Bioaccessibility of lipophilic and hydrophilic marine biotoxins in seafood: an *in vitro* digestion approach

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Abstract

This study aimed to assess the bioaccessibility of different marine biotoxins in naturally contaminated shellfish and fish gonads using an *in vitro* digestion methodology. In general, hydrophilic toxins (domoic acid, paralytic shellfish poisoning toxins and tetrodotoxins) showed higher bioaccessibility than lipophilic ones (okadaic acid and azaspiracids). The bioaccessibility of toxins from the okadaic acid group ranged from 69 % (raw European razor clams) to 74 % (raw donax clams). Regarding azaspiracids, 47 % of the initial content was bioaccessible in steamed blue mussel. As for hydrophilic toxins,

100 % of the initial content was bioaccessible after digestion in raw shellfish and puffer fish gonads. The total tetrodotoxin bioaccessibility in puffer fish gonads decreased significantly after steaming. The profile of tetrodotoxins changed during the digestion process: TTX and 11-norTTX-6*S*-ol analogues decreased significantly after digestion, but the 5,6,11-trideoxy TTX analogue increased in both raw and steamed puffer fish gonads. These preliminary findings confirm the need to consider bioaccessibility data in future seafood risk assessment, as such information enables a more accurate and realistic estimation of potential seafood hazards, particularly in what concerns lipophilic toxins, therefore, constituting a crucial tool in the refinement of regulatory limits for the presence of biotoxins in seafood.

Keywords: seafood, bioaccessibility, okadaic acid, azaspiracids, domoic acid, PSP toxins, tetrodotoxins

1. Introduction

Some marine biotoxins are produced by specific harmful algae which may proliferate causing harmful algal blooms (HABs) under specific climatic and environmental conditions (Gerssen et al., 2010; Zingone and Enevoldsen, 2000). These toxins have been considered as a global problem, since they may represent a threat to human health, particularly due to human food-borne illnesses. In addition, HABs outbreaks can cause severe economic losses to the shellfish and fish industries due to the closure of harvesting areas (Berdalet et al., 2015; Visciano et al., 2016).

Some marine biotoxins present a complex chemical structure and toxicity, and may be classified in two large groups: lipophilic and hydrophilic toxins (Chen et al., 2016a; Gerssen et al., 2010; Toyofuku, 2006). A detailed description of the different

marine biotoxins including their chemical structure, mode of action and respective poisoning symptoms was recently revised by Murk et al., (2019). Okadaic acid (OA) along with its analogues dinophysistoxins (DTX1 and DTX2) and their ester derivatives constituting the OA-group toxins are among the most common and abundant lipophilic biotoxins in European countries (Marr et al., 1992; Vale and Sampayo, 2002a), being responsible for diarrhetic shellfish poisoning (DSP) (EFSA, 2008a; Valdiglesias et al., 2013). Cases of DSP caused by OA-group toxins have been reported in Portugal, Italy, Spain, Norway and Ireland (Carmody et al., 1996; Ciminiello et al., 2014; Lee et al., 1989; Moita et al., 2016; Morono et al., 2003; Rodriguez et al., 2015). This health disorder is generally associated to blooms of dinoflagellates, particularly from the genus *Dinophysis*, but also from *Prorocentrum* and *Phalachroma*, which are ingested by shellfish species, including mussels, cockles, clams, scallops and oysters (Braga et al., 2016; Chen et al., 2016a; Gerssen et al., 2010; Manita et al., 2017; Torgersen et al., 2008; Vale and Sampayo, 2002b; Wu et al., 2015). Toxicological studies have revealed that OA-group toxins can have a carcinogenic, immunotoxic and neurotoxic effect in humans (Ferreiro et al., 2015; Valdiglesias et al., 2013).

Apart from the OA-group toxins, shellfish can also accumulate other lipophilic toxins, such as azaspiracids (AZAs) (Aasen et al., 2010; Salas et al., 2011; Tillmann et al., 2010). AZAs are a group of fast-acting polyether toxins produced by dinoflagellates from the genera *Azadinium* and *Amphidoma*. Symptoms caused by AZAs are similar to those elicited by OA-group toxins, and cases of AZAs-related human poisoning have been reported in Ireland, Portugal, Spain, France, Norway and Italy (Berdalet et al., 2015; Jauffrais et al., 2013; Percopo et al., 2013; Tillmann et al., 2010; Vale and Botana., 2008). Based on their frequent occurrence and toxicity to humans, the European Commission has set regulatory limits for the presence of both OA-group toxins and AZAs in seafood

(i.e. 160 μg OA or AZA equivalents per kg of shellfish meat; EFSA 2008a; 2008b; European Commission, 2004a).

The exposure to hydrophilic toxins also represents hazards to human health, including amnesic shellfish poisoning (ASP) and paralytic shellfish poisoning (PSP) (EFSA, 2009a; EFSA, 2009b; Gerssen et al., 2010; Quilliam and Wright, 1989). Domoic acid (DA) is a water-soluble cyclic amino acid produced by several species of diatoms from the genus *Pseudo-nitzschia* that structurally resembles the neurotransmitter glutamic acid. Thus, DA acts in the central nervous system by binding to glutamate receptors, leading to cell dysfunction due to the depolarization of neurons caused by the increase of calcium ions permeability (Berman and Murray, 1997). ASP symptoms include neurological and gastrointestinal disorders, involving short-term memory loss, incapacitating headaches, diarrhea, vomiting and in severe cases seizures, coma and death (EFSA, 2009a; Quilliam and Wright, 1989). Several cases of ASP outbreaks associated to the presence of DA have been reported around the world (Bates et al., 1989; Paredes et al., 2011; Pulido, 2008).

On the other hand, PSP is caused by neurotoxins, such as saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (Neo) and gonyautoxins 1-4 (GTXs). These toxins, also known as PSP toxins or PSTs, are produced by dinoflagellates from the genera *Pyrodinium*, *Gymnodinium* and *Alexandrium*. Generally, they act by blocking the voltage-gated sodium channels in both muscle and nerve cells (Deeds et al., 2008; Oshima et al., 1993; Wiese et al., 2010). Symptoms of PSP appear within 30 to 60 minutes after the ingestion of PSTs contaminated shellfish, and include vertigo, blocking of respiration, loss of coordination, facial numbness, vomiting, diarrhea and abdominal pain (reviewed by Ciminiello et al., 2014). PSP toxins have been detected in several coastal regions around the world, including the Atlantic coast from Norway to Portugal, and the

Mediterranean region (Bernd and Bernd, 2008; Costa et al., 2015; Nakashima et al., 2004; Ngy et al., 2008). Therefore, given the risk to public health these two groups of toxins represent, the European legislation has set a maximum toxicity threshold of 20 mg of DA per kg of shellfish meat and 800 µg of PSP toxins (in STX equivalents) per kg of shellfish meat (European Commission, 2004a). Tetrodotoxin (TTX) is another potent neurotoxin that has a structure similar to STX, and blocks cell membranes' voltage gated sodium channels. The symptoms of TTX poisoning are often identical to those elicited by PSP toxins (How et al., 2003). The mode of action of TTX and STX is similar but with differences in the affinity of some subtypes of Nav channels. In particular at human nociceptive voltage-gated sodium channel (Nav1.7), as demonstrated by Walker et al (2012). The TTX group has more than 30 congeners and its poisoning is generally associated to the consumption of several puffer fish species, but it has also been detected in other marine organisms, including gastropods, bivalves, starfish and sea slugs (reviewed by Chen et al., 2016b). TTX is commonly found in different oceans including the South-East Asia region. Recently, based on the theory of "Lessepsian migration" of marine species from the Red Sea to the Mediterranean, puffer fish containing TTXs have also been observed in several locations in the Mediterranean Sea (Bentur et al., 2008). Recently, TTX has been detected in gastropods, mussels and oysters harvested in Portugal, Greece, the Netherlands and the United Kingdom (Nzoughet et al., 2013; Rodriguez et al., 2012; Silva et al., 2012). Currently, no regulatory limit has been set at European level for TTX, despite being a toxin of emerging concern (Ajani et al., 2017). In Europe, European Food Safety Authority (EFSA) has raised an opinion to establish 44 µg of TTX and/or the equivalent toxic amount of its analogues per kg shellfish meat as the possible regulatory level for TTXs (EFSA, 2017). In Japan, a maximum level of 2 mg

TTX.kg^{-1} has been established for the commercialization of puffer fish (Noguchi & Ebesu, 2001).

To date, based on toxicological and risk assessment studies of marine toxins, regulatory limits are established expressing the maximum concentration of a given toxin in raw seafood (EFSA 2008a, 2008a, 2009a, 2009b, 2010). However, such estimations do not always reflect the amount of toxin that becomes available for absorption at the intestinal epithelium level, defined as compound bioaccessibility (Versantvoort et al., 2005). Several *in vitro* methods were developed to simulate the gastrointestinal digestion process in humans (Cardoso et al., 2015; Marques et al., 2011), being used to evaluate nutrients and contaminants' bioaccessibility in seafood (Alves et al., 2017, 2018; Cabanero et al., 2004, 2007; Maulvault et al., 2011). Yet, information about bioaccessibility of marine biotoxins after the human gastrointestinal digestion process is scarce. To the best of our knowledge, only two studies have assessed the bioaccessibility of marine toxins, both of them focusing on OA-group toxins (Braga et al., 2016; Manita et al., 2017), and only the later study considered the effect of shellfish cooking procedures on compound bioaccessibility (Manita et al., 2017). These studies reported not only the conversion of OA-group toxins into more toxic compounds during human digestion, but also a significant reduction of their bioaccessibility upon cooking seafood, reflecting the need to develop further studies covering bioaccessibility of other toxins and derivatives.

In this context, the aim of this study was to assess the bioaccessibility of a range of marine lipophilic and hydrophilic biotoxins (OA-group toxins, AZAs, DA, PSP toxins and TTXs) and their derivatives in naturally contaminated seafood species (blue mussels, donax clams, European razor clams, Mediterranean mussels, surf clams and puffer fish gonads), using an *in vitro* digestion methodology and accounting for the potential

biotransformation of toxins throughout the digestive process. The effects of steaming on TTXs bioaccessibility in puffer fish gonads was also studied.

2. Materials and methods

2.1. Species sampling and sample preparation

Twenty naturally contaminated seafood samples were obtained from laboratories conducting monitoring programs in different European countries. Samples included: i) one steamed sample of vacuum packed blue mussels (*Mytilus edulis*) from Ireland ii) eighteen shellfish samples collected from shellfish production areas along the Portuguese coast (i.e. European razor clams, *Ensis arcuatus*, n = 4; donax clams, *Donax sp.*, n = 3; Mediterranean mussels, *Mytilus galloprovincialis*, n = 7; cockles, *Cerastoderma edule*, n = 2; and surf clams, *Spisula solida*, n = 2 and iii) pufferfish (*Lagocephalus sceleratus;* n = 1) gonads from Denia (Spain). For each seafood species, origin, number of analyzed samples, number of specimens from each sample, and biotoxins group analyzed (target compounds were selected according to seafood species levels/profile of natural biotoxin contamination) are described in **Table 1**.

Table 1.

Seafood species used for assessment of marine biotoxins bioaccessibility.

Seafood	Species	Origin	N	*n*	Raw/Steamed	Marine biotoxins analysed
Blue mussels	*Mytilus edulis*	Ireland	1	n/a	Steamed	AZAs
European razor clams	*Ensis arcuatus*	Portugal	4	30	Raw	OA-group
Donax clam	*Donax sp.*	Portugal	3	30	Raw	OA-group
Mediterranean mussels	*Mytilus galloprovincialis*	Portugal	3	30	Raw	PSP toxins
			4	30	Raw	DA
Cockles	*Cerastoderma edule*	Portugal	2	30	Raw	DA
Surf clams	*Spisula solida*	Portugal	2	30	Raw	DA
Pufferfish gonads	*Lagocephalus sceleratus*	Spain	1	1	Raw/Steamed	TTXs

N – number of samples used to analyze the biotoxins; n – number of specimens composing each analyzed sample; n/a – not available

Thirty bivalve specimens per sample were removed from the shell, washed with running tap water to remove any salt water and sand residues, properly drained and homogenized with a blender. All shellfish samples were analyzed as a raw product, except mussels from Ireland (already provided as steamed product; specimens were heated in a water bath for 3 minutes). For the puffer fish sample, female gonads were selected as target tissue based on the fact that tissue has been previously described to accumulate high levels of TTX (Rambla-Alegre et al., 2017). Female gonads were dissected from puffer fish, then divided into two portions, one to be analysed as raw product and another to be analysed after steaming [i.e. steaming performed in an oven (Combi-Master CM 6, Rational GroßkÜcken Technik GmbH, Germany) at 105 °C during 10 min], in order to evaluate the effect of culinary treatment in TTX bioaccessibility. Raw and steamed gonads were subsequently homogenized with a grinder (Retasch Grindomix GM200, Germany) using polypropylene cups and stainless-steel knives at 10,000 g until complete visual disruption of the tissue. All homogenized shellfish and puffer fish gonads were stored at -20 °C until further analyses.

2.2. *In vitro* digestion model

The bioaccessibility of OA-group toxins, AZAs, DA, PSP toxins in bivalves and TTXs in puffer fish gonad was assessed using an *in vitro* digestion methodology previously described by Versantvoort et al. (2005) and modified by Braga et al. (2016). Briefly, the simulated human digestion was performed in three different phases (oral, gastric and intestinal) using four digestive fluids (salivary, gastric, duodenal and bile), and each sample was digested in triplicate. For each sample, 1.5-2.0 g of shellfish/fish gonad samples was digested at 37 °C using a Rotary Tube Mixer with Disc (25 rpm; LSCI, Portugal). The simulated digestion was assessed using the following protocol: oral

phase (4 mL of saliva fluid at pH 7.0 ± 0.2; 5 min), gastric phase (8 mL of gastric fluid at pH 2 ± 0.2; 120 min) and intestinal phase (8 mL of duodenal fluid and 4 mL of bile fluid at pH 7 ± 0.2; 120 min). Each digestion fluid was prepared just before starting the digestion protocol in order to avoid enzyme degradation/inhibition. At the end of the digestive process, digested samples were placed on ice to stop the digestion process, and centrifuged at 2,750 x g at 10 °C during 10 min to separate the bioaccessible (BIO) and non-bioaccessible (NBIO) fractions.

Considering that TTX analogues in puffer fish female gonads revealed extremely low concentrations during a preliminary bioaccessibility optimization assay, in this case, sub-samples (of both raw and steamed gonads) were also collected at the end of each digestive step, in order to evaluate the potential effects of each digestive phase (namely, pH conditions in the digestive fluids) on TTXs' bioaccessibility.

Digestion efficiency was confirmed by analysing total protein levels in samples before digestion (BD) and in both BIO and NBIO fractions, as detailed in Alves et al. (2017). Protein digestibility was always above 65 %.

2.3. Toxins analysis

Methodologies used to analyse each marine biotoxin group in BD, BIO and NBIO fractions are described in **Appendix A as supplementary data**. **Table 2** shows for each biotoxins group the methodology used, the limit of detection (LOD), the limit of quantification (LOQ), and the toxicity equivalency factors (TEFs) when applicable.

OA-group toxins and AZAs in BD samples and NBIO fractions were extracted following the Standardized Operating Procedure (SOP) of the European Reference Laboratory for Marine Biotoxins for the determination of marine lipophilic biotoxins in bivalve mollusks (EURLMB, 2015; Braga et al., 2016). In the case of BIO fractions, these

toxins were extracted following Braga et al. (2016) and Manita et al. (2017). All fractions (BD, BIO and NBIO) were analysed by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) following Braga et al. (2016) and García-Altares et al. (2013). DA extraction and quantification in BD samples and NBIO fractions was carried out by reversed phase high-performance liquid chromatography (RP-HPLC) using UV detection (EURLMB, 2008), following the EU Harmonised Standard Operating Procedure for determination of domoic acid in shellfish and finfish, modified by Vale & Sampayo (2001). In the case of the BIO samples, fractions were extracted and determined following the same EURLMB, (2008) protocol, with minor modifications. PSP toxins extraction and determination in BD samples and in NBIO fractions were performed according to the AOAC Official Method 2005.06 (the so-called Lawrence method) modified by Costa et al. (2014), and BIO fractions were analysed by liquid chromatography and fluorescence detection (LC-FLD) as described in the official method (AOAC Official Method 2005.06). The extraction of TTXs in the BD samples and NBIO fractions of puffer fish gonads was performed following the protocol previously described by Reverté et al. (2015) for puffer fish tissues. For BIO samples, analyses were performed after filtration by nylon 0.2µm. All three fractions (BD, BIO and NBIO) were analysed by LC-MS/MS detection, following the protocol previously described in detail by Rambla-Alegre et al. (2017) by a TSQ Quantum system (Thermo Fisher Scientific, Bremen, Germany).

Percentages of toxins in the bioaccessible fraction (%) were calculated as follows: BIO x 100 / BD, where BIO corresponds to the toxin amount detected in the bioaccessible fraction and BD is the toxin amount detected in the sample before digestion.

Table 2. Method description, limit of detection (LOD), limit of quantification (LOQ) and toxicity equivalency factors (TEFs) for each marine biotoxin group.

	Method description	LOD	LOQ	TEFs (EFSA)
Okadaic Acid - group (OA-group) toxins	SOP for lipophilic toxins from EURLMB, 2015	OA - 15 µg.kg^{-1} DTX1 - 12 µg.kg^{-1} DXT2 - 12 µg.kg^{-1}	OA - 40 µg.kg^{-1} DTX1 - 40 µg.kg^{-1} DXT2 - 40 µg.kg^{-1}	OA=1 DTX1=1 DTX2=0.6
Azaspiracids (AZAs)	SOP for lipophilic toxins from EURLMB, 2015	AZA1 - 9 µg.kg^{-1} AZA2 - 9 µg.kg^{-1} AZA3 - 12 µg.kg^{-1}	AZA1 - 30 µg.kg^{-1} AZA2 - 30 µg.kg^{-1} AZA3 - 40 µg.kg^{-1}	-
Domoic acid (DA)	EU SOP for ASP toxins, EURLMB, 2008	DA - 0.7 µg·kg^{-1}	DA - 2 µg·kg^{-1}	-
Paralytic shellfish poisoning toxins (PSP toxins)	AOAC Official Method 2005.06	STX - 12 µg.kg^{-1} dcSTX - 11 µg.kg^{-1} C1+2 - 76 µg.kg^{-1} dcGTX2+3 - 62 µg.kg^{-1} GTX2+3 - 14 µg.kg^{-1} GTX5 - 13 µg.kg^{-1}	STX - 36 µg.kg^{-1} dcSTX - 32 µg.kg^{-1} C1+2 - 228 µg.kg^{-1} dcGTX2+3 - 187 µg.kg^{-1} GTX2+3 - 59 µg.kg^{-1} GTX5 - 57 µg.kg^{-1}	STX=1 dcSTX=1 C1+ 2=0.1 dcGTX2+3=0.4 GTX2+3=0.6 GTX5=0.1
Tetrodotoxins (TTXs)	Rambla-Alegre et al., 2017	TTX - 0.05 mg·kg^{-1}	TTX - 0.1 mg· kg^{-1}	-

2.4. Statistics

For both lipophilic and hydrophilic toxins groups, differences in bioaccessibility between seafood species were analysed by one-way analysis of variance (ANOVA) with the significance level set at 5%. Tukey's post-hoc test was used for pair wise multiple comparisons. Prior to ANOVA analysis, normality and variance homogeneity were checked (SigmaPlot v10.0, Systat software, Inc., CA, USA). For each toxin group, differences in the toxin molar fractions between BD and BIO fractions were evaluated by t-student test with the significance level set at 5%.

3. Results

3.1. Baseline levels of lipophilic and hydrophilic toxins in seafood

Toxicity levels for OA-group toxins and AZAs are presented in **Table 3**. OA-group toxicity ranged from 116 to 231 µg OA eq. kg^{-1} in raw donax clams and from 93 to 254 µg OA eq. kg^{-1} in raw European razor clams. The steamed mussel sample was naturally contaminated with high levels of AZAs (i.e. 12,529 µg AZA eq.kg^{-1}) (**Table 3**).

The hydrophilic toxin DA was detected in mussels, cockles and surf clams. The highest (68 mg DA kg^{-1}) and lowest (17 mg DA kg^{-1}) DA concentrations were recorded in mussel samples. In cockles and surf clams, the highest DA levels were 56 mg DA kg^{-1} and 66 mg DA kg^{-1}, respectively (**Table 3**).

Toxicity levels of PSP toxins in mussels varied from 1,064 to 1,922 µg STX eq. kg^{-1} (**Table 3**). All samples analysed were above the maximum permitted level (MPL) of 800 µg of PSP toxins equivalents per kg of shellfish meat. TTXs levels in raw and steamed puffer fish gonads were 141 and 213 ∑mg TTXs.kg^{-1}, respectively (**Table 3**).

Table 3. Lipophilic (OA-group toxins, AZAs) and hydrophilic (DA, PSP toxins, TTXs) marine toxins in seafood samples. Concentrations for OA-group toxins, AZAs and PSP toxins were based on toxicity equivalency factors (TEFs). Concentrations for TTXs were the sum of individual TTX and TTX analogues.

		Toxin concentration
Lipophilic toxins	**Okadaic Acid - group (OA-group) toxins**	**µg OA eq. kg^{-1}**
	Donax clam raw	231
		116
		203
	European razor clam raw	93
		170
		103
		254
	Azaspiracids (AZAs)	**µg AZA eq. kg^{-1}**
	Blue mussel steamed	12,529
Hydrophilic toxins	**Domoic acid (DA)**	**mg DA kg^{-1}**
	Mediterranean mussel raw	32
		29
		68
		17
	Cockle raw	56
		48
	Surf clam raw	66
		65

Paralytic shellfish poisoning toxins (PSP toxins)	µg STXeq. kg^{-1}
Mediterranean mussel raw	1,922
	1,468
	1,064
Tetrodotoxins (TTXs)	**∑mg TTXs kg^{-1}**
Puffer fish gonad raw	141
Puffer fish gonad steamed	213

3.2. Bioaccessibility of marine toxins

Figure 1 shows the total lipophilic (A) and hydrophilic (B) toxins bioaccessibility in seafood. In general, hydrophilic toxins showed higher bioaccessibility than the lipophilic ones. For OA-group toxins, high percentages of bioaccessibility were observed in both raw donax clam (74 %) and razor clam (69 %). Azaspiracids in steamed mussels revealed low toxins' bioaccessibility (47 %, $p < 0.05$), (**Fig. 1A**). In the case of hydrophilic toxins, high bioaccessibility was observed for DA and PSP toxins in raw shellfish, as well as, for total TTX in raw gonad (107 %). DA and PSP toxins were 100 % bioaccessible in Mediterranean mussels, cockles and surf clams ($p > 0.05$; **Fig. 1B**). In puffer fish gonads, total TTX bioaccessibility decreased significantly after steaming (59 %; $p < 0.05$), (**Fig.1B**). When analysing individual TTX analogues, different bioaccessibilities were observed: while for some TTX analogues the bioaccessibility increased, interestingly, for parent TTX the bioaccessibility decreased to regardless of the processing (15 % for raw and 14 % for steamed).

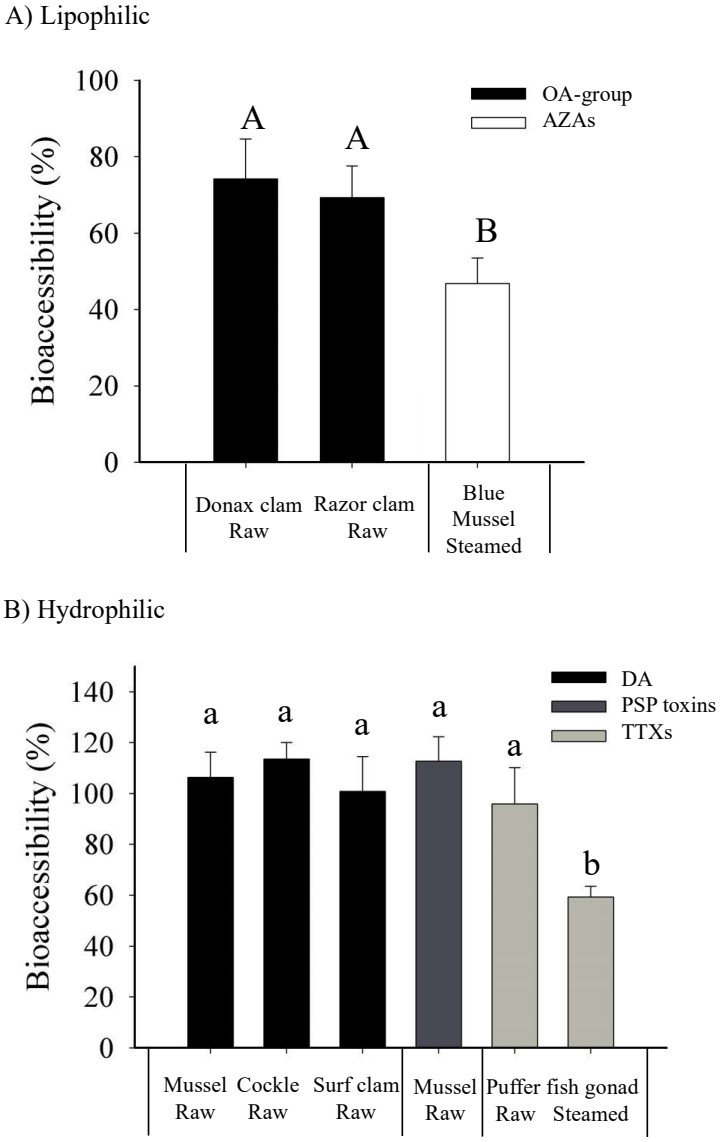

Fig.1. Bioaccessibility (%, average ± standard deviation) of (A) lipophilic (OA-group, AZAs) and (B) hydrophilic toxins in seafood (DA, PSP toxins, TTXs). A - Uppercase letters represent differences in lipophilic toxins bioaccessibility between seafood species (ANOVA, $p < 0.05$). B - Lowercase letters represent differences in hydrophilic toxins bioaccessibility between seafood species (ANOVA, $p < 0.05$).

The profile of the toxins in samples before (BD) and after the *in vitro* digestion (BIO) is shown in **Fig. 2**. Profile of OA-toxins group varied between shellfish species. OA-toxins group profile in donax clams was characterized by the following analogues: free DTX2 (35 %) > esterified OA (29 %) > free OA (25 %) > esterified DTX2 (11 %). Razor clams had a toxin profile mainly composed by esterified OA (84 %). No significant

changes in toxins profile were observed after *in vitro* digestion neither in donax clams and nor in razor clams (t-student, $p > 0.05$), (**Fig. 2A**). In the case of AZA, toxins mass fractions were maintained after digestion (t-student, $p > 0.05$), and varied according to the following order: AZA1 > AZA3 > AZA2 (**Fig. 2B**).

Figure2C shows the PSP toxins profile in raw mussels, and the most abundant forms in this sample were saxitoxins analogues, such as N-sulfocarbamoyl toxins C1+2 (74 %) and GTX5 (21 %), followed by decarbamoyl toxins (dcGTX2+3, dcSTX) and gonyautoxins (GTX2+3). Saxitoxin (STX) was not detected in the mussel samples. No significant changes were observed in toxins profiles between BD and BIO fractions ($p > 0.05$), (**Fig. 2C**). Out of the nine TTX analogues analysed, 5,6,11-trideoxyTTX was the most abundant in the puffer fish gonads (raw - 67 %, steamed – 81 %). On the other hand, TTX (raw - 16 %, steamed - 9 %) and 11-norTTX-6*S*-ol (raw – 12 %, steamed – 6 %) were present at much lower concentrations. TTX and 11-norTTX-6*S*-ol analogues decreased significantly after digestion (t-student, $p < 0.05$), nevertheless the 5,6,11-trideoxy TTX analogue showed a significant increase ($p < 0.05$) in both raw and steamed puffer fish gonads (**Fig. 2D**).

Taking into account the bioaccessibility percentage of the parent TTX toxin in raw pufferfish gonad (20% in the whole *in vitro* digestion model; data not shown), TTX bioaccessibility was evaluated throughout the *in vitro* digestion process (oral phase pH 7, gastric phase pH 2 and intestinal phase pH 7). Percentages of BIO and NBIO fractions are presented in **Fig. 3**. In both oral and intestinal phases only around 20 % of TTX was bioaccessible. No significant differences were observed in the TTX concentrations before and after the gastric phase. The same trend of bioaccessibility percentages was observed for the other TTX analogues 5,6,11-trideoxyTTX, 4-*epi*TTX and 11-norTTX-6*S*-ol (data not shown).

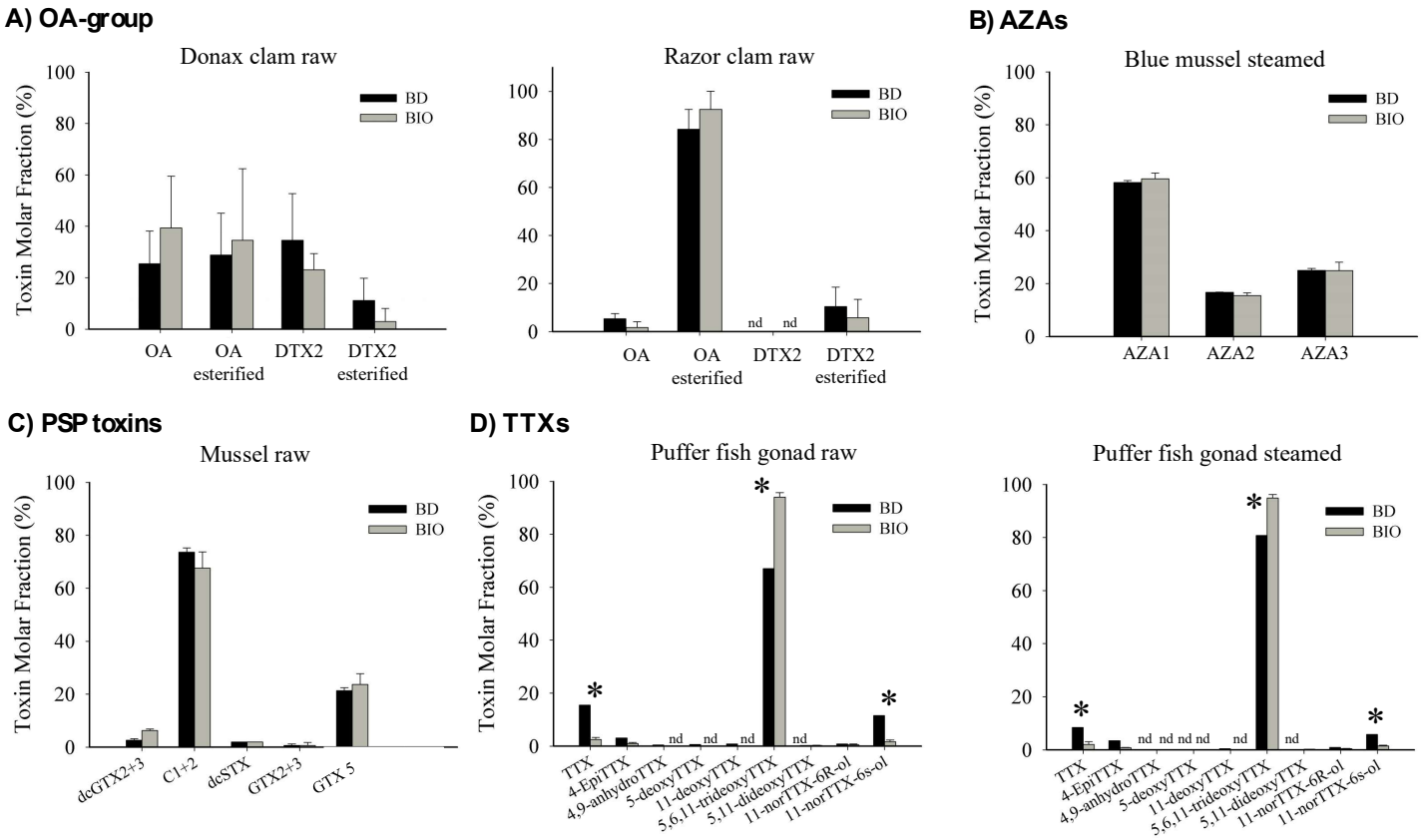

Fig. 2. Profile of lipophilic (A-B) and hydrophilic (C-D) marine toxins (%, average ± standard deviation) in seafood samples before digestion (BD) and in the bioaccessible fractions (BIO). A) OA-group toxins in donax clams and European razor clams and in steamed blue mussel; B) AZAs group in steamed blue mussel; C) PSP toxins in Mediterranean mussels and D) TTXs in puffer fish gonads. Asterisk (*) represent differences for the toxins profile between BD and BIO (t-student, $p < 0.05$).

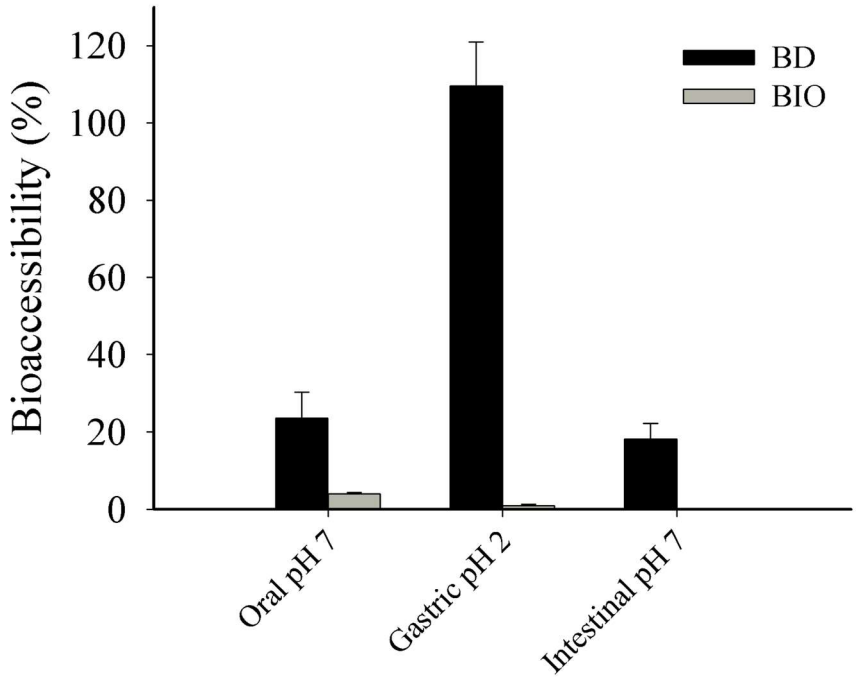

Fig. 3. Bioaccessibility (%) of parent TTX in pufferfish gonads during the digestion phases (oral, gastric and intestinal) in bioaccessible (BIO) and non-bioaccessible (NBIO) fractions.

4. Discussion

4.1. Occurrence and profiles of lipophilic and hydrophilic toxins in seafood

The production of toxins by phytoplankton and or symbiotic bacteria, as well as their accumulation in bivalve and fish species has been well recognized throughout European countries (Costa et al., 2017; McNamee et al., 2016). Yet, the consumption of seafood contaminated with these toxins leads to known food-borne human diseases, which represents the main health hazard associated with marine toxins (Berdalet et al., 2015).

Two lipophilic toxins groups were studied, i.e. OA-group toxins and AZAs. In the present study, five out of eight shellfish samples showed OA levels at concentrations above the maximum permitted level (MPL) of 160 µg OA eq. kg^{-1} (European

Commission, 2004a), therefore, representing a risk for seafood consumers. The OA toxins concentration obtained in this study in donax clams and European razor clams was within the range of values previously reported for harvested natural contaminated shellfish (between 44 and 892 µg OA eq. kg^{-1}; e.g. Rodríguez et al., 2015; Braga et al., 2016). For example, donax clams collected in Portuguese coast during 2014 showed an OA toxicity ranging between 229 and 395 OA eq. kg^{-1} (Braga et al., 2016). In shellfish from Galicia coast, total OA concentration showed a high variability between samples (44 up to 892 µg OA eq. kg^{-1}) (Rodríguez et al., 2015). In Europe, OA and DTX2 are most commonly found in free or esterified forms, though the proportion of these compounds may greatly vary between samples. Moreover, the fact that DTX1 was not detected in any sample is in agreement with previous findings (Johnson et al., 2016; Rodríguez et al., 2015; Vale & Sampayo, 2002a).

Differences in the OA-group toxins profile were observed between shellfish species, showing the following profiles, higher levels of esterified DTX2 (razor clam) and equal proportions of both free and esterified OA (donax clam). Such results were not surprising as OA-group toxins profile can change according to algae and shellfish species, sampling location and seasonality (EFSA, 2008a; Moita et al., 2016; Yasumoto et al., 1985). For example, three different OA-group toxins profiles were identified in mussels (*Mytilus* sp.) from Great Britain collected between 2011 and 2015. Mussels were predominantly composed by free OA, free DTX2 or even esterified OA as observed in mussel samples from that study (Johnson et al., 2016). As previously reported, mussels and donax clams have low ability to efficiently biotransform OA into fatty acid ester derivatives compared to razor clams (Rossignoli et al., 2011; Vale and Sampayo, 2002b).

Although OA group usually predominate (in terms of quantity and frequency) within the lipophilic toxins, co-occurrence with other lipophilic toxins such as AZAs can

occur (Regueiro et al., 2011; Villar-González et al., 2007), as observed in the steamed blue mussel sample. The AZAs content in the steamed mussel sample from Ireland was above the maximum MPL of 160 µg AZA eq. kg^{-1} (EFSA, 2008b). This result matched the previously reported maximum AZA levels in raw blue mussels from Ireland (8,970 µg AZA1 eq. kg^{-1}) (Salas et al., 2011). Moreover, an increase in AZAs concentration in mussels after steaming was also expected due to the loss of water (Hess et al., 2005), and may justify the high toxicity observed in the present study for these toxins. Although AZAs have been identified in shellfish from several European countries, toxin levels were generally very low, therefore, suggesting reduced risks of human disease outbreaks due to azaspiracids poisoning (AZP) (Amzil et al., 2008; Bacchiocchi et al., 2015; Twiner et al., 2008). More than 10 AZA analogues have been reported in shellfish, but AZA1, AZA2 and AZA3 are the predominant analogues detected in European shellfish (James et al., 2002). Similarly, to the present study, James et al. (2005) reported the, overall, predominance of AZA1 toxin (50-65%) over AZA2 (20-30%) and AZA3 (5-20%).

Three hydrophilic toxins groups were also investigated in this study, i.e. DA and PSP toxins in shellfish and TTX in fish. DA concentrations observed in the eight shellfish samples from the Portuguese coast were close or above the established DA MPL (20 mg DA kg^{-1}) (European Commission, 2004a, 2004b). Such variability, even within the same species, has also been reported in other studies (EFSA, 2009a; James et al., 2005; Ujević et al., 2010; Vale & Sampayo, 2001). Unintended ingestion of PSP toxins is the main cause of PSP incidents (i.e. around 2,000 reported cases worldwide), often having devastating consequences. For instance, in the Philippines, between 1983 and 2002, more than 115 PSP cases resulted in death (Ching et al., 2015), mostly associated with STX and some GTX, showing severe toxicity. In our study, mussels from the Portuguese coast showed PSP toxins levels above the MPL for the safe consumption of shellfish (i.e. 800

µg equiv. STX kg^{-1}; European Commission, 2004a, 2004b). According to the EFSA, mussels seem to be the bivalve organisms that are mostly susceptible to PSP toxins contamination (EFSA, 2009b). Portugal and Spain have been considered two areas that are greatly affected by PSP toxins, with these toxins showing values as high as 67,616 and 40,800 µg STX equiv. kg^{-1} of shellfish meat, respectively (EFSA, 2009a). Additionally, results obtained in the present study are in accordance with previous PSP toxins occurrence in Iceland, Chile and New Zealand (Burrell et al., 2013; MacKenzie, 2014; Zamorano et al., 2013). Out of the several identified STX-like congeners, C1+2, GTX5 and the decarbamoyl toxins dcGTX2+3 and dcSTX represented more than 90 % of the toxin profile detected in the Mediterranean mussel samples. Although they represent more than 90% of toxins profile in mussels and their presence is often associated with outbreaks of paralytic shellfish poisoning in Portugal, these toxins have been described to be moderately toxic to humans (Botelho et al., 2015; Costa et al., 2015; Etheridge, 2010; Negri et al., 2007).

TTX is considered to be one of the most dangerous and lethal toxin in the marine environment, and the number of poisoning cases in European countries is increasing (Fernández-Ortega et al., 2010). Presently, legislation in Europe establishes that poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the markets (European Commission, 2004a, 2004b). So far, no regulatory limit has been established in the EU for the presence of this toxin in seafood, although EFSA has set a value of 44 $ug.kg^{-1}$ as a possible limit (EFSA, 2017). The levels obtained in the present study (raw and steamed gonads) were within the range of values previously reported for TTX (0.17-239.32 mg kg^{-1} in gonads; Rambla-Alegre et al. 2017). Yet, these values are 10-fold above the Japanese acceptability criterion value of 2 mg TTX equiv. kg^{-1} pufferfish tissue for human consumption (Noguchi & Ebesu, 2001).

In the current study, parent TTX represented less than 16 % of the total TTX toxins. More than 30 congeners of TTX with different levels toxicity have been isolated and identified in marine organisms to date (reviewed by Bane et al., 2014). The 5,6,11-trideoxy TTX was the predominant TTX congener observed in both raw (molar fraction, 67 %) and steamed (molar fraction, 81 %) puffer fish gonad. In fact, 5,6,11-trideoxy TTX was reported as the predominant congener in *Lagocephalus sceleratus* and other fish species, such as *Fugu pardalis*, *Fugu niphobles* and *Tetraodon nigroviridis* (Jang et al., 2006, 2010; Rodríguez et al., 2012). In addition, 5,6,11-trideoxy TTX is considered to be less toxic compared to the TTX parental toxin, given the fact that this derivative exhibits a lower number of hydroxyl groups (Yotsu-Yamashita, 2007).

4.2. Toxins bioaccessibility and changes in toxins profile after *in vitro* digestion

So far, information on marine toxins' bioaccessibility is scarce, and only data on OA-group toxins has been previously reported (Braga et al., 2016; Manita et al., 2017). To the best of our knowledge, this is the first study reporting bioaccessibility percentages for AZAs, DA, PSP toxins and TTXs in seafood.

Matching the present results, bioaccessibility of OA, DTX2 and their ester derivatives has been previously reported in raw mussels (87%), donax clams (75%) and cockles (59%), (Braga et al., 2016; Manita et al., 2017). The bioaccessibility value in European razor clams in the present study was closer to the one observed in donax clams, despite the toxin profiles in the two species being remarkably different, with European razor clams exhibiting a profile where over 80 % of the toxin was esterified, and donax clams profile reaching only 40 %. Little is known about the absorption and metabolism of AZAs in mammals. The results obtained in the present study show that AZAs bioaccessibility in steamed mussel was around 45%, as observed for other lipophilic

toxins in steamed shellfish (Manita et al. 2017). Our findings are in accordance with previous observations done by Kilcoyne et al. (2014), that reported AZAs bioaccessibility values between 30-54% in raw mussel. However, only 10% of bioaccessible AZAs was observed in the same steamed samples. These authors suggested that thermal denaturation of proteins in mussel tissues occurred and this may have been the main reason for such low bioaccessibility, as AZAs seem to be weakly bound to 45 kDa proteins. This difference between our results and those observed by Kilcoyne et al. (2014) can be explained by the use of different *in vitro* digestion protocols. In contrast to the OA-group toxins, no conversion between AZA analogues was observed during the *in vitro* digestion. Nevertheless, it has been suggested in an *in vivo* experiment with mini pigs orally exposed to AZAs that the metabolism of AZAs can occur during digestion (Geraghty et al., 2014).

To our knowledge, this is the first study reporting hydrophilic toxins (DA, PSP toxins) bioaccessibility, and results showed that almost 100 % of toxins were available to be absorbed by the gastrointestinal tract after digestion. This complete bioaccessibility was not surprising due the hydrophilic nature of these toxins, as it has been reported that less hydrophobic compounds may be more easily transferred to the bioaccessible digestion fluids during digestion (Alves et al., 2017). No conversion between PSP analogues was observed after the digestion process. Some studies revealed that PSP toxins metabolism occurs in humans during digestion. Hepatic conversion of STX and GTX3/GTX2 epimers into neoSTX or GTX4/GTX1 epimers, respectively, by oxidation and glucuronidation of PSP toxins can occur (García et al., 2004, 2009, 2010). However, these PSP toxins analogues were not the most abundant in the toxins' profile of mussel samples.

TTXs, which are Na^+ channel blockers (Lee and Ruben, 2008), also showed high bioaccessibility (100 %, for raw and 59 % for steamed), but parent TTX showed very low

bioaccessible values (14-15 %). This could be due to the digestion conditions carried out during the *in vitro* bioaccessibility process. In this work, the three main digestive steps were individually evaluated in order to better understand this biochemical mechanism. The high TTX bioaccessibility observed (around 100 %) at the end of the gastric step demonstrates that the decrease in the pH during this phase is not the main factor for the low TTX bioaccessible values observed at the end of the digestion process. In addition, at the gastric phase, a higher pH was tested, in order to evaluate the stability of the TTX regarding pH conditions. A bioaccessible percentage of 37 % was obtained at pH 7 (data not shown), while around 100 % was observed at pH 2, showing the importance of the pH in the bioaccessibility study. The decrease in the TTX bioaccessibility should occur somewhere during the intestinal phase, and TTX analogues conversion during this step can be a possible explanation. 5,6,11-TrideoxyTTX, 11-norTTX-(*S/R*)-ol and 4-*epi*TTX were the main analogues of TTX observed in the BIO and NBIO fractions, with the latter compound being chemically interchangeable with TTX. Some conversion in the bioaccessible fractions was observed in TTX analogues, resulting in TTX decreases (2.5%) and 5,6,11-trideoxyTTX increases (94%). This could be attributed to the dehydrogenation of some hydroxyl groups during the digestion process (Yotsu-Yamashita et al. 1995). The biosynthetic and metabolic pathways of TTX remain to be elucidated. Yotsu-Yamashita et al. 2013 described the oxidation process from 5,6,11-trideoxyTTX to TTX where 5,6,11-trideoxyTTX is first oxidized to 5,11-dideoxyTTX, which would be oxidized to both 5-deoxyTTX and 11-deoxyTTX. These monodeoxy TTXs are predicted to be the exact precursors of TTX (Yotsu-Yamashita et al. 2013).

5. Conclusions

The current study provided new insights on marine biotoxins that may be of interest to evaluate potential dietary exposure to some marine toxins through the consumption of seafood. The bioaccessibility of toxins from the okadaic acid group ranged from 69 up to 74 %. Regarding azaspiracids, 47 % of the initial content was bioaccessible in steamed blue mussel after the *in vitro* digestion. Unlike lipophilic OA-group toxins and AZAs, after *in vitro* digestion, the initial content of hydrophilic toxins (DA, PSP toxins and total TTXs) was almost totally bioaccessible for absorption by the human intestine epithelia. In puffer fish gonads, steaming significantly reduced the bioaccessibility of total TTXs. Hence, these results raise the interest for conducting future studies on the effects of different culinary treatments, including steaming, in other toxins groups. The present data also provided an insight on the possible transformation of some toxins after digestion, including those from TTX analogues, but the mechanisms underlying this transformation still remain to be elucidated. In future studies, it will also be interesting to evaluate the role of the gastrointestinal tract microflora in the biotransformation of toxins analogues.

As for lipophilic toxins, the present bioaccessibility results suggest that previous data on the exposure to these toxins may be overestimated. In contrast, for the majority of the analysed hydrophilic toxins, the total toxin concentration detected in seafood can be considered as the amount of the toxin that will become available for absorption after digestion. In this way, these preliminary results prove that compound bioaccessibility can be a powerful tool towards a more accurate evaluation of risks and benefits associated with seafood consumption, and call for the need to define new and more realistic guidelines and regulations for the presence of marine toxins in seafood. Finally, taking into consideration the limitations of the present study (i.e. number of seafood

species/samples analysed), further studies are urgently required to diversify and increase data robustness on toxins' bioaccessibility.

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