312.0632   | >98        | Merck (Darmstadt, Germany)                       |
| Ochratoxin-A           | Mycotoxin     | C20H18CINO6      | 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-Deoxygeninol              |
| 184.1332         | C10 H17 N O2  | 35 | 3-Acetoxytropine             |
| 197.1536         | C12H20O2      | 35 | 3-Thujyl acetate             |
| 646.3221         | C34H47NO11    | 35 | Aconitine                    |
| 313.0706         | C17 H12 O6    | 70 | Aflatoxin B                  |
| 315.0863         | C17 H14 O6    | 35 | Aflatoxin B                  |
| 329.065          | C17 H12 O7    | 35 | Aflatoxin G                  |
| 331.0812         | C17H14O7      | 35 | Aflatoxin G2                 |
| 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         | C27H35N4O     | 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                   |
| Molecular Formula | Scientific Name | Description |
|------------------|-----------------|-------------|
| C8H17NO          | Conhydrine      |             |
| C6H6O3           | Coumarin        |             |
| C16 H29 N O4     | Curassavine     |             |
| C13H24N2O        | Cuscohygrine    |             |
| C15H21N5O7S      | Cylindrospermopsin |         |
| C15H1004         | Daidzein (DAI)  |             |
| C21H2009         | Daidzin        |             |
| C29H32O13        | Dalbin          |             |
| C23H22O8         | Dalbinol        |             |
| C15H2003         | Damsin          |             |
| C16H18O5         | Dehydrocurcularin |          |
| C20H18O6         | Deoxynivalenol  |             |
| C22H18O8         | Desertorin A    |             |
| C19H26O7         | Diacetoxyscirpenol |        |
| C41H64O13        | Digitoxin       |             |
| C27H42O3         | Diosgenin       |             |
| C17 H26 O4       | Embelin         |             |
| C15H1005         | Emodin          |             |
| C60H74N10O10     | Ergocladin      |             |
| C18H23NO6        | Erucifoline     |             |
| C16H12O4         | Formononetin (FOR) |        |
| C10H8O5          | Fraxetin        |             |
| C15H1005         | Genistein or baicalein |       |
| C10H18O          | Geraniol        |             |
| C41H64O14        | Gitoxin         |             |
| C8 H13 N O2      | Heliotridine    |             |
| C17H21NO4        | Hyoscine        |             |
| C6H6O4           | Kojic acid      |             |
| C34 H52 O5       | Lantadene D     |             |
| C18H27 N O4      | Laudanosine     |             |
| C46H67N7O12      | MC-LA           |             |
| C49H74N10O12     | MC-LR           |             |
| C54H72N8O12      | MC-LW           |             |
| C52H72N10O13     | MC-YR           |             |
| C11H12O3         | Myristicin      |             |
| C41 H60 N8 O10   | Nodularin       |             |
| C7H13NO          | Norhygrine      |             |
| C5H5N5O          | Nostocine       |             |
| C20H18CINO6      | Ochratoxin-a    |             |
| C11H18O4         | Pestalotin      |             |
| C8H8N2O2         | Riciné          |             |
| C11H15NO2        | Salsoline       |             |
| C45H73NO15       | Solanine        |             |
| C42H67NO10       | Spirolide       |             |
| C8H6O5           | Stipitatic acid |             |
| C8H15N03         | Swainsoneine    |             |
| C10H16O          | Thujone         |             |
| C5H6O3           | Tulipalin B     |             |
| C9H6O3           | Umbelliferone   |             |
| C22 H30 N2 O2    | Vincaminorein   |             |
|                  | (Aspidospermine) |            |
Table A3. Calibration curve parameters for the quantification of the confirmed compounds.

| Toxins     | Molecular Formula | [M+H]+ (µg/L) | Recovery % | RSD % | LOD (µg/L) | LOQ (µg/L) | R²     |
|------------|-------------------|---------------|------------|-------|-------------|-------------|--------|
| Ana        | C₁₀H₁₅NO          | 166.1234      | 84         | 8.0   | 0.2         | 0.5         | 0.989  |
| AflB₁      | C₁₇H₁₂O₆          | 416.1242      | 86         | 9.9   | 0.2         | 0.7         | 0.999  |
| MC-LR      | C₂₀H₃₀N₁₂O₁₀      | 995.5568      | 78         | 3.3   | 0.2         | 0.5         | 0.995  |
| MC-LW      | C₂₄H₃₄N₁₆O₁₀      | 1025.534      | 55         | 5.8   | 0.1         | 0.5         | 0.991  |
| Nod        | C₁₃H₁₈N₈O₈        | 825.4512      | 94         | 16.2  | 0.2         | 0.8         | 0.992  |
| MC-YR      | C₂₄H₃₄N₁₆O₁₀      | 1045.536      | 84         | 16.9  | 0.4         | 1.5         | 0.943  |
| Kja        | C₁₂H₁₈O₃          | 208.1093      | 85         | 6.4   | 0.02        | 0.08        | 0.990  |
| 7-methoxycoumarin | C₁₀H₈O₃     | 177.0546      | 82         | 7     | 0.002       | 0.007       | 0.999  |
| Umbelliferone | C₁₃H₁₆O₃    | 163.0388      | 79         | 11.2  | 0.009       | 0.03        | 0.998  |

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