***In vitro* assessment of cyanotoxins bioaccessibility in raw and cooked mussels**

Leticia Díez-Quijada Jiménez, Remedios Guzmán-Guillén*, Antonio Cascajosa Lira, Ángeles Jos, Ana M. Cameán

Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Sevilla, Spain.

*Corresponding author: Remedios Guzmán-Guillén

Area of Toxicology, Faculty of Pharmacy, University of Sevilla.C/ Profesor García González, nº 2. C.P. 41012, Seville. Spain.

E-mail address: rguzman1@us.es

Abstract

The oral route by ingestion of water and food contaminated with cyanotoxins is the main route of exposure to these toxins. This study addresses for the first time the bioaccessibility of some of the most common Microcystins (MC-LR, MC-RR and MC-YR) and Cylindrospermopsin (CYN) simultaneously in raw and steamed mussels spiked at 250 ng/g fresh weight of each cyanotoxin, after an *in vitro* digestion, including the salivary (incubation with artificial saliva, 30s), gastric (with pepsin, 2h, pH 2), duodenal (with pancreatin and bile salts, 2h, pH 6.5) and colonic phases (with lactic-acid bacteria, 48h, pH 7.2). The results obtained suggest that the potential absorption of these cyanotoxins by consumption of contaminated mussels is lower than expected. After the total effect of cooking and digestion, the mean bioaccessibility levels recorded were 24.65% (CYN), 31.51% (MC-RR), 17.51% (MC-YR) and 13.20% (MC-LR). Moreover, toxins were transferred to the steaming waters at 3.77±0.24 μg L^{-1} CYN, 2.29±0.13 μg L^{-1} MC-LR, 6.60±0.25 μg L^{-1} MC-RR and 3.83±0.22 μg L^{-1} MC-YR. These bioaccessibility results should be considered for a more accurate risk assessment related to these cyanotoxins in mussels, including the fact that the steaming waters could also represent a risk after human consumption.

Keywords: Microcystins; Cylindrospermopsin; bioaccessibility; *in vitro* digestion; mussels; steaming

1. Introduction

The occurrence of cyanobacterial blooms has increased worldwide mainly due to anthropogenic activities, climate change and eutrophication (Buratti et al., 2017; Bormans et al., 2019). One of the problems generated by this growth is the production of cyanotoxins, considered as secondary metabolites (Carmichael, 1992) that cause a series of damages at environmental and economic levels, including human and animal health risks (Dodds et al., 2013; Codd et al., 2017).

Microcystins (MCs) are a group of cyclic heptapeptides with a specific beta amino acid-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) and two variable amino acids in positions 2 and 4, leading to more than 246 variants (Catherine et al., 2017), although the most commonly found in the environment are MC-LR, MC-RR and MC-YR (Svircev et al., 2017). They are potent inhibitors of protein phosphatases 1 and 2A, leading to diverse toxic effects *in vitro* (Pichardo et al., 2007; Puerto et al., 2009) and *in vivo*, mainly in the liver, but also in the lung, nervous and reproductive systems (Díez-Quijada et al., 2019a). Cylindrospermopsin (CYN) is an emerging cyanotoxin (Pichardo et al., 2017), a hydrophilic alkaloid containing a tricyclic guanidine moiety linked to a hydroxymethyluracil group (Ohtani et al., 1992). It is known to induce inhibition of glutathione and protein synthesis (Terao et al., 1994), oxidative stress mainly in liver and kidney (Puerto et al., 2011; Guzmán-Guillén et al., 2013), and potential neurotoxic, and progenotoxic effects (Guzmán-Guillén et al., 2015; Hercog et al., 2017; Puerto et al., 2018; Díez-Quijada et al., 2019b).

Unlike MCs, which are mainly intracellular, the extracellular concentrations of CYN are much higher, due to its hydrophilic nature (van Apeldoorn et al., 2007). The main route of exposure to these toxins is by consumption of contaminated water, although it can also occur through the intake of food (fish, shellfish or vegetables) or cyanobacterial

food supplements contaminated with cyanotoxins (Buratti et al., 2017). In fact, several authors have reported accumulation of MCs (Vasconcelos, 1995; Vareli et al., 2012; Gibble et al., 2016; Baralla et al., 2017; Kim et al., 2017) and CYN (Anderson et al., 2003; Saker et al., 2004) in mussels, resulting in potential human risks after their consumption (Ibelings and Chorus, 2007; Gutiérrez-Praena et al., 2013; Testai et al., 2016).

Based on the potential human health risks, a guideline value of 1 µg L^{-1} (in MC-LR equivalents) in drinking water was fixed by the World Health Organization for MCs (WHO, 1998). For CYN, due to its insufficient relevant toxicological data, only a proposed guideline of 1 µg L^{-1} has been adopted until now, and some countries set their own health advisories (Hoeger et al., 2005; Chorus, 2012). Besides, provisional tolerable daily intakes (TDI) of 0.04 and 0.03 µg/kg of body weight/day have been established for MCs and CYN, respectively (Sivonen and Jones, 1999; Humpage and Falconer, 2003).

Risk assessment evaluations on human consumption of cyanotoxin-contaminated food have normally compared the toxin concentrations in raw organisms directly with their respective TDI, but the influence that processing and cooking of these organisms may have on the concentration of cyanotoxins should be taken into account, because most of them are not usually consumed raw (Freitas et al., 2014; Maisanaba et al., 2017). In this sense, the amount ingested does not always coincide with the fraction of the contaminant that is released from the food matrix by digestive juices (known as bioaccessibility), that would lead to the amount available to be absorbed by the organism, since some metabolic processes might modify this fraction (Meca et al., 2012; Bordin et al., 2017). Otherwise, there may be an over-estimation of exposure levels via consumption of these contaminated foods.

Bioaccessibility can be calculated for several contaminants or ingredients in different food matrices by simulating *in vitro* the physiological conditions occurring *in vivo* (Guerra et al., 2012; Ménard et al., 2018). These methods usually include the oral, gastric and duodenal phases, and sometimes also a colonic fermentation, evaluating the presence of digestive enzymes, salts, pH, and digestion time (Minekus et al., 2014).

MCs stability in solutions has been evaluated after exposure to simulated gastric and intestinal conditions (Moreno et al., 2004; Smith et al., 2010). Focusing on food matrices, the bioaccessibility of lipophilic and hydrophilic marine toxins has been studied on seafood (including mollusks and pufferfish gonads) (Braga et al., 2016; Manita et al., 2017; Alves et al., 2019), and only the last two studies evaluated the effect of cooking processes on bioaccessibility. Concerning cyanotoxins, bioaccessibility assessment for MC-LR has been performed in clams (Freitas et al., 2014), and for CYN in mussels, fish and vegetables (Freitas et al., 2016; Maisanaba et al., 2017, 2018). However, the assessment of bioaccessibility in mussels did not include the colonic digestion; nonetheless a whole digestion should be simulated in order to investigate the passage through the entire human digestive tract and to obtain a more accurate human exposure scenario, following the EFSA (European Food Safety Authority) recommendations (Testai et al., 2016). Moreover, as far as we are concerned, there are currently no studies on the bioaccessibility of MCs and CYN together coexisting in a contaminated organism, which is an important aspect to consider, due to the existence of mixed blooms in nature (Minasyan et al., 2018).

In view of these reports, the aim of the present study was to investigate for the first time the influence of an *in vitro* digestion model including salivary, gastric, duodenal and colonic phases, to elucidate MCs (MC-LR, MC-RR and MC-YR) and CYN bioaccessibility on raw and steamed mussels contaminated with a combination of these

toxins under laboratory conditions, by a multitoxin method using Ultra performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS).

2. Materials and Methods

2.1. Chemical and reagents

Three congeners of MCs (MC-LR, MC-RR and MC-YR) (99% purity) and CYN (95% purity) were purchased from Enzo Life Sciences (Lausen, Switzerland) and resuspended in 100% methanol (MeOH) (MC-LR and –YR), 80% MeOH (MC–RR) and Milli-Q water (resistivity < 18 MΩ.cm; Millipore, Bedford, MA, USA) (CYN). Dichloromethane (DCM), Methanol (MeOH), formic acid (FA) and sodium hydroxide (NaOH) were purchased from Merck (Darmstadt, Germany). The graphitized carbon cartridges BOND ELUT® (500 mg, 6 mL) and C18 Bakerbond® (500 mg, 6 mL) were purchased from Agilent Technologies (Amstelveen, The Netherlands) and Dicsa (Andalucia, Spain), respectively. For the *in vitro* digestion model: FA (HCOOH), potassium chloride (KCl), potassium thiocyanate (KSCN), sodium dihydrogen-phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, mucin, chloride acid (HCl), pepsin, pancreatin and bile salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bacteria culture medium MRS was supplied by Oxoid (Madrid, España), and Microbiology Anaerocult® A, used to generate an oxygen-depleted and CO_2-enriched atmosphere, by Merck (Darmstadt, Germany). For UPLC–MS/MS analyses, reagents were of LC–MS grade: water and acetonitrile were supplied by VWR International (Fontenay-sous-Bois, France) and FA by Fluka (Steinheim, Germany).

2.2. *Experimental Setup and Sample Treatment*

Commercial mussels were bought in a supermarket and transferred to the laboratory. In the present work, uncooked and cooked mussels were tested as different matrices (3 samples per matrix). First of all, mussels were washed and separated from their shells, homogenized and then 9 portions of the homogenate (1 g fresh weight -f.w.- each) were spiked with 500 µL of a multitoxin solution (MC-LR, MC-RR, MC-YR and CYN) containing 500 ng/mL of each toxin, in order to obtain 250 ng/g f.w. in the mussel. This concentration was selected taking into account those reported in the literature for MCs (Kim et al., 2017; Turner et al., 2018) and CYN (Saker et al., 2004) in bivalves. Then, 3 of these samples were cooked by steaming for 2 min, as described in Guzmán-Guillén et al. (2017), once the water began to boil (100ºC), and afterwards they were submitted to the *in vitro* digestion model to assess toxins bioaccessibility (see section 2.3). Other 3 samples were used as controls to know the corresponding 100% of toxins for the uncooked and undigested mussels and, finally, the other 3 samples were cooked by steaming for 2 min,, to know the effect of this cooking practice on the cyanotoxins in mussels. The mussel samples were left at room temperature for 30 min, frozen at -80ºC and lyophilized (Cryodos 80, model 204, Telstar, Tarrasa, Spain) before toxins extraction and purification (see section 2.4). The mean weight loss after lyophilization was approximately of 85%. Moreover, one non-spiked mussel was submitted to the *vitro* digestion process to obtain the matrix necessary for the calibration curves in each digestion phase. After steaming the mussels, the waters were collected and extracted for toxin determination.

2.3. In vitro digestion model

The 3 cooked samples for digestion and the non-spiked mussel were submitted to an *in vitro* digestion model according to Maisanaba et al. (2017, 2018) (Figure 1). Hence, 2.5 g per sample were mixed with 1.5 mL of artificial saliva and 21 mL of water

into the plastic bag for digestion, then, these mixtures were homogenized by Stomacher IUL Instruments (Barcelona, Spain) for 30 s to simulate the human mastication process. Secondly, a pepsin solution was added and the pH was lowered to a value of 2 in order to activate the enzyme, then, water was added to reach 25 g and the mixture was incubated for 2h in an orbital shaker (37ºC, 250 rpm). Next, the pH was raised to 6.5 and pancreatin and bile salts were added, leaving the mixture for another 2h in the orbital shaker (37ºC, 250 rpm). Finally, to simulate the anaerobic conditions in the colon, a selection of lactic-acid bacteria (LAB) was added to this duodenal intestinal fluid at 10^8 CFU/mL, and incubated for 48h at 37ºC in anaerobic conditions (5% CO_2/95% air). The selected bacterial strains included some of the most predominant bacteria in the large intestine, namely *Lactobacillus casei* CECT 4180, *Lb. casei rhamnosus* CECT 278T, *Lb. plantorum* CECT 220, *Lb. delbur sub bulgaricus* CECT 4005, *Lb. salivarius* CECT 4305, *Lb. johnsonii* CECT 289, *Bifidobacterium breve* CECT 4839T and *B. bifidum* CECT 870T. All of them were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) in sterile 18% glycerol. After each digestion step, aliquots of each replicate sample were taken and centrifuged at 4000 rpm, 4ºC, 10 min. The supernatants obtained were filtered by 0.22 µm filters previous to the analysis to determine and quantify MCs and CYN bioaccessibility in each digestion phase.

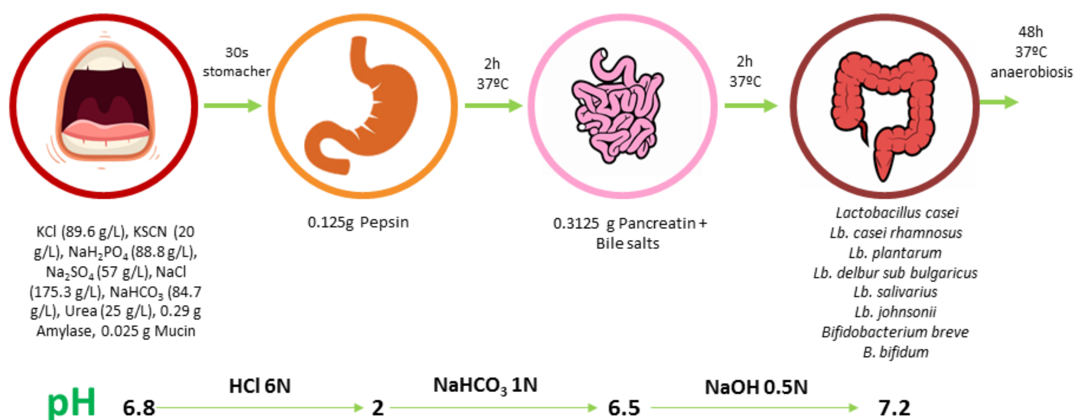

Figure 1. Procedure of the *in vitro* digestion model and composition of the digestion phases (salivary, gastric, duodenal and colonic).

2.4. Microcystin and Cylindrospermopsin extraction

The 6 mussel samples previously lyophilized were extracted following the multitoxin method recently validated by Díez-Quijada et al. (2020, in press). For this, 1 g f.w. was extracted with 90% MeOH (11.25 mL), sonicated and stirred for 15 min each step, centrifuged (3700 r.p.m., 15 min, 25ºC); the whole process was repeated once again. Then, the supernatants were pooled and concentrated in a rotary evaporator to reach 15% MeOH and, after pH adjustment to 11, the extracts were cleaned through a dual solid phase extraction (SPE) system consisting on a C18 cartridge (Bakerbond® 500 mg, 6 mL; Dicsa, Andalucia, Spain) and a porous graphitic carbon cartridge (BOND ELUT®, 500 mg, 6 mL, Agilent Technologies, Amstelveen, The Netherlands), as described in the cited work. At the end, the extracts were evaporated to dryness and resuspended in 1 mL 20% MeOH. For toxins extraction from waters, the method described by Zervou et al. (2017) with some modifications was followed. Briefly, 50 mL of water was mixed with 500 µL 100% MeOH, and the pH was risen to 11. Next, the extract was subjected to the same purification process with SPE cartridges used for mussels.

2.5. Toxin analysis by UPLC-MS/MS

Chromatographic separation was performed using a UPLC Acquity (Waters) coupled to a Xevo TQ-S micro (Waters, Milford, MA, USA) with a triple quadrupole mass spectrometer and an electrospray ion source operating in positive mode. The column was a 100 x 2.1 mm XSelect HSS T3 2.5 µm, working at a flow rate of 0.45 mL/min, and with a binary gradient consisting of (A) water and (B) acetonitrile, both containing a 0.1% FA (v/v). The injection volume was 5 µL, and the elution profile was: 2% B (0.8 min), linear gradient to 70% B (6.2 min), 100% B (1min), and finally, 2% B (2min). Multiple Reaction Monitoring (MRM) was applied, where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. Capillarity voltage: 1 Kv; source temperature: 500 ºC; source desolvation gas flow: 1000 L/h; and source cone gas flow: 50 L/h.

The transitions employed were: for MC-LR 996.5/135.0, 996.5/213.1 and 996.5/996.5; for MC-RR 520.2/135.0 and 1039.5/135.0; for MC-YR 1046.5/135.0, 1046.5/213.0 and 1046.5/1046.5; and for CYN 416.2/194.0 and 416.2/176.0. The first transitions were employed for quantification and the following for confirmation of each toxin.

2.6. Statistical analysis

Results were analyzed with the statistical package INSTANT, GraphPad™, applying a one-way analysis of variance (ANOVA), representing mean ± standard deviation (SD) of samples per group. Differences in mean values between groups were assessed by the Tukey´s test and were considered statistically significant from $p < 0.05$ level (99.5% confidence interval).

3. Results

3.1. Effects of cooking on contaminated mussels

The concentrations of the four toxins were determined in the cooked and undigested mussels, representing 73.01% (CYN), 79.19% (MC-LR), 69.72% (MC-RR) and 84.96% (MC-YR) of the total toxin concentrations in the uncooked controls, which represent 100% (250 ng/g f.w.) (Figure 2). It is important to keep in mind that the quantification method detects the free toxin, not the fraction bound to proteins.

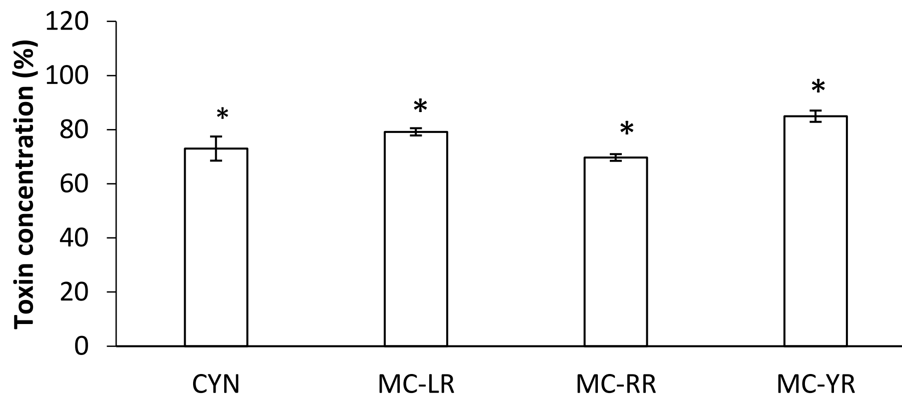

Figure 2. Effects of cooking on contaminated mussels compared to the uncooked control. The percentages reported are calculated considering the values detected in the cooked undigested mussels compared to that of the control (uncooked sample) (250 ng/g f.w.). *Statistical differences ($p < 0.001$) compared to the control.

3.2. Effects of digestion on contaminated mussels

After digestion of cooked mussels, toxin concentrations contained in the salivary, gastric, duodenal and colonic fluids were determined and compared to the corresponding toxin concentrations detected in cooked and undigested mussels, in order to know the effect that the digestion process alone would have on the mussels destined for human consumption.

In the case of CYN, its concentration in the salivary fluid fell to 69.60%, so the losses due to the salivary phase were of 30.40% CYN compared to the cooked and undigested mussels. A similar value was detected in the gastric phase (69.71%), and in the subsequent digestion phases, CYN bioaccessibility decreased to 44.65% and 33.77% in the duodenal and colonic fluids, respectively. Statistical differences ($p<0.001$) were found in all phases compared to the cooked and undigested mussels, and in the duodenal and colonic phases compared to the salivary and gastric phases (Figure 3a).

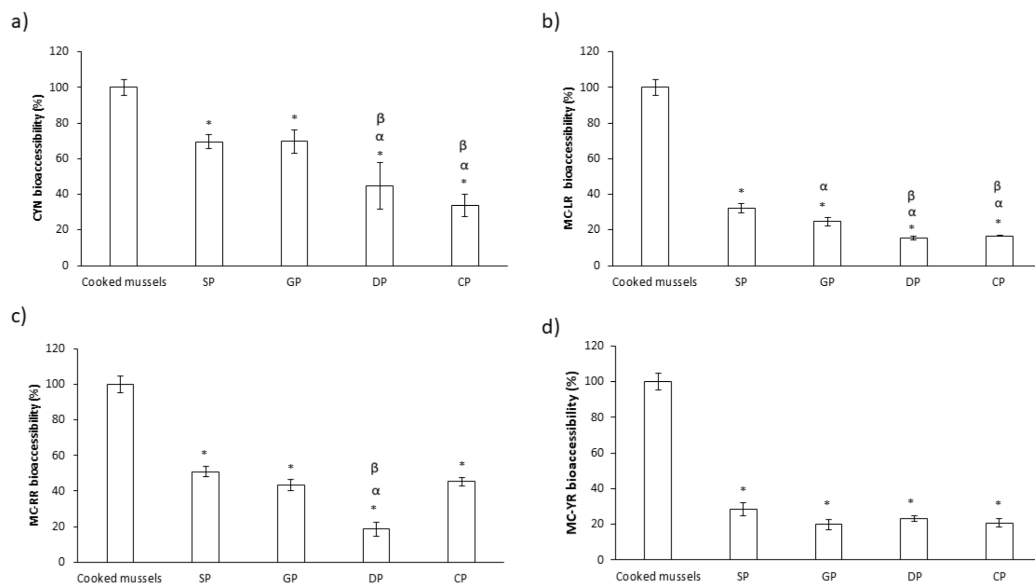

Figure 3. Bioaccessibility of CYN (a), MC-LR (b), MC-RR (c) and MC-YR (d) in cooked mussels compared to cooked and undigested mussels. SP: salivary phase; GP: gastric phase; DP: duodenal phase; CP: colonic phase. *Statistical differences ($p<0.001$) compared to cooked and undigested mussels; $^{\alpha}$ statistical differences ($p<0.001$) compared to salivary phase; $^{\beta}$ statistical differences ($p<0.001$) compared to gastric phase.

MC-LR concentration in the salivary phase decreased to 32.03% compared to the cooked and undigested mussels, meaning a reduction of 67.97%. Following with the digestion process, these values continued to decrease to 24.65%, 15.35% and 16.67%,

in the gastric, duodenal and colonic phases, respectively. Statistical differences ($p<0.001$) were found in all phases compared to the cooked and undigested mussels; in the gastric, duodenal and colonic phases compared to the salivary phase; and in the duodenal and colonic phases compared to the gastric one (Figure 3b).

For MC-RR, cooked mussels showed bioaccessibility values of 50.86% in the salivary fluid, 43.20% in the gastric phase, 18.51% in the duodenal phase, and 45.20% in the colonic phase. Statistical differences ($p<0.001$) were found in all phases compared to the cooked and undigested mussels; and only in the duodenal phase compared to the salivary and gastric ones (Figure 3c).

Bioaccessibility values for MC-YR were similar in the four digestion phases, showing values of 28.31% (salivary phase), 19.94% (gastric phase), 23.06% (duodenal phase) and 20.61% (colonic phase), representing the lowest values compared to the other cyanotoxins. Statistical differences ($p<0.001$) were only found in all phases compared to the cooked and undigested mussels (Figure 3d).

Figure 4 represents the total effect of cooking plus the digestion process for the four cyanotoxins, comparing all of them. For that purpose, the bioaccessibility values of cooked mussels in each phase have been compared to the value obtained for the control (uncooked and undigested mussels). As it can be observed, the highest bioaccessibility levels were recorded for CYN, followed by MC-RR, MC-YR and, finally, MC-LR, which showed the lowest values in all digestion phases. Statistical differences ($p<0.001$) were observed for all MCs versus CYN, for MC-LR and –YR compared to –RR only in the salivary and gastric phases, and for MC-LR compared to –YR in the duodenal and colonic phases.

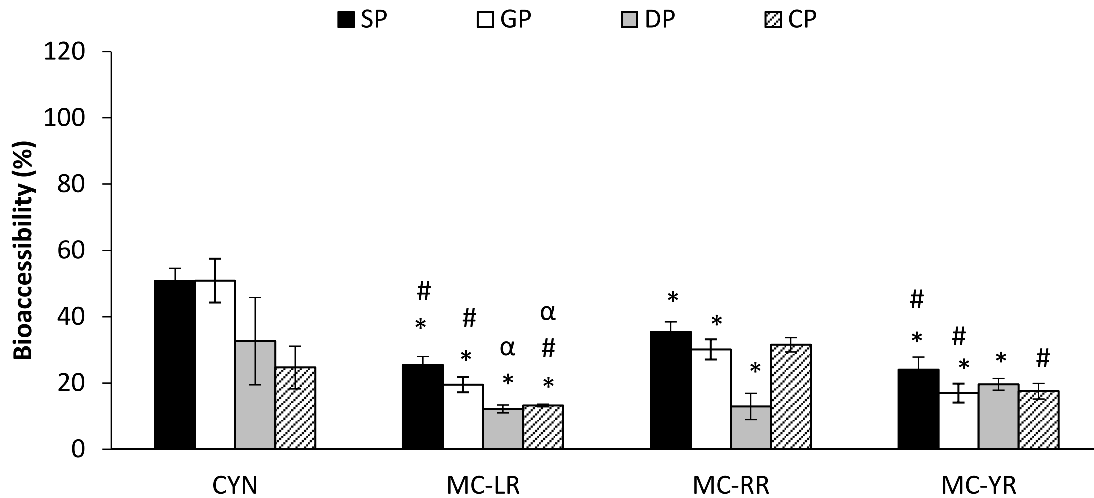

Figure 4 . Bioaccessibility of CYN, MC-LR, MC-RR and MC-YR in cooked mussels. SP: salivary phase; GP: gastric phase; DP: duodenal phase; CP: colonic phase. *Statistical differences (p<0.001) compared to CYN in the same digestion phase; #statistical differences (p<0.001) compared to MC-RR in the same digestion phase; αstatistical differences (p<0.001) compared to MC-YR in the same digestion phase.

3.3. MCs and CYN concentrations in the boiling waters

Cyanotoxins were detected in the waters used for steaming the mussels, at the following concentrations: 3.77 ± 0.24 μg L^{-1} CYN; 2.29 ± 0.13 μg L^{-1} MC-LR; 6.60 ± 0.25 μg L^{-1} MC-RR; 3.83 ± 0.22 μg L^{-1} MC-YR.

4. Discussion

Bioaccessibility is the amount of an ingested substance that is released from the matrix and solubilized into the water phase, becoming available for absorption in the systematic circulation through the digestive tract (Bordin et al., 2017). It is an important parameter to take into account in order to investigate a more realistic exposure to cyanotoxins in the frame of risk assessment (Meca et al., 2012), together with the effect of cooking processes, depending on the nature and the type of food (Domingo et al., 2011). The ingestion of food contaminated with cyanotoxins is the most common route

of chronic exposure to them, after ingestion of contaminated drinking water (Ibelings and Chorus, 2007). However, the fact that cyanotoxins are present in food does not mean that they could be absorbed, depending on various aspects, for example if the toxins are present on their free form or not. Indeed, food is submitted to processing and/or cooking before ingestion, and the heat may cause significant changes in the food matrix. In general, the effect of food processing on the bioaccessibility of compounds of interest depends on different aspects, such as the type of processing, the type of compound considered, the composition and structures of the matrix, and the potential presence of other components that may affect the absorption of the cited compound (Cilla et al., 2018). In fact, Alves et al. (2019) who studied the bioaccessibility of marine toxins in seafood, highlighted the importance of evaluating the effects of different culinary treatments, such as steaming, in other toxins groups.

In comparison with other models like *in vivo* feeding methods, that use animals or humans as model, and generally offer the most precise results, *in vitro* models are simpler, and represent lower costs and less ethical issues (Lucas-Gonzalez et al., 2018). Hence, recent studies about the correlation between *in vivo* and *in vitro* data on food digestion concludes that although *in vitro* static models are oversimplified and do not reproduce all the dynamic aspects of the gastrointestinal tract (only the main conditions: pH, enzymes and salts concentrations), they are increasingly useful in predicting *in vivo* digestion in some cases and have numerous advantages (Bohn et al., 2017). Some studies on marine toxins have been carried out using *in vitro* static models (Braga et al., 2016; Manita et al., 2017; Alves et al, 2019). The ideal *in vitro* digestion technique should provide precise results in a short time and help as an instrument for rapid analysis of food/s models with different structures and compositions (Coles et al., 2005).

This is the first work in which the bioaccessibility of MCs and CYN is investigated in mussels exposed simultaneously to both cyanotoxins with an *in vitro* dynamic digestion model from the salivary to the colonic phase. In fact, regarding cyanotoxins, the EFSA recommends the need to address the effects of food cooking and digestion on cyanotoxins, to know their bioaccessibility after an *in vitro* digestion model (Testai et al., 2016).

Globally, a progressive decrease in the bioaccessibility of the four toxins has been observed from the initial salivary phase to the final colonic step. Taking into account the whole process, which implies cooking and digestion, the bioaccessibility of cyanotoxins in mussels decreased in the following order: CYN > MC-RR > MC-YR > MC-LR. These results indicate that higher bioaccessibility values were obtained for the more hydrophilic cyanotoxins (CYN and MC-RR), and lower bioaccessibility values for the more lipophilic ones (MC-LR and MC-YR), in agreement with Alves et al. (2019), who reported the same trend for hydrophilic and lipophilic marine toxins.

Moreover, in the present work, toxins have been transferred from the mussels to the water used for steaming, in agreement with Freitas et al. (2016); in fact, heat process causes loss of water to the food matrix and, consequently, loss of water soluble compounds and/or some modifications in hydrophobic compounds too. Soaking and cooking of legumes could drain off the minerals, such as Fe and Zn, decreasing their bioaccessibility, probably because of mineral lost in the water (Sebastiá et al., 2001; Pereira et al., 2016). In the present study, regarding the effects of bioaccessibility in cooked mussels, the decrease observed in toxin bioaccessibility seems to be due to the combined effects of cooking and digestion, but mainly to the digestion process, not to the cooking itself. The main components of the food matrix in mussels are proteins and some fatty acids. Alves et al. (2019) suggested that thermal denaturation of proteins in

mussel tissues could explain the low bioaccessibility values obtained for the marine toxins azaspiracids (AZAs) in steamed mussels. Several authors have studied the effects of these matrix components on the bioaccessibility of bioactive compounds. For example, chlorogenic acid associated with milk caseins rather than with β-lactoglobulin, being this complex relatively stable in simulated gastric and intestinal phases, or flavonoids double bounded with the proteins present in the milk chocolate, decreasing their bioaccessibility and consequently their absorption (Lucas-González et al., 2018).

Regarding our results by comparing the effect of each digestive phase for individual cyanotoxins, MC-LR is the most sensitive to be reduced in cooked mussels, showing the lowest value at the end of the digestion process, and with significant differences from the gastric phase. The higher susceptibility of MCs in comparison to CYN may be due to their peptide structure, making them more sensitive to the pH conditions and enzymes (Moreno et al., 2004).

A survey performed about the *in vitro* digestion models applied to different type of foods revealed that the most common ones tested were vegetables (26%), dairy foods (23%), bakery foods (17%), meat products (13%), marine foods (12%) and eggs (7%) (Lucas-González et al., 2018). Certainly, there is a need to study cyanotoxins bioaccessibility on marine organisms, as they share the same habitat than cyanotoxins-producing cyanobacteria. Previously, bioaccessibility of both MCs and CYN has been studied separately by other authors in mollusks (Freitas et al., 2014, 2016), fish (Maisanaba et al., 2017) and vegetables (Maisanaba et al., 2018), showing different degrees of affectation. Among them, up to date, the only two works carried out in mollusks were focused in isolated cyanotoxins, such as MC-LR in clams (Freitas et al., 2014), and CYN in mussels (Freitas et al., 2016), but they did not take into account the colonic fermentation. So, this is the first study investigating the bioaccessibility of the

four cyanotoxins simultaneously in mussels by a complete digestion model, from mouth to colon. The application of gastric conditions to clams resulted in increased MC-LR bioaccessibility (Freitas et al., 2014), in contrast with our results for all MCs congeners, which showed diminished bioaccessibility values in this phase. Similarly, the concentrations of the three MC congeners (MC-LR, -RR and –YR) in water samples were decreased by pepsin and the lower pH of the stomach (Moreno et al., 2004). Regarding the duodenal phase, MC-LR bioaccessibility in mussels was reduced to 83% after proteolytic digestion by pancreatic enzymes (Freitas et al., 2014), although higher decreases were obtained in this work, showing a bioaccessibility value of 15.35% in this phase for the same cyanotoxin, if we only take into account the effect of digestion; and a similar value of 12.15% if cooking effects are also added. Pancreatin contains several enzymes (amylase, ribonuclease, lipase and trypsin) which could be involved in the hydrolysis of peptide bonds (Calatayud et al., 2012). Nevertheless, other authors suggested the stability of MCs congeners to intestinal enzymes, lowering its bioaccessibility only in 1-14% (Moreno et al., 2004) or causing no affectation after 6h of incubation. Our results obtained for the more lipophilic cyanotoxins (MC-LR and –YR) in steamed mussels were lower than those obtained for AZAs in steamed mussels (around 45%) (Alves et al., 2019), but agree with those obtained for the same lipophilic marine toxin in steamed mussels (around 10%) (Kilcoyne et al., 2014).

In previous studies carried out with marine toxins and with MCs where *in vitro* digestion is implicated, the colonic step is not taken into account, because *in vivo* food digestion and absorption of compounds mainly take place in the small intestine (Freitas et al., 2014; Braga et al., 2016; Freitas et al., 2016; Manita et al., 2017; Alves et al., 2019). Nevertheless, bacteria fermentation could be implicated in decreasing or increasing bioaccessibility, since several studies have reported that bacteria can free the

molecules from the food matrix (Lucas-Gonzalez et al., 2018). In this sense, fiber-entrapped polyphenols were poorly extractable and hardly soluble in gastrointestinal fluids, but the action of bacteria enzymes dramatically increased the antioxidant potential of the food residues in the lower gastrointestinal tract (Lucas-Gonzalez et al., 2018). Mercury was reduced from soluble fractions in solutions of this metal from mushrooms by application of LAB (Jadán-Piedra et al., 2017). Concerning cyanotoxins, Manubolu et al. (2014) suggested that the rumen microbial flora could protect ruminants from cyanotoxin intoxications due to the observed decrease in MC-LR, -RR and –YR and nodularin after incubation with rumen fluid for 3h. This finding suggested the need to include the bacteria fermentation in the *in vitro* digestion model and the harmonization of the different methods as Minekus et al. (2014) proposed with a consensus protocol, within the COST FA 1005 Action INFOGEST Network, based on human physiological conditions. The present work is the first one evaluating the effect of colonic conditions on MCs in exposed mussels. In this sense, it seems that LAB were not the main responsible for the highest decreases in MCs bioaccessibility in the present study, as their lowest values were shown in phases previous to the colonic one, especially for MC-LR and MC-YR, where the most drastic decrease is shown in the salivary phase, compared to MC-RR.

Moreover, in this work, in contrast to MCs, CYN showed to be more stable under the influence of the digestion process, as the bioaccessibility values are higher than those obtained for MCs for all digestion phases. When only the effect of digestion is taken into account, the lowest values for CYN were shown in the duodenal and colonic phases, with statistical differences compared to the previous ones. However, when the whole process of cooking and digestion is considered, minimum changes have been observed among salivary, gastric and duodenal phases in cooked mussels, despite

the different pH conditions suffered through the gastrointestinal tract, in agreement with Maisanaba et al. (2017, 2018) for CYN in fish and vegetables. This behavior was also reported by Alves et al. (2017, 2019) who observed higher bioaccessibility values for hydrophilic marine toxins compared to the lipophilic ones, being the first ones more easily transferred to the bioaccessible digestion fluids along the digestion process. In fact, those authors reported low values for tetrodotoxin after the duodenal phase, reaching values of around 20% (Alves et al., 2019), similar to the 25% we obtained in CYN values after the whole digestion process.

Adamski et al. (2016) reported the stability of CYN at 40ºC at several pH values including acidic conditions. The decrease begins to be significant in the duodenal phase compared to the salivary and gastric phases, with a bioaccessibility of 44.65%, similarly to the behavior of CYN in this phase obtained in fish muscle (Maisanaba et al., 2017). This lower CYN bioaccessibility from the duodenal intestinal phase could be explained by the adsorption of CYN in food. Our results also agree with those reported by Freitas et al. (2016) for CYN in steamed mussels, showing a sequential decrease on its bioaccessibility from the mouth to the small intestine. In the present work, there were no statistical differences on the effects on CYN bioaccessibility among the colonic and duodenal phases, in contrast to previous results obtained in other matrices (fish, vegetables) where the most drastic decreases were shown by the action of LAB (Maisanaba et al., 2017, 2018). Other authors have described a binding and degradation of some plant alkaloids by digestion enzymes similar to those employed in the present work, for example fungal α-amylase and bovine α-chymotrypsin (Zsila et al., 2011; Tintu et al., 2012). As CYN is an alkaloid, it is possible that these enzymes are able to degrade CYN too. CYN bioaccessibility decreases in the present work after cooking and digestion to a minimum of 24.65% in the colonic phase, similarly to the lowest value of

12.50% observed in steamed fish (Maisanaba et al., 2017), and the resemblance shown between mussels and fish matrices mainly due to the high protein content, may explain the similar value.

The results obtained contribute to a better understanding of the behavior of these cyanotoxins in mussels, by application of an *in vitro* digestion model, representing a useful tool for a more accurate risk assessment in humans exposed to cyanotoxin-contaminated mussels. Further studies would be useful to elucidate if the decrease observed in cyanotoxins bioaccessibility could result in the formation of decomposition products of the toxins.

5. Conclusions

In summary, this work evaluates for the first time the role of the digestion process on contents of MCs and CYN in raw and cooked mussels. At the concentration tested, the sequential pass through the digestive tract (including the four steps: salivary, gastric, duodenal and colonic) may be effective in the reduction of bioaccessibility in mussels. Globally, taking into account the total effect of cooking and digestion, MCs seems to be more sensitive than CYN in the presence of digestive juices, reaching bioaccessibility values around 22%. These bioaccessibility results suggest that previous data on cyanotoxins exposure could be over-estimated, if the total processes of cooking and digestion are not taken into account. Moreover, toxins have been transferred from the mussels to the water used for steaming, with values in the range 2.29-6.60 $\mu g\ L^{-1}$. Therefore, this fact should be also considered for a more realistic risk assessment of cyanotoxins exposure through consumption of contaminated mussels.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to acknowledge the Ministerio de Economía y Competitividad of Spain (AGL2015-64558-R, MINECO/FEDER, UE) for its financial support, and the grant FPI (BES-2016–078773) awarded to Leticia Díez-Quijada Jiménez.

References

Adamski, M., Zmudzki, P., Chrapusta, E., Kaminski, A., Bober, B., Zabaglo, K., Bialczyk, J., 2016. Characterization of cylindrospermopsin decomposition products formed under irradiation conditions. Algal Res. 18, 1–6. https://doi.org/10.1016/j.algal.2016.05.027.

Alves, R.N., Maulvault, A.L., Barbosa, V.L., Cunha, S., Kwadijk, C.J.A.F., Álvarez-Muñoz, D., Rodríguez-Mozaz, S., Aznar-Alemany, Ò., Eljarrat, E., Barceló, D., Fernandez-Tejedor, M., Tediosi, A., Marques, A., 2017. Preliminary assessment on the bioaccessibility of contaminants of emerging concern in raw and cooked seafood. Food Chem. Toxicol. 104, 69–78. https://doi.org/10.1016/j.fct.2017.01.029.

Alves, R.N., Rambla-Alegre, M., Braga, A.C., Maulvault, A.L., Barbosa, V., Campàs, M., Reverté, L., Flores, C., Caixach, J., Kilcoyne, J., Costa, P.R., Diogène, J., Marques, A., 2019. Bioaccessibility of lipophilic and hydrophilic marine biotoxins in seafood: An *in vitro* digestion Approach. Food Chem. Toxicol. 129, 153-161. https://doi.org/10.1016/j.fct.2019.04.041.

Anderson, L., Fabbro, L.D., Cowden, K., 2003. Assessment of Blue-Green Algal Toxins in Barramundi, Red Clay and Mussels from Awoonga Dam. Central Queensland University, Gladstone, Australia.

Baralla, E., Varoni, M.V., Sedda, T., Pasciu, V., Floris, A., Demontis, M.P., 2017. Microcystins presence in mussels (*M. galloprovincialis*) and water of two productive mediterranean's lagoons (Sardinia, Italy). BioMed Res. Int. 2017, 1–7. https://doi.org/10.1155/2017/3769245.

Bordin, K., Saladino, F., Fernández-Blanco, C., Ruiz, M.J., Mañes, J., Fernández-Franzón, M., Meca, G., Luciano, F.B., 2017. Reaction of zearalenone and a-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro*. Food Chem. 217, 648–654. https://doi.org/10.1016/j.foodchem.2016.09.044.

Bohn, T., Carriere, F., Day, L., Deglaire, A., Egger, L., Freitas, D., Golding, M., Le Feunteun, S., Macierzanka, A., Menard, O., Miralles, B., Moscovici, A., Portmann, R., Recio, I., Rémond, D., Santé-Lhoutelier, V., Wooster, T.J., Lesmes, U., Mackie, A.R., Dupont, D., 2017. Correlation between *in vitro* and *in vivo* data on food digestion. What can we predict with static in vitro digestion models? Crit. Rev. Food Sci. Nutr., 59, 1307–1323. https://doi.org/10.1080/10408398.2017.1315362.

Bormans, M., Amzil, Z., Mineaud, E., Brient, L., Savar, V., Robert, E., Lance, E., 2019. Demonstrated transfer of cyanobacteria and cyanotoxins along a freshwater-marine continuum in France. Harmful Algae. 87- 101639. https://doi.org/10.1016/j.hal.2019.101639.

Braga, A.C., Alves, R.N., Maulvault, A.L., Barbosa, V., Marques, A., Costa, P.R., 2016. *In vitro* bioaccessibility of the marine biotoxin okadaic acid in shellfish. Food Chem. Toxicol. 89, 54-59. http://dx.doi.org/10.1016/j.fct.2016.01.008.

Buratti, F.M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., Funari, E., 2017. Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch. Toxicol. 91, 1049-1130. https://doi.org/10.1007/s00204-016-1913-6.

Carmichael, W.W., 1992. Cyanobacteria secondary metabolites- the cyanotoxins. J. Appl. Microbiol. 2, 445–59. https://doi.org/10.1111/j.1365-2672.1992.tb01858.x.

Catherine, A., Bernard, C., Spoof, L., Bruno, M., 2017. Mycrocistins and Nodularins. In: Meriluoto, J., Spoof, L., Cood, G.A. (Eds.), Handbook of cyanobacterial monitoring and cyanotoxin analysis, first ed. John Wiley & Sons. Pp. 109-126. https://doi.org/10.1002/9781119068761.ch11.

Calatayud, M., Devesa, V., Virseda, J.R., Barberá, R., Montoro, R., Vélez, D., 2012. Mercury and selenium in fish and shellfish: occurrence, bioaccessibility and uptake by Caco-2 cells. Food Chem. Toxicol. 50, 2696–2702. https://doi.org/10.1016/j.fct.2012.05.028.

Chorus, I., 2012. Current Approaches to Cyanotoxin Risk Assessment, Risk Management and Regulations in Different Countries. Federal environment Agency, Germany.

Cilla, A., Bosch, L., Barberá, R., Alegría, A., 2018. Effect of processing on the bioaccessibility of bioactive compounds- A review focusing on carotenoids, minerals, ascorbic acid, tocopherols and polyphenols. J. Food Compos. Anal. 63, 3-15. https://doi.org/10.1016/j.jfca.2017.01.009.

Codd, G.A., Meriluoto, J., Metcalf, J.S., 2017. Introduction: Cyanobacteria, Cyanotoxins, their human impact, and risk management. In: Meriluoto, J., Spoof, L., Cood, G.A. (Eds.), Handbook of cyanobacterial monitoring and cyanotoxin analysis, first ed. John Wiley & Sons. pp. 3-18. https://doi.org/10.1002/9781119068761.ch11.

Coles, L. T., Moughab, P. J., Darragh, A. J., 2005. *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. Animal Feed Sci. Tech. 123–124, 421–444. https://doi.org/10.1016/j.anifeedsci.2005.04.021.

Díez-Quijada, L., Puerto, M., Guitiérrez-Praena, D., Llana-Ruiz-Cabello, M., Jos, Á., Cameán, A.M., 2019a. Microcystin-RR: Occurrence, content in water and food and toxicological studies. A review. Environ. Research. 168, 467-489. https://doi.org/10.1016/j.envres.2018.07.019.

Díez-Quijada, L., Llana-Ruiz-Cabello, M., Cătunescu, M.G., Puerto, M., Moyano, R., Jos, Á., Cameán, A.M., 2019b. *In vivo* genotoxicity evaluation of cylindrospermopsin in rats using a combined micronucleus and comet assay. Food Chem. Toxicol. 132, 110664. https://doi.org/10.1016/j.fct.2019.110664.

Diez-Quijada, L., Guzmán-Guillén, R., Cătunescu, G.M., Campos, A., Vasconcelos, V., Jos, Á., Cameán, A.M., 2020. New method for the simultaneous determination of cyanotoxins (Microcystins and Cylindrospermopsin) in mussels using SPE-UPLC-MS/MS. Environ. Res. (in press). https://doi.org/10.1016/j.envres.2020.109284,

Dodds, W.K., Perkin, J.S., Gerken, J.E., 2013. Human Impact on Freshwater Ecosystem Services: A Global Perspective. Environ. Sci. Technol. 47, 9061−9068. https://doi.org/10.1021/es4021052.

Domingo, J.L., 2011. Influence of Cooking Processes on the Concentrations of Toxic Metals and Various Organic Environmental Pollutants in Food: A Review of the Published Literature. Crit. Rev. Food Sci. Nutr. 51, 29-37. https://doi.org/10.1080/10408390903044511.

Freitas, M., Azevedo, J., Carvalho, A.P., Campos, A., Vasconcelos, V., 2014. Effects of storage, processing and proteolytic digestion on microcystin-LR concentration in edible clams. Food Chem. Toxicol. 66, 217–23. https://doi.org/10.1016/j.fct.2014.01.041.

Freitas, M., Azevedo, J., Carvalho, A.P., Mendes, V.M., Manadas, B., Campos, A., Vasconcelos, V., 2016. Bioaccessibility and changes on cylindrospermopsin

concentration in edible mussels with storage and processing time. Food Control. 59, 567-574. https://doi.org/10.1016/j.foodcont.2015.06.025.

Gibble, C.M., Peacock, M.B., Kudela, R.M., 2016. Evidence of freshwater algal toxins in marine shellfish: Implications for human and aquatic health. Harmful Algae. 59, 59-66. https://doi.org/10.1016/j.hal.2016.09.007.

Guerra, A., Eienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., Alric, Mo., 2012. Relevance and challenges in modeling human gastric and small intestinal digestion. Trends Biotechnol. 30, 591-600. https://doi.org/10.1016/j.tibtech.2012.08.001.

Gutiérrez-Praena, D., Jos, Á., Pichardo, S., Moreno, I.M., Cameán, A.M., 2013. Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A review. Food Chem. Toxicol. 53, 139-152. https://doi.org/10.1016/j.fct.2012.10.062.

Guzmán-Guillén, R., Prieto, A.I., Vasconcelos, V.M., Cameán A.M., 2013. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*). Chemosphere. 90, 1184-1194. https://doi.org/10.1016/j.chemosphere.2012.09.027.

Guzmán-Guillén, R., Lomares, I., Moreno, I.M., Prieto-Ortega, A.I., Moyano, R., Blanco, A., Cameán, A.M., 2015. Cylindrospermopsin induces neurotoxicity in tilapia fish (*Oreochromis niloticus*) exposed to *Aphanizomenon ovalisporum*. Aquat. Toxicol. 161, 17-24. https://doi.org/10.1016/j.aquatox.2015.01.

Guzmán-Guillén, R., Maisanaba, S., Prieto, A.I., Valderrama-Fernández, R., Jos, Á., Cameán, A.M., 2017. Changes on cylindrospermopsin concentration and

characterization of decomposition products in fish muscle (*Oreochromis niloticus*) by boiling and steaming. Food Control. 77, 210-220. https://doi.org/10.1016/j.foodcont.2017.02.035.

Hercog, K., Maisanaba, S., Filipic, M., Jos, Á., Cameán, A.M., Zegura, B., 2017. Genotoxic potential of the binary mixture of cyanotoxins microcystin-LR and cylindrospermopsin. Chemosphere. 189, 319-329. https://doi.org/10.1016/j.chemosphere.2017.09.075.

Hoeger, S.J., Hitzfeld, B.C., Dietrich, D.R., 2005. Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants. Toxicol. Appl. Pharmacol. 203, 231–242. https://doi.org/10.1016/j.taap.2004.04.015.

Humpage, A.R., Falconer, I.R., 2003. Oral toxicity of the cyanobacterial toxin Cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ. Toxicol. 18, 94-103. https://doi.org/10.1002/tox.10104.

Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater “seafood” and its consequences for public health: A review. Environ. Pollut. 150, 177–192. https://doi.org/10.1016/j.envpol.2007.04.012.

Jadán-Piedra, C., Alcántara, C., Monedero, V., Zúñiga, M., Vélez, D., Devesa, V., 2017. The use of lactic acid bacteria to reduce mercury bioaccesibility. Food Chem. 228, 158–166. https://doi.org/10.1016/j.foodchem.2017.01.157.

Kilcoyne, J., Jauffrais, T., Twiner, M.J., Doucette, G.J., Aasen, J.A., Sosa, S., Krock, B., Séhet, V., Nulty, C., Salas, R., Clarke, D., Geraghty, J., Duffy, C., Foley, B., John, U., Quilliam, M.A., McCarron, P., Miles, C.O., Silke, J., Cembella, A., Tillmann, U., Hess, P., 2014. Azaspiracids—Toxicological Evaluation, Test Methods and

Identification of the Source Organisms (ASTOX II). Technical Report. Marine Institute, Galway, Ireland (ISSN:2009-3195. 188 pp).

Kim, M.S., Lee, Y.J., Ha, S.Y., Kim, B.H., Hwang, S.J., Kown, J.T., Choi, J.W., Shin, K.H., 2017. Accumulation of Microcystin (LR, RR and YR) in Three Freshwater Bivalves in *Microcystis aeruginosa* bloom using dual isotope tracer. Mar. Drugs. 15, 226. https://doi.org/10.3390/md15070226.

Lucas-González, R., Viuda-Martos, M., Pérez-Álvarez, J.A., Fernández-López, J., 2018. *In vitro* digestion models suitable for foods: Opportunities for new fields of application and challenges. Food Res. Int. 107, 423-436. https://doi.org/10.1016/j.foodres.2018.02.055.

Maisanaba, S., Saladino, F., Font, G., Jos, Á., Cameán, A.M., Meca, G., 2017. Bioaccesibility of Cylindrospermopsin from cooked fish muscle after the application of an *in vitro* digestion model and its bioavailability. Food Chem. Toxicol. 110, 360-370. http://dx.doi.org/10.1016/j.fct.2017.10.052.

Maisanaba, S., Guzmán-Guillén, R., Valderrama, R., Meca, G., Font, G., Jos, Á., Cameán, A.M., 2018. Bioaccessibility and decomposition of cylindrospermopsin in vegetables matrices after the application of an *in vitro* digestion model. Food Chem. Toxicol., 120, 164-171. https://doi.org/10.1016/j.fct.2018.07.013.

Manita, D., Alves, R.N., Braga, A.C., Fogaça, F.H.S., Marques, A., Costa, P.R., 2017. *In vitro* bioaccessibility of the marine biotoxins okadaic acid, dinophysistoxin-2 and their 7-O-acyl fatty acid ester derivatives in raw and steamed shellfish. Food Chem. Toxicol. 101, 121-127. http://dx.doi.org/10.1016/j.fct.2017.01.008.

Manubolu, M., Madawala, S.R.P., Dutta, P.C., Malmlof, K., 2014. *In vitro* biodegradation of cyanotoxins in the rumen fluid of cattle. BMC Veter. Res. 10, 110–117. doi: 10.1186/1746-6148-10-110.

Meca, G., Mañes, J., Font, G., Ruiz, M.J., 2012. Study of the potential toxicity of enniatins A, A_1, B, B_1 by evaluation of duodenal and colonic bioavailability applying an *in vitro* method by Caco-2 cells. Toxicon. 59, 1-11. https://doi.org/10.1016/j.toxicon.2011.10.004.

Menárd, O., Bourlieu, C., De Oliveira, S.C., Dellarosa, N., Laghi, L., Carrière, F., Capozzi, F., Dupont, D., Deglaire, A., 2018. A first step towards a consensus status *in vitro* model for simulating full-term infant digestion. Food Chem. 240, 338–345. https://doi.org/10.1016/j.foodchem.2017.07.145.

Minasyan, A., Christophoridis, C., Wilson, A.E., Zervou, S.K., Kaloudis, T., Hiskia, A., 2018.Diversity of cyanobacteria and the presence of cyanotoxins in the epilimnion of Lake Yerevan (Armenia). Toxicon. 150, 28–38. https://doi.org/10.1016/j.toxicon.2018.04.021

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Eggeg, L., Golding, M., Korakaya, S., Kirklus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickman, M.S., Weitchies, W., Brodkorb, A., 2014. A standardised static *in vitro* digestion method suitable for food – an international consensus. Food Funct. 5, 1113–1124. https:// doi: 10.1039/c3fo60702j.

Moreno, I.M., Maraver, J., Aguete, E.C., Leao, M., Gago-Martínez, A., Cameán, A.M., 2004. Decomposition of Microcystin-LR, Microcystin-RR, and Microcystin-YR in

Water Samples Submitted to in Vitro Dissolution Tests. J. Agric. Food Chem. 52, 5933-5938. https://doi.org/10.1021/jf0489668.

Ohtani, I., Moore, R.E., Runnegar, M.T.C., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. J. Am. Chem. Soc. 114, 7941–7942. https://doi.org/10.1021/ja00046a067.

Pereira, E.J., Carvalho, L.M., Dellamora-Ortiz, G.M., Cardoso, F.S., Carvalho, J.L., 2016. Effect of different home-cooking methods on the bioaccessibility of zinc and iron in conventionally bred cowpea (*Vigna unguiculata* L. Walp) consumed in Brazil. Food Nutr. Res. 60, 29082. https://doi.org/10.3402/fnr.v60.29082.

Pichardo, S., Jos, A., Zurita, J.L., SaIguero, M., Camean, A.M., Repetto, G., 2007. Acute and subacute toxic effects produced by microcystin-YR on the fish cell lines RTG-2 and PLHC-1. Toxicol. in vitro 21, 1460-1467. https://doi.org/10.1016/j.tiv.2007.06.012.

Pichardo, S., Cameán, A.M., Jos, Á., 2017. *In vitro* toxicological assessment of cylindrospermopsin: A review. Toxins. 9, 402. https://doi.org/10.3390/toxins9120402.

Puerto, M., Pichardo, S., Jos, Á., Cameán, A.M., 2009. Comparison of the toxicity induced by microcystin-RR and microcystin-YR in differentiated and undifferentiated Caco-2 cells. Toxicon. 54, 161-169. https://doi.org/10.1016/j.toxicon.2009.03.030.

Puerto, M., Jos, Á., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M., 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology. 20, 1852-1860. https://doi.org/10.1007/s10646-011-0723-0.

Puerto, M., Prieto, A.I., Maisanaba, S., Gutiérrez-Praena, D., Mellado-García, P., Jos, Á., Cameán, A.M., 2018. Mutagenic and genotoxic potential of pure Cylindrospermopsin by a battery of *in vitro tests*. Food Chem. Toxicol. 121, 413-422. https://doi.org/10.1016/j.fct.2018.09.013.

Saker, M.L., Metcalf, J.S., Codd, G.A., Vasconcelos, V.M., 2004. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel *Anodonta cygnea*. Toxicon. 43, 185-194. https://doi.org/10.1016/j.toxicon.2003.11.022.

Sebastiá, V., Barberá, R., Farré, R., Lagarda, M.J., 2001. Effects of legume processing on calcium: iron and zinc contents and dialysabilites. J. Sci. Food Agric. 81, 1180–1185. https://doi.org/10.1080/10408398.2017.1315362.

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management. E and FN Spon, London/New York, pp. 41–111. http://

apps.who.int/iris/bitstream/10665/42827/1/0419239308_eng.pdf.

Smith, J.L., Schulz, K.L., Zimba, P.V., Boyer, G.L., 2010. Possible mechanism for the foodweb transfer of covalently bound microcystins. Ecotoxicol Environ Saf. 73, 757–761. https://doi.org/10.1016/j.ecoenv.2009.12.003.

Svirčev, Z., Drobac, D., Tokodi, N., Mijović, B., Codd, G.A., Meriluoto, J., 2017. Toxicology of microcystins with reference to cases of human intoxications and epidemiological investigations of exposures to cyanobacteria and cyanotoxins. Arch. Toxicol. 91, 621–650. https://doi: 10.1007/s00204-016-1921-6.

Terao, K., Ohmori, S., Igarashi, K., Ohtani, I., Watanabe, M.F., Harada, K.I., Ito, E., Watanabe, M., 1994. Electron microscopic studies on experimental poisoning in mice

induced by cylindrospermopsin isolated from blue-green alga *Umezakia natans*. Toxicon. 32, 833–843. https://doi.org/10.1016/0041-0101(94)90008-6.

Testai, E., Buratti, F.M., Funari, E., Manganelli, M., Vichi, S., Arnich, N., Biré, R., Fessard, V., Sialehaamoa, A., 2016. Review and analysis of occurrence, exposure and toxicity of cyanobacteria toxins in food. EFSA Supporting Publication 2016: EN-998. 309 pp. https://doi.org/10.2903/sp.efsa.2016.EN-998.

Tintu, I., Dileep, K.V., Augustine, A., Sadasivan, C., 2012. An isoquinoline alkaloid, berberine, can inhibit fungal alpha amylase: enzyme kinetic and molecular modeling studies. Chem. Biol. Drug. Des. 80, 554-560. https://doi.org/10.1111/j.1747-0285.2012.01426.x.

Turner, A.D., Waack, J., Lewis, A., Edwards, C., Lawton, L., 2018. Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders. J. Chromatogr. B 1074-1075, 111-123. https://doi.org/10.1016/j.jchromb.2017.12.032.

van Apeldoorn, M.E., van Egmond, H.P., Speijers, G.J.A., Bakker, G.J.I., 2007. Toxins of cyanobacteria. Mol. Nutr. Food Res. 51, 7-60. https://doi.org/10.1002/mnfr.200600185.

Vareli, K., Zarali, E., Zacharioudakis, G.S.A., Vagenas, G., Varelis, V., Pilidis, G., Briasoulis, E., Sainis, I., 2012. Microcystin producing cyanobacterial communities in Amvrakikos Gulf (Mediterranean Sea, NW Greece) and toxin accumulation in mussels (*Mytilus galloprovincialis*). Harmful Algae. 15, 109–118. https://doi.org/10.1016/j.hal.2011.12.005.

Vasconcelos, V.M., 1995. Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis*. Aquat. Toxicol. 32, 227–237. https://doi.org/10.1016/0166-445X(94)00085-5.

World Health Organization. Division of Operational Support in Environmental Health. (1998). Guidelines for drinking-water quality. Vol. 2, Health criteria and other supporting information: addendum, 2nd ed. World Health Organization. https://apps.who.int/iris/handle/10665/63844.

Zervou, S.K., Christophoridis, C., Kaloudis, T., Triantis, T.M., Hiskia, A., 2017. New SPE-LC-MS/MS method for simultaneous determination of multi-class cyanobacterial and algal toxins.J. Hazard. Mater. 323, 56–66. https://doi.org/10.1016/j.jhazmat.2016.07.020.

Zsila, F., Kámán, J., Bogányi, B., Józsvai, D., 2011. Binding of alkaloids into the S1 specificity pocket of a-chymotrypsin: evidence from induced circular dichroism spectra. Org. Biomol. Chem. 9,4127-4137. DOI:10.1039/c0ob01221a.

Figure captions

Figure 1. Procedure of the *in vitro* digestion model and composition of the digestion phases (salivary, gastric, duodenal and colonic).

Figure 2. Effects of cooking on contaminated mussels compared to the uncooked control. The percentages reported are calculated considering the values detected in the cooked undigested mussels compared to that of the control (uncooked sample) (250 ng/g f.w.). *Statistical differences ($p < 0.001$) compared to the control.

Figure 3. Bioaccessibility of CYN (a), MC-LR (b), MC-RR (c) and MC-YR (d) in cooked mussels compared to cooked and undigested mussels. SP: salivary phase; GP: gastric phase; DP: duodenal phase; CP: colonic phase. *Statistical differences ($p<0.001$) compared to cooked and undigested mussels; $^{\alpha}$ statistical differences ($p<0.001$) compared to salivary phase; $^{\beta}$ statistical differences ($p<0.001$) compared to gastric phase.

Figure 4. Bioaccessibility of CYN, MC-LR, MC-RR and MC-YR in cooked mussels. SP: salivary phase; GP: gastric phase; DP: duodenal phase; CP: colonic phase. *Statistical differences ($p<0.001$) compared to CYN in the same digestion phase; $^{\#}$statistical differences ($p<0.001$) compared to MC-RR in the same digestion phase; $^{\alpha}$statistical differences ($p<0.001$) compared to -YR in the same digestion phase.

Supplementary material

Table S1. Toxins concentrations (CYN, MC-LR, MC-RR and MC-YR) detected in control mussels (uncooked), in cooked and undigested mussels and in cooked and digested mussels. SP: salivary phase; GP: gastric phase; DP: duodenal phase; CP: colonic phase.

	Control mussels (uncooked) (ng/g f.w.)	**Cooked and undigested mussels (ng/g f.w.)** (mean ± SD)	**Cooked and digested mussels (ng/g f.w.)** (mean ± SD)			
			SP	**GP**	**DP**	**CP**
CYN	250	182.52 ± 11.15	127.03 ± 6.93	127.23 ± 12.08	81.50 ± 24.05	61.63 ± 11.78
MC-LR	250	197.99 ± 3.35	63.41 ± 5.20	48.81 ± 4.66	30.38 ± 2.36	33.01 ± 0.77
MC-RR	250	174.29 ± 3.10	88.65 ± 5.18	75.29 ± 5.30	32.26 ± 6.93	78.77 ± 3.77
MC-YR	250	212.41 ± 5.20	60.14 ± 7.99	42.35 ± 6.08	49.00 ± 3.77	43.77 ± 5.02