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Grau de Química

Treball Final de Grau

Suspects screening and Target analysis of emerging toxins in aquatic environment by high resolution mass spectrometry

***Suspects Screening* i anàlisi *Target* mitjançant espectrometria de masses d'alta resolució per toxines emergents al medi aquàtic**

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L'accés a un font segura, garantida i suficient d'aigua és un requeriment fonamental per a la supervivència, el benestar i el desenvolupament socioeconòmic de tota la humanitat. Encara continuem actuant com si l'aigua dolça fos un recurs abundant i perpetu. No és així.

Kofi Annan

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REPORT

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1. SUMMARY

The nutrients' rising in waterbodies caused by the agricultural and industrial waste, can stimulate the growth and bloom of cyanobacteria. This can be especially dangerous if in the bloom contain certain species capable of produce toxins noxious as well as for animals as for humans. The widest and most studied cyanotoxins family are the microcystins, a cyclic heptapeptides with a general formula that changing amino acids would define a different variant with extensive range of toxicity.

In this project, we stablished high-resolution mass spectrometry criteria as well levels of confidence in compounds' identification. Based on those, databases have been created with the exact mass of diverse microcystins and other related toxins, in order of develop an analytic methodology based in full scan of environmental samples (through High-Resolution Mass Spectrometry and Liquid Chromatography separation) and then processing it with the databases, thus identifying more than 200 compounds among microcystins, peptides and pigments produced by cyanobacteria.

Keywords: Cyanobacteria, Microcystins, Liquid Chromatography, High-Resolution Mass Spectrometry, Target analysis, Suspects Screening, Databases.

2. RESUM

L'augment de nutrients a masses d'aigua provocat pels abocaments agrícoles i industrials, pot estimular el creixement i "aflorament" de cianobacteris. Aquest fet pot ser especialment perillós si hi ha presencia de certes especies capaces de produir toxines nocives tant per animals com pels humans. La família de cianotoxines més extensa i estudiada són les microcistines, uns heptapèptids cíclics amb una formula general on canviar aminoàcids de la seva estructura conforma una variant diferent amb un ampli rang de toxicitat.

En aquest projecte s'han establert criteris d'espectrometria de masses d'alta resolució i uns nivells de confiança en la identificació de compostos. En base a aquests, s'han creat bases de dades de massa exacta amb diverses microcistines i altres toxines relacionades, per desenvolupar una metodologia analítica basada en l'escombratge complet de mostres ambientals (mitjançant espectrometria de masses d'alta resolució i una separació amb cromatografia líquida) i el processament amb les bases de dades per així identificar més de 200 compostos entre microcistines, pèptids i pigments produïts pels cianobacteris.

Paraules clau: Cianobacteris, Microcistines, Cromatografia líquida, Espectrometria de masses d'alta resolució, Anàlisi *target*, *Suspects Screening,* Bases de dades.

3. INTRODUCTION

3.1. CYANOBACTERIAL TOXINS

Harmful Algal Blooms (HABs) are the natural process of massive growth of detrimental algae. It occurs under special environmental variables such as enough sunlight, increased water temperature, low predation, lake stratification (which reduce water flow) and high nutrient ambient (known as eutrophication): specifically high concentration of Phosphorus and low nitrogen-to-phosphorus ratio are thought to be critical for development of the bloom. In those blooms, algae species from the phylum *Cyanobacteria* grow excessively, a noxious kind of bacteria that can produces toxins at the same rate that changes the water coloration, creating a foul odour and a pernicious impact on health to animals and humans [1]–[3].

Actually, Cyanobacteria are not genuine algae, because algae are photosynthetic eukaryotic organism: which means that algae can convert light into chemical energy and also they contain the nucleus and other organelles enclosed within membranes. On the other hand, bacteria are prokaryotic that have not membrane-bound organelles and nucleoid with DNA directly in the cytoplasm. Blue-green algae (BGA) is another name for cyanobacteria, since its etymology comes from the word *kyanós*, which means colour blue in Greek and these cyanobacteria can also perform photosynthesis in plastids, producing energy and obtaining their green colour.

There are hundreds of toxins produced by BGA, all they are called cyanotoxins, and they could differ so much in structure and toxicity. We could classify those products by the specific target organs they affect [4], [5]:

- Hepatotoxins: chemical that causes liver injury.
- Neurotoxins: chemical that destructs nerve tissue in peripheral or/and central nervous system.
- Dermatoxins: chemical that wounds skin or/and mucous membranes.

Also cyanotoxins may be genotoxic, cytotoxic, or carcinogenic: that could imply mutations, cell damage in multiples organs like liver, kidneys, adrenal glands and small intestine or tumour promotion.

One of the most common and also detrimental family of toxins are Microcystins, which have a worldwide distribution and can cause from gastrointestinal disturbances to serious damage to the liver, their structure consist on a cyclic heptapeptide with a specific lateral chain: an unique non-proteinogenic amino acid (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid known as ADDA that is only found in other group of cyclic pentapeptide toxins, Nodularins [4], [6].

There are a large number of Microcystin variants, but their levels of toxicity are very different, being the Microcystin-LR the considered most toxic one; with a LD_{50} Mice Intraperitoneal of 43.0 μg/kg according Díez-Quijada [7]. Other microcystins ordered in descending toxicity are MC-LR > [DMAdda5]MC-LR > MC-YR = MC-WR > MC-FR = MC-AR> [Dha7]MC-LR > [Mser7] MC-LR > MC-RR > MC-M(O)R] [7], [8]. MC-LR is also the most frequent and also the most studied microcystin, so is crucial it correct and precise identification in order to control water supplies quality [9].

The term Microcystin-LR corresponds to a microcystin containing Leucine and Arginine, with these amino acids in position 2 and 4 respectively, as is showed in Figure 1. Alternatives microcystins appear by changing the amino acids assigning, with a general formula MC-XZ (X and Z amino acids). In the most common microcystins, for which standards are available, Y stands for tyrosine, W for tryptophan, A for alanine and F for phenylalanine. Also the demethylation/methylation of positions 3 in methylaspartate, 5 in ADDA or 7 in methyl-dehydroalanine, as well as the methylesterification of position 6 in D-Glutamic acid can also produce variants of MCs. Even some modifications in ADDA like O-acetylation will produce new MCs. Rare microcystin congeners include strange amino acids as: aminoheptanoic acid, aminoisobutyric acid, dehydroalanine, dehydrobutyrine, glutamic acid methylester, homoarginine, homoisoleucine, homophenylalanine, homotyrosine, kynurenine, methionine-S-oxide, N-methylserine, N-formylkynurenine, oxindolyalanine, methoxytyrosine, …[10]

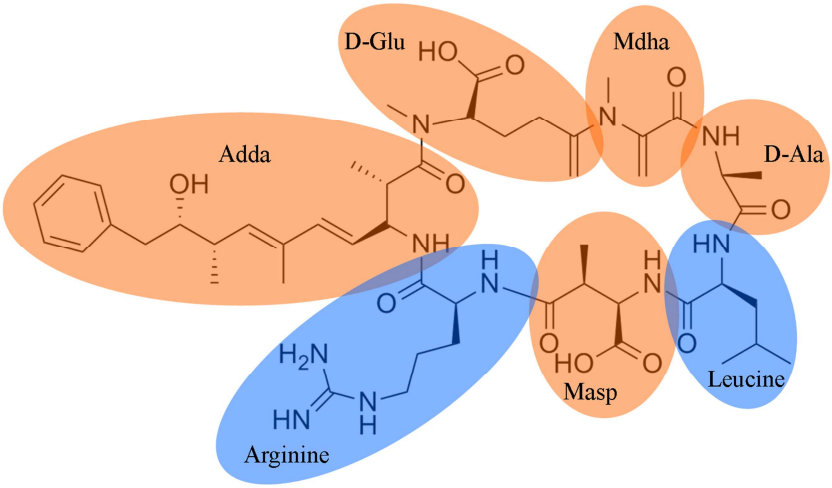

Figure 1. General structure of microcystin: MC-LR, in blue the frequently changed amino acids. *(ToxMais, 3/12/19 via Wikimedia Commons, Creative Commons Attribution)*

As mentioned above, microcystins are the most common cyanotoxins, but cyanobacteria can also produce other noxious substances with different structure, toxicity and proprieties. Toxin families like anabaenopeptins, cylindrospermopsins, microviridins, oscillapeptins are also common [11], [12].

Other associated by-products from BGA's are pigments, they usually have a blue-green colour but other colouration are possible depending on the genus of the algae.

The World Health Organization had established a preliminary provisional guideline valour (GV) for Microcystin-LR concentration: 1 μg L^{-1} is the limit in drinking water, considering a Tolerable Daily Intake of 0.4 μg/kg bw/day without any toxicological effect, and an Allocation Factor that drinking water would account the 80% of the total ingestion of MCs [8].

$$GV = \frac{TDI * body\ weight * AF}{2\ L\ of\ drinking\ water\ a\ day} = 1\ \frac{\mu g}{L}$$

Conventional water treatment methods such sand filtration, coagulation and flocculation are effective to remove other contaminants, even the cyanobacterial cells, but not to remove the dissolved toxins. So we have to distinguish between intracellular toxins known as sestonic fraction and extracellular toxins dissolved in the water fraction. New technologies for water treatment are implemented in large water supplies because they can remove MCs [13], [14].

Several countries have implemented legislation in order to control microcystins in water (as showed in Table 1): the Spanish Government Official State Gazette (BOE) had laid down tough standards for water quality and set a maximum of 1 µg microcystin L^{-1}, without specifying which MC variable is considered for the evaluation of total MCs. Also the Official Journal of the European Union is looking for improve public health in recreational waters taking especial care with HABs [15]–[17].

Table 1. Cyanotoxins legislation in different countries at 2014.

*(data extracted from B. W. Ibelings et al. Harmful Algae. **2014**, 40, 63-74)*

Country	Microcystins in drinking water supplies [µg L^{-1}]	Microcystins in recreational water [µg L^{-1}]	Other Cyanotoxins regulated in drinking water, recreational water and/or freshwater sea-food
Argentina	1[a]	–	–
Australia	1.3[b]	10[b]	Nodularin, Cylindrospermpsin, Saxitoxin
Brazil	1[b]	–	Cylindrospermpsin, Saxitoxin
Canada	1.5[a]	20[a]	Anatoxin-a
Czech Republic	1[b]	–	–
Finland	1[b]	–	Any potentially toxic cyanobateria
France	1[b]	25[b]	–
Germany	1[b]	10[b]	Cylindrospermpsin
Hungary	–	20[a]	–
Italy	–	25[b]	–
New Zealand	1.3[b]	12[b]	Nodularin, Cylindrospermpsin, Saxitoxin, Anatoxin-a, Homoanatoxin-a
Singapore	1[a]	–	–
South Africa	1[a]	–	–
Spain	1[b,d]	–	–
Turkey	1[b]	10[a]	–
United States of America	1[b]	Varies in different states	Cylindrospermpsin[c], Anatoxin-a[c]
Uruguay	1[a]	–	–

(a) Refers only to MC-LR
(b) Value of the sum of all microcystins
(c) Only in California
(d) Only will be measured when exist suspect of eutrophication in the water
(–) Means no data available in the source

Because the diverse nature of the different cyanotoxins is not easy to have a general analytical method to detect and identify all of them simultaneously. Some methods for the analysis of microcystins are showed in Figure 2. For instance, unspecialised methods such as the toxicity essays on mouse can give us information about the toxicity level, methods based on Nuclear Magnetic Resonance can provide us with structural information, even though but its limited sensitivity. HPLC-UV is a valuable method since amino acid ADDA has a conjugated diene, which according the Woodward-Fiesser rules shows a local maximum of absorption at 238 nm. In addition, phosphatase assay and ELISA are very sensitive methods since the toxins analysed affects directly to the protein phosphatase enzymes (PP1 and PP2A) but cannot distinguish from other toxicants [9], [18]–[21].

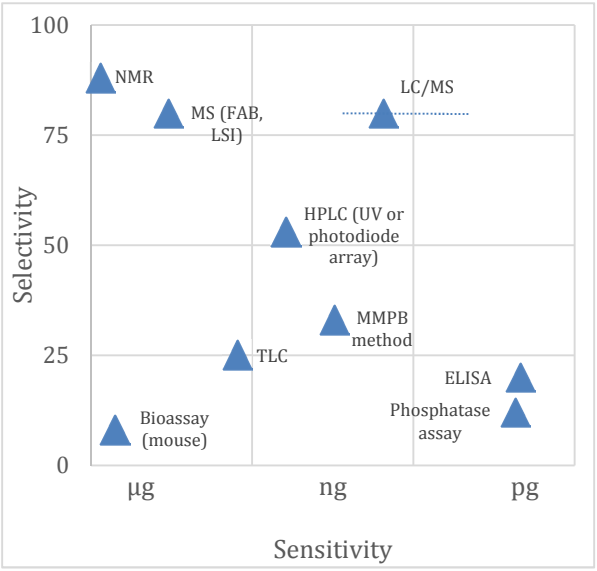

Figure 2. Comparison of selectivity and sensitivity of methods of analysis for microcystins.
*(data extracted from K. Harada et al. "Laboratory Analysis of cyanotoxins," Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management. WHO, **1999**, 13, 362-400)*

Finally, as showed in Figure 2, liquid chromatography coupled to mass spectrometry is the technique, which shows enough sensitivity and excellent selectivity to differentiate most of the substances based on their *m/z*. Several analytical methods have been described working with triple quadrupole mass spectrometers for in the target analysis of some MCs. The aim of our study is to develop a suspects screening method to detect unknown toxins by LC-HRMS based on accurate mass measurement [5].

3.2. LC-HRMS

3.2.1. Liquid Chromatography

The chromatographic separation of cyanotoxins are usually performed through reversed phase liquid chromatography using C18 column and binary mobile phase composed by solvent A acidified acetonitrile and solvent B acidified water, both solvents acidified with formic acid [9].

3.2.2. High Resolution Mass Spectrometry

Mass spectrometry (MS) is the analytical technique that generates ions in the gas phase from neutrals compounds, separates them based on their mass-to-charge ratio (*m/z*) and detect them qualitatively and quantitatively based on their abundance. A mass spectrometer consists of an ion source, a mass analyser and a detector.

Mass Spectrometry is a very sensitive technique with detection limits several orders of magnitude below the low nanogram range. MS is a fast, sensitive and selective technique that can process a large number of samples so quick, with a minimal pre-treatment or manipulation of complex samples.

MS could be used for the analysis of any contaminant that can produce characteristic ions in their ionization process. This technique is a powerful tool for the unequivocal identification of compounds, which provides information about the molecular mass and the chemical formula. Additionally, it allows the simultaneous identification of a variety of molecules [22].

Mass spectrometry can be run at different mass resolution:

- Low-Resolution Mass Spectrometry: it allows obtaining the nominal mass (with no decimal, a resolution of a unit of mass), running in Full Scan and Selected Ion Monitoring (SIM) operation modes. Full Scan is used for screening detection and SIM for quantitative analysis.
- High-Resolution Mass Spectrometry (HRMS), it allows obtaining the accurate mass (up to 4/5 decimals without a significant error in the measurement, usually below 5 ppm) of ions. That measurement is adequate for the search of unknowns in a full scan (Total Ion Chromatogram, TIC).

Additionally, chemical structural information can be obtained using hybrid instruments by tandem mass spectrometry (MS/MS).

A mass spectrometer usually detects ions that are produced in an ion source; in that work we used Electrospray ionization (ESI). Is an ionization technique in which a high electrical potential is applied to the needle by which the sample flows through in order to generate a spray of charged droplets that contain the ionic analytes. Finally, the ions are moving from the condensed-phase to the gas-phase by ion evaporation in order to be analysed. ESI allows production of multiply charged ions and operate in positive and negative mode. In the positive ion mode protonated and/or alkali adduct analyte molecules are observed in the mass spectra. On the other hand, in the negative ion mode operation peaks corresponding to deprotonated analyte molecules are observed [23].

The screening of toxins by LC-HRMS, provides the accurate mass of the analytes with high accuracy in the mass measurement (< 5 ppm) and enough specificity, which allows both the identification and the quantification. The operation in Full Scan mode also allows to detect other contaminants in addition to the family of compounds in study, since in the acquisition saves data of all mass-to-charge ratios of the ions detected in the screening and not only the known *m/z* in consideration. In addition, HRMS allows to resolve signals of analytes that co-elute, even with complex matrixes, enhancing selectivity, sensitivity and robustness [24].

3.2.2.1. High Resolution Criteria

Accurate mass measurement permits the correct individual isotopic assignment of *m/z* values, which allows the determination of the elemental composition molecular formula of each ion detected. There are some important factors that have to be considered in order to determinate the molecular formula and the chemical structure, which guarantee a high degree of confidence in the ion assignment [25], [26]:

- Relative mass accuracy: Accuracy defines how close are the measured value to the real one. The relative accuracy is more meaningful to the mass measurement because some mass analysers can gradually lose accuracy when enlarging the *m/z* range to be scrutinized. Relative mass accuracy (*Δm/m*) is measured in parts per million (ppm). In HRMS an error higher than 5 ppm make doubtful the reliable identification of a compound, since it is considered that there is too much uncertainty in the measurement to guarantee the assignment.

$$\frac{\Delta m}{m} = \frac{(m/z)_{experimental} - (m/z)_{calculated}}{(m/z)}$$

- Isotope pattern: The mass spectrum of compound is not as easy as only one exact mass; in LC-MS each compound can produce different adduct ions, being the simplest the protonated one. Additionally, a signal is composed by a distribution of multiple peaks, which depends on the isotopes of the elements of the substance, being the monoisotopic mass the most intense peak of the distribution. Since natural occurrence of ^{13}C is approximately 1.1% front ^{12}C, the probability of having a peak X+1, X+2, X+3 (nomenclature according J. Gross [25]), …, increases as the number of carbons increases in the molecule, as shown in Figure 3. Other elements such oxygen, chlorine, bromine or sulphur can generate diverse isotope patterns, according their isotopes.

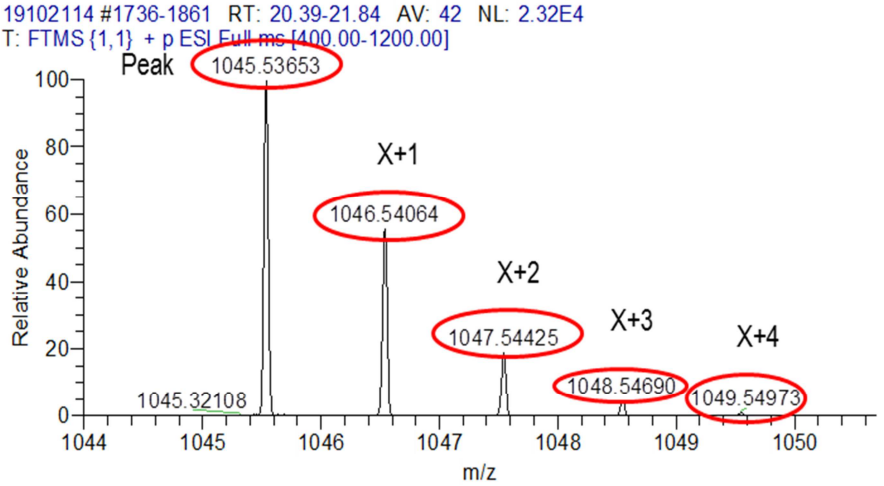

Figure 3. Example of the isotopic pattern of MC-YR ($C_{52}H_{72}N_{10}O_{13}$) in protonated adduct *(own work with Thermo Scientific™ Xcalibur™ Qual Browser)*

- Fragment ions: Mass spectrometry give reliable information about molecular formula but not so much chemical structural information. In an Exactive Orbitrap instrument, multiple experiments like fragmentation of the analytes through higher-energy collisional dissociation are needed to generate fragment ions, which allows the assignment of a correct chemical structure.
- Ring Double Bond Equivalents (RDBE): The number of instaurations of a molecule is defined by the following equation:

$$RDBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

For the toxin compounds under study and taking into account the kind of ionization process used (electrospray) the ions generated are ions with even number of electrons (even ions), so only decimal values are possible.

3.2.2.2. Orbitrap mass spectrometer

In this work the mass analyser used for accurate-mass measurements is an Orbitrap (barrel shaped electrode and inner spindle that traps ion converting into spectrum with Fourier transform) interfaced with an heated electrospray ionization (H-ESI II) and equipped with a attached higher-energy collisional dissociation cell (HCD) after a appended to the C-trap to collect and stabilized the ion beam as shown in Figure 4 [27].

- Higher-energy collisional dissociation cell: In this cell the fragmentation of ions is induced by collision-induced dissociation when applying there is enough energy.
- C-trap: A chamber that trap ions before sending them to the mass analyser, the amount of ions inside the C-trap is regulated by the Automatic Gain Control (AGC, the pack of ions is sent when a number of ions are accumulated) and the Maximum Injection Time (IT, The pack of ions inside the C-trap is sent when the accumulation time is over).
- Mass analyser: In this work an Orbitrap mass analyser has been used. It detects the angular frequency of the ions and converts it into a mass spectrum through a Fourier transform.

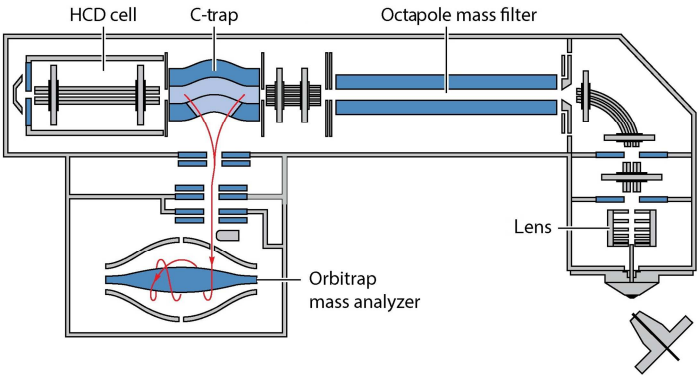

Figure 4. Schematic of the Exactive™ Orbitrap mass spectrometer.
(image modified from S. Eliuk and A. Makarov Annu. Rev. Anal. ***2015****, 8, 61-80)*

The Orbitrap follow the next equation were ω is the angular frequency that is proportional to the mass resolution, and it is inversely proportional to mass-to-charge ratio, so at higher mass-to-charge ratio the accuracy will go down, and not be the resolution set in the method.

$$\omega = \sqrt{k/(m/z)}$$

Total Ion Current (TIC) Full Scan that scrutinize a wide range of mass-to-charge ratio is necessary in order to expose new and modified toxins, as well as other adducts ions besides only detecting the protonated one (some MCs could be ionized as alkali metal adduct ions because the presence of trace of metal ions in the ambient or in the container used in the sampling) [28].

3.3. DATA ACQUISITION STRATEGIES

There is no information in bibliography about clear definitions of suspects screening and non-target analysis and that can head for confusion between different methodologies. In this project we performed target analysis and suspects screening according our following interpretations:

- Target analysis: Where the aim of the search is the detection of specific substances that are well characterized and which behaviour is well-known through the use of standards. This analysis is frequently performed through Selected Ion Monitoring (SIM) or Selected Reaction Monitoring (SRM), where the mass analyser only considers the mass-to-charge ratio of the target compounds.
- Suspects screening: That analysis is based on a pre-defined list of analytes that could be in the samples. The MS information of these analytes is obtained from the literature and because the large number of suspects' databases (on-line or home-made) are generally used.
- Non-target analysis: Is the deeper scrutinize where an investigation without knowing what to search is performed. It tries to identify signals and assign ions and chemical structures based on the accurate masses and the tandem/fragmentation mass spectra. That method requires a lot of additional manual processing to assign signals to described compounds [29].

4. OBJECTIVES

The main aim in this work is the analyse cyanobacterial toxins on continental waters. To achieve this goal, we combine physical separations like chromatography and high-resolution mass analysis (LC-HRMS) to allow the characterization of both the microcystins (MCs) commercially available and other MCs recently described for which there are not standards until now. Additionally, this project also aim to identify simultaneously other signals from different cyanobacterial peptides and pigments susceptible to be found in the same samples.

Specific purposes of this project are:

- Achieve theoretical knowledge about mass spectrometry as well as the experimental skills with this specialised technique.
- Be able to distinguish between target analysis, suspects screening and non-target analysis and to choose the most suitable in each case.
- Identify the factors which influence in the accuracy and precision of Accurate Mass Measurements.
- Search and identify in bibliography reliable mass spectrometry information about toxins.
- Learn the use of mass spectrometry databases and the process to create them.
- Learn about common methods of extraction analytes from environmental samples.
- Develop a screening method for suspects' cyanotoxins screening.
- Stablish confidence levels of identification for Microcystins to allow their robust characterisation.

5. EXPERIMENTAL AND METHODS

5.1. Chemicals, standards and materials

All the reagents and solvents used were of analytical quality and HPLC grade.

Ultra-pure Water for chromatography LC-MS Grade and Acetonitrile hypergrade for LC-MS (LiChrosolv® Merk).

Methanol for gas chromatography with ECD and FID (SupraSolv® Merk).

Formic acid 98-100% for analysis (EMSURE® Merk).

Acetone RPE - For analysis - ISO - ACS - Reag.Ph.Eur. (Carlo Erba Reagents)

Nitrogen gas purity ≥ 99.999% (ALPHAGAZ™ Air Liquide)

High Purity Standard (≥ 95%) Isolated from *Nodularia spumigena;* Nodularin-R was purchased from Alexis Biochemicals® Enzo Life Sciences. High Purity Standards (≥ 95%) Isolated from *Microcystis aeruginosa*: [D-Asp3]microcystin-RR, Microcystin-RR, [D-Asp3]microcystin-LR, Microcystin-YR, Microcystin-LR, Microcystin-WR, Microcystin-LA, Microcystin-LY, Microcystin-LW and Microcystin-LF.

The instruments used to measure water properties were: Electrical conductivity meter DiST® 3 Waterproof EC Tester (0-2000 μS cm^{-1}) of Hanna Instruments™, Whatman® Panpeha™ pH indicator strips (0-14 pH) and Chlorine reagent Cl_2-1 (liquid) for chlorine test (0.10-2.00 mg L^{-1} Cl_2 free chlorine) MColortest™ from Merk Millipore.

A Luer-Lok syringe with disposable needles and Polytetrafluoroethylene (PTFE) Uptidisc filters 4mm x 0.20 μm were used to filter samples before injection.

5.2. Sampling

Two different sets of samples were received for detecting cyanobacterial toxins. The first ones sent by Eurecat formerly Centre Tecnològic de Manresa, were six bottles of superficial water, these samples could contain extracellular toxins (water fraction), those that were supposed to be dissolved in the water. The other set of sample was sent by Univesidade de Santiago de Compostela, who sent eighteen dry algae samples, each one was dried on a filter which could contain intracellular toxins (sestonic fraction), that were inside the cell of the cyanobacteria [30].

5.3. Treatment

5.3.1. Sample pre-treatment

The diverse nature of samples made necessary to apply different sample treatment methods. First, electrical conductance, their pH and their free chlorine were measured for each water samples. A low electrical conductance is needed for the LC-HRMS as well neutral pH, free chlorine should be zero, because peptides such as microcystins are easily to decompose by chlorination with hypochlorite and that decay depends on the free chlorine dose. [31], [32]

One sample showed a slightly upper amount of free chlorine that was quenched with adding ascorbic acid solution.

$$C_5H_5O_5CH_2OH + HClO \rightarrow C_5H_3O_5CH_2OH + HCl + H_2O$$

Ascorbic acid + Hypochlorous acid → Dehydroascorbic acid + Hydrochloric acid + water

The algae in sestonic fraction for release the toxins the cells have to be broken, following the standardized method for microcystin extraction optimized by M. Barco et al [33].The whole samples were frozen and thawed three times in order to break the cell wall and allow the release of toxins. After that, the algae were transferred to screwed-cap test tube and weighed.

5.3.2. Toxin extraction from samples

- Extracellular samples (water fraction): Since the suspect toxins were thought to be dissolved in the water, these samples were injected directly into the LC-HRMS system without previous extraction.
  For each sample, 990 µL of water sample were transferred to an injection vial and 10 µL of Nodularin-R 0.09483 ng μL^{-1} in MeOH were added as internal standard. Nodularin were selected as internal standard because it was supposed to show a similar behaviour than the analytes under study. Nodularins were not expected to be found in the samples since it is produced in brackish water (slightly salty), while microcystins are produced in fresh water.
  After the addition of internal standard, through syringe filtration and PTFE 4 mm x 0.20 µm filter, particles are removed from the liquid media in order to avoid the damage of the chromatograph column and the mass spectrometer.
- Intracellular samples (sestonic fraction): a solution of methanol acidified with formic acid is added to soak the algae; an ultrasonic bath for continuous shaking that eventually extracted the toxins into the solution. Next, the tubes with the samples were centrifuged and the supernatant was evaporated to 500 µL with N_2.
  In order to obtain a good recovery, the process (the addition of solution, the sonication and evaporation) is done by triplicate. All fraction concentrate in the same rotating evaporator tube to a volume 500 µL, until ending transferring the rich remaining solution to a vial. Each final vial is filtrated with syringe and Uptidisc PTFE 4 mm x 0.20 µm, as can be observed in Figure 5, those some extracts with

more suspended particles had to been processed twice in order to get a proper extract that were able to be injected into the LC-HRMS system.

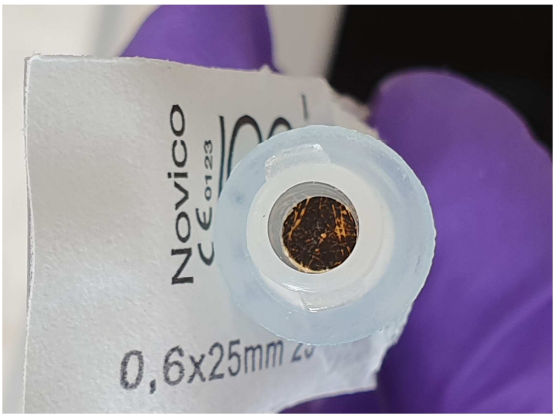

Figure 5. A syringe PTFE filter after processing an algae extract.
(own work)

5.4. LC-ESI-HRMS

As have been detailed in the *Section 3.2.2.* in this work we used a mass spectrometer Orbitrap Exactive™ from Thermo Scientific (Bremen, Germany), equipped with an electrospray ionization source and coupled to HPLC system equipped with a quaternary pump (Thermo Scientific Surveyor MS Plus) and an Accela Open AutoSampler (Thermo Fisher Scientific, San Jose, California) that were able to a refrigerate vial at 6 ºC.

The conditions of the H-ESI was the following: 42 psi in sheath gas flow rate, 10 for the auxiliary gas, heater temperature of 30 ºC, capillary temperature of 275 ºC, capillary voltage of 35 V, tube lens voltage of 186 V, skimmer voltage of 18 V.

To achieve the maximum information for the reliable identification of toxins, three experiments were performed simultaneously when analysing the sample: 1) ESI Full Scan (positive ion mode 400-1200 *m/z*), 2) HCD All Ion Fragmentation (AIF) at 30 eV (positive ion mode 60-1200 *m/z*) and 3) HCD AIF at 70 eV (positive ion mode 60-1200 *m/z*). For ion fragmentation in the HCD cell nitrogen was used as a collision gas.

Each type of extract (water fraction and sestonic fraction) was injected using a different injection method. For the extracts of the intracellular fraction (sestonic fraction, algae extract in methanol), 5 µL were injected directly in the LC-HRMS system; while for the extracellular

fraction (water), 95 µL were injected directly due their low concentration level of toxins in this fraction. The configuration of the autosampler for the injection of big volume is easy, only the loop of the autosampler has to be changed by a loop with higher volume and the new sample injection volume has to be set the microsyringe.

The chromatographic separation was performed in a Phenomenex® Luna® C18 (2) column (150 mm length and 2 mm internal diameter, 5 µm of particle size and 100 Å pore size). A precolumn (a couple fitted in series of cartridges of Phenomenex® Security Guard C18 4 x 2.0 mm) was also used to protect the actual analytical column from contamination of the samples. Ultra-pure water was used as solvent A and acetonitrile as solvent B, both acidified at 0.1% v/v with formic acid. For the screening of more than 160 toxins the gradient elution program was the following as shown in Figure 6: 10-30% B 10 min, 30-35% B 20 min, 35-55% B 15 min, 55% B 5 min, 55-90% B 2 min, 90% B 3 min and then the system was back to the initial conditions for re-equilibration (10% B) during 10 min before the next injection. The having a total chromatograph time was 60 minutes per sample using a flow rate of 200 µL min^{-1}[34].

Pump 2

	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	200.0
1	10.00	70.0	30.0	0.0	0.0	200.0
2	30.00	65.0	35.0	0.0	0.0	200.0
3	45.00	45.0	55.0	0.0	0.0	200.0
4	50.00	45.0	55.0	0.0	0.0	200.0
5	52.00	10.0	90.0	0.0	0.0	200.0
6	55.00	10.0	90.0	0.0	0.0	200.0
7	55.10	90.0	10.0	0.0	0.0	200.0
8	60.00	90.0	10.0	0.0	0.0	200.0
9		100.0	0.0	0.0	0.0	200.0

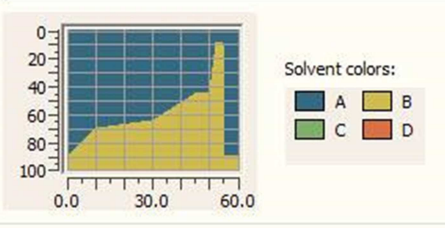

Figure 6. LC gradient setup, A: H_2O, B: ACN.
(own work with Thermo™ Xcalibur™)

C-trap was set as balanced with AGC of maximum 10^6 ions and IT of 250 ms. The Orbitrap mass spectrometer operated in all three experiments at a mass-resolution resolution of 50000 at Full Width at Half Maximum (at *m/z* 200, 2 Hz). The mass accuracy was always better than 2 ppm using external calibration (Pierce™ LTQ ESI Positive Ion Calibration Solution, Thermo Scientific™) but mass-resolution could become lower at higher *m/z* ratios according their ion motion presented in this work in *Section 3.2.2.2.* [27].

5.5. Data processing software

Liquid chromatography coupled to mass spectrometry is a powerful technique to analyse organic compounds. Modern autosamplers and HPLC systems can process much more samples than decades ago, while cutting-edge spectrometers could detect thousands of different mass-to-charge ratios within milliseconds, at the same time that also perform multiple HRMS experiments (like full scan and ion fragmentation).

With all these new features, a simple analysis could generate big files that a single technician could expend many hours to process and to interpret them. In order to obtain the maximum information from a LC-HRMS analysis, we need to process the spectral data. The software used to analyse, codify and interpret the data was the following:

- Thermo Scientific™ Xcalibur™ Qual Browser - Ver3.1.66.10: Verifies and figures out data obtained from the LC-HRMS system, allowing the user browse through the chromatogram to look for peaks and also to search the mass at an exact time. It allows data acquisition and processing.
- Thermo Scientific™ TraceFinder™ EFS - Ver3.3: Optimized for Environmental and Food Safety, it works for the search of specific compounds within a database. Working with data bases it can automate the file processing, to search compound by their exact mass, their adducts, the retention time, their precursor and fragment ions, … It's a very useful tool if you know what to search in the samples and for quantitative purposes.

6. RESULTS AND DISCUSSION

6.1. Development of an HRMS Database

The samples have been screened by acquiring the mass spectral data in Full Scan and submitted to a manual search by using Xcalibur™ Qual Browser in order to detect the most common Microcystins. When using the target methodology, the XIC (Extracted Ion Chromatogram) allowed the extraction of the chromatogram for a specific *m/z* (requested analyte) using an isolation window of ±5 ppm. The mass analyser worked in full scan, it had

detected hundreds of signals at diverse masses-to-charge ratio, but the relationship between the signals and compounds was unknown to us. This relationship may have been detected and described in scientific literature: a valuable way to identify compounds is through databases, which can storage information acquired from accurate mass measurement and contrast the experimental evidence with the theoretical data.

In order to detect multiple toxins by using software TraceFinder™ EFS, we built an in-house database that included toxins detected in blue-green algae species such as *Microcystis, Nostoc, Anabaena, Oscillatoria, Planktohrix,* etc. Diverse genera of cyanobacteria known by their secondary metabolites: cyanotoxins, a hepatotoxic by-product like Microcystins, but other related peptides which are also produced which also show diverse toxicity: some of them are neurotoxic or dermatotoxic [35].

The TraceFinder™ EFS software allows monitoring different parameters (mass accuracy, isotope pattern and fragment ions) to identify and to confirm detected molecular formulas with their substances; but first of all, we need to know which compounds are we looking for.

Microcystin-LR is the most studied variety of microcystin, even though there are hundreds of molecules described in literature with similar structure but different molecular formula. In order to integrate in the database all the compounds selected, we had to calculate the molecular formula for each compound and their monoisotopic mass of each expected adduct ion such as $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+2H]^{2+}$.

A first "alpha" database was implemented with the Microcystins that we had standards for (dmMC-RR, MC-RR, dmMC-LR, MC-LR, MC-YR, MC-WR, MC-LA, MC-LY, MC-LW, MC-LF) along with Nodularin-R (NOD-R), as shown in Table 2. This set of substances allowed us to get used to the workflow of the TraceFinder™ software and the creation of databases.

Table 2. Extract of "alpha" database.

Compound	Molecular Formula (M)	*m/z* expected adduct ion	Retention Time[c] [min]	*m/z* fragment ion[d]
dmMC-RR	$C_{48}H_{73}N_{13}O_{12}$	512.7824[b]	13.6	135.0804
MC-RR	$C_{49}H_{75}N_{13}O_{12}$	519.7902[b]	13.8	135.0804
Nodularin R	$C_{41}H_{60}N_8O_{10}$	825.4505[a]	17.0	135.0804
MC-LA	$C_{46}H_{67}N_7O_{12}$	910.4921[a]	44.4	135.0804
dmMC-LR	$C_{48}H_{72}N_{10}O_{12}$	981.5404[a]	20.0	135.0804

MC-LF	$C_{52}H_{71}N_7O_{12}$	986.5233[a]	53.8	135.0804
MC-LR	$C_{49}H_{74}N_{10}O_{12}$	995.5560[a]	20.7	135.0804
MC-LY	$C_{52}H_{71}N_7O_{13}$	1002.5183[a]	46.5	135.0804
MC-LW	$C_{54}H_{72}N_8O_{12}$	1025.5342[a]	52.5	135.0804
MC-YR	$C_{52}H_{72}N_{10}O_{13}$	1045.5353[a]	20.1	135.0804
MC-WR	$C_{54}H_{73}N_{11}O_{12}$	1068.5513[a]	25.1	135.0804

(a) $[M+H]^+$
(b) $[M+2H]^{2+}$
(c) Standard mix in our column, using the methodology proposed in this work
(d) Fragmentation of ADDA

An exhaustive bibliography work was necessary with the purpose to include in the database not only the most common microcystin, but also those microcystins with the most frequent amino acid (L, R, Y, …) changed, others with modifications in the ADDA, even those that have been seldom found [10], [19], [36]–[39].

The final database included 167 cyanotoxins: 10 nodularins and 157 microcystins. An additional supplementary database was built including 30 additional cyanobacterial peptide from algae but with other structure, neither like Nodularins (cyclic pentapeptides) nor Microcystins (cyclic heptapeptides) such as anabaenopeptins or oscillamides [11], [12], [38]. Finally, a last database was also created with 39 pigments usually found in algae samples, for assigning signals that are not related with noxious substances, like chlorophyll by-products, as shown in Figure 7 [40], [41].

Pheophorbide A

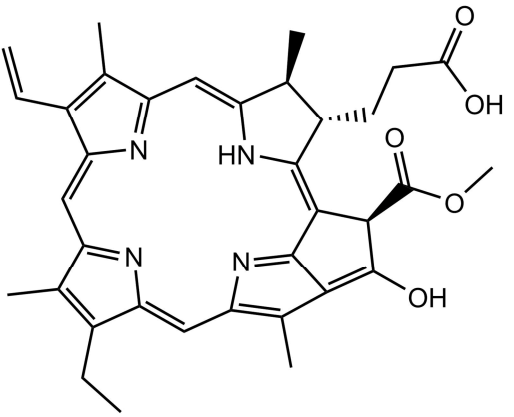

Figure 7. A breakdown product of chlorophyll, notice the porphyrin group present also in mammal blood.
(own work with PerkinElmer, Inc. ChemDraw® Pro16.0)

6.2. Microcystin identification confidence levels

The optimal scenario to confirm the presence of an analyte in a sample by LC-HRMS would through an analytical standard of the specific substance to compare its chromatogram and mass spectrum with that observed in the sample. Criteria like the chromatographic retention time of the compound in the analytical column used, the accurate mass and the isotopic pattern, are used for identification purposes and must be the same, assuming a certain tolerance, in both the standard and the sample for confirmation purposes.

However, for more than 160 referenced microcystins, there is not Certified Reference Materials and only 14 commercial standards are available, since toxins are natural products synthesized by cyanobacterial cells and it is difficult to isolate different compounds from algae cultures.

According to the confidence levels proposed by Schymanski et al. [42] we have proposed three confidence levels for identification purposes, a distinctive restructuration to give more importance to the presence of a particular fragment ion found in the microcystins and nodularins mass spectra.

- Level 3 Suspects: The exact mass (within a 5 ppm mass relative error) of a toxin is found in the mass spectrum, with a chromatographic peak area larger than 3000 counts, the isotope pattern should have a minimum score fit of 70% (compared to the theoretical one) and the RDBE value should be a non-integer number (for example in MC-LR is 16.5). These criteria must be met to get the molecular formula of a compound.
- Level 2 Probable: Additionally of level 3, the Higher-energy Collisional Dissociation (HCD) is used to fragment the ions detected in an Orbitrap instrument. Two fragmentation experiments at 30 eV and at 70 eV (different peptides fragmentised better at one or other energy) are performed in order to identify the product ion $[C_9H_{11}O]^+$ corresponding to the fragmentation of the amino acid ADDA commonly found in cyanobacterial toxins, as shown in Figure 8. The minimal threshold to consider the presence of ADDA is an area of 500 counts and a mass relative error below 5 ppm for the fragment ion *m/z* 135.0804.

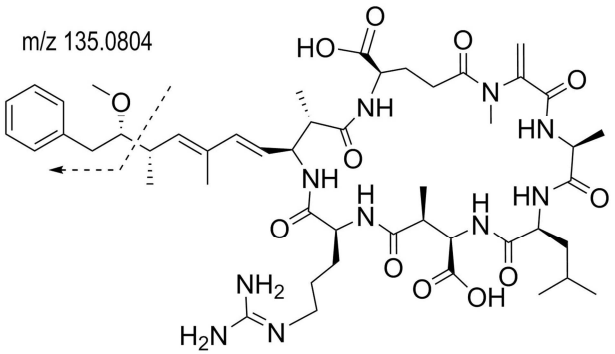

Figure 8. Characteristic fragmentation of Microcystin LR.
(own work with PerkinElmer, Inc. ChemDraw® Pro16.0)

- Level 1 Unequivocal: The microcystin has been detected with the previous levels, its standard is available and its retention time matches with that of the standard in the same conditions. Once achieved that confidence level, we can state the unequivocal presence of that microcystin in the sample.

6.3. Suspects screening in standard mix

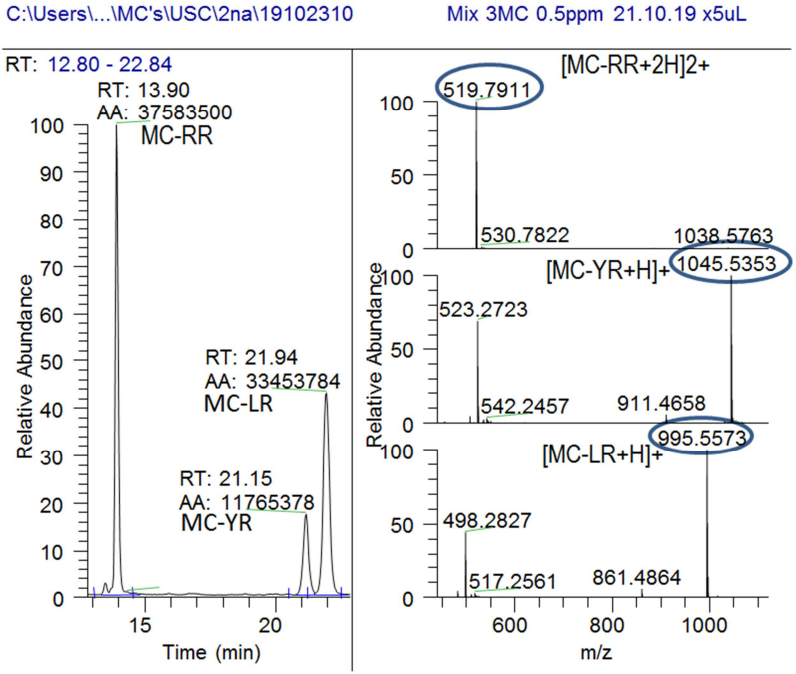

Figure 9. Chromatogram and mass spectra from standards of MC-RR, MC-YR and MC-LR.
(own work with Thermo Scientific™ Xcalibur™ Qual Browser)

Microcystins and Nodularin-R standards were purchased from Alexis Biochemicals® Enzo Life Sciences; the brand stands that these products were at least 95% pure. They have been used in routine analysis of environmental samples to detect that 11 target toxins.

The standard solution was injected in the LC-HRMS. The 10 MCs and NOD-R can be extracted from the full screening manually in Xcalibur™ (Figure 9 shows 3 of them), but they also were searched using the "alpha" database in TraceFinder™ EFS. Resulting of finding the 11 compounds identified satisfactorily fulfilling the criteria previously stablished, each one with their exact mass, their fragmentation of 135.0804 *m/z* and their identical retention time. These all factors represent the first level of confidence (that confirms the proper functioning of database since it was built with those standards and match the retention time).

The mass spectral data of the standard mixture were also processed using the complete microcystin database that includes 167 substances. New signals were detected in the LC-HRMS chromatogram, up to 20 different *m/z* values match with toxins in the database (relative mass error < 5 ppm and isotope pattern score fit > 70%). Those 20 mass-to-charge ratios with unique chemical formula can correspond up to 54 different toxins but it is not possible to characterize which one could be without knowing the retention times of the unknown compounds to compare with the experimental retention times we get. Among the 20 signals detected, 11 matched with compounds with the standards and the other 9 signals were considered suspect microcystins that were not reported in the standard as impurities and that could correspond up to 21 different toxin conformations.

The identification of more toxins that those specified in the standards is possible because these natural products are extracted from algae cultures and purified to get a purity ≥ 95%. However, the other 5% of impurities are easily detectable using HRMS and our database, and they could be characterized if analytical standards existed.

6.4. Detection of microcystins in continental waters

6.4.1. Extracellular samples (water fraction)

Following the procedures indicated in the *Experimental and Methods (Sample pre-treatment)*, for each water sample properties such as electrical conductance, pH and free chlorine were measured (Table 4) in order to check the suitability of the water sample for its direct injection into the LC/HRMS system.

Table 4. Water sample properties measured before the sample pre-treatment.

Sample reference	Electrical conductance [µS cm^{-1}]	pH	Free Chlorine [mg Cl_2 L^{-1}]
4854	402	7	0
4855	400	6.5	0
4856	478	7	0
4857	476	7	0
4858	479	7	~0-0.10
4859	466	7	0

If the electrical conductance was much more elevated, the water sample would need to be diluted. The pH would be adjusted in case of be far from the neutral value. Finally, the free Chlorine was inactivated quenching them with ascorbic acid added to the sample.

Water samples were analysed by LC-HRMS and not toxins or pigments were detected, probably because they were water samples without algae rests. Since we spiked the same amount of NOD-R in each sample as an internal standard, the only compound detected was that Nodularin-R. Having a similar behaviour than Microcystins, this internal standard is added to correct the possible loss of analyte during the injection process; since the levels of nodularin have remained constant, we can state that these toxins will not be lost by volatilization nor degradation.

Through a Full Scan screening, not many signals were detected in the Total Ion Current (TIC), so the Extracted Ion Chromatogram (XIC) of the ion at *m/z* 135.0804 (characteristic fragment ion of ADDA in microcystins) in the HCD fragmentation is requested to selectively detect the MCs. As can be seen in Figure 10, in the chromatograms of HCD fragmentations at 30 eV and 70 eV we have detected a signal at 16.93 min also present in the TIC (16.91 min), but it was not a microcystin, this signal corresponded to Nodularin-R (IS).

Nodularin added to the samples would be used for quantify the microcystins levels if they would be detected in the water fraction.

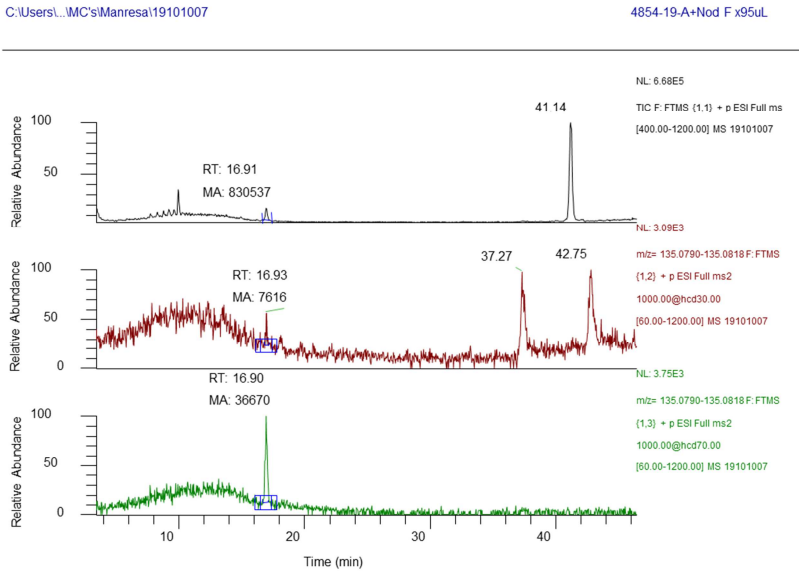

Figure 10. Chromatogram of a water sample (TIC and XIC 135.0804 at HCD 30 eV and HCD 70 eV). *(own work with Thermo Scientific™ Xcalibur™ Qual Browser)*

None of the target toxins had been detected at the Detection Limit and Quantification Limit previously stablished for the current LC-ESI-HRMS method for water fraction [34] (Table 5). Additionally, must be taken into account that the guideline values proposed by the WHO are 1 µg of MC-LR L^{-1}.

Table 5. Limits of Detection and Quantification according to C. Flores and J. Caixach [34].

Compound	**LOD** [µg L^{-1}]	**LOQ** [µg L^{-1}]
dmMC-RR	0.01	0.20
MC-RR	0.01	0.06
MC-LA	0.01	0.06
dmMC-LR	0.05	0.20
MC-LF	0.05	0.06
MC-LR	0.01	0.06
MC-LY	0.03	0.06
MC-LW	0.05	0.06
MC-YR	0.04	0.06
MC-WR	0.04	0.06

6.4.2. Intracellular samples (sestonic fraction)

6.4.2.1. Microcystins and Nodularins

The algae samples were intracellular and richer than the extracellular ones in many compounds, including microcystins, cyanobacterial peptides and pigments. First of all, the toxins identified by our database were the potent hepatotoxins such as Microcystins and Nodularins. It must be indicated that some signals had firstly identified as compounds in study, but after a detailed inspection, a few of them were discarded and considered false positive for the following reasons:

- Non-Gaussian peak: the signals in a chromatogram must have at least 10 data points across the chromatographic peak and the peak shaper should be close to a Gaussian function (a bit of fronting or tailing allowed). Some software can mislead modelling the peaks through smoothing, but that signals have to be reviewed thoroughly. Figure 11 shows an example of a non-Gaussian peak detected.

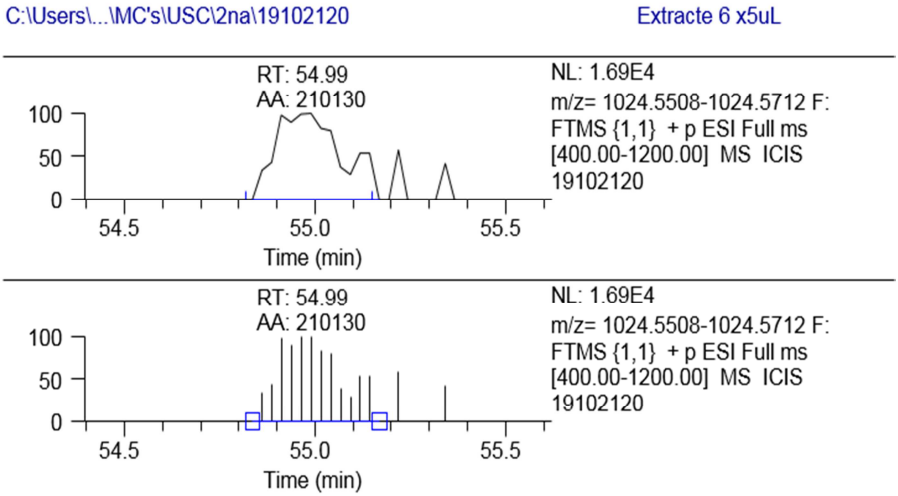

Figure 11. Example of Non-Gaussian peak in different views, being the first one a point to point representation and the other a stick representation.

(own work with Thermo Scientific™ Xcalibur™ Qual Browser)

- Being part of isotope pattern of other compound: According the isotopic distribution the signal detected by the software (chromatographic peaks) as a mass in the database can be an X+1 from another compound (Figure 12)

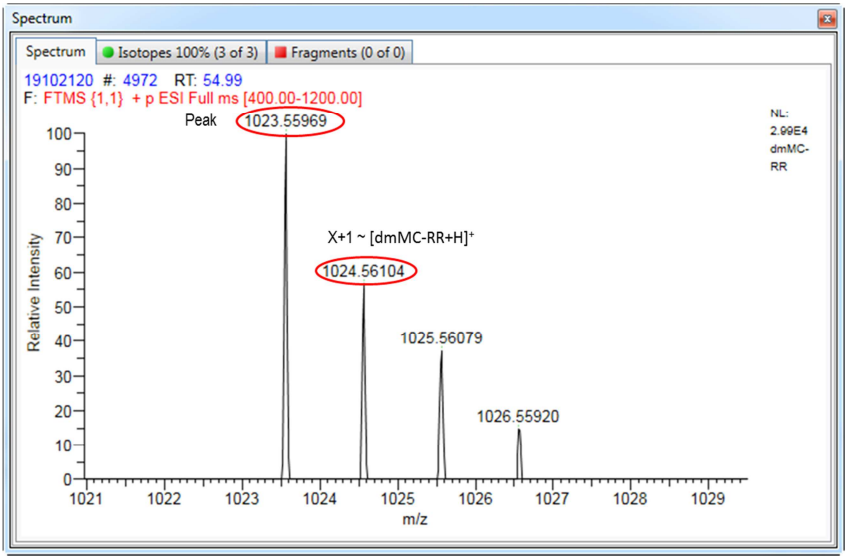

Figure 12. Example of false positive of an analogous of $[dmMC\text{-}RR+H]^+$ (*m/z* 1024.55774) but in fact is an X+1 in the isotope cluster another unknown compound.
(own work with Thermo Scientific™ TraceFinder™ EFS)

After these filters, the final reliable signals to be identified and confirmed as microcystins were classified into the 3 levels of confidence set in section *6.2. Microcystin identification confidence levels.* Table 6 summarizes this classification.

Table 6. Classification into level of confidence of Microcystins and Nodularins identified in the samples.

Level 3 Suspects	Level 2 Probable	Level 1 Unequivocal
48	35	1

We have also detected several adducts of these toxins. This could be explained according that the continental water where the cyanobacterial had grown contain ammonium and alkali metals salts that can form adduct ions with microcystins in electrospray ionization. Although double-charged ions were barely the most intense peak, their signal is characteristic in Microcystins with arginine like MC-RR, which contains more nitrogen atoms able to be charged by protonation.

Table 7. Adduct ions identified of Microcystins and Nodularins.

$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M+K]^+$	$[M+2H]^{2+}$
17	7	21	2	1

The results in Table 7 are dependent on the alkali and ammonium composition of the waters where the cyanobacteria were grown, or according Yuan et al. also in salts in the sampling container that could contaminated samples [28].

Some of the signals detected in this work could be assigned to not usual microcystins so they will be considered as provisional results and until following experiments were performed to confirm the chemical structure of the molecular formula found by measuring the accurate mass in HRMS. Few *m/z* were detected at different retention times for different samples, while for those substances we have no standards we cannot confirm a retention time, just assuming that they are analogue microcystin with different structural conformation but same formula and exact mass. Up to 4 of the signals interpreted have never been described in scientific literature and their characterization would be the first time being identified; according to our criteria they could be microcystins or other related peptides. One found in several samples are suspect to be a cyanobacterial peptide with more degree of unsaturation, according their RDBE (double bonds and/or cycles) than microcystins used to have. In Table 8 some distinctive results are described.

Table 8. Example of tentatively assigned toxins with the database.

Compound	tR [min]	Adduct ion	Proposed empirical formula	Exact mass *m/z*	Samples[a]	Accuracy [ppm]	Isotope pattern score [%]
MC-OiaAba	57.4	$[M+H]^+$	$C_{52}H_{68}N_8O_{13}$	1013.4979	2, 5, 13	-0.6, -0.3, -1.9	93, 100, 100
[Gly^1,Asp^3, ADMAdda^5]MC-LR	9.1	$[M+2H]^{2+}$	$C_{48}H_{70}N_{10}O_{13}$	498.2635	10	-1.7	99
MC-FA	8.6	$[M+NH_4]^+$	$C_{49}H_{65}N_7O_{12}$	961.5029	10	0.4	72
[L-Ser^7]MC-E(OMe)E(OMe)	8.9	$[M+Na]^+$	$C_{48}H_{69}N_7O_{17}$	1038.4642	14	-0.8	99
Not referenced toxin[b]	39.1	$[M+K]^+$	$C_{46}H_{67}N_7O_{12}$	948.4479	16	-0.1	99
[(6Z)-Adda^3]NOD	48	$[M+H]^+$	$C_{41}H_{60}N_8O_{10}$	825.4505	16	0.6	100

(a) Reminder that is an 18 samples set

(b) Compound with the same formula that MC-LA but the retention time does not match with the available standard of MC-LA and it have analysed in mix.

6.4.2.2. Cyanobacterial peptides

Other cyanobacterial peptides produced by green blue algae have been detected in some intracellular samples and in the standard mix. As mentioned above in *Section 6.4.1*, in water samples none of the analytes in study had found.

Anabaenopeptins have been identified, ABPN D, G and I in the samples while ABPN B, E and F are present only in the standards. Agardhipeptin A, Cylindrospermopsin, Oscillacyclin and Oscillapeptin C and F have also been detected in some samples (chemical structures of some compounds are shown in Figure 13, ABPN B is a representative member of their family). It will be interesting to monitor samples combining bio-toxicological assay with high-resolution mass spectrometry analyses prior liquid chromatography separation, because cyanotoxins show a wide variety in their toxicity and we do not know about how hazardous could be new characterized peptides.

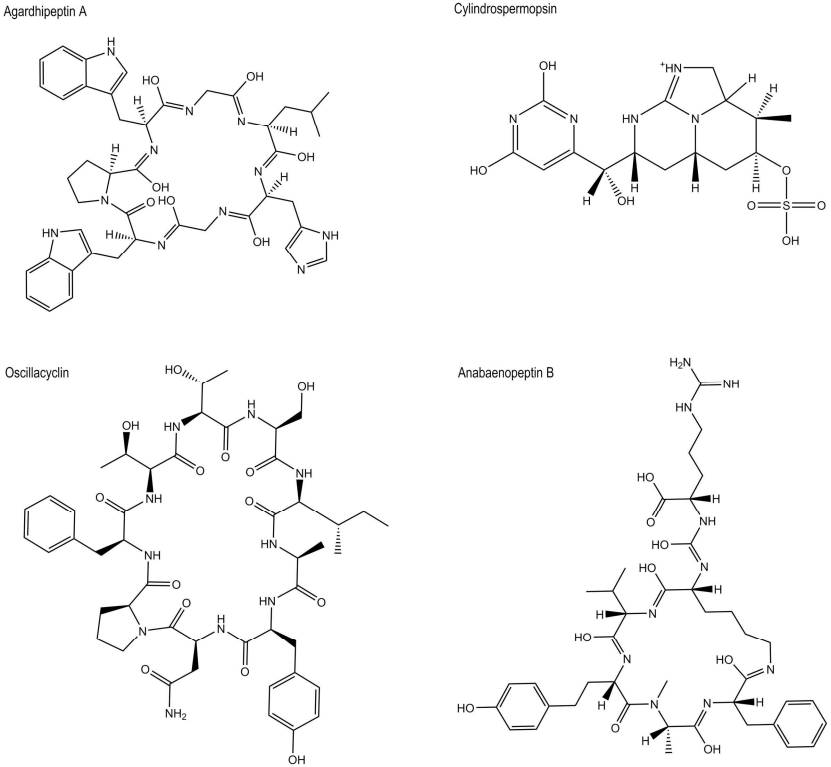

Figure 13. Cyanobacterial peptides identified.
(own work with PerkinElmer, Inc. ChemDraw® Pro16.0)

6.4.2.3. Cyanobacterial pigments

27 pigments among the 39 present in the database have been identified in sestonic fraction in algae samples, being the most frequent Crocoxanthin and Echinenone (present in 13 of the 18 samples), Fucoxanthin (present in 8 samples), Monadoxanthin and Pheophorbide A (present in 7 samples). Being Crocoxanthin and Monadoxanthin a representative green pigment from plastids, Echinenone and Fucoxanthin are xanthophylls, yellow pigments that give a brownish coloration to algae and Pheophorbide A is a sub-product from chlorophyll in its breakdown. Also there have been found a relation between the amounts of pigments detected in an extract and the colour of the sample, been more of the pigments common to the intracellular samples by the time all of them are cyanobacteria allowed to perform photosynthesis.

The pigments detected are feasible to the nature of the samples (algae); the best way to identify that kind of compounds would be a different gradient for the chromatographic separation and detect them by UV/visible since different pigments have a diverse range of absorption, nevertheless a reliable characterization of pigments is not the main scope of this work, as it is the characterization of toxins. In Figure 14 the structure of some pigments identified are showed to make aware of their multiple chromophore groups based in conjugated pi-bond systems.

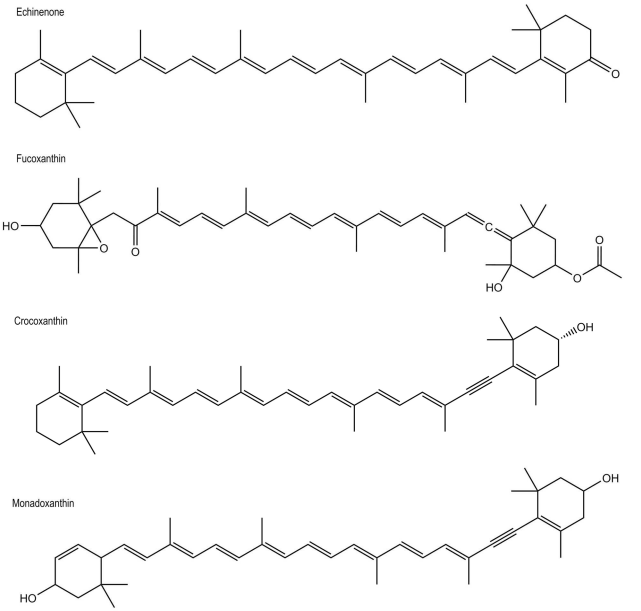

Figure 14. Cyanobacterial pigments found most frequently in the samples.
(own work with PerkinElmer, Inc. ChemDraw® Pro16.0)

7. CONCLUSIONS

High resolution mass spectrometry is a powerful technique to get reliable information about analytes' composition through accurate mass measurements. Setting restrictive criteria is decisive in unequivocal identification of both target and unknown compounds.

We have built a total of four databases with information of 1) 10 target microcystins and a nodularin (the ones with standards available), 2) 157 MCs and 10 NODs, 3) 30 cyanobacterial peptides and toxins like Cylindrospermopsin and 4) 39 pigments characteristics of blue green algae.

HRMS criterion for detection of toxins has been defined, and we put forward levels of confidence for the identification. We have tuned up an analytical method for suspects screening and it have been applied in environmental samples such as water and algae.

For water fraction none of the microcystins of which the standard is available (dmRR, RR, dmLR, YR, LR, WR, LA, LY, LW, LF) neither other microcystins or nodularin in databases had been detected. In algae samples, a large number of tentatively microcystins have been found in sestonic fraction, along other cyanobacterial peptides and pigments.

8. REFERENCES AND NOTES

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9. ACRONYMS AND ABBREVIATIONS

(6Z)-Adda: Stereoisomer of Adda at the Δ^6 double bond

A: Alanine

Aba: L-2-aminobutanoic acid

ABPN: Anabaenopeptin

ADMAdda: O-Acetyl-O-demethylAdda

ADDA: (all-S,all-E)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

AGC: Automatic Gain Control

AIF: All Ion Fragmentation

AMM: Accurate Mass Measurement

BGA: Blue-green algae

D-Asp: D-methylaspartic acid

D-Dha: N-methyldehydroalanine

Dha: Dehydroalanine

DMAdda: O-DemethylAdda

E(OMe): Glutamic acid methyl ester

ESI: Electrospray Ionization

ELISA: Enzyme-Linked Immunosorbent Assay

GV: Guideline value

HAB: Harmful algal bloom

Har: Homoarginine

HCD: Higher-energy Collisional Dissociation

H-ESI: Heated Electrospray Ionization

Hil: Homoisoleucine

Hty: Homotyrosine

HRMS: High-Resolution Mass Spectrometry

IS: Internal Standard

IT: Injection Time

L: Leucine

LC: Liquid chromatography

LD_{50}/LC_{50}: Lethal dose/concentration required to kill have of the tested population

MC: Microcystin

MMPB: 3-methoxy-2-methyl-4-phenylbutyric acid

M(O): Methionine-S-oxide

MS: Mass Spectrometry

MSer: N-Methylserine

NOD-R: Nodularin with arginine

m/z: Mass-to-charge ratio

Oia: Oxindolylalanine

PTFE: Polytetrafluoroethylene

R: Arginine

RDBE: Ring Double Bond Equivalents

SIM: Selected Ion Monitoring

SN: Signal to noise ratio

SRM: Selected Reaction Monitoring

TDI: Tolerable daily intake

TIC: Total Ion Current

TLC: Thin Layer Chromatography

WHO: World Health Organization

XIC: Extracted Ion Chromatogram

Y: Tyrosine

APPENDICES

APPENDIX 1: SAFETY MEASURES

The compounds in study are mainly hepatotoxins, which produce severe damage to the liver if it arrives to our organism. The way to avoid this for happening is working correctly and protecting ourselves. Besides following the Good Laboratory Practice that guarantee certain degree of quality and safety, the nature of analytes requests special measures.

As a Personal Protective Equipment, CSIC has provide me with a couple of laboratory white coats preventing chemical stains and washing them in their specialized laundry to destroy contaminants, also, safety glasses to avoid eye damage in solutions' splatter. Nitrile gloves had been used to not contaminate samples, but an additional pair of special fabric-gloves were used below the rubber ones to protect us against possible punctures with syringe. Is crucial not to expose open wounds or pricking our skin with contaminated needles as the intravenous or intraperitoneal way of entering the body is most toxic than the oral ingestion.

Preventing other future poisoning, all the material in contact with toxins (syringe tips, filters, vials, pipettes and gloves) are safely disposed of in cytostatic container for their correct waste management.

APPENDIX 2: SOFTWARE DEMONSTRATION

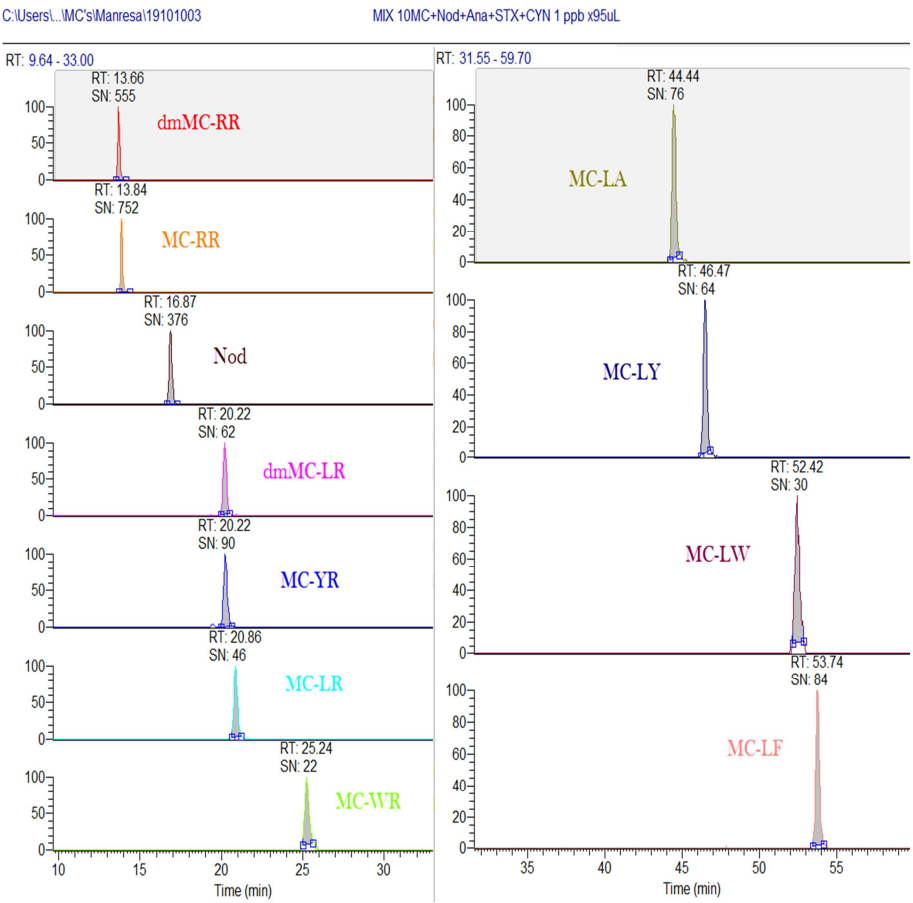

Figure 15. Extracted Ion Chromatograms for the standards of 10 MCs and Nod.
(own work with Thermo Scientific™ Xcalibur™ Qual Browser)

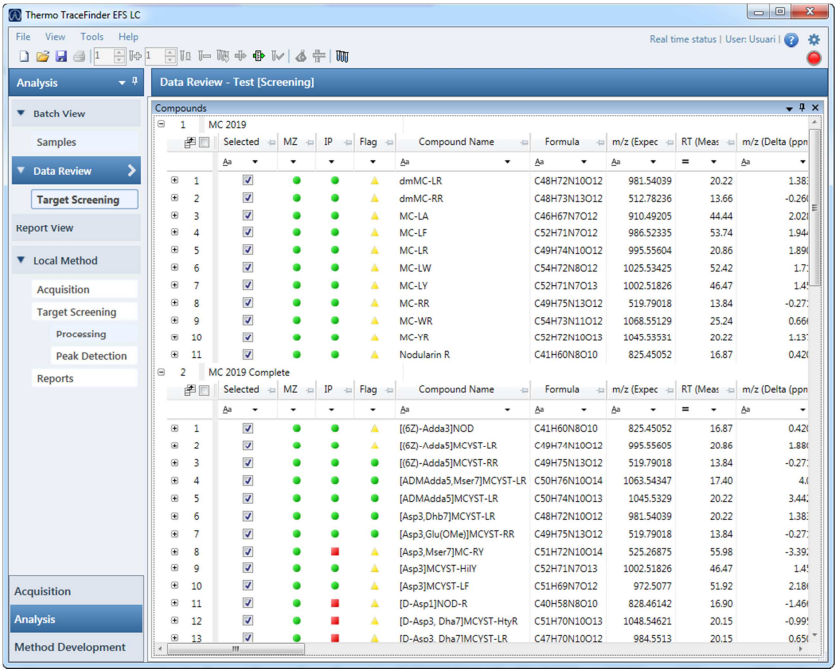

Figure 16. Same data that in Figure 15, processed with standards and complete MCs databases.
(own work with Thermo Scientific™ TraceFinder™ EFS)

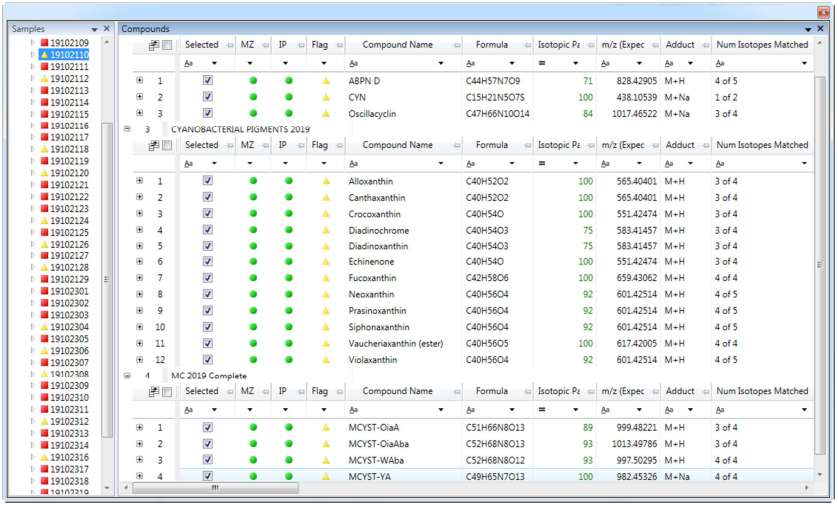

Figure 17. Diverse toxins and pigments detected applying databases to a sample.
(own work with Thermo Scientific™ TraceFinder™ EFS)

APPENDIX 3: EXTRACTION

Figure 18. Example of set of algae samples.
(own work)

Figure 19. Same samples in acidified methanol.
(own work)

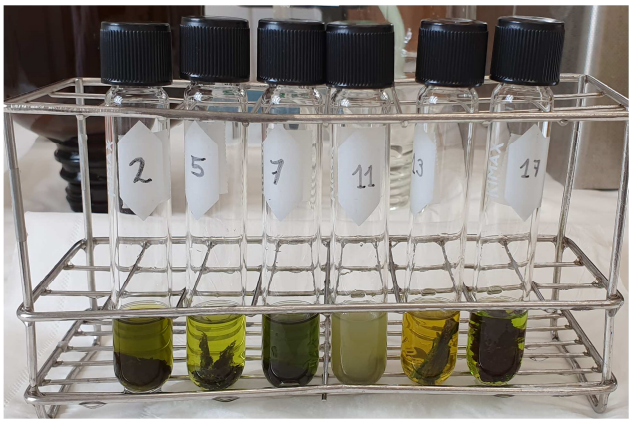

Figure 20. Same algae samples after extraction.

(own work)

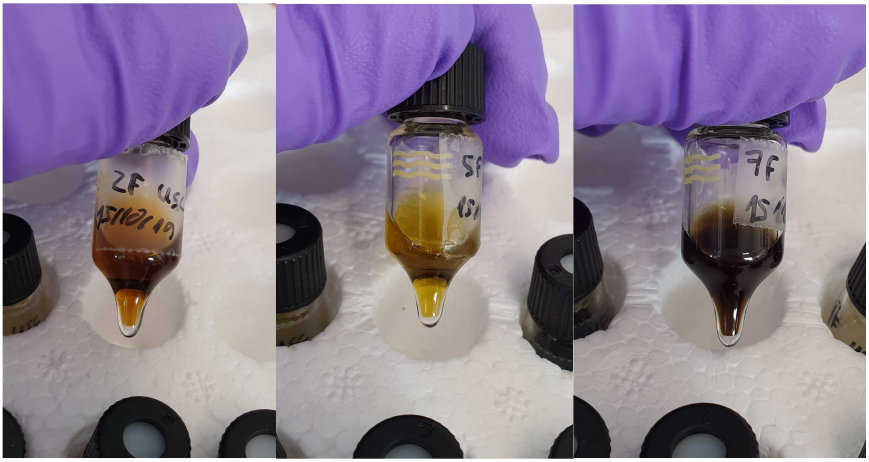

Figure 21. Final extracts of algae in injection vials.

(own work)