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Alterations in Mediterranean mussel (*Mytilus galloprovincialis*) composition exposed to cyanotoxins as revealed by analytical pyrolysis

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ABSTRACT

Cylindrospermopsin (CYN) and Microcystin-LR (MC-LR) are biotoxins produced by cyanobacteria species that, due to climate change and water eutrophication, proliferate together with an increase of the associated cyanotoxins. Both toxins are usually found in the aquatic environment and filter feeding organisms such as mussels are particularly exposed which may be the cause of metabolic alterations. In this work, pyrolysis gas chromatography mass spectrometry (Py-GC/MS) is used to evaluate compositional changes in mussel (*M. galloprovincialis*) after exposure for 14 days to cyanotoxins of *Chrysosporum ovalisporum* (CYN), *Microcystis aeruginosa* (MC-LR) and to a combination of both toxins. The pyrolysis of mussel flesh produced complex chromatograms, with up to 100 different compounds, but very similar between treatments. Major groups found were N compounds (pyridine, N-alkyl molecules) (18.2 % ± 1.5) and peptide/protein derived compounds that include alkyl indols and diketopiperazines (DKPs) (17.7 % ± 2.4), series of medium chain length (C_{14}-C_{22}) saturated, mono and polyunsaturated fatty acids (45.1 % ± 5.0), steroids (7.5 % ± 0.6) and aromatics with undetermined origin (8.9 % ± 1.1). A chemometric treatment of the chromatographic data allowed the discrimination between the different mussel populations exposed to cyanotoxins. The results are discussed in terms of the probable effects exerted by the biotoxins in mussel composition and the possible metabolic pathways affected.

1. Introduction

Cyanotoxins are toxic secondary metabolites produced by cyanobacteria algae that typically thrive in warm and nutrient-rich environments. Therefore, in a global warming and increased eutrophication scenario it is predicted that the problems caused by microalga blooms and associated toxins will intensify, both in frequency and duration [1,2]. Aquatic animals such as molluscs, fish and crustaceans are able to live in the presence of these biotoxins and therefore accumulate them in the food chain [3]. Humans can be exposed to cyanotoxins by different routes such as recreational and professional activities, inhalation or dermal exposure. However, the main route for cyanotoxin intake is through the consumption of contaminated drinking water and food (molluscs, fish, crustaceans, vegetable, crops and food supplements) [1,3,4].

Amongst the cyanotoxins, Microcystins (MCs) and Cylindrospermopsin (CYN) are the most investigated due to their high toxicity and extended distribution. The MCs encompass an extensive family of cyanotoxins produced principally by freshwater cyanobacteria genera such as *Microcystis*, *Aphanizomenon* and *Dolichospermum* [1]. MCs are cyclic heptapeptides with the structure: (D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda-Arg^5-D-Glu^6-Mdha^7), where D-MeAsp is D-erythro-β-methylaspartic acid, Mdha is N-methyldehydroalanine, Adda is the β-amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca- 4,6-dienoic acid, exceptional for cyanobacteria and X and Z are variable amino acids [5,6]. Up to now 246 congeners of MCs have been identified [7]. Between all of them, MC-LR, with leucine (L) at position X and arginine (R) at position Z, is the congener most studied due to its presence, prevalence and toxicity, in comparison to other variants, such as MC-RR or MC-YR [6,8–10]. The MCs are classified as hepatotoxins mainly due

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to their transport into the hepatocytes by the organic anion transport polypeptides (OATP) [11]. With respect to the toxicity mechanisms of MC-LR, the most extensively investigated is the specific inhibition of serine/threonine phosphatases, particularly types 1 and 2A (PP1 and PP2A), by covalent binding [12]. Moreover, apoptosis, induction of oxidative stress, reduced DNA repair and tumour promotion are also reported among others [13–15]. In addition, although the mechanisms implicated are not totally understood, it has been demonstrated that MC-LR can cause genotoxic effects and cancer development [16]. In view of the tumour-promoting activity of MC-LR, the International Agency for Research on Cancer (IARC) classified MC-LR as possible carcinogen to human (Group 2B) [17].

The cyanotoxin CYN is synthesized by diverse cyanobacterial species (*Umezakia natans, Aphanizomenon ovalisporum, Raphidiopsis curvata*, etc) with *Cylindrospermopsis raciborskii* as its main producer [18,19]. Structurally, CYN is an alkaloid with a tricyclic guanidine moiety linked through a hydroxyl bridge to a hydroxymethyluracil group [20]. It is recognized that the liver and kidney are the primarily organs of CYN acute toxicity, but also cytotoxicity has been observed in other organs [21–23]. Among the mechanisms implicated in CYN toxicity are an irreversible protein synthesis inhibition [21,24] and oxidative stress and apoptosis [25–27], which have been recently compiled and revised [1, 19]. However, CYN has not been yet classified as carcinogenic by the IARC and more studies are needed to get a better understanding of its toxicity.

As previously mentioned, exposure of humans to cyanotoxins consists in ingestion of contaminated drinking water or food. The bioaccumulation of MCs and CYN in edible aquatic organisms such as molluscs, fish, bivalves, crustaceans, etc., could represent a serious hazard for human health [3,10] but data about the bioaccumulation of MCs in mussels is limited [28–30]. With respect to CYN, the studies are even scarcer [31,32]. Despite the fact that a concomitant occurrence of MCs and CYN is increasing [33], only limited studies on their combination have been found in the scientific literature, that is mainly focused on plants [34–36]. Following the recommendations of the European Food Safety Authority (EFSA), there is the need to estimate potential additive or synergistic effects and interactions between cyanotoxins for a more realistic risk assessment of exposure to a multi-toxin scenario [4]. In this direction, some *in vitro* assays have been recently carried by our group, in an attempt to assess the toxicological profile of MC-LR/CYN combinations [37–39].

In relation to toxin determination, liquid chromatography-mass spectrometry protocols (LC–MS, LC–MS/MS) have been used in the identification and quantification of individual MCs or CYN in several matrices, including molluscs and fish, [30,40–42], and more recently multi-toxin analytical approaches have been developed for detection in water [43,44] and vegetables [45]. In all these methods, intermediate steps i.e. previous extraction of MCs or CYN and clean-up are required, and consequently, they are time-consuming and may introduce unwanted analytical artefacts. As an alternative approach, the use of conventional analytical pyrolysis (Py-GC/MS) has been previously applied for the analysis of MCs or CYN in algal blooms [46,47]. Analytical pyrolysis is a technique with well-known advantages such as the need of small samples, minimum sample preparation or manipulation requirements and relatively short analytical times. Moreover, the technique usually yields a wide diversity of pyrolysis products that can be assigned to different biogenic origins and are suitable for chemometric analyses [48–50].

To the best of our knowledge no data describing simultaneous bioaccumulation of MCs and CYN and its effect in mussels have been reported to date. Therefore, in this work our aim was to investigate possible changes in the composition of the Mediterranean mussel (*M. galloprovincialis*) exposed to cyanotoxins. For this, we used conventional pyrolysis gas chromatography mass spectroscopy (Py-GC/MS) combined with appropriate chemometric statistics as screening technique to elucidate the effects of a potential mollusc exposure to MCs produced by *M. Aeruginosa* or CYN produced by *C. ovalisporum*, individually and in combination.

2. Material and methods

2.1. Mussels farming and keeping

Specimens of *Mytilus galloprovincialis* (L.) (dimensions 60 mm ± 15 mm) were collected in Memória Beach situated in Matosinhos (Porto, North Portugal) [51,52]. Mussels were carried to the laboratory in thermal boxes together with the natural sea water. Ninety-three mussels per aquarium were placed randomly in 30 L filtered sea water, with a total of eight aquaria. For 21 days, mussels were kept in aquaria under natural light conditions, water temperature was 15 ± 2 °C, pH 7.90 ± 0.10, salinity 34 ± 2% and dissolved oxygen 8.0 ± 0.5 mg/L. The animals were fed daily with 1×10^5 cell/mL of the green microalgae *Parachlorella kessleri* [51] and the water of aquariums was renewed every two days. *Microcystis aeruginosa* (LEGE 91094), *Chrysosporum ovalisporum* (LEGE X-001) and *Parachlorella kessleri*(LEGE Z-001) were obtained from Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) [53].

2.2. Experimental design

After acclimation, 8 aquariums (2 aquaria per experimental group) with 93 animals each were used to carry out the exposure experiments. For this, mussels were splited up in four experimental groups and fed with algae at a density of 1×10^5 cell/mL during 14 days. The groups consisted of a control group (C) with mussels fed with the non-toxic microalgae *P. Kessleri*, groups (MCs) and (CYN) that were fed individually with the toxic microalgae *Microcystis aeruginosa* and *Chrysosporum ovalisporum* respectively and a group (MCs + CYN) with mussels fed with a combination of both toxic cyanobacteria. During the experiments, all the conditions were adjusted according to the number of mussels in each aquarium. At the end of the experiment, four animals were collected randomly as replicates for each treatment and for the control group, immediately clan, freeze dried and finely grind before analysis.

2.3. Conventional analytical pyrolysis (Py-GC/MS)

The thermochemical decomposition of organic materials at elevated temperature in the absence of oxygen produces a pyrolysate that is amenable to chromatographic separation. When combined with a mass spectrometer (Py-GC/MS), a molecular structural fingerprint can be obtained, even for very complex matrices of natural and synthetic origin [54]. To obtain molecular information and unambiguously characterize the main products of pyrolysis, direct pyrolysis-gas chromatography--mass spectrometry (Py-GC/MS) analysis was conducted using a micro-furnace double-shot pyrolyzer (Frontier Laboratories model 2020iD, Fukushima, Japan) connected to a GC system (Agilent Technologies Inc., model 6890 N, Santa Clara, CA, USA) fitted with a mass selective detector (Agilent Technologies Inc., model 5973 N, Santa Clara, CA, USA). The mussel samples (0.7 mg) were introduced in small deactivated steel crucible capsules and dropped in a preheated micro-oven for pyrolysis during 1 min at 400 °C. The volatile pyrolysates were then directly moved into the GC/MS for analysis. The gas chromatograph was equipped with a low polar-fused silica (5% phenyl-methylpolysiloxane) capillary column (Agilent J&W HP–5 ms Ultra Inert, of 30 m x 250 μm x 0.25 μm film thickness). Helium was used as carrier gas with a controlled flow of 1 mL min^{-1}. The oven temperature was set at 50 °C for 1 min and then increased to 100 °C at 30 °C min^{-1}, from 100 °C to 300 °C at 10 °C min^{-1}, and then constant at 300 °C for the last 10 min. The total chromatographic time was 32 min. The mass spectra were acquired at 70 eV ionizing energy. Compound designation was accomplished via single-ion monitoring (SIM) for diverse homologous series, published mass spectra available in the literature and stored in digital libraries

data (NIST and Wiley).

2.4. Statistical analysis

The chromatographic peak areas (as total area counts) were integrated and calculated as total abundances. Only compounds representing at last a 0.25 % of the total chromatographic area were considered in the analysis. A first exploratory data analysis was conducted to evaluate experimental repeatability. Firstly, all the compounds with no information in all the clusters were removed. Then, compounds with more than two no reported information (no data in Table S1) were carefully analysed in the whole context of the dataset - prior to be accepted or removed - in order to avoid that these data could distort the results. Relative standard deviation (RSD) of each group was calculated for the initial set of selected compounds. Chemical compounds with a high relative standard deviation (> 100 %) in all the clusters or combined with (RSD = 0) were qualified as outliers and proposed to be discarded in the following studies with multivariate techniques.

Brown-Forsythe test was used because it allows determining the homogeneity of the variances and the selection of variables with univariate discriminate ability [55]. In addition, the Brown-Forsythe test gives quite accurate error rates even when data deviate significantly from a normal distribution [56]. The procedure allowed selecting compounds that showed significant differences in their relative concentration ($p < 0.05$) among the groups. ANOVA test was also used as it is the most common applied variable reduction technique. No disagreement was found among their results.

However, because this study is based in multivariate information, which means that information from chemical compounds may be interacting, a Stepwise Linear Discriminant Analysis (SLDA) was also applied to the chromatographic areas. Thus, the compounds selected by the Brown-Forsythe test were filtered from a multivariate viewpoint by SLDA applied under strict conditions due to the small number of samples. The objective was to reduce the number of selected compounds to avoid hyper-optimist results in the light of the number of samples of the experiment. Finally, Principal Component Analysis (PCA) was used with the last set of the selected compounds by SLDA. PCA is an unsupervised statistical procedure that infers the natural structure Present in the dataset within no previous hypothesis classifying or defining the data [57], which is one of the objectives of this work.

The univariate and multivariate statistics were applied using Statistica 8.0 (StatSoft, Tulsa, OK).

3. Results and discussion

The pyrolysis of lyophilized mussel flesh produced well resolved and complex chromatograms (Fig. 1A). A reconstructed model pyrogram with the average intensity and deviation of all the pyrolysis compounds found in the entire sample set (Table S1) is depicted in Fig. 1B. Up to 100 different compounds were identified in the mussel pyrolysates and as expected from an animal biomass with similar origin, the compounds distribution in the pyrograms were very similar between repetitions and treatments. The major groups of compounds identified were: N bearing compounds like pyridine and N-alkyl molecules (18.2 % ± 1.5) and peptide/protein derived compounds (17.7 % ± 2.4) that include alkyl indols and diketopiperazines (DKPs) that are normally formed as pyrolysis product from amino acid condensation [58], series of medium chain length (C_{14}-C_{22}) saturated, mono and polyunsaturated fatty acids (45.1 % ± 5.0), steroids (7.5 % ± 0.6) and aromatic unspecific compounds of unknown origin (8.9 % ± 1.1) (Fig. 2).

In a multivariate statistical procedure with less samples than variables, a selection of variables must be operated to avoid possible hyper-optimist results, even for non-supervised procedures such is principal component analysis (PCA) [59]. Thus, in a first steep attending to the relative standard deviation in each group (C, MC, CYN, MIX), the initial number of variables (100 compounds) was reduced to 61 and further reduced to 13 after applying the Brown-Forsythe and ANOVA univariate test. Finally, after applying the SLDA multivariate procedure, only 8 variables were selected and considered with the highest discriminant value. This number was considered adequate for our study that include

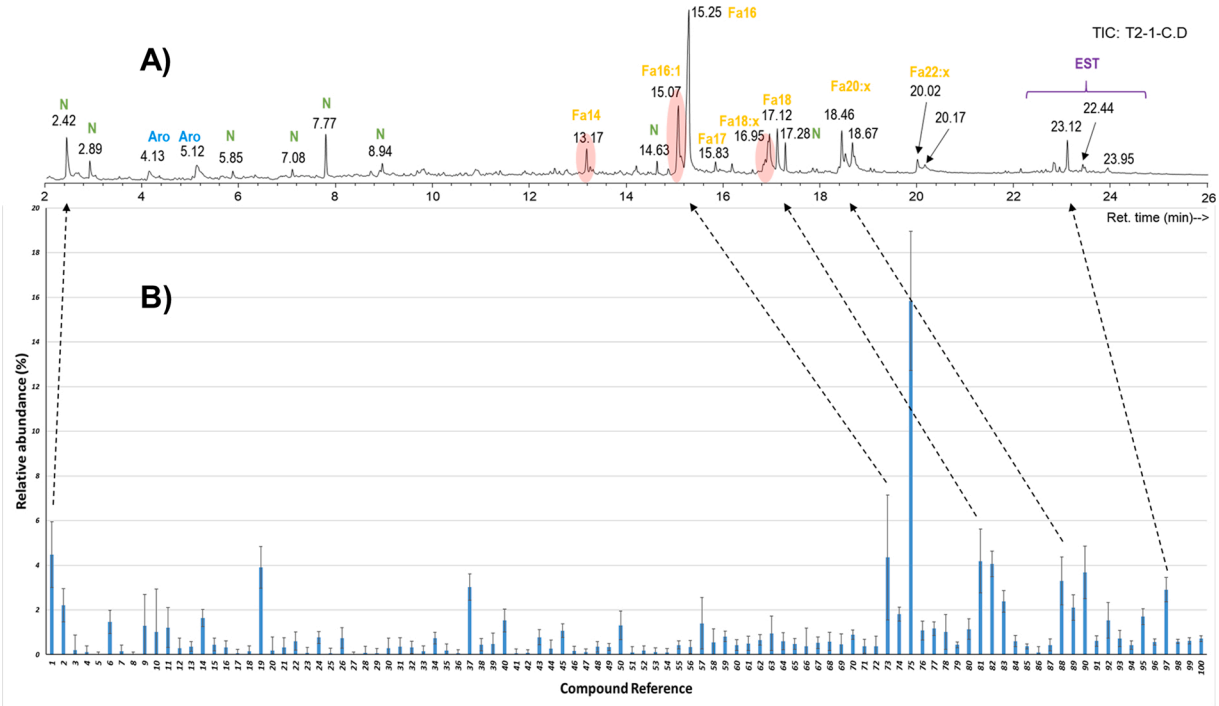

Fig. 1. Analytical pyrolysis of mussel flesh. A): Example of a pyrogram (Py-GC/MS) labelled with the major peaks, the chromatogram corresponds to the TIC from a control treatment (C1 in Table S1); B) Reconstructed chromatogram with the average relative abundances referred to total chromatographic area, compound references correspond to those is Table 1S, arrows point to the corresponding major peaks or groups of peaks and the error bars are STD values (n = 16). Compound reference corresponds with those listed in Table S1.

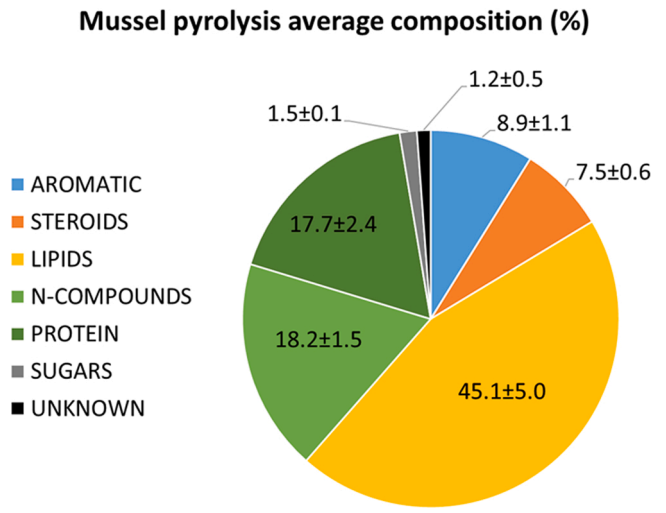

Fig. 2. Average composition of mussel flesh calculated from the relative abundance of products released after pyrolysis at 400 °C (average ± STD; n = 16).

16 mathematical samples (4 treatments and 4 replicates per treatment). The final selected 8 compounds were: [**2**] toluene (benzene, methyl), [**16**] trimethyl-pyrazine (2,3,5-Trimethylpyrazine), [**40**] methyl-indole (1H-Indole, 1-methyl-), [**47**] pentadecene (1-pentadecene), [**58**] dimethyl-carbazole (2,6-dimethyl-9H-carbazole), [**68**] pentadecanoic acid, [**82**] octadecanoic acid and [**98**] desmosterol acetate (cholesta-5, 24-dien-3ol, acetate. (3.β)-).

The chemometric analysis of the chromatographic data by univariate and multivariate statistical procedures (Brown-Forsythe test, stepwise linear discriminant analysis and principal component analysis) allowed a robust discrimination between the different mussel populations exposed to the two cyanotoxins and the control unexposed populations. The mussels exposed to the mixture of both toxins did not group with any of the three main clusters and were scattered by only 8 chemical compounds (Fig. 3). The 2D plot with the first two PCA factors explains 66.99 % of the variance (factor 3 explains an additional 16.55 % variance) and logically separated the treatments (C, MC, CYN) in clusters at different quarters of the PCA space (Fig. 3A). The ellipses of confidence of each cluster did not overlap meaning neat differences among treatments. It is remarkable the fact that C samples are not only placed in-between the treated clusters, but also forming a more homogenous group indicating a less variable composition. Among the 8 compounds selected out of the total pyrolysis structures that contributed with higher values to the discrimination of CYN treated samples were [**2**] toluene, [**47**] pentadecene and [**58**] 2,6-dimethylcarbazole; for MC samples other 3 compounds, probably related with the fat metabolism, the sterane [**98**] and the fatty acids [**68**] and [**82**] were the ones with higher discriminant value and higher values. The control untreated samples (C) were separated based in their relative high contents of the pyrazine [**16**] that may be related with the protein metabolism, and lower values for the other 7 compounds. The loadings plot of the chemical compounds - inserted in Fig. 3A - corroborates the just explained conclusions from a multivariate viewpoint analysis. When pooling the MIX samples into the model, the two first PCA factors explain now 63.87 % of the variance (Fig. 3B) (factor 3 explains an additional 17.08 % variance). One MIX sample was located inside the ellipse of confidence of CYN samples while other two, although outside the ellipses of confidence, were placed near MC & C groups and other sample was located in between samples MC and CYN, opposed to C samples. Results also show, from a general viewpoint, that the inclusion of more samples (25 %) to the dataset slightly modifies the initial PCA (Fig. 3A). This variability observed for the samples exposed to mixtures of the toxins may indicate a differential mechanism by which the molluscs absorb or respond to the different

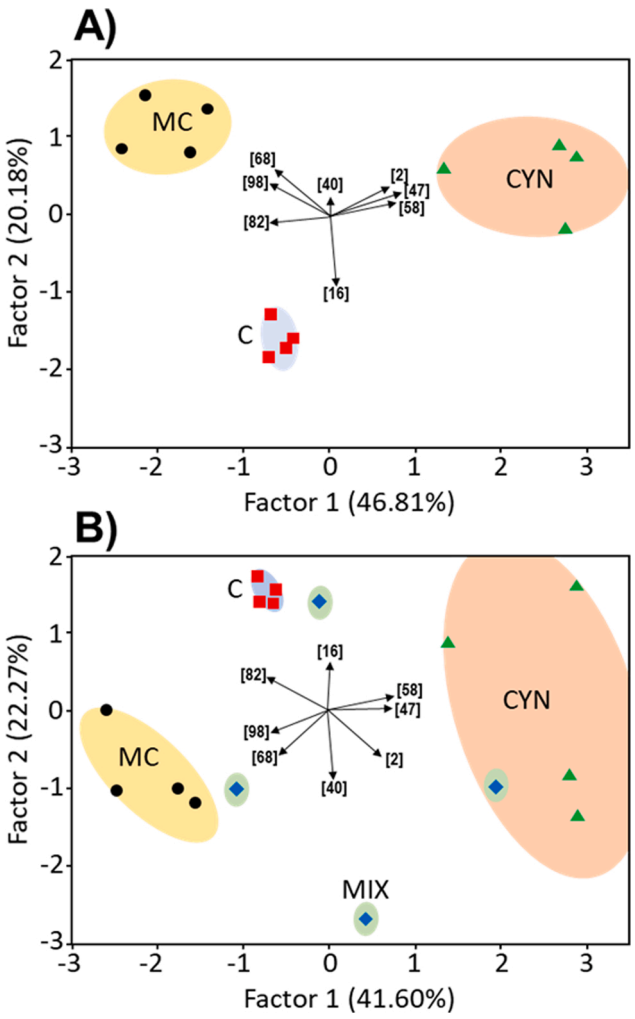

Fig. 3. Results of PCA analysis carried out with 8 selected compounds: [**2**] toluene (benzene, methyl), [**16**] trimethyl-pyrazine (2,3,5-Trimethylpyrazine), [**40**] methyl-indole (1H-Indole, 1-methyl-), [**47**] pentadecene (1-pentadecene), [**58**] dimethyl-carbazole (2,6-dimethyl-9H-carbazole), [**68**] pentadecanoic acid, [**82**] octadecanoic acid and [**98**] desmosterol acetate (cholesta-5,24-dien-3ol, acetate. (3.β)-). A) excluding the MIX; treatment and B) including the MIX treatment. These two figures include inserts with the PCA representation of the selected eight compounds. The place of these compounds in the quadrants explains their importance characterizing the three sets of treated mussels.

toxins.

In this respect, only a few studies based on *in vitro* assays have been conducted so far in relation to the potential interactions of MC-LR/CYN mixtures [37–39,59]. Firstly, Hercog et al. [60] studied the genotoxic effects of MC-LR/CYN mixtures in HepG2 cells and reported an antagonist interaction between these cyanotoxins under the conditions assayed, being the global effects comparable to those of CYN alone. Similar results were obtained by Diez-Quijada et al. [39] that assessed the genotoxicity and mutagenicity of MC-LR /CYN mixtures by a battery of *in vitro* assays. In this case, only positive results were obtained in the micronucleus test (MN) in the presence of the metabolic fraction S9, attributed mainly to the individual CYN. Regarding cytotoxicity studies, a higher effect was observed in HepG2 cells exposed to MC-LR/CYN combinations in comparison to the toxins alone after 24 h of exposure, although the calculated combination index revealed an antagonistic response [37]. In this work, the morphological changes were found more pronounced with the mixture of both cyanotoxins. These results are also in good agreement with those reported in undifferentiated and differentiated SH-SY5Y cells exposed to MC-LR and CYN individually and in

combination: although the mixture presented the highest cytotoxicity, the isobolograms method established that these toxins together induced an antagonistic response [38].

Due to the lack of studies about the simultaneous exposure to cyanotoxins mixtures, their toxicological profile, effects and mechanisms of action are still poorly known. Nonetheless, it is known that cyanotoxins mixtures can interact and regulate pertinent canonical pathways, biological processes and upstream regulators involved in cellular dysfunction, morbidity and development [61]. Therefore, studies like this one about potential effects of toxin exposure to MCs, CYN and their combination in aquatic animals such as molluscs are desirable. This relies in that these organisms are bioaccumulators, that are in fact in continuous contact with cyanotoxins [3]. Among these organisms, mussels are economically relevant filter animals, under constant exposure to cyanotoxins and their mixtures that, in turn, may cause alterations to their composition and metabolic pathways. To the best of our knowledge, there is no data on coincident bioaccumulation and potential interaction of MCs and CYN in mussels, even though the interest of the subject to assess their effects and interactions due to their simultaneous occurrence in the environment [1,33].

With respect to a possible effect of specific cyanotoxins and their mixtures on mussel fat metabolism, as observed here, it is known that exposure to MC-LR can induce hepatic lipid metabolism disorders in mice, hepatic lipid vacuoles accumulation and relative liver weight increase, which may be mediated by unsaturated fatty acid (UFA) biosynthesis and peroxisome proliferator-activated receptor (PPAR) activation [62]. Also, MC-LR it is well known to cause serious public health problems, including various liver diseases such as hepatitis, primary liver cancer, liver lipid peroxidation, and chronic liver injury [63]. He et al. [64] have also indicated that MC-LR can affect hepatic lipid metabolism and induce hepatic lipid metabolism abnormalities, but the potential mechanism of action remains unclear. Qin et al. [65] revealed that Endoplasmic reticulum (ER) stress plays a significant role in hepatic lipid metabolism abnormalities in mice exposed to MC-LR.

4. Conclusions

The combination of direct analytical pyrolysis (Py-GC/MS) of mussel flesh and appropriate chemometric treatments was useful in discriminating populations of molluscs not-exposed and exposed to different types of cyanotoxins and to mixtures. Specific pyrolysis compounds were found to have particular discrimination value and therefore with the potential as markers surrogated to the presence of cyanotoxins in mussel populations. Some of the compounds found as having the highest discriminant value can be also related with specific metabolic pathways that may be affected in the mollusc exposed to the toxins, including the fat and the protein metabolism. The results also suggest the existence of differential mechanism, may be exclusionary, by which the molluscs absorb or respond to the different toxins.

CRediT authorship contribution statement

Leticia Diez-Quijada: Investigation, Writing - review & editing. **Flavio L. de Oliveira:** Investigation, Writing - original draft. **Ángeles Jos:** Investigation, Methodology, Resources, Writing - original draft. **Ana M. Cameán:** Funding acquisition, Methodology, Supervision, Writing - review & editing. **Ramón Aparicio-Ruiz:** Formal analysis, Investigation, Visualization, Writing - review & editing. **Victor Vasconcelos:** Funding acquisition, Methodology, Writing - review & editing. **Alexandre Campos:** Investigation, Writing - original draft. **Francisco J. González-Vila:** Conceptualization, Methodology, Writing - review & editing. **José A. González-Pérez:** Conceptualization, Investigation, Methodology, Visualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jaap.2020.104970.

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