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Oral bioaccessibility of toxic and essential elements in raw and cooked commercial seafood species available in European markets

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Abstract

The oral bioaccessibility of several essential and toxic elements was investigated in raw and cooked commercially available seafood species from European markets. Bioaccessibility varied between seafood species and elements. For example, methylmercury bioaccessibility varied between 10 (octopus) and 60 % (monkfish). Arsenic (> 64%) was the toxic element showing the highest bioaccessibility. Concerning essential elements bioaccessibility in raw seafood, selenium (73 %) and iodine (71 %) revealed the highest percentages. The bioaccessibility of elements in steamed products increased or decreased according to species. For example, methylmercury bioaccessibility decreased significantly after steaming in all species, while zinc bioaccessibility increased in fish (tuna and plaice) but decreased in molluscs (mussel and octopus).

Together with human exposure assessment and risk characterization, this study could contribute to the establishment of new maximum permissible concentrations for toxic elements in seafood by the European food safety authorities, as well as recommended intakes for essential elements.

Keywords: seafood, toxic/essential elements, steaming, bioaccessibility

1. Introduction

Seafood is considered an important dietary source of energy, proteins with high biological value and long chain polyunsaturated n-3 fatty acids (LCn-3-PUFA). LCn-3-PUFAs are considered key players in the prevention against cardiovascular diseases (CVD), (Larsen et al., 2011). The high nutritional value associated to seafood is also due to the presence of considerable amounts of essential trace elements, including selenium (Se), iodine (I), iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn). Essential elements are generally involved in metabolism and several biological processes acting as cofactors of enzymes (Fraga, 2005). A balanced diet based on seafood has been extensively recommended due to its health benefits (Larsen et al., 2011).

Nevertheless, marine seafood can also accumulate high levels of chemical contaminants, including toxic elements such as cadmium (Cd), mercury (Hg), arsenic (As) and lead (Pb), raising human health-related concerns (Bosch et al., 2016; Chiocchetti et al., 2016; Maulvaut et al., 2015). The World Health Organization (WHO) defined these four toxic elements as toxic chemicals of major public health concern (WHO, 2012), and the European Safety Authority (EFSA) has in recent years published toxicological guidelines for these toxic elements (EFSA, 2009, 2012).

Regulatory authorities and the scientific community have been focusing their attention on these toxic and essential elements in seafood (Bosch et al., 2015; Chiocchetti et al., 2016; European Commission, 2016; Figueira and Freitas 2103; Salvo et al., 2016; Vandermeersch et al., 2015). For example, in the Southern Spain coastal waters, high levels of essential Zn, Mn and Cu elements were observed in mussels, common sole, and crustaceans, respectively. In the case of Se, higher levels were associated to predatory fish species (Olmedo et al. 2013). Qin and colleagues (2015)
analysed the levels of 28 trace elements in farmed cyprinid fish from Northeast China, and Zn (7.9 mg kg\(^{-1}\) wet weight w.w), Fe (6.71 mg kg\(^{-1}\) w.w) and Sr (1.17 mg kg\(^{-1}\) w.w) were the main detected elements. Furthermore, these authors suggested that Cd, As and Pb should be monitored frequently in this farmed fish species. Similar findings were reported in a study with more than 30 fish species from France markets (Zn – 5.43 mg kg\(^{-1}\) w.w, Fe – 4.42 mg kg\(^{-1}\) w.w and Sr – 1.42 mg kg\(^{-1}\) w.w). The predominant toxic elements detected in that study were the aluminium (Al), barium (Ba), silver (Ag) and lead (Pb), (Guérin et al. 2011). High and low Cd contamination is associated to molluscs/algae and fish, respectively (Moreda-Piñeiro et al., 2012, Wang et al. 2014). Within the group of toxic elements, methylmercury (MeHg) is the mercury compound most commonly found in fish, being recognized as a neurotoxic and carcinogenic pollutant. Due to the high stability and long-term elimination from tissues, this persistent pollutant has been one of the most studied contaminants, and high concentrations are usually associated with predatory and carnivorous fish species (Burger and Gochfeld, 2011; Storelli and Barone, 2013; Teffer et al., 2014). Several studies have shown that Se has an ability to decrease the toxicity of MeHg, and the molar ratio between Se and MeHg (Se:MeHg), and Se health benefit values (HBV\(_{Se}\)) has been used to assess Se-dependent health benefits and the impact of Hg exposure in seafood (Ralston et al., 2016).

The total nutrients and contaminants concentration detected in seafood does not always reflect the amount of the nutrients and contaminants that will become available for absorption at the intestinal epithelium level, defined as bioaccessibility. Bioaccessibility is an important tool to estimate the oral availability, i.e. the proportion of the bioaccessible fraction that potentially reaches the systemic circulation (Versantvoort et al., 2005). The assessment of bioaccessibility is
fundamental for risk-benefit analysis of nutrients and contaminants associated to food consumption, providing more accurate information and guidelines for food consumption to authorities, industry and consumers (Maulvault et al., 2011; 2013). Several in vitro models were developed to simulate the gastrointestinal digestion process in humans (Cardoso et al., 2015; Minekus et al., 2014). These in vitro digestion methodologies have been used to evaluate essential and toxic elements bioaccessibility in seafood, mainly in molluscs and fish (Costa et al., 2015; Laird & Chan, 2013; Lei et al., 2013; Torres-Escribano et al., 2011a). For example, in farmed Hong Kong oysters Crassostrea hongkongensis, bioaccessibility of elements such as Ag, Cu and Zn varied between 38.9 and 93.1 % (Gao and Wang, 2014). High Hg bioaccessibility was reported for shellfish (Cano-Sancho et al., 2015) MeHg bioaccessibility is dependent from the seafood matrix (Cabañero et al., 2007). As example, MeHg bioaccessibility ranged from 26 to 62% in five predatory fish species from the Baltic Sea (Kwasniak et al., 2012). Most studies on the bioaccessibility of essential and toxic elements were performed in raw products, despite most seafood is only consumed after culinary preparation. Therefore, risk assessment of chemical contaminants is currently evaluated mainly for raw seafood products, potentially becoming a bottleneck in public health safety guidelines (Maulvault et al., 2013). Recent studies described the effect of culinary treatments in bioaccessibility, and indeed it has been demonstrated that culinary treatment generally reduces toxic elements bioaccessibility in seafood (e.g. MeHg) (Cano-Sancho et al., 2015; Matos et al., 2015; Ouedraogo and Amyot, 2011). Furthermore, the existing information regarding the effects of culinary treatments in essential and toxic elements bioaccessibility from seafood species available in European markets is still scarce.

In this context, this study aims to: (1) assess the bioaccessibility of several essential (Zn, Se, Cu, Mn, Sr, I, Fe) and toxic (Hg, MeHg, As, Cd) elements in marine
seafood species (including fish, molluscs, crustaceans and seaweed) available in different European markets. The choice of the essential elements to be addressed in this study was based on the existent literature for seafood. Toxic elements were selected based on the recent European database generated for the emerging contaminant levels in seafood. We decided to exclude Pb from the analysis as seafood in Europe is not at present considered a major contributor to lead dietary intake (Vandermeersch et al. 2015); (2) evaluate the effects of culinary treatment (steaming) on the bioaccessibility of essential/toxic elements; and (3) evaluate the differences in bioaccessibility of the analysed elements according to seafood type.

2. Material and methods

2.1. Sampling species and culinary treatment

Nine seafood species were collected in different European markets including hake (Merlucius australis), tuna (Katsuonus pelamis), monkfish (Lophius piscatorius), mackerel (Scomber scombrus), plaice (Pleuronectes platessa), mussel (Mytilus edulis), octopus (Octopus vulgaris), shrimp (Litopenaeus vannamei) and one seaweed species (Laminaria digitata). All samples were collected in different seasons between April 2014 and November 2015. For each species, specimens were of commercial sizes, with uniform sizes and weights. Origin, market country, number of specimens, total length (mean mm) and total weight (g) (mean ± SD) and moisture (%) are described in Table 1.

Insert table 1
For fish, muscle tissue was collected without skin from 25 specimens. For cephalopods (n = 25) and crustaceans (n = 50), mantle and abdominal muscle tissue were sampled, respectively. The edible part with the intervalvar liquid of bivalves was collected from 50 individuals, whereas in seaweed, only the frond (leaf) was collected from 20 *L. digitata* specimens, separating the newer part of the leafs (closer to the stipe and designated hereafter as new) and the older part of the leafs (more distant to the stipe and designated as old). For each species, specimens were divided in three different pools (each pool was composed by 8-9 individuals for fish and octopus, 16-17 individuals for mussels and crustaceans and 6-7 individuals for seaweed).

The individuals from each pool were divided into two portions, one used for the culinary treatment (steaming at 105 °C wrapped up in aluminium foil), and one for raw assessment. Each sample was divided into two portions, one used for the culinary treatment (steaming at 105 °C wrapped up in aluminium foil), and one for raw assessment. Fish, cephalopods and seaweeds were steamed for 15 minutes whereas bivalves and crustaceans were steamed for 5 minutes. Raw and steamed samples were homogenised with a grinder (Retasch Grindomix GM200, Germany) using polypropylene cups and stainless steel knives at 5,000 rpm until complete visual disruption of the tissue and further kept at -20°C until the *in vitro* digestion.

2.2. *In vitro* human digestion model

2.2.1. Reagents
The reagents used to prepare the digestion fluids solution were the following:

Inorganic: NaCl (Merck, 99.5% m/v), NaHCO₃ (Merck, 99.5% m/v), CaCl₂·2H₂O (Sigma, C3881), KCl (Merck, 99.5% m/v), KSCN (Sigma, P2713), NaH₂PO₄ (Merck, 99.5% m/v), Na₂SO₄ (Merck, 90% m/v), NH₄Cl (Riedel-de Haen, 99.5% m/v), K₂HPO₄ (Merck, 99.5%), MgCl₂ (Riedel-de Haen, 99.5% m/v), HCl (Merck, 37% m/v);

Organic: urea (Sigma, U5128), glucose (Sigma, G5400), glucuronic acid (Sigma, G5269), D-(-)-Glucosamine hydrochloride (Sigma, G4875), uric acid (Sigma, U2625), albumin from bovine serum (Sigma, A7906), α-amylase, from Aspergillus oryzae (Sigma, 86250), mucin from porcine stomach (Sigma, M2378), pepsin from porcine stomach mucosa (Sigma, P7125), lipase from porcine pancreas type II (Sigma, L3126), pancreatin from porcine pancreas (Sigma, P8096), trypsin from porcine pancreas (Sigma, T6567), α-chymotrypsin from bovine pancreas (Sigma, C4129) and bile porcine extract (Sigma, B8631).

2.2.2. *In vitro* digestion protocol

The *in vitro* digestion protocol used to study the elements bioaccessibility was is schematized in Appendix A (Supplementary Fig. S1). The *in vitro* digestion protocol adapted from Versantvoort et al. (2005) and Minekus et al. (2014) was used to assess the elements bioaccessibility. Raw and steamed seafood samples were *in vitro* digested with four digestion fluids: saliva, gastric, duodenal and bile. Each digestion fluid is composed by several inorganic and organic components (Appendix A, Supplementary Table S1).

Briefly, for each homogenized sample, 1.5 to 2.0 grams were digested in triplicate (Nalgene™ high-speed PPCO centrifuge tubes) at 37°C using a Rotary Tube
Mixer with Disc (25 rpm; LSCI, Portugal) inserted in an incubator. The digestion was performed using the following protocol: oral phase (4 ml of saliva fluid for 5 min at pH 7.0 ± 0.2), gastric phase (8 ml of gastric fluid for 2 hrs at pH 2 ± 0.2) and intestinal phase (8 ml of duodenal fluid and 4 ml of bile fluid for 2 hrs at pH 7 ± 0.2). To avoid enzyme degradation/inhibition, each digestion fluid was prepared just before starting the digestion protocol. In each digestion phase, pH was adjusted immediately before the digestion. In the end, reaction tubes were placed on ice, and centrifuged at 2,750x g at 10°C for 10 minutes to separate the bioaccessible fraction (BIO) from the sample residues (non-bioaccessible fraction - NBIO). Negative controls containing the digestion fluids without seafood were performed. These control reactions were used to correct the proteins, toxic and essential elements concentrations determined in each sample.

2.2.3. Digestion efficiency

To assess the in vitro digestion efficiency, total protein levels were determined using an FP-528 DSP LECO nitrogen analyser (LECO, St. Joseph, USA). Protein levels were measured in wet weight (raw and steamed) – before digestion (BD), and in the bioaccessible (BIO) and non-bioaccessible (NBIO) fractions. The calibration standard curve was performed with EDTA following the methodology described by Saint-Denis & Goupy (2004).

Protein recovery (%) was defined as the following ratio:

\[
\frac{(\text{BIO} + \text{NBIO}) \times 100}{\text{BD}},
\]
where BIO + NBIO are the sum of protein levels detected in the bioaccessible (BIO) and non-bioaccessible (NBIO) fractions, and BD is the amount of protein detected in the sample before digestion.

Bioaccessible protein (%) was defined as the following ratio:

\[
\frac{\text{BIO} \times 100}{\text{BD}},
\]

where BIO corresponds to the protein levels detected in the bioaccessible fraction (BIO), and BD is the amount of protein detected in sample before digestion.

2.3. Essential and toxic element analysis

2.3.1. Mercury and methylmercury

Bioaccessibility of total mercury (Hg) and methylmercury (MeHg) was assessed in several seafood species including hake, tuna, mackerel, monkfish and octopus. Hg levels in the remaining species were below the limit of quantification. The methodology used to extract MeHg has been previously described by Maulvault et al. (2015). Briefly, each BD sample was freeze-dried (at -50 °C, low pressure – 10^{-1} atm) for MeHg analysis prior to the *in vitro* digestion. Then, approximately 150 mg of BD sample, and 5 g of BIO were hydrolysed with hydrobromic acid (10 ml, 47 % w/w; Merck), and then MeHg extraction was performed with two steps of purification with toluene (99.8 % w/w, Merck). In the end, cysteine solution (1 % L-cystein chloride in 12.5 % anhydrous sodium sulphate and 0.8 % sodium acetate) was added to extract MeHg (Scerbo and Barghigiani, 1998). Hg and MeHg (through cysteine extracts) levels were determined by atomic absorption spectrometry using the Hg analyser (Leco, AMA 254, St. Joseph,
MI, USA). This technique is based on Hg cold vapour generation, where samples are decomposed by combustion. Subsequently, Hg is concentrated by amalgamation with gold and detected at 254 nm. Mercury concentrations were calculated from linear calibration with a Hg(II) nitrate standard solution (1,000 mg L\(^{-1}\), Merck) diluted in nitric acid (0.5 mol L\(^{-1}\), Merck). For total Hg, 10 mg of wet weight BD or NBIO, and 100 to 800 µl of BIO were used.

Total Hg recovery (%) was defined as the following ratio:

\[
\frac{(\text{Hg BIO} + \text{Hg NBIO}) \times 100}{\text{Hg BD}}
\]

where Hg BIO + Hg NBIO are the sum of Hg levels detected in BIO and NBIO fractions, and Hg BD is the amount of Hg detected in BD sample.

Bioaccessible Hg (MeHg) (%) was defined as the following ratio:

\[
\frac{\text{Hg (MeHg) BIO} \times 100}{\text{Hg (MeHg) BD}}
\]

where Hg (MeHg) BIO corresponds to Hg levels detected in BIO, and Hg (MeHg) BD is the amount of Hg (MeHg) levels detected in BD sample.

2.3.2. Zinc, selenium, copper, manganese, strontium, iodine, iron, arsenic and cadmium bioaccessibility of Zn, Se, Cu, Mn, Sr, I, Fe, As and Cd was assessed in different seafood matrices, including hake, tuna, mackerel, monkfish, plaice, octopus, mussel, shrimp and seaweed (old and new segments).

The water used was ultra-purified (<18 MΩ cm) using a Milli-Q-Integral system (Millipore, Milford, MA, USA) and all reagents used were “per analysis” quality or
better. Nitric acid (HNO₃), hydrogen peroxide (H₂O₂) (Merck) and hydrochloric acid (HCl) (PlasmaPure, SCP Science, Courtaboeuf, France) were used for sample preparation and analysis. Standard stock solutions of all elements were prepared at 1000 mg ml⁻¹ (PlasmaCal, SCP Science, Courtaboeuf, France).

For Zn, Se, Cu, Mn, Sr, Fe, As and Cd, subsamples of seafood before digestion (1.0 g wet weight), BIO fraction (1.0 ml) and CRM (0.20 g dry weight) were digested in closed vessels in a microwave oven (MARS5, CEMNC, USA) with 4 ml nitric acid (65% v/v) and 2 ml hydrogen peroxide (30% v/v). The digests were diluted to a volume of 25 ml with milliQ water. Prior to analysis sample aliquots were further diluted (seafood: 5 and 100 times; digestive solutions: 2 times; and CRM extracts: 5 and 50 times) with diluted acids to obtain 2% HNO₃ and 1% HCl (w/v) aqueous measurement solutions.

For iodine (I), subsamples of homogenized seafood BD, NBIO (1.0 g of wet sample) and BIO fraction (1.0 ml) were digested with the alkaline tetramethylammonium hydroxide (25% w/w; TMAH = (CH₃)₄N⁺OH⁻, 99.9999%, Thermofisher, Germany) reagent. Samples were homogenized with MilliQ water. 1 mL of TMAH was added to the samples, mixed thoroughly and incubated at 90 ± 3 °C during 3 h. After cooling, the digests were diluted to a volume of 25 ml with MilliQ water.

An inductive coupled plasma mass spectrometer (ICP-MS) (Agilent 8800 ICP-QQQ-MS, Santa Clara, USA) equipped with a micromist concentric quartz nebulizer and a Scott type double-pass water-cooled spray chamber was run in no gas (Cd, I), helium (Zn, Cu, Mn, Sr and Fe) and oxygen (As, Se) mode, respectively, with 0.2 s integration time per mass. Typical plasma conditions were 1550 W RF power, 15 L min⁻¹ plasma gas, L min⁻¹ carrier gas and 0 L min⁻¹ makeup gas. Cell gas flows were 5
mL min\(^{-1}\) for helium and 30 % oxygen with stabilization times of 30 s, 10 s and 30 s for helium, no gas, and oxygen mode, respectively. The auto sampler (ASX-500, Agilent Technologies, Waldbronn, Germany) introduced the samples into the ICP-MS with a sample uptake time of 50 s (0.4 rps) and a stabilization time of 40 s (0.1 rps). Rinse programme between samples: port (water) 10 s (0.2 rps), succeeded by rinsing in 2% HNO\(_3\) w/v during 30 s (0.1 rps) and 60 s (0.4 rps). Internal standard (ISTD) was added on-line (5 µg L\(^{-1}\) Rh and Bi) via a t-piece using a peristaltic pump.

The determination of iodine was performed in a separate analytical sequence using the same instrumental settings as above with iodine analysed in no-gas mode and using tellurium (Te) as internal standard. All calibration solutions were diluted in 0.25% TMAH using an iodine stock solution (999 µg/ml ammonium iodide, Teknolab, Sweden). The rinse programme between samples was as follows: port (water) 10 s (0.2 rps) succeeded by rinse steps 1 and 2 (0.25% TMAH c/v) 30 s (0.1 rps) and 60 s (0.4 rps), respectively.

To verify instrument stability a low calibration standard was repeatedly analysed throughout the sequence for least after every 10 samples for all elements except iodine (every 33 samples). Blank samples were analysed together with the samples and subtracted to all results. The LOD was assigned to the detection limit (DL) of the calibration curve (DL = 3 x standard deviation (σ) of response at the zero concentration level) and the LOQ was calculated as (3 x LOD). The quantification was done by external linear calibration (ng/ml: Cd (0.01-6), As (0.15-200), Mn (0.3-90), Fe (2-1000), Cu (0.3-120), Zn (3-200), Sr (0.3-90), Se (0.3-30) and I (0.75-45).

Essential and toxic elements in the bioaccessible fraction (%) were calculated as the following ratio:

\[
\frac{E \text{ BIO} \times 100}{E \text{ BD}}
\]
where E BIO corresponds to the element levels detected in BIO fraction, and E BD is the amount of element detected in the sample before digestion.

2.3.3. Quality control

Replicate measurements were done for each sample. The detection limits for each element are given in Appendix A, Supplementary Table S2. Certified reference material (CRM), TORT-2 (lobster hepatopancreas) and DORM-4 (fish muscle) (both National Research Council Canada, Ontario, Canada) was used to check the method accuracy for Hg, MeHg, Zn, Se, I, Cu, Mn, Sr, Fe, As and Cd quantification. Detailed information on the quality assurance data can be found in Appendix A, Supplementary Table S2.

2.3.4. Se:Hg and Se:MeHg molar ratios and Selenium Health Benefit Value (HBV_{Se})

Se:Hg and Se:MeHg molar ratios were calculated by converting Se, Hg and MeHg concentrations in µmol kg\(^{-1}\) (µM), and the Selenium Health Benefit Value (HBV_{Se}) was determined according to Ralston et al. (2016):

\[
HBV_{Se} = \frac{Se - Hg(\text{MeHg})}{Se} \cdot (Se + Hg(\text{MeHg})),
\]

where Se and Hg (or MeHg) were the concentrations expressed in µmol/kg.

2.3.5. Benefit-Risk balance

Percentages of the average requirements (AR) or adequate intakes (AI) for Zn, Se, Cu, Mn, I and Fe were calculated according to reference values set for individual
adults (>18 years old) by the European Safety Authority (EFSA, 2006, 2013, 2014a, 2014b, 2014c, 2015a, 2015b) and considering a daily meal composed by 150 g of seafood (raw or cooked). A smaller portion (i.e. 50 g) was considered for seaweed given the fact that vegetable food items are usually consumed as a side dish. For Zn, Cu and Fe, a range of values, instead of one single reference value, is defined as AR or AI, according to gender or vulnerable group [i.e. women or men, pregnant or breastfeeding women; EFSA, 2014a (Zn), 2015a (Cu), 2015b (Fe)]. In these cases, the lowest AR or AI set by EFSA was selected as reference value for calculation purposes. Strontium was not considered in this analysis, as no reference value is available for the intake of this element. In cases where the percentage of AR or AI was above 100 %, upper tolerable levels (ULs), i.e. maximum levels of intake of essential elements unlikely to result in adverse effects to the general population, were also calculated.

The percentage of tolerable weekly intakes (TWIs) for MeHg, Cd and As accomplished with the consumption of 150 g of fish, molluscs and crustaceans (white meat, i.e. excluding hepatopancreas and gonads) or 50 g of seaweeds were also calculated according to the values established by EFSA (2011, 2012), and considering an average adult body weight (bw) of 70 kg. Currently, there is no tolerable intake level set for As, since the latest EFSA scientific opinion on this element concluded that the previous PTWI of 15 µg kg\(^{-1}\) bw was no longer appropriate (EFSA, 2009). For this reason, along with the fact that the most toxic and previously regulated form of As (i.e. inorganic As) was not analysed in the present study, this element was not included in this risk-benefit assessment. Calculations regarding ARs or AIs, ULs, as well as TWIs were performed either including or excluding elements’ bioaccessibility.

2.3.6. Statistical analysis
For each culinary treatment (raw or steamed), differences in elements concentration/elements bioaccessibility/Se:Hg/Se:MeHg/\( \text{HBV}_{\text{Se}} \) were analysed by One-way ANOVA with the significance level set at 5% after checking for normality and variance homogeneity.

For each measurement mentioned above (e.g. elements concentration), differences between raw and steamed for the same seafood species, a t-test student for dependent samples with the significance level set at 5% was used.

Differences in elements concentration, bioaccessibility, Se:Hg, Se:MeHg and \( \text{HBV}_{\text{Se}} \) between samples and between raw and steamed seafood were analyzed by Two-way ANOVA with the significance level set at 5% using SigmaPlot v10.0 (Systat Software, Inc., CA, USA) after checking for normality and variance homogeneity. Tukey’s post-hoc test was used for pair wise multiple comparisons. When ANOVA assumptions were not met, ANOVA by Ranks followed by Dunn’s test for pairwise comparisons was performed. Statistical significance was set at \( p < 0.05 \).

3. Results

3.1. Elements in raw seafood

Concentrations of toxic elements Hg, MeHg, As and Cd were determined in seafood species and are represented as mean ± standard deviation. Hg concentration varied between 0.148 ± 0.000 (mackerel) and 0.376 ± 0.003 µg g\(^{-1}\) ww (octopus). MeHg concentration in seafood ranged between 0.049 ± 0.000 µg g\(^{-1}\) ww (plaice) and 0.266 ± 0.003 µg g\(^{-1}\)ww (octopus). Hg and MeHg varied according to the following order:
octopus > hake > monkfish > tuna > mackerel, (p < 0.05), (Fig. 1, Appendix A
Supplementary Table S3). The highest (48.9 ± 0.15 µg g⁻¹ ww) and lowest (0.51 ± 0.03 µg g⁻¹ ww) As levels were observed in octopus and shrimp, respectively. Significant differences were observed between the two seaweed leaf portions (p < 0.001), with the oldest part showing higher As levels (12.0 ± 0.24 µg g⁻¹ ww) (Fig. 1, Appendix A, Supplementary Table S3). Fig. 1 reveals that Cd concentration in seafood ranged between 1.03 ± 0.29 (monkfish) and 72.8 ± 1.2 (mussel) µg kg⁻¹ ww. Higher Cd levels were observed in bivalves, crustaceans and seaweed compared to fish (p < 0.05). In relation to seaweed, the old segment (53.0 ± 4.5 µg kg⁻¹ ww) revealed 50 times higher Cd levels than those observed in the newer part (7.11 ± 0.52 µg kg⁻¹ ww) (p < 0.001) (Appendix A, Supplementary Table S3).

In general, molluscs and crustaceans showed the highest levels of the essential elements Zn, Cu, Mn and Fe. For example, the highest Zn and Cu concentrations were found in shrimp and were respectively, 18.4 ± 0.4 and 8.78 ± 0.47 µg g⁻¹ ww. High levels of Mn (2.82 ± 0.04 µg g⁻¹ ww) and Fe (84.9 ± 0.9 µg g⁻¹ ww) were registered in mussel (Fig. 1, Supplementary Table S4). Seaweed was the richest seafood in I (433 ± 17 µg g⁻¹ ww) and Sr (old leafs - 154 ± 0 µg g⁻¹ ww; new leafs - 222 ± 36 µg g⁻¹ ww). Hake showed the lowest concentration of most elements (Zn, Cu, Mn, Sr, I, Fe). Tuna showed high concentration of Zn (15.5 ± 1.0 µg g⁻¹ ww), Se (2.07 ± 0.02 µg g⁻¹ ww), Fe (33.0 ± 0.4 µg g⁻¹ ww) and Cu (2.12 ± 0.06 µg g⁻¹ ww) (Fig. 1, Appendix A, Supplementary Table S4).

3.2. Elements in steamed seafood

Hg, MeHg, Cd and Zn were the elements most affected by the steaming, where levels significantly changed in almost all the analysed seafood species (p < 0.05). Hg,
MeHg, Cd and Zn were the elements most affected by the steaming, where levels significantly changed in least at 65% of the total analysed species (p < 0.05) (Fig. 1, Appendix A, Supplementary Tables S3 and S4). MeHg concentration significantly increased 20-30% after steaming in monkfish, tuna and octopus (p < 0.05). In the case of Cd, concentrations decreased after steaming in tuna, octopus and shrimp (p < 0.05), but increased at least 50% in monkfish, mussel, plaice and seaweed old (p < 0.05) (Fig. 1, Appendix A, Supplementary Table S3). Zn was the unique essential element affected by steaming in the majority analysed seafood species. This element generally increased after the culinary treatment and this increment ranged from 15% (mackerel) and 120% (mussel) (Fig. 1, Appendix A, Supplementary Table S4).

As, Se, Cu, Mn, Sr, I and Fe were generally unaffected by the steaming. For example, arsenic content only in monkfish, plaice, octopus and in seaweed old leafs was significantly changed after steaming compared with the raw product (p < 0.001). In the case of Mn and Fe, only one of the ten seafood species showed significant differences between raw and steamed products (p < 0.05) (Fig. 1, Appendix A, Supplementary Table S4). Steaming resulted in a significant increase of I concentration only in the old and newer parts of the seaweeds (p < 0.05) (Fig. 1, Appendix A, Supplementary Table S3).

Insert Figure 1

3.3. Bioaccessibility

3.3.1 Bioaccessibility of proteins
In general, high protein digestibility was observed in both raw and steamed samples from all commercial species analysed, ranging from 61.0 % (steamed mussel) and 98.9 % (raw octopus). Seaweed showed the lowest protein bioaccessibility (raw 15.2 %; steamed 8.8 %) (Appendix A, Supplementary Table S5).

3.3.2 Bioaccessibility of toxic and essential elements in raw seafood

Hg bioaccessibility ranged between 11 ± 3 % (octopus) and 61 ± 3 % (hake), with most species presenting a bioaccessibility below 50 % (Fig. 2, Appendix A, Supplementary Table S6). Hg recovery after the digestion process ranged between 70 % in monkfish and 95 % in octopus. Regarding MeHg, the highest concentrations in the bioaccessible fractions were found in monkfish (61 ± 1 %) and hake (52 ± 4), whereas the lowest MeHg bioaccessibility was observed in octopus (10 ± 1 %, p < 0.05) (Fig. 2). In general, As bioaccessibility was high in all analysed species, ranging between 54 ± 2 % (seaweeds) and 98 % (monkfish and octopus). Cd bioaccessibility was high for mussel (103 ± 10 %) and shrimp (75 ± 7 %), and low for tuna (41 ± 6 %) (Fig. 2, Appendix A, Supplementary Table S6).

Zn bioaccessibility ranged between 28 ± 2 % (tuna) and 77 ± 17 % (old seaweed). Bioaccessible Zn values for mackerel and monkfish could not be determined as the results were below the limit of detection (Fig. 2, Appendix A, Supplementary Table S7). The bioaccessibility of Se was always higher than 59 %, showing low variability between seafood species (72 ± 13 %, average ± standard deviation). The highest Se bioaccessibility was observed in shrimp (97 ± 3 %). Fig. 2 reveals that Cu bioaccessibility ranged between 43 ± 20 % (new seaweed leaves) and 93 ± 8 % (octopus). This high variability in bioaccessibility among species was also observed for Mn, with
seaweed old showing the lowest Mn bioaccessibility (22 ± 12%). In octopus, Mn was 113 ± 6 % bioaccessible (Fig. 2, Appendix A, Supplementary Table S7). The bioaccessibility of Cu and Mn was statistically higher in the newer part of the seaweed (p < 0.05). Sr bioaccessibility was highly variable between species and ranged between 18 ± 0.3 % (newer seaweed leafs) and 96 ± 11 % (mussel). In general, fish Sr bioaccessible values were below the LOQ. High I bioaccessibility was obtained for octopus (86 ± 3 %), mussel (84 ± 2 %) and the newer seaweed leafs (74 ± 4 %), whereas only 47 ± 1 % of I was bioaccessible in tuna. Almost all Fe bioaccessible concentrations were below the limit of quantification, except for mussel (26 ± 3 %) and tuna (69 ± 7 %) respectively (Fig. 2, Appendix A, Supplementary Table S7).

3.3.3. Effect of steaming in the elements bioaccessibility

For all analysed species, steaming significantly decreased total Hg bioaccessibility (p < 0.001), ranging between 1 ± 0 % (octopus) and 19 ± 2 % (hake). Additionally, MeHg bioaccessibility also significantly decreased in all species after steaming (p < 0.001). MeHg bioaccessibility varied between 2 ± 0 % (octopus) and 22 ± 5 % (hake) in steamed seafood (Fig. 2, Appendix A, Supplementary Table S6). No statistical differences were registered in the bioaccessibility of As in raw and steamed seafood (Appendix A, Supplementary table S6). In contrast, Cd bioaccessibility decreased around 20 % in mussel and shrimp after steaming (p < 0.05) (Fig. 2, Appendix A, Supplementary Table S6).

In fish (tuna and plaice), Zn bioaccessibility increased after steaming (p < 0.05), whereas Zn bioaccessibility decreased in molluscs after the culinary treatment. Se bioaccessibility decreased after steaming in shrimp (74 ± 2 %) and plaice (54 ± 8 %),
whereas for mackerel, Se bioaccessibility increased after steaming (84 ± 24 %) (p < 0.05). No significant differences were found between raw and steamed bioaccessible Se in hake, tuna, monkfish, octopus and mussel (p > 0.05) (Fig. 2). In the newer seaweed leaves, steaming increased and decreased Cu and Mn, respectively (Fig. 2, Appendix A, Supplementary Table S7). In contrast, the culinary treatment did not significantly change Cu bioaccessibility in octopus, mussel and shrimp (p > 0.05). Concerning Sr bioaccessibility, only plaice (increased – 84 ± 4 %) and mussel (decreased – 69 ± 6 %) revealed statistically changes after the culinary treatment (p < 0.05). Steaming only significantly affected I bioaccessibility in the older seaweed part (66 ± 1 % - raw, 81 ± 4 % - steamed) (p < 0.05). Fe bioaccessibility only decreased after steaming in mussel, around 50 % (p < 0.05) (Fig. 2, Appendix A, Supplementary Table S7).

Insert Figure 2

3.4. Impact on toxic/essential elements exposure in seafood

3.4.1. Se-dependant benefits

Before digestion, Se:Hg or Se:MeHg molar ratios were higher than 1 in all raw and steamed seafood. The highest Se:Hg and Se:MeHg were observed in tuna, while the lowest occurred in octopus (p < 0.05). Before digestion, steaming only led to a significant increase in Se:MeHg for hake, whereas significant decrease in Se:Hg was observed for hake, tuna, monkfish and mackerel, as well as in Se:MeHg for tuna and monkfish (Table 2). In the bioaccessible samples, tuna and octopus showed the highest Se:Hg and Se:MeHg ratios (p < 0.05), and in both species Se:Hg and Se:MeHg ratios increased after steaming (p < 0.01, Table 2). Steaming induced statistical increased
Se:Hg and Se:MeHg ratios in the bioaccessible fraction of tuna, mackerel and octopus (Table 2).

Few significant differences were observed in HBV$_{Se}$ values in relation to Hg and MeHg. Before digestion, as well as in bioaccessible samples the highest HBV$_{Se}$ value was observed in tuna (p < 0.05). The HBV$_{Se}$ (for Hg and MeHg) values in bioaccessible samples were not significant different between in hake, mackerel and octopus (p > 0.05, Table 2). Before digestion, as well, in bioaccessible samples the highest HBV$_{Se}$ values were observed in tuna (p < 0.05). In bioaccessible samples, HBV$_{Se}$ (for Hg and MeHg) values were not significant different in hake, mackerel and octopus (p > 0.05, Table 2). The culinary treatment only affected HBV$_{Se}$ before digestion in tuna and octopus (p < 0.05; increasing in octopus and decreasing in tuna), whereas no effects of steaming were observed in bioaccessible samples (Table 2).
3.4.2. Benefit-Risk balance

Table 3 shows the benefit-risk balance for the consumption of seafood species (raw and cooked) based on average requirements (AR) or adequate intakes (AI) and upper limits (UL) set for each essential element. Percentages of AR/AI and ULs for a consumption of 150 g seafood varied according to species and element. Crustaceans and cephalopods revealed high intakes of Zn (between 27 and 44 % of the AR) and Cu (over 92 %), whereas remarkably higher Se intakes were observed in fish species, particularly tuna (over 100 % of AI and 82 % of UL). Mussels revealed the highest % AR of Mn and Fe, respectively, especially after steaming (Mn = 30 % and Fe = 71 %). Mussels and seaweed also provided over 100% of AI set for I. In what concerns toxic elements, the consumption of 150 g octopus revealed the highest intake of MeHg (44 % and 60 % of TWI in raw and cooked samples, respectively), whereas the highest Cd % TWI was registered with mussels (Table 4).

In terms of cooking effect, no clear pattern was observed (Tables 3 and 4), as the contents of some elements increased in some seafood species after steaming (e.g. Zn and Se, except in tuna and monkfish; MeHg, except in plaice), while in others contents drastically decreased (e.g. Fe in octopus). Despite some exceptions (i.e. Cu and Mn in octopus), overall, the inclusion of elements’ bioaccessibility tended to decrease the
percentages of both AR or AI (and ULs) of each essential element, as well as the percentages of the TWIs for toxic elements, with the consumption of 150 g of seafood (Tables 3 and 4).

Insert table 4

4. Discussion

4.1. Protein bioaccessibility – in vitro digestion efficiency

Nutrients and contaminants available in seafood and their bioaccessibility have been studied during the last years to evaluate the risks and benefits associated with seafood consumption (Afonso et al., 2016; Cardoso et al., 2015; He et al., 2019). The in vitro digestion procedure adapted from Versantvoort et al. (2005) and Minekus et al. (2014) optimized for different seafood matrices enabled a proper digestion of seafood samples, as the vast majority of proteins were hydrolysed and released to the bioaccessible fraction, in the range of reported values for the digestible fish proteins (77 to 99 %), (Usydus et al., 2009). In steamed samples, protein bioaccessibility decreased as expected due to the thermal treatment, and consequent protein denaturation and configuration changes (Matos et al., 2015). The in vitro digestion protocol used in this study revealed lower protein bioaccessibility for seaweeds. Seaweeds have a complex carbohydrate matrix structure that is difficult to enable the access of proteins to digestive enzymes and thus to ensure a proper digestion.

4.2. Toxic elements bioaccessible in seafood
In the present study, analysis of total Hg (including MeHg) in seafood revealed octopus as the species showing the highest concentration, and where both Hg and MeHg concentrations increased after steaming. For example, steamed octopus (Hg, 0.553 µg g⁻¹ ww) showed levels higher than the maximum levels (MLs) set by EU for Hg. The effects of culinary treatments are relevant, as in general, they induce an increase in Hg as a result of water loss during steaming (Maulvaut et al., 2011). Nevertheless it was demonstrated by Schimdt et al. (2015) that boiling, frying or roasting fish does not change the Hg concentration present in raw fish. Nevertheless, Hg or MeHg concentrations do not always reflect the bioaccessible fraction that is released from seafood during the digestive process and potentially reaches the systemic circulation, and the results obtained in the current study support this statement. In fact, it has been reported that an overestimation of health risks for humans associated to Hg and MeHg is likely to occur (Cano-Sancho et al., 2015). To our knowledge this is the first study reporting Hg and MeHg bioaccessibility in octopus, showing the lowest bioaccessibility (~ 11%). Bioaccessibility of Hg/MeHg in fish is generally very variable, ranging from 13 up to 87 % (reviewed by Chiocchetti et al. (2016). The high variability between species reported in literature may be due to different in vitro digestion protocols used, distinct nutritional composition of the food matrix, different Hg/MeHg accumulation rates in seafood, seafood feeding habitats and other biotic parameters (Cabañero et al., 2007; Calatayud et al., 2012; Cano-Sancho et al., 2015; Torres-Escribano et al., 2011b). For example, total Hg bioaccessibility in commercial fish from Hong Kong varied between 19 % (bigeye) and 51.7 % (orange-spotted grouper), (Wang et al., 2013). These authors considered that the conditions of sample storage can be potential factors
affecting Hg bioaccessibility, such as thawing conditions, freezing rates and storage temperature, which are related to protein denaturation.

In the case of As, octopus and plaice showed the highest concentrations, and this was not surprising as benthic species that live in direct contact with the sea bottom are potentially more exposed to contamination (Cano-Sancho et al., 2015). Low arsenic levels in mussels were revealed by other studies. For example, total As in mussels from different China regions ranged from 1.60 to 2.94 (µg/g w.w.), (He and Wang, 2013). Both old and news seaweed leafs accumulated significant levels of As (7.30 – 12.0 µg g\(^{-1}\) ww). The values obtained in the present study were lower than those reported for the same seaweed species in a previous study (Maehre et al., 2016), whereas for other seaweed species might contain higher As levels have been reported, such as the seaweed Laver, *Porphyra abottae* (33.0 µg g\(^{-1}\) ww). In a previous study, the culinary treatment (steaming, boiling) did not affect total As in *Cancer pagurus* muscle and brown meat (Maulvault et al., 2011), and similar results were observed in the current study for 6 species. Contrary to what was observed for Hg, As bioaccessibility was high in almost all species, in accordance with previous findings (He et al., 2010; Laird and Chan, 2013; Laparra et al., 2007; Maulvault et al., 2011; Peng et al., 2016). In fact, the low pH observed during the stomach digestion phase (gastric fluid) can be one of the factors responsible for the high As solubilisation. Other factors such as enzymatic activity, As interaction with soluble compounds can also be related with the high As bioaccessibility observed in the present study. Therefore, future studies should assess if the inorganic fraction of As (most toxic form) in these seafood species follows the same trend of total As.

Mussel was the seafood species showing the highest Cd concentration, and populations where bivalves play a central role in the diet can be more exposed to
toxicological effects associated with Cd (Amiard et al., 2008; Leufroy et al., 2012; Vandermeersch et al., 2015). High and low Cd contamination is associated to molluscs/algae and fish, respectively (Moreda-Piñeiro et al., 2012). Levels of Cd in mussel, plaice and the older part of the seaweed increased after steaming. Similar findings were observed in steamed and boiled Cancer pagurus (Maulvault et al. 2011). Cadmium bioaccessibility was higher in mussel and octopus (shellfish) than in tuna, and similar findings were previously reported for mussel and shrimp (Leufroy et al., 2012). The same parameters that influenced MeHg bioaccessibility such as differences in moisture, food composition (proteins, fibres) and metal and cellular components interaction can explain the variability found in Cd bioaccessibility (Wang et al., 2014).

The influence of steaming in the toxic elements bioaccessibility provides more evidences concerning the accurate risks of seafood consumption. Indeed, steaming significantly reduced Hg and MeHg bioaccessibility in all seafood analysed. Similar findings have also been reported for other fish species (Cano-Sancho et al., 2015; Maulvault et al., 2011; Torres-Escribano et al., 2011b). Protein structure modification and loss of the native form by heating during culinary treatments can make the complexes Hg-protein less accessible to digestive enzymes, and subsequently reduce their solubilisation during digestion. MeHg is bound to different proteins in the tissues, including albumin, glutathione or cysteine-rich proteins (Ouedraogo and Amyot, 2011). The reduction of Cd bioaccessibility observed in steamed mussel and octopus is in accordance with previous studies performed in shellfish, including mussels, oysters, clams and scallops (Gao and Wang, 2014). As observed for MeHg, the decrease in Cd bioaccessibility after steaming is likely due to the loss of highly digestible proteins during the steaming process (Amiard et al. 2008). Additionally, the formation of insoluble components due to the denaturation can reduce protein digestibility and
subsequently decreases in Cd bioaccessibility (Kulp et al., 2003). In contrast to MeHg and Cd, steaming has not affected As bioaccessibility (except seaweed), and is in agreement with previous studies in seafood subjected to different culinary treatments (Laparra et al., 2007). Indeed, the amount of As bioaccessible was not correlated with the protein content of the seafood (Moreda-Piñero et al., 2012).

4.3. Essential elements bioaccessible in seafood

Shellfish (mussels, octopus and shrimp) are a source of Zn, Mn, Cu and Fe in comparison to fish. Guérin et al. (2011). High Zn levels were also observed in mussels from Galicia (Spain), but shrimps and tuna from this region revealed low Zn values (Olmedo et al., 2013). Moreover, Cu concentrations were low compared to levels obtained in other studies (Leufroy et al. 2012, Guérin et al. 2011), but similar with those observed by Olmedo et al. (2013). In the present study, steaming generally increased Zn concentration in almost all seafood species, whereas Cu, Mn and Fe levels only increased in mussel, octopus and shrimp.

Excluding the low Mn and Fe bioaccessibility observed in shrimp and mussels, respectively, the bioaccessibility of essential elements in shellfish were always higher than 60 %. The high variability found in Zn bioaccessibility has already been observed in previous studies (He et al., 2015). Furthermore, previous findings also reported similar element (Zn, Cu, Mn) bioaccessibility in seafood (Amiard et al. 2008; Leufroy et al 2012). Additionally, the varying level of Mn bioaccessibility observed for shellfish in the current study has also been previously reported (Leufroy et al., 2012).

Se content revealed a great variability, as previously reported by Marval-Léon et al (2014), and Se levels were similar to the current study. Steaming increased
significantly the Se content in octopus and mussels in accordance with previous findings reported for blue shark after grilling and steaming (Matos et al., 2015). The increase in Se content is likely related to water loss during culinary treatment (Afonso et al., 2015). Se bioaccessibility was high in all seafood species, and similar Se bioaccessibility has been reported for fish (Calatayud et al., 2012; Matos et al., 2015) and shellfish (Calatayud et al., 2012). These authors suggested that the gastrointestinal fluid composition and the food matrix composition, can explain the variation in essential elements bioaccessibility between species.

Steaming induced an increase in Zn bioaccessibility for tuna and plaice, but a reduction was observed for octopus and mussel. This is consistent with previous findings of Amiard et al. (2008) in shellfish. Differences between fish and shellfish in Zn bioaccessibility are likely associated with the distinct chemical composition of these matrices. In contrast, Cu bioaccessibility seems not to be affected by steaming, contrarily to previous results obtained for fish and shellfish (He et al., 2010). Cu is mainly associated to metallothioneins and insoluble ligands in the form of less degradable complexes. However, in most severe heating conditions (e.g. frying or grilling) it has been reported to cause a decrease in Cu bioaccessibility (Amiard et al., 2008). In hake, tuna, monkfish, mussel and shrimp, steaming does not seem to affect Se bioaccessibility, as observed for blue shark (Matos et al. 2015), seabass and red seabream (He et al., 2010). In contrast, steaming decreased Se bioaccessibility in plaice and shrimp (He & Wang, 2013).

Besides the high levels of Zn and Fe observed for the seaweed *L. digitata*, this species can also be a good source of Sr and I, though the concentrations were higher in the newer leafs. *Laminaria digitata* and *Laminaria hyperborea* seaweeds have been described as very important sources of I (Maehre et al., 2016). In fact, I is an essential
bioactive element used in biosynthesis of thyroid hormones, and a lack of I leads to thyroid disorders (Zimmermann, 2010). A previous study performed with boiled Japanese tangle (Laminaria japonica) revealed 54% of bioaccessible I (Fukushima and Chatt, 2012), which was lower than values obtained in the current study. However, the current study reveals that the part of seaweed being analysed play an important role in I bioaccessibility, as the levels were in the older part increased (80%) significantly after steaming. The present results will contribute with relevant data concerning the bioaccessible profile of a broad range of elements in different seafood species. Nevertheless, the research for reference material for most of elements in a broad range of different seafood species, should be in the future addressed in order to improve the elements bioaccessibility quality control data in seafood.

4.4. Selenium and mercury balance

Selenium has been associated to the reduction of Hg toxicity (Ralston et al., 2016). The molar ratio between Se and Hg (Se:Hg; Se:MeHg) has been suggested as an essential criteria to evaluate the health risks raised by Hg (Ralston et al., 2007). In the present study, all five predatory species had Se:Hg and Se:MeHg molar ratios above 1, showing that Se molar content exceeded Hg molar content. Similar findings were described in other species, such as meagre (Afonso et al., 2015). Se:Hg and Se:MeHg increased in the bioaccessible fractions, due to the reduction of MeHg bioaccessibility after steaming but without changing Se bioaccessibility. Cabañero and co-authors (2007) observed a similar increase in Se:Hg ratio in bioaccessible swordfish, tuna and sardine. Moreover, steaming increased the Se:MeHg in tuna, mackerel and octopus, suggesting that this culinary treatment is a good strategy to reduce the toxic effect of
MeHg. Selenium health benefit value ($HBV_{Se}$), based on the molar concentrations of Hg/MeHg and Se found in seafood is a risk assessment tool used to evaluate the effects of MeHg exposure after seafood consumption (Ralston et al., 2016). Indeed, a low Se intake is generally associated with a high MeHg exposure, and during pregnancy it can result in severe negative effects for fetal tissues (Crump et al., 1998). In this study, the analysed seafood species presented positive $HBV_{Se}$ values, suggesting that consuming these five seafood species can reduce the risks associated with the inhibition of selenoenzymes by MeHg. The molar ratio excess of Se in comparison to Hg has also been verified in other pelagic fish (Ralston et al., 2007).

4.5. Benefit-risk balance

Zn, Cu, Se, Fe, I and Mn are essential micronutrients for the human body and appropriate intake of these elements should be provided in a balanced diet to meet the consumers’ daily requirements (EFSA, 2006). It should be noted that the present risk-benefit balance was based on the adults requirements, regardless of gender or being included in a vulnerable group (elderly, pregnant or lactating women), and calculations do not include infants and children requirements. Since some elements’ requirements vary among demographic and vulnerable groups, the results and conclusions here presented should be seen as estimations. In this sense, considering seafood’s elemental profiles and the minimum daily dose of essential elements required for a healthy diet of adult individuals (including pregnant women, to which the same AI value was defined), the studied fish, mollusc and crustacean species showed to be good sources of Se, which plays an important role against oxidative stress, in the regulation of thyroid hormones’ action, and as an antagonist in MeHg exposure (e.g. Ralston et al., 2016). Yet,
considering the dichotomy in essential elements benefit-risk assessment, i.e. the need to accomplish appropriate element intakes that are neither too low, causing nutritional deficiencies, nor too high, being toxic to consumers, some fish species such as tuna should be consumed with limitations to avoid exceeding the UL set for Se (EFSA, 2006). The same principle can also be applied for Cu in octopus and shrimps, as well as in mussels and seaweeds due to the remarkably high levels of I. It should be further stressed that: i) The percentages of Se AI (and UL) obtained through the consumption of seafood may be higher for infants and children because, although no specific indicators of Se requirements were available for this demographic group, in the latest EFSA report it is extrapolated an AI value of 15 µg day$^{-1}$ for this element (EFSA, 2014b); ii) An additional dose of 15 µg day$^{-1}$ of Se was also added as a daily requirement of breast feeding women, thus, in this case, percentages of Se AI provided with the consumption of seafood may be lower (EFSA, 2014a); iii) percentages of Cu AI accomplished with seafood consumption may be lower for men and pregnant women, but should be higher for infants and children, given the range of values set for the element according to demographic and vulnerable group (EFSA, 2015a); Given the UL set for Cu, a parsimonious consumption of octopus by early aged children is advisable (EFSA, 2006); iv) the percentages of I AI acquired through seafood consumption may be lower for pregnant and breast feed women, but should be higher for infants and children, considering the specific requirements of these vulnerable groups (EFSA, 2014b); When it comes to children, closer attention should be paid to the consumption of bivalves and seaweed, as this group is likely above the threshold set for I daily intake, when consuming these seafood species (EFSA, 2006). The same principle can also be applied for Cu in octopus and shrimps, as well as mussels and for seaweeds due to the remarkably high levels of I. It should be further stressed that: i) The
percentages of Se AI (and UL) fulfilled with the consumption of seafood may be higher for infants and children because, although no specific indicators of Se requirements were available for this demographic group, in the latest EFSA report it is extrapolated an AI value of 15 µg/day of this element (EFSA, 2014b); ii) An additional dose of 15 µg/day of Se was also added as daily requirements of breast feeding women, thus, in this case, percentages of Se AI provided with the consumption of seafood may be lower (EFSA, 2014a); iii) percentages of Cu AI accomplished with seafood consumption may be lower for men and pregnant women, but higher for infants and children, given the range of values set for the element according to demographic and vulnerable group (EFSA, 2015a); Given the UL set for Cu, a parsimonious consumption of octopus by early-aged children is advisable (EFSA, 2006); iv) the percentages of I AI accomplished with seafood consumption may be lower for pregnant and breast feed women, but higher for infants and children, considering the specific requirements of these vulnerable groups (EFSA, 2014b). When it comes to children, closer attention should be paid to the consumption of bivalves and seaweed, as this group is likely above the threshold set for I daily intake when consuming these seafood species (EFSA, 2006).

Regarding MeHg and Cd, which have no known biological role in the human body, out of the studied seafood species, the consumption of octopus showed to place consumers at a higher risk of exceeding the TWI set for MeHg, whereas the consumption of 150 g of mussels revealed Cd intakes closest to the TWI. This could be particularly worrying for children, which have a lower body weight, as well as pregnant and breast feeding women. Hence, it is advisable a parsimonious consumption of octopus and mussels by these vulnerable groups.

Despite the changes occurred in seafood chemical composition upon cooking, results do not point out to a negative effect of steaming on seafood elemental profiles. In fact,
when considering more realistic data based on element bioaccessibility, overall steaming showed to increase the percentages of the ARs (or AIs) for most essential elements, and decreased the percentages of MeHg and Cd TWIs (Tables 3 and 4). Noteworthy, compared to other culinary procedures which generally induce more drastic changes in seafood chemical composition (e.g. in lipid, protein and elemental profiles) such as frying or grilling, steaming has been pointed out as an healthy option when it comes to cooking seafood, maintaining seafood’s nutritional attributes closer to the original (i.e. in raw products) (e.g. Maulvault et al., 2011, 2013). Consumers should also bear in mind that other seafood quality and safety aspects, apart from the elemental profile, should also be accounted when balancing the risks and benefits of cooking procedures (involving heat treatment), such as the drawbacks in terms of long-chain polyunsaturated fatty acids oxidation (e.g. Maulvault et al., 2011), or the positive effects in the elimination of seafood pathogens (virus, bacteria a parasites) which can be hazardous to humans. To sum up, as final take home message, the studied steamed seafood species can be considered good choices, in what concerns the elemental profiles, and can be included in a well-balanced and diversified diet. Although the benefits seem to outcome the risks in most cases, for specific individual groups, such as children and pregnant women the consumption of seafood species with element intake levels close to the UL or the TWI should be done with further prudence.

5. Conclusions

The bioaccessibility of toxic and essential elements in different seafood matrices, including fish, shellfish and seaweeds, was influenced by species and greatly varied between elements. MeHg revealed low bioaccessibility in all fish species. In
contrast, As bioaccessibility was high in all species. Therefore, future studies should assess if inorganic As bioaccessibility follows the same trend. In the case of essential elements, overall bioaccessibility showed high values in fish and seaweed (for Zn, Mn and I), whereas lower values and wider variation was found among shellfish.

Steaming affected differentially the elements bioaccessibility. MeHg and Cd levels were reduced in steamed seafood, thus lowering the health risks when seafood is consumed with this culinary practice. In contrast, for essential elements, steaming increased (e.g. Zn in fish and seaweed; Sr in plaice, Mn in seaweed old), decreased (e.g. Zn in shellfish, Se in plaice and shrimp, Mn, Sr and Fe in mussel) or unchanged (e.g. Se in some fish and mollusc; Cu and I for almost seafood species) the bioaccessibility, according to seafood species.

In general, fish, shellfish and seaweed species can be considered as reasonable sources of essential elements. Despite data based on elemental profiles indicate that steamed seafood can potentially add value to human diet, some species (e.g. mussels and tuna) can also contain high levels of toxic elements such as iAs and MeHg. Hake was the species in the present study with lowest essential elements bioaccessible concentrations. Tuna can be a reasonable source of Zn, Se, Cu and I, and low MeHg and Cd bioaccessibility was observed in this species. Newer segments of the seaweed showed to be more enriched in essential elements but lower in arsenic content. Moreover, a low health hazard was associated to the five predatory species consumption as shown by the positive HBV\textsubscript{Se} and high molar Se:MeHg ratio. The present seafood species are a valuable source of iodine particularly in the geographical areas where iodine intakes from other foods are insufficient, however it is desirable to do it in an equilibrated and balanced diet, as some of these species showed very high I levels.
Finally, as far as elements are concerned, the steamed seafood species studied in this work are recommended to be regularly consumed as this culinary method reduces the bioaccessibility of toxic elements and, whenever available, most essential elements are maintained at high concentrations after digestion. Additional studies will be interesting to conduct in the future in order to evaluate the effect of other culinary treatments, such as grilling, boiling and frying that are also generally culinary procedures used by the European consumers. In addition, the evaluation of the effects of simulating a full meal by mixing other food and drink types in the elements bioaccessibility will be another line of investigation we want to address in the near future.

This study clearly reveals that food risk and benefit assessment should take in the future into consideration the diversity of seafood species, the effects of culinary treatment and the bioaccessibility of the compounds under study to provide more accurate indications about health effects to consumers, refinements of food safety legislation (MPCs and TWIs/RDIs) and guidelines for consumers regarding seafood consumption, thus minimizing under- or overestimations of risks/benefits, and providing more realistic information.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Legend of figures

**Fig. 1** – Concentrations of toxic (A – mercury, B – methylmercury, C – arsenic, D - cadmium) and essential (E -zinc, F - selenium, G - copper, H - manganese, I - strontium, J – iodine and K- iron) elements in raw and steamed samples prior to *in vitro* digestion (µg g⁻¹ ww or µg kg⁻¹ ww, average ± standard deviation). * represent differences between raw and steamed for each seafood species (t-student for dependent variables, p < 0.05), please see detailed information in Appendix A, Supplementary tables 3 and 4.

**Fig. 2** – Bioaccessibility (%) of toxic (A – mercury, B – methylmercury, C – arsenic, D - cadmium) and essential (E -zinc, F - selenium, G - copper, H - manganese, I - strontium, J – iodine and K - iron) elements in raw and steamed samples (average ± standard deviation). * represent differences between raw and steamed for each seafood species (t-student for dependent variables, p < 0.05), please see detailed information in Appendix A, Supplementary tables 6 and 7.
Table 1.
Selected species from European seafood markets used for the essential and toxic elements bioaccessibility assessment.

<table>
<thead>
<tr>
<th>Seafood</th>
<th>Species</th>
<th>Origin</th>
<th>Market country</th>
<th>n</th>
<th>Total length (mm)</th>
<th>Total weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hake</td>
<td><em>Merluccius australis</em></td>
<td>Pacific Ocean</td>
<td>Portugal</td>
<td>25</td>
<td>n.a</td>
<td>2,500-3,500</td>
</tr>
<tr>
<td>Tuna</td>
<td><em>Katsuwonus pelamis</em></td>
<td>Pacific Ocean</td>
<td>Portugal</td>
<td>25</td>
<td>n.a</td>
<td>&gt;3,400</td>
</tr>
<tr>
<td>Monkfish</td>
<td><em>Lophius piscatorius</em></td>
<td>Atlantic Ocean</td>
<td>Portugal</td>
<td>25</td>
<td>570-590</td>
<td>3,365-3,448</td>
</tr>
<tr>
<td>Mackerel</td>
<td><em>Scomber scombrus</em></td>
<td>Adriatic Sea</td>
<td>Italy</td>
<td>25</td>
<td>189-285</td>
<td>47.7-268.7</td>
</tr>
<tr>
<td>Plaice</td>
<td><em>Pleuronectes platessa</em></td>
<td>North Sea</td>
<td>The Netherlands</td>
<td>25</td>
<td>347-411</td>
<td>376-635</td>
</tr>
<tr>
<td>Mussel</td>
<td><em>Mytilus edulis</em></td>
<td>Atlantic Ocean</td>
<td>The Netherlands</td>
<td>50</td>
<td>44-68</td>
<td>5.9-18.5</td>
</tr>
<tr>
<td>Octopus</td>
<td><em>Octopus vulgaris</em></td>
<td>Mediterranean</td>
<td>Spain</td>
<td>25</td>
<td>920¹</td>
<td>2,600²</td>
</tr>
<tr>
<td>Shrimp</td>
<td><em>Litopenaeus vannamei</em></td>
<td>Indic Ocean</td>
<td>Portugal</td>
<td>50</td>
<td>11-14</td>
<td>11-28</td>
</tr>
<tr>
<td>Seaweed</td>
<td><em>Laminaria digitata</em></td>
<td>North Sea</td>
<td>Norway</td>
<td>20</td>
<td>n.a</td>
<td>n.a</td>
</tr>
</tbody>
</table>

n, number of specimens analyzed; total length (mm) and total weight (g) – range minimum and maximum; ¹ total length (mm) presented as mean; ² total weight (g) presented as mean; ³ Moisture was determined in the samples used for MeHg extraction after freeze-drying; n.a, data not available as part of the specimens were provided by the suppliers; n.d, not determined.
<table>
<thead>
<tr>
<th></th>
<th>Se:Hg</th>
<th>Se:MeHg</th>
<th>HBV&lt;sub&gt;Se&lt;/sub&gt; for Hg</th>
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<tr>
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<td>BD&lt;sup&gt;1&lt;/sup&gt;</td>
<td>BIO&lt;sup&gt;2&lt;/sup&gt;</td>
<td>BD&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.93±0.00b</td>
<td>5.92±0.64b</td>
<td>6.00±0.00c</td>
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<tr>
<td>Steamed</td>
<td>4.54±0.00B</td>
<td>17.52±1.27C</td>
<td>6.91±0.00C</td>
</tr>
<tr>
<td>Tuna</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Raw</td>
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<td>63.71±5.35a</td>
<td>38.13±0.00a</td>
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<tr>
<td>Steamed</td>
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<td>195.25±46.97A</td>
<td>28.70±0.00A</td>
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<td>Monkfish</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>3.95±0.25c</td>
<td>10.65±1.52b</td>
<td>8.91±0.00b</td>
</tr>
<tr>
<td>Steamed</td>
<td>3.18±0.01C</td>
<td>25.36±5.59C</td>
<td>7.30±0.00B</td>
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<td>Mackerel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>4.79±0.36b</td>
<td>14.30±1.31b</td>
<td>5.87±0.44c</td>
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<tr>
<td>Steamed</td>
<td>4.43±0.00B</td>
<td>53.14±7.85C</td>
<td>5.90±0.00D</td>
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<td>Octopus</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Raw</td>
<td>2.67±0.00d</td>
<td>25.67±3.63ab</td>
<td>4.05±0.00d</td>
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<tr>
<td>Steamed</td>
<td>2.45±0.00D</td>
<td>118.82±10.8B</td>
<td>3.98±0.00E</td>
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</tbody>
</table>

<sup>1</sup>BD – before digestion; <sup>2</sup>BIO – bioaccessible element

Different lower case letters (a-d) in each column indicate significantly differences between species in raw seafood (One-way ANOVA; p < 0.05)

Different upper case letters (A-D) in each column indicate significantly differences between species in steamed seafood (One-way ANOVA; p < 0.05)

Values highlighted in light gray represent differences between raw and steamed for each seafood species (t-student for dependent variables; p < 0.05).

n = 25 for fish and cephalopods species; n = 20 for seaweed; n = 50 for bivalves and crustaceans
Table 3.
Percentage of the recommended average requirements (AR) or adequate intakes (AI) and upper limits (UL) of each element set by EFSA, accomplished with the consumption of 150 g of fish, mollusks and crustaceans, or 50 g of seaweed.

<table>
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<tr>
<th></th>
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<th>Zn&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Se&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Se&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Cu&lt;sup&gt;1&lt;/sup&gt;</th>
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<td>Steamed</td>
<td>Raw</td>
<td>Steamed</td>
<td>Raw</td>
<td>Steamed</td>
</tr>
<tr>
<td>Hake</td>
<td>7.0</td>
<td>8.6</td>
<td>5.0</td>
<td>6.2</td>
<td>&gt;AI (0.2)</td>
<td>66.0</td>
</tr>
<tr>
<td>Tuna</td>
<td>36.3</td>
<td>20.1</td>
<td>10.1</td>
<td>10.7</td>
<td>&gt;AI (81.7)</td>
<td>&gt;AI (77.4)</td>
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<td>72.9</td>
<td>79.7</td>
<td>46.2</td>
<td>67.1</td>
<td>5.1</td>
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</tr>
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<td>11.6</td>
<td>13.5</td>
<td>0.0</td>
<td>0.0</td>
<td>&gt;AI (5.2)</td>
<td>&gt;AI (4.3)</td>
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<td></td>
<td>72.9</td>
<td>79.7</td>
<td>46.2</td>
<td>67.1</td>
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<td>0.0</td>
<td>&gt;AI (1.4)</td>
<td>&gt;AI (3.0)</td>
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<tr>
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<td>4.9</td>
<td>13.1</td>
<td>&gt;AI (3.4)</td>
<td>&gt;AI (12.0)</td>
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<td>61.8</td>
<td>52.3</td>
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<tr>
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<td>33.9</td>
<td>61.4</td>
<td>23.0</td>
<td>28.9</td>
<td>&gt;AI (6.6)</td>
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<td></td>
<td>85.7</td>
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Table 3 (cont.)

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<th>Mn&lt;sup&gt;2&lt;/sup&gt;</th>
<th>I&lt;sup&gt;1&lt;/sup&gt;</th>
<th>I&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Fe&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Fe&lt;sup&gt;2&lt;/sup&gt;</th>
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<td>Raw</td>
<td>Steamed</td>
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</tr>
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</tr>
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<td>&gt;AI (&gt;UL)</td>
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</tr>
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<td>&gt;AI (&gt;UL)</td>
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<td>6.9</td>
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53
<table>
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<th>0.8</th>
<th>0.6</th>
<th>0.4</th>
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<th>&gt;AI</th>
<th>&gt;AI</th>
<th>&gt;AI</th>
<th>13.4</th>
<th>12.4</th>
<th>0.0</th>
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</tr>
</thead>
</table>

1 – Values calculated without considering element bioaccessibility; 2 – Values calculated considering element bioaccessibility.

Values in parenthesis represent the percentage of the upper limit (UL). UL percentages only calculated whenever the RDA were exceeded.

Percentages were calculated according to the average requirements (AR) or adequate intakes (AI), as well as the upper limits (UL) set by EFSA (2006, 2013, 2014a, 2014b, 2014c, 2015a, 2015b). The following AR/AI and UL values were used as reference, respectively: 6.2 mg/day (AR) and 25 mg/day (UL) for Zn, 70µg/day (AI) and 300 µg/day (UL) for Se, 1.3 mg/day (AI) and 5 mg/day (UL) for Cu, 3 mg/day (AI) for Mn, 150 µg/day (AI) and 600µg/day for I (UL), and 6 mg/day (AR) for Fe. In what concerns Mn, and Fe, no upper limit has been proposed by EFSA so far. Regarding Sr, no AR/AI nor UL have been set yet for this element.
Table 4. 
Percentage of the tolerable weekly intakes (TWI) set for MeHg and Cd, accomplished with the consumption of 150 g of fish, mollusks and crustaceans, or 50 g of seaweed.

<table>
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<th>MeHg$^2$</th>
<th>Cd$^1$</th>
<th>Cd$^2$</th>
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<td>Raw</td>
<td>Steamed</td>
</tr>
<tr>
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<td>33.1</td>
<td>18.6</td>
<td>7.5</td>
</tr>
<tr>
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<td>24.4</td>
<td>31.6</td>
<td>7.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Mackerel</td>
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<td>16.3</td>
<td>8.0</td>
<td>3.2</td>
</tr>
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<td>Monkfish</td>
<td>28.8</td>
<td>34.1</td>
<td>5.7</td>
<td>2.6</td>
</tr>
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<td>Plaice</td>
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<td>43.8</td>
<td>60.3</td>
<td>4.3</td>
<td>1.2</td>
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<td>0.0</td>
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</tr>
<tr>
<td>Shrimp</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Seaweed old</td>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Seaweed new</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
</tbody>
</table>

1 – Values calculated without considering element bioaccessibility; 2 – Values calculated considering element bioaccessibility.

Percentages were calculated according to the TWI set by EFSA for the exposure to Cd (2.5 µg kg$^{-1}$ of individual body weight; EFSA, 2011) and MeHg (1.3 µg kg$^{-1}$ of individual body weight; EFSA, 2012), and considering an adult average body weight of 70 kg.
Highlights:

- MeHg showed the lowest bioaccessibility
- MeHg and Cd bioaccessibility decreased after steaming
- Bioaccessibility of elements increased or decreased after steaming
- The youngest segments of seaweed revealed higher levels of essential elements
- Fish, shellfish and seaweed species are good sources of essential elements