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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 bioaccessibility of essential (zinc, selenium, copper, manganese, strontium, iron iodine) and toxic (mercury, methylmercury, arsenic, cadmium) elements was investigated in raw and cooked commercially available seafood species from European markets.

Bioaccessibility varied between seafood species and elements. Low MeHg bioaccessibility was observed in all species (10-60 %) and decreased significantly after steaming. Overall, As (> 64%) were the toxic elements showing the highest bioaccessibility. Concerning essential elements bioaccessibility in raw seafood, Se (72.6 %) and I (70.5 %) revealed the highest percentages, whereas Fe, Cu and I (> 73 %) showed the highest bioaccessibility in steamed seafood. In addition, high selenium:methylmercury ratio and positive selenium health benefit values (HBV_{Se}) were observed. The bioaccessibility of essential elements in steamed products increased or decreased according to species.

This study provides new insights and relevant toxicological data that will 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. These results will strengthen stakeholders' confidence towards commercially available seafood, and help consumers to wisely select what they eat in a more informed way.

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Highlights:

- Low MeHg bioaccessibility in seafood
- MeHg and Cd bioaccessibility decreased after steaming
- The bioaccessibility of essential elements increased (fish) and decreased (shellfish) after steaming
- The youngest segments of seaweed revealed higher levels of essential elements
- Fish, shellfish and seaweed species are good sources of essential elements

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

Abbreviations: AL – action of limits, ANOVA - analysis of variance, As – arsenic, BD - sample before digestion, BIO - bioaccessible fraction after *in vitro* digestion, Cd – cadmium, CRM - certified reference biological material, CVD - cardiovascular diseases, Cu – copper, DORM-4 - dogfish muscle reference material, Fe – iron, HBV_{Se} - selenium health benefit value, Hg – mercury, ICP-MS - inductive coupled plasma mass spectrometer, I - iodine, LCn-3-PUFA - long chain polyunsaturated n-3 fatty acids, LOD - limit of detection, LOQ - limit of quantification, MeHg – methylmercury, Mn – manganese, MPC – maximum permissible concentration, NBIO - non-bioaccessible fraction after *in vitro* digestion, RDAs - recommended dietary allowances, Se – selenium, Se:Hg - Molar ratio between selenium and mercury, Se:MeHg - Molar ratio between selenium and methylmercury, Sr – strontium, TORT-2 - lobster hepatopancreas reference material, TWIs - tolerable weekly intakes, ULs – upper limits, WHO - World Health Organization, Zn – zinc

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), (Larsen et al., 2011; Lund, 2013), which LCn-3-PUFA are considered as key players in the prevention against cardiovascular diseases (CVD), (He et al., 2004). The high nutritional value associated to seafood is also due to the presence of considerable amount 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 (Celik and Oehlenschlager, 2007; 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 (Maulvault et al., 2015; Vazquez 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, 2009a, 2009b, 2012).

Regulatory authorities and the scientific community have been focusing their attention on these toxic and essential elements in seafood (Vandermeersch et al., 2015; Vazquez et al., 2015). 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 (Chapman and Chan, 2000), this persistent pollutant has been one of the most studied contaminants, and high concentrations are usually associated with predatory and

carnivorous fish species (Costa et al., 2016; Zmozinski 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 (Cabanero et al., 2007; Ralston et al., 2016).

The total contaminant concentration detected in seafood does not always reflect the amount of the contaminant 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 (Collins et al., 2015; 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 (Cardoso et al., 2013; Maulvault et al., 2013; Maulvault et al., 2011). Several *in vitro* models were developed to simulate the gastrointestinal digestion process in humans (Cardoso et al., 2015; Marques et al., 2011; 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. 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 (Cardoso et al., 2010; Maulvault et al., 2013). Recent studies described the effect of culinary treatments in MeHg bioaccessibility, and indeed it has been demonstrated that culinary treatment generally reduces toxic elements bioaccessibility in seafood (e.g. mercury) (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; (2) evaluate the effects of culinary treatment (steaming) on the bioaccessibility of essential/toxic elements; (3) explore the correlation between the elements bioaccessibility and the overall concentration in seafood; and (4) 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 (Lito*penaeus vannamei*) and a 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.

Table 1.
Selected species from European seafood markets used for the essential and toxic elements bioaccessibility assessment.

Seafood	Species	Origin	Market country	*n*	Total length (mm)	Total weight (g)	Moisture (%)	
							Raw	Steamed
Hake	*Merlucius australis*	Pacific Ocean	Portugal	25	n.a	2,500-3,500	74.66	67.13
Tuna	*Katsuwonus pelamis*	Pacific Ocean	Portugal	25	n.a	>3,400	67.6	56.2
Monkfish	*Lophius piscatorius*	Atlantic Ocean	Portugal	25	570-590	3,365-3,448	82.41	77.17
Mackerel	*Scomber scombrus*	Adriatic Sea	Italy	25	189-285	47.7-268.7	75.2	72.5
Plaice	*Pleuronectes platessa*	North Sea	The Netherlands	25	347-411	376-635	n.d	n.d
Mussel	*Mytilus edulis*	Atlantic Ocean	The Netherlands	50	44-68	5.9-18.5	n.d	n.d
Octopus	*Octopus vulgaris*	Mediterranean	Spain	25	920[1]	2,600[2]	80.1	72.66
Shrimp	*Litopenaeus vannamei*	Indic Ocean	Portugal	50	11-14	11-28	n.d	n.d
Seaweed	*Laminaria digitata*	North Sea	Norway	20	n.a	n.a	n.d	n.d

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; n.a, data not available; n.d, not determined.

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).

Each sample was divided into two portions, one used for the culinary treatment (steaming at 105 ºC 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 -20ºC 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), $NaHCO_3$ (Merck, 99.5% m/v), $CaCl_2.2H_2O$ (Sigma, C3881), KCl (Merck, 99.5% m/v), KSCN (Sigma, P2713), NaH_2PO_4 (Merck, 99.5% m/v), Na_2SO_4 (Merck, 90% m/v), NH_4Cl (Riedel-de Haen, 99.5% m/v), KH_2PO_4 (Merck, 99.5%), $MgCl_2$ (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 described in Braga et al. (2016) and schematized in 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 in triplicate with four digestion fluids: saliva, gastric, duodenal and bile. Each digestion fluid is composed by several inorganic and organic components (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 37ºC 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 10ºC for 10 minutes to separate the bioaccessible fraction (BIO) from the sample residues (non-bioaccessible fraction - NBIO).

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:

$$(\mathbf{BIO} + \mathbf{NBIO})\ \mathbf{x}\ \mathbf{100} / \mathbf{BD},$$

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:

$$\mathbf{BIO}\ \mathbf{x}\ \mathbf{100} / \mathbf{BD},$$

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 ºC, 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:

$$(\mathbf{Hg\ BIO} + \mathbf{Hg\ NBIO})\ \mathbf{x}\ \mathbf{100} \Big/ \mathbf{Hg\ BD},$$

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:

$$\mathbf{Hg\ (MeHg)\ BIO\ x\ 100} \Big/ \mathbf{Hg\ (MeHg)\ BD},$$

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 (HNO_3) 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, Merck) and 2 ml hydrogen peroxide (30% v/v, Merck). 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) resulting in 2% HNO_3 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 = $(CH_3)4N^+OH^-$, 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,), helium (Zn, Se, Cu, Mn, Sr and Fe) and oxygen (As (mass shift 75->91) mode, respectively, with 0.2 sec integration time per mass. Typical plasma conditions were 1550 W RF power, 15 L min^{-1}plasma gas, 1.05 L/min 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 sec. (0.4 rps) and a stabilization time of 40 sec (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 sec (0.1 rps) and 60 sec (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 sec (0.1 rps) and 60 sec (0.4 rps), respectively.

A low calibration standard was repeatedly analysed throughout the sequence sequence in order to verify instrument stability. 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), and As (0.15-200).

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

$$\mathbf{E\ BIO\ x\ 100} / \mathbf{E\ BD},$$

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 Table 2. Certified reference material (CRM), Lobster hepatopancreas - 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 Table 2.

Table 2.

Target values (certificate ± range) and mean measured concentrations (mg/kg dry weight) and the associated relative standard deviation (RSD) in certified reference materials (CRM). For all elements mean values were reported for CRM samples extracted in duplicates and analysed in two dilutions (5 and 50 times) except for iodine, Hg and MeHg were extracted in duplicates and analysed. Limits of detection (LOD) for each element in the seafood sample before digestion (BD) and in the bioaccessible fraction (BIO).

		CRM				LOD		
Element	**Type**	**Certificate (mg/kg)**	**Measured (mg/kg)**	**RSD (%)**	**Recovery (%)**	**Measure-ment solution (ng/ml)**	**Raw or steamed seafood (BD) (ng/g)**	**Bioacces-sible fraction (BIO) (ng/ml)**
Hg	TORT-2	0.27 ± 0.06	0.332 ± 0.004	1	123	1.93-3.24	1.93-3.24	1.93-3.24
MeHg	TORT-2	0.152 ± 0.013	0.140 ± 0.009	6	92	1.93-3.24	1.93-3.24	1.93-3.24
As	DORM-4	6.80 ± 0.64	6.73	1	99	0.06	8	3
	TORT-2	21.6 ± 1. 8	22	1	102			
Cd	DORM-4	0.306 ± 0.015	0.317	3	104	0.0003	0.04	0.02
	TORT-2	26.7 ± 0.6	28.5	0	107			
Zn	DORM-4	52.2+/-3.2	51.8	6	99	1.22	153	61
	TORT-2	180+/-6	188	3	104			
Se	DORM-4	3.56+/-0.34	3.58	8	101	0.16	20	8
	TORT-2	5.63+/-0.67	5.51	6	98			
Cu	DORM-4	15.9+/-0.9	16.3	10	103	0.11	14	5.5
	TORT-2	106+/-10	103	5	97			
Mn	DORM-4	-	3	8	-	0.65	81	32.5
	TORT-2	13.6+/-1.2	13.4	4	99			
Sr	DORM-4	-	7.88	5		0.16	20	8
	TORT-2	45.2+/-1.9	43.9	5	97			
I	DORM-4	-	2.75	1	-	0.04	4	4
	TORT-2	-	44.71	2	-			
	Seaweed*	631+/47	587	1	93			
Fe	DORM-4	341+/-27	364	4	107	5.44	680	272
	TORT-2*	105+/-13	105	7	100			

* Material characterized in a collaborative trial (CENFEEDiod-S1).

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 Raltson & Raymond (2014).

$$HBV_{Se} = \frac{\mathbf{Se - Hg(MeHg)}}{\mathbf{Se}} * (\mathbf{Se + Hg(MeHg)}),$$

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

2.3.5. Benefit-Risk balance

Percentages of recommended dietary allowances (RDAs) for Zn, Se, Cu, I, Fe, as well as of action limits (AL) set for Mn were calculated according to reference values set by the US National Academy of Sciences for individual adults aged between 19 and 50 (USNAS, 2010) 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. 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 RDA 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 and Cd accomplished with the consumption of 150 g of fish, mollusks and crustaceans 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. Arsenic was not included in

the analysis since the reference value set by EFSA only concerns the intake inorganic As (As speciation not analysed in the present study). Furthermore, the last Scientific Opinion released by EFSA suggested that, given the recent toxicological data on this toxic element, the previous provisional TWI (PTWI = 15 μg kg^{-1} bw) set for iAs requires update (EFSA, 2009).

Calculations regarding RDAs (and ALs), ULs, as well as TWIs were performed either including or excluding elements' bioaccessibility.

2.3.6. Statistical analysis

Differences in elements concentration, bioaccessibility, Se:Hg, Se:MeHg and HBV_{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 $p < 0.05$. Additionally, for each element the regression coefficient between the total element concentration and bioaccessibility was analysed by SigmaPlot v10.0 (Systat Software, Inc., CA, USA).

3. Results

3.1. Elements in raw seafood

Concentrations of toxic elements Hg, MeHg, As and Cd were determined in seafood species. Hg concentration varied between 0.146 (mackerel) and 0.376 µg g^{-1} ww (octopus). MeHg concentration in seafood ranged between 0.079 µg g^{-1} ww (mackerel) and 0.266 µg g^{-1}ww (octopus). Hg and MeHg varied according to the following order: octopus > hake > monkfish > tuna > mackerel, ($p < 0.05$), (Fig. 1, Supplementary Table S2). The highest and lowest 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 µg g^{-1} ww.) (Fig. 1, Supplementary Table S2). Fig. 1 reveals that Cd concentration in seafood ranged between 1.03 (monkfish) and 72.8 (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 revealed 50 times higher Cd levels than those observed in the newer part ($p < 0.001$) (Supplementary Table S2).

In general, molluscs and crustaceans showed the highest levels of Zn, Cu, Mn and Fe, whereas seaweed was the richest seafood in I and Sr. Hake showed the lowest concentration of most elements (Zn, Cu, Mn, Sr, I, Fe). Tuna showed high concentration of Zn, Se, Fe and Cu (Fig. 1, Supplementary Table S3). The highest Zn concentrations were found in shrimp (18.4 µg g^{-1} ww), whereas the lowest Zn concentration was registered in hake (2.91 µg g^{-1} ww). The Se content ranged between <0.020 (seaweeds) and 2.076 ± 0.02 µg g^{-1} ww (tuna), (Fig. 1, $p < 0.05$). Shrimp (8.78 µg g^{-1} ww) and octopus (8.00 µg g^{-1} ww) revealed the highest levels of Cu, whereas hake showed the lowest levels (0.08 µg g^{-1} ww). Levels of Mn ranged between 0.13 µg g^{-1} ww (monkfish) and 2.82 µg g^{-1} ww (mussel). Highest levels of Sr were detected in the old (154 µg g^{-1} ww) and new (222 µg g^{-1} ww) seaweed leafs, whereas the lowest levels were found in monkfish (0.22 µg g^{-1} ww), (Fig. 1, Supplementary Table S3). A similar

trend was found for I, with the new seaweed leafs showing the highest concentration (433 µg g^{-1} ww), whereas hake revealed the lowest I content (0.12 µg g^{-1} ww), (Fig. 1, Supplementary Table 3). As observed in Fig.1, mussel (84.9 µg g^{-1} ww) showed the highest Fe levels among all raw seafood species, whereas hake showed the lowest Fe levels (Supplementary Table S3).

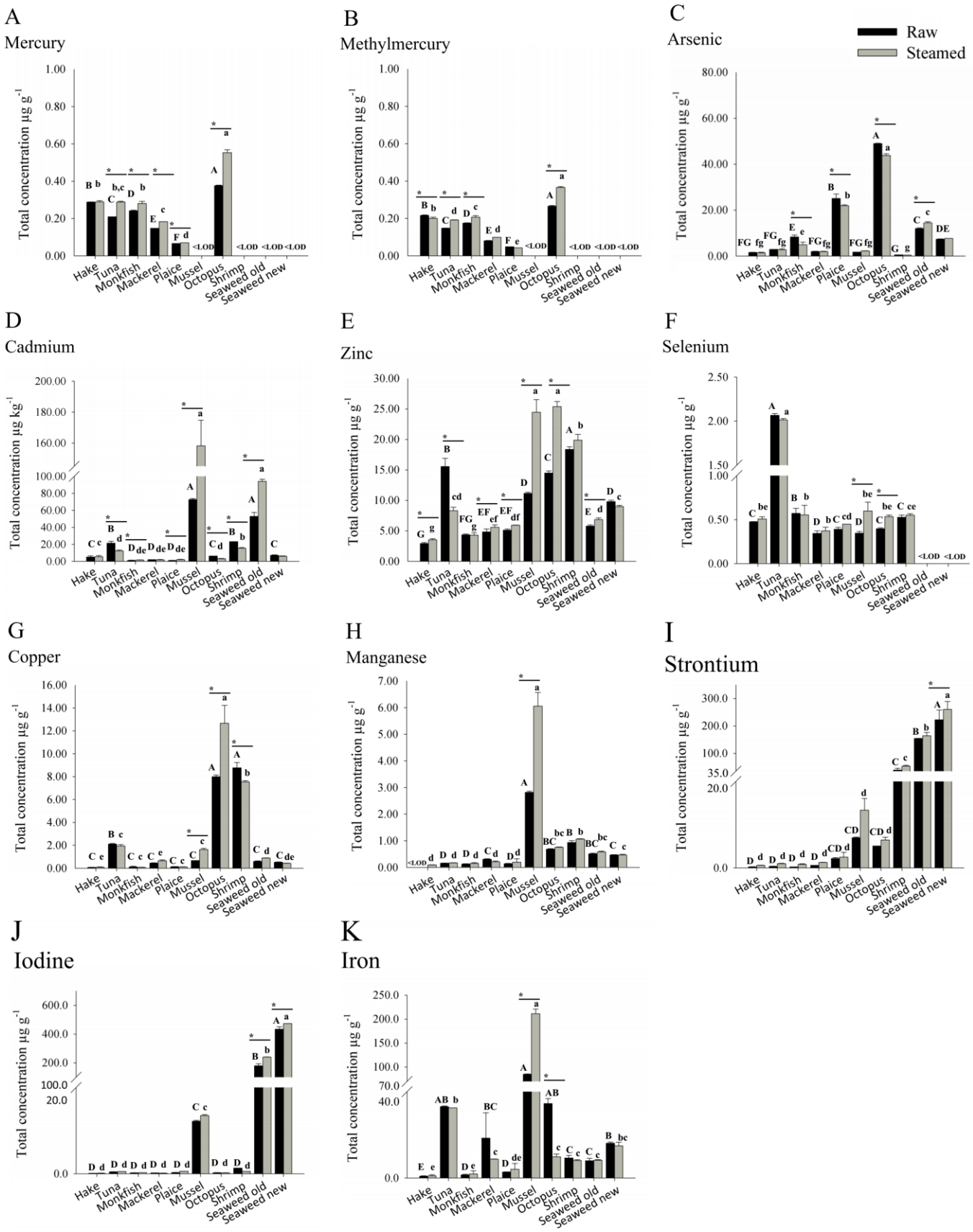
A
Mercury
B
Methylmercury
C
Arsenic
Raw
Steamed
D
Cadmium
E
Zinc
F
Selenium
G
Copper
H
Manganese
I
Strontium
J
Iodine
K
Iron
Total concentration µg g-1
Total concentration µg kg-1
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new
<LOD

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). Different upper case letters indicate significantly differences between species for each element in raw seafood ($p < 0.05$); different lower case letters (a-g) indicate significantly differences between species for each element in steamed seafood ($p < 0.05$); * represent differences between raw and steamed for each seafood species ($p < 0.05$), please see detailed information in Supplementary tables 2 and 3.

3.2. Elements in steamed seafood

Steaming led to an increase in Hg and MeHg concentrations, except in hake. For example, Hg and MeHg significantly increased approximately 30% after steaming in tuna and octopus ($p < 0.05$). In mackerel and monkfish, the increase was around 20% ($p < 0.05$), (Fig. 1, Supplementary Table S2). Arsenic content in monkfish, plaice and octopus was significantly lower after steaming compared with the raw product ($p < 0.001$). The As content in the remaining species was not significantly affected by steaming (Fig. 1). Steaming induced statistically higher levels of Cd in mussel, plaice and in the old part of seaweed ($p < 0.05$). In contrast, tuna, monkfish, shrimp and octopus levels of Cd were significantly lower in steamed products ($p < 0.05$), whereas no differences were observed between raw and steamed hake, mackerel and the newer seaweed segment (Fig. 1, Supplementary Table S2).

Zn content increased significantly after steaming in hake, mackerel, plaice, octopus, mussel, shrimp and old seaweed leafs (Fig. 1, $p < 0.05$). In general no

significant differences were detected in Se between raw and steamed samples ($p > 0.05$), except in octopus and mussels. Octopus and mussels were the only species revealing a significant increase in Cu concentration after steaming (Fig. 1, Supplementary Table S3, $p < 0.05$). After steaming, mussel was the only species revealing significant changes in Mn levels, increasing to 6.06 µg g^{-1} ($p < 0.05$). Once steamed, only the new seaweed revealed a significant increase in Sr concentration reaching 260 µg g^{-1} ww (Supplementary Table S3). Steaming resulted in a significant increase of I concentration only in the old and newer parts of the seaweeds ($p < 0.05$). Steaming only significantly affected Fe levels in mussel (increasing to 211 µg g^{-1} ww) and octopus (decreasing to 9.85 µg g^{-1} ww) (Fig. 1, Supplementary Table S3).

3.3. Bioaccessibility of elements

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) (Supplementary Table S4). Seaweed showed the lowest protein bioaccessibility (raw 15.2 %; steamed 8.8 %)

3.3.2 Seafood species

Hg bioaccessibility ranged between 61 % (hake) and 11 % (octopus), with most species presenting a bioaccessibility below 50 % (Fig. 2, Supplementary Table S5). 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 hake and monkfish, whereas the lowest MeHg bioaccessibility was observed in octopus (10 %, $p < 0.05$) (Fig. 2). In general, As bioaccessibility was high in all analysed species, ranging between 54 % (seaweeds) and 98.3 % (monkfish and octopus). Cd bioaccessibility was high for mussel (102 ± 10 %) and shrimp (75 ± 7 %), and low for tuna (41 ± 6 %) (Fig. 2, Supplementary Table S5).

Zn bioaccessibility ranged between 28 % (tuna) and 77 % (old seaweed). Bioaccessible Zn values for mackerel and monkfish could not be determined as the results were below the limit of detection (Fig. 2, Supplementary Table S6). The bioaccessibility of Se was always higher than 59 %, showing low variability between seafood species. The highest Se bioaccessibility was observed in shrimp (97 %). Fig. 2 reveals that Cu bioaccessibility ranged between 44 % (newer seaweed leafs) and 94 % (octopus). This high variability in bioaccessibility among species was also observed for manganese, with shrimp showing the lowest Mn bioaccessibility (24 %). In octopus, Mn was 100 % bioaccessible (Fig. 2, Supplementary Table S6). The bioaccessibility of both elements was statistically higher in the newer part of the seaweed ($p < 0.05$). Sr bioaccessibility was highly variable between species and ranged between 18 % (newer seaweed leafs) and 96 % (mussel). In general, fish Sr bioaccessible values were below the LOQ. High I bioaccessibility was obtained for octopus (86 %), mussel (84 %) and the newer seaweed leafs (73 %), whereas only 46 % of I was bioaccessible in tuna. Almost all Fe bioaccessible concentrations were below the limit of quantification, except for mussel (26%) and tuna (69%) respectively (Fig. 2, Supplementary Table S6).

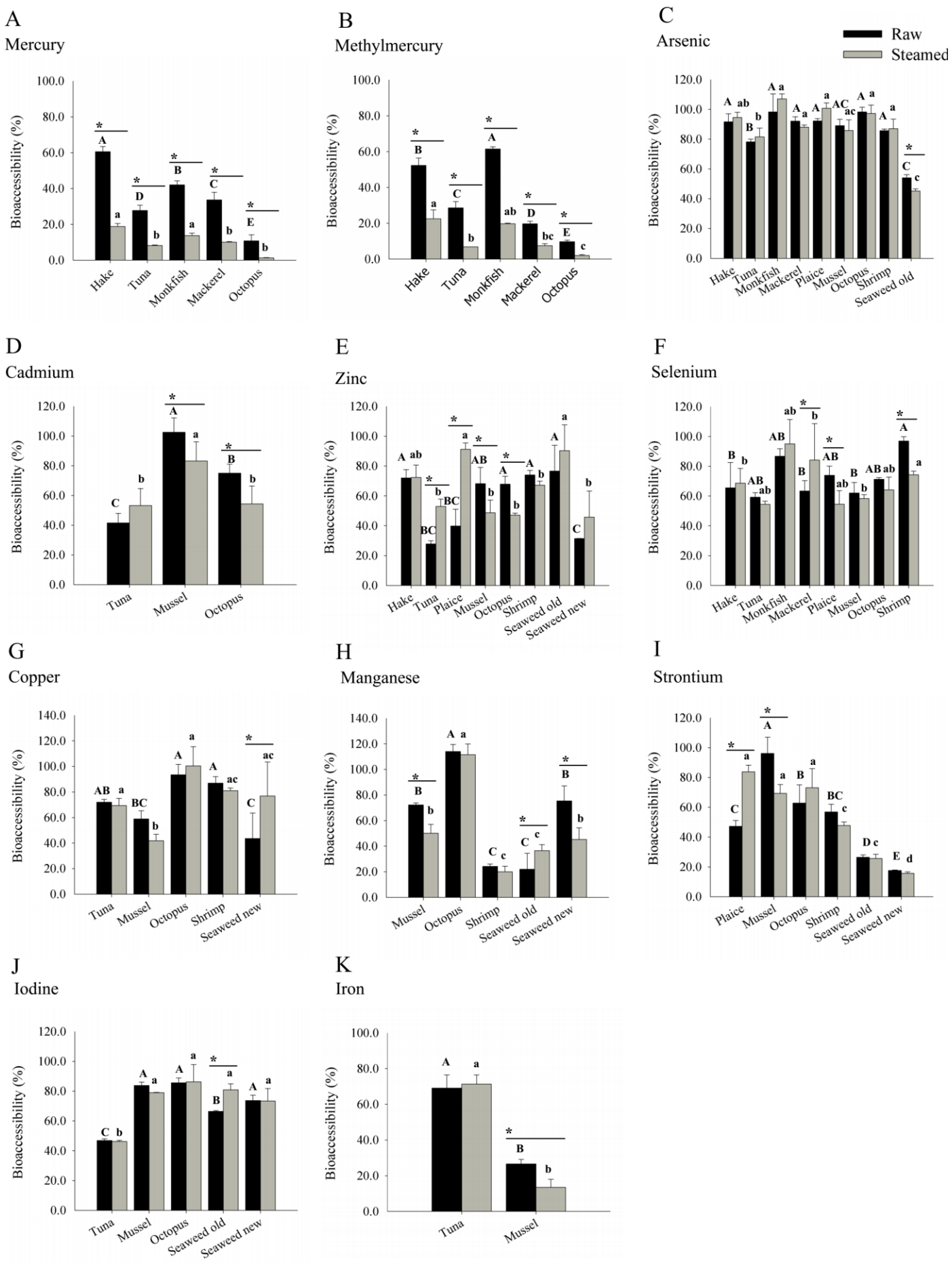
A
Mercury
B
Methylmercury
C
Arsenic
Raw
Steamed
Bioaccessibility (%)
Hake
Tuna
Monkfish
Mackerel
Octopus
Plaice
Mussel
Shrimp
Seaweed old
Seaweed new
D
Cadmium
E
Zinc
F
Selenium
G
Copper
H
Manganese
I
Strontium
J
Iodine
K
Iron

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). Different upper case letters indicate significantly differences between species for each element in raw seafood ($p < 0.05$); different lower case letters (a-g) indicate significantly differences between species for each element in steamed seafood ($p < 0.05$); * represent differences between raw and steamed for each seafood species ($p < 0.05$), please see detailed information in Supplementary tables 5 and 6.

3.3.3. Effect of steaming

For all analysed species, steaming significantly decreased total Hg bioaccessibility ($p < 0.001$), ranging between 1 % (octopus) and 19 % (hake). Additionally, MeHg bioaccessibility also significantly decreased in all species after steaming ($p < 0.001$). MeHg bioaccessibility varied between 2.1 % (octopus) and 22.5 % (hake) in steamed seafood (Fig. 2, Supplementary Table S5). No statistical differences were registered in the bioaccessibility of As in raw and steamed seafood (Supplementary table 5). In contrast, Cd bioaccessibility decreased around 20 % in mussel and shrimp after steaming ($p < 0.05$) (Fig. 2, Supplementary Table S5).

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 %) and plaice (54 %), whereas for mackerel, Se bioaccessibility increased after steaming (84 %) ($p < 0.05$). 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 leafs,

steaming increased and decreased Cu and Mn, respectively (Fig. 2, Supplementary Table S6). 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 %) and mussel (decreased – 69 %) revealed statistically changes after the culinary treatment ($p < 0.05$). Steaming only significantly affected I bioaccessibility in the older seaweed part (66 % - raw, 81 % - steamed) ($p < 0.05$). Fe bioaccessibility only decreased after steaming in mussel ($p < 0.05$) (Fig. 2, Supplementary Table S6).

Analysis of correlations between total element concentration (mg kg^{-1} ww or µg kg^{-1} ww) and bioaccessibility (%) in raw and steamed seafood are shown in Table 3. For almost all elements, no correlation was observed between elements concentration and bioaccessibility ($p > 0.05$). The exceptions were Cd and Zn, where bioaccessibility was positively correlated with the total elemental concentration (Cd, $r = 1.000$ in steamed, $p < 0.01$; Cu, $r = 0.895$ in raw, $p < 0.05$). In contrast, Sr was negatively correlated in raw and steamed samples (raw $r = 0.811$, $p < 0.05$; steamed $r = 0.950$, $p < 0.01$) (Table 3).

Table 3.
Correlation coefficient between element concentration (mg/kg w.w or µg/kg w.w) and bioaccessibility (%) in raw and steamed seafood.

	Raw	**Steamed**
Hg	0.491	0.661
MeHg	0.1	0.327
As	0.0451	0.0047
Cd	0.853	1.000 ** ↑
Zn	0.0741	0.575
Se	0.298	0.422
Cu	0.895* ↑	0.751
Mn	0.114	0.0563
Sr	0.811 * ↓	0.950** ↓
I	0.0663	0.0771
Fe	n.d	0.0384

Statistical significance: *$p < 0.05$; ** $p < 0.01$; ↓ negative correlation and ↑ positive correlation

3.4. Impact on toxic 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 4). 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 4). Steaming induced statistical increased Se:Hg and Se:MeHg ratios in the bioaccessible fraction of tuna, mackerel and octopus (Table 4).

Few significant differences were observed in HBV_{Se} values in relation to Hg and MeHg. 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 4). 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 4).

Table 4.

Se:Hg and Se:MeHg molar ratios and selenium health benefit value (HBV_{Se}) in raw and steamed seafood (average ± standard deviation).

		Se:Hg		Se:MeHg		HBV_{Se} for Hg		HBV_{Se} for MeHg	
		BD[1]	**BIO**[2]	**BD**	**BIO**	**BD**	**BIO**	**BD**	**BIO**
Hake	**Raw**	4.93±0.00b	5.92±0.64b	6.00±0.00c	7.45±1.02b	5.77±0.00c	3.81±0.72bc	5.88±0.00c	3.86±0.72bc
	Steamed	4.54±0.00B	17.52±1.27C	6.91±0.00C	21.47±3.67C	5.97±0.00B	4.40±0.63B	6.18±0.07B	4.40±0.63B
Tuna	**Raw**	24.23±0.03a	63.71±5.35a	38.13±0.00a	79.62±6.68a	26.62±1.00a	15.50±0.77a	26.65±1.00a	15.50±0.77a
	Steamed	21.44±0.01A	195.25±46.97A	28.70±0.00A	235.08±41.63A	25.44±0.95A	15.35±2.58A	25.47±0.95A	15.35±2.58A
Monkfish	**Raw**	3.95±0.25c	10.65±1.52b	8.91±0.00b	13.73±2.07b	6.93±0.29b	6.78±1.04b	7.37±0.28b	6.81±1.03b
	Steamed	3.18±0.01C	25.36±5.59C	7.30±0.00B	31.42±8.28CD	6.18±0.18B	5.94±1.48B	6.82±0.20B	5.94±1.48B
Mackerel	**Raw**	4.79±0.36b	14.30±1.31b	5.87±0.44c	18.84±1.90b	4.26±0.20d	2.73±0.100c	4.36±0.19d	2.73±0.10c
	Steamed	4.43±0.00B	53.14±7.85C	5.90±0.00D	68.52±29.18BD	4.32±0.02C	3.97±1.15B	4.45±0.02C	3.97±3.97B
Octopus	**Raw**	2.67±0.00d	25.67±3.63ab	4.05±0.00d	28.30±0.28ab	3.09±0.13e	4.31±1.30bc	3.49±0.14d	4.31±1.30bc
	Steamed	2.45±0.00D	118.82±10.8B	3.98±0.00E	109.80±29.85B	4.06±0.01C	4.34±0.57B	4.75±0.01C	4.34±0.57B

[1] BD – before digestion; [2] BIO – bioaccessible element

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

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

Values highlighted in light gray represent differences between raw and steamed for each seafood species ($p < 0.05$).

n = 25 for fish and cephalopods species; n = 20 for seaweed; n = 50 for bivalves and crustaceans

3.4.2. Benefit-Risk balance

Table 5 shows the benefit-risk balance for the consumption of seafood species (raw and cooked) based on RDAs and ULs set for each essential element. Percentages of RDAs and ULs for a consumption of 150 g seafood varied according to species and element. Crustaceans and cephalopods revealed high % RDAs for Zn (between 19 and 35 %) and Cu (over 100 %), whereas remarkably higher Se intakes were observed in fish species, particularly tuna (over 100 % of RDA and 65 % of UL). Mussels revealed the highest % AL and % RDA of Mn and Fe, respectively, especially after steaming (Mn = 39.5 % and Fe > 100 %). Mussels and macroalgae also provided over 100% of RDA 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 6).

Table 5.
Percentage of the recommended dietary allowances (RDA)of each element considering the consumption of a portion size of 150 g of fish, mollusks and crustaceans, or 50 g of seaweed. In some cases action limits (AL) and upper limits (UL) are taken into account and / or specified (see note at the bottom of the table for more details).

	Zn[1]		Zn[2]		Se[1]		Se[2]		Cu[1]		Cu[2]	
	Raw	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**
Hake	4.0	4.8	2.8	3.5	>RDA (3.9)	>RDA (5.3)	84.0	94.9	1.3	1.6	0.0	0.0
Tuna	20.5	11.3	5.7	6.0	>RDA (65.0)	>RDA (61.8)	>RDA (32.9)	>RDA (27.3)	35.3	32.5	25.4	22.6
Mackerel	6.5	7.6	0.0	0.0	92.7	>RDA (0.2)	58.8	85.4	7.4	1.1	0.0	0.0
Monkfish	6.0	5.9	0.0	0.0	>RDA (7.6)	>RDA (7.0)	>RDA (4.8)	>RDA (6.0)	1.9	1.3	0.0	0.0
Plaice	7.0	8.1	2.8	7.4	>RDA (0.9)	>RDA (3.1)	78.7	66.6	2.4	2.1	0.0	0.0
Octopus	19.1	34.6	13.0	16.3	>RDA (1.3)	>RDA (6.3)	77.7	93.5	>RDA (3.0)	>RDA (10.0)	>RDA (2.2)	>RDA (10.1)
Mussel	15.0	33.3	10.2	16.2	95.5	>RDA (8.7)	59.3	95.3	11.3	27.2	6.7	11.4
Shrimp	24.5	27.1	18.2	18.2	>RDA (6.1)	>RDA (6.8)	>RDA (5.5)	>RDA (1.5)	>RDA (4.2)	>RDA (2.3)	>RDA (2.4)	>RDA (0.2)
Seaweed old	2.6	3.1	2.0	2.8	0.0	0.0	0.0	0.0	3.3	5.0	0.0	0.0
Seaweed new	4.5	4.1	1.4	1.9	0.0	0.0	0.0	0.0	2.9	2.4	1.3	1.8

Table5 cont.

	Mn[1]		Mn[2]		I[1]		I[2]		Fe[1]		Fe[2]	
	Raw	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**
Hake	**0.0***	**0.6***	**0.0***	**0.0***	12.0	13.8	0.0	0.0	0.6	1.0	0.0	0.0
Tuna	**1.0***	**1.1***	**0.0***	**0.0***	44.2	51.7	20.7	23.9	27.5	27.1	19.0	19.3
Mackerel	**2.0***	**1.4***	**0.0***	**0.0***	18.1	13.9	0.0	0.0	8.5	7.3	0.0	0.0
Monkfish	**0.8***	**1.0***	**0.0***	**0.0***	20.3	26.7	0.0	0.0	1.2	2.4	0.0	0.0
Plaice	**0.9***	**1.3***	**0.0***	**0.0***	28.2	58.3	0.0	0.0	2.3	3.4	0.0	0.0
Octopus	**4.4***	**4.9***	**5.0***	**5.5***	22.9	21.4	19.6	18.5	28.8	8.2	0.0	0.0
Mussel	**18.4***	**39.5***	**13.3***	**19.8***	>RDA (>UL)	>RDA (>UL)	>RDA (>UL)	>RDA (>UL)	70.7	>RDA (30.4)	18.8	23.8
Shrimp	**6.1***	**6.9***	**1.5***	**1.4***	>RDA (4.4)	54.9	0.0	0.0	7.8	6.8	0.0	0.0
Seaweed old	**1.1***	**1.3***	**0.2***	**0.5***	>RDA (>UL)	>RDA (>UL)	>RDA (>UL)	>RDA (>UL)	2.2	2.3	0.0	0.0
Seaweed new	**1.0***	**1.0***	**0.8***	**0.5***	>RDA (>UL)	>RDA (>UL)	>RDA (>UL)	>RDA (>UL)	4.5	4.1	0.0	0.0

1 – Values calculated without considering element bioaccessibility; 2 – Values calculated considering element bioaccessibility. Values in bold followed by an asterisk represent the percentage of the action limit (AL) considering the consumption of 150 g of seafood, whereas 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 RDA (or **AL***, for Mn) and UL values, respectively, for Zn (11 mg day^{-1} and 40 mg day^{-1}), Se (55 µg day^{-1} and 400 µg day^{-1}), Cu (0.9 mg day^{-1} and 10 mg day^{-1}), Mn (2.3 mg day^{-1} and 11 mg day^{-1}), I (150 µg day^{-1} and 1,100 µg day^{-1}) and Fe (18 mg day^{-1} and 45 mg day^{-1}), set by the US National Academy of Sciences for individual adults aged between 19 and 50 years (USNAS, 2010).

In terms of cooking effect, no clear pattern was observed (Tables 5 and 6), as 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 RDAs (and ULs) and TWIs for each element with the consumption of 150 g of seafood (Tables 5 and 6).

Table 6.
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.

	MeHg[1]		MeHg[2]		Cd[1]		Cd[2]	
	Raw	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**	**Raw**	**Steamed**
Hake	35.6	33.1	18.6	7.5	0.4	0.5	0.0	0.0
Tuna	24.4	31.6	7.0	2.2	1.8	1.1	0.8	0.6
Mackerel	13.0	16.3	8.0	3.2	0.2	0.2	0.0	0.0
Monkfish	28.8	34.1	5.7	2.6	0.1	0.1	0.0	0.0
Plaice	8.1	7.1	0.0	0.0	0.1	0.2	0.0	0.0
Octopus	43.8	60.3	4.3	1.2	0.5	0.3	0.0	0.0
Mussel	0.0	0.0	0.0	0.0	6.2	13.6	6.4	11.3
Shrimp	0.0	0.0	0.0	0.0	2.0	1.3	1.5	0.7
Seaweed old	0.0	0.0	0.0	0.0	1.5	2.7	0.0	0.0
Seaweed new	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.0

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 and adult average body weight of 70 kg.

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 (Cardoso et al., 2015; He et al., 2010; Vazquez et al., 2015). 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 (Afonso et al., 2015; 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 MeHg concentrations increased after steaming. For example, steamed octopus (0.553 µg g^{-1} ww) showed levels higher than the maximum levels (MLs) set by EU. The effects of culinary treatments are relevant, as in general, they induce an increase in Hg as a result of water loss during steaming (Maulvault et al., 2011; Torres-Escribano et al., 2010). 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), and in

this study, low Hg and MeHg bioaccessibility was observed supporting this statement. To our knowledge this is the first study reporting Hg and MeHg bioaccessibility in octopus, showing the lowest bioaccessibility (~ 11%). 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 (Cabanero et al., 2004; Jadan-Piedra et al., 2016; Kwasniak et al., 2012; Siedlikowski et al., 2015; Torres-Escribano et al., 2010; Torres-Escribano et al., 2011).

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 more exposed to As contamination (Anacleto et al., 2009; Cano-Sancho et al., 2015). Both old and news seaweed leafs accumulated significant levels of As (7.30 – 12.0). 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 (Laird and Chan, 2013; Laparra et al., 2007; Laparra et al., 2003; Maulvault et al., 2011; Peng et al., 2016). In fact, the low pH observed during the stomach digestion phase (gastric fluid) is responsible for the high As solubilisation (Oomen et al. 2003). Therefore, future studies should assess if the inorganic fraction of As (most toxic form) in seafood 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; Metian et al., 2009; Qin et al., 2015; Vandermeersch et al., 2015). High and low Cd contamination is associated to molluscs/algae and fish, respectively (Moreda-Pineiro 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 (Afonso et al., 2015; Cano-Sancho et al., 2015; Maulvault et al., 2011; Torres-Escribano et al., 2011). 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; He and

Wang, 2013; Metian et al., 2009). 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; Cabañero et al. 2004). 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; Laparra et al., 2003). 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 good 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 these three 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; Peng et al., 2016). Furthermore, previous findings also reported similar element (Zn,

Cu, Mn) bioaccessibility in seafood (Amiard et al. 2008; Leufroy et al 2012; Metian et al. 2009, Peng et al., 2016). Additionally, the varying level of Mn bioaccessibility observed for shellfish in the current study has also been previously reported (He and Wang, 2013; 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 (Cabanero et al., 2004; Calatayud et al., 2012; Jadan-Piedra et al., 2016; Matos et al., 2015) and shellfish (Calatayud et al., 2012; Laird and Chan, 2013). 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. In contrast, Cu bioaccessibility seems not to be affected by steaming, in contrast to previous results obtained for fish and shellfish (He et al., 2010; He and Wang, 2013). Differences in seafood matrix composition can be a reliable justification for this unchanged of Cu bioaccessibility, as Cu is mainly associated to metallothioneins and insoluble ligands in the form of less degradable complexes. However, the most severe heating conditions (e.g. frying or grilling) have been reported to cause a decrease in Cu and Zn 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 reasonable 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. Low Sr concentrations were also observed in fish and shellfish (Agusa et al., 2007; Guerin et al., 2011; Qin et al., 2015). *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, 2009, 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.

4.4. Relationship between trace elements concentration and bioaccessibility

Previous studies in fish and shellfish showed no correlation between element concentration and bioaccessibility (He and Wang, 2013; Laird and Chan, 2013), which is in accordance to results obtained for 8 elements in the current study. In contrast, other authors demonstrated a significant positive correlation between the element concentration and bioaccessibility for Cd in fish (He et al., 2010) and mussel digestive gland (Amiard et al., 2008), which was also observed in the current work for steamed seafood. However, a negative correlation was observed between the bioaccessibility and

Cd concentration in oysters (Gao et al., 2014). In mussels and clams, Cu concentration revealed significant negative correlation between the element concentration and its bioaccessibility. The food matrix composition and variability between the seafood species can justify the existence or absence of correlation between concentration and bioaccessibility (He & Wang , 2013).

4.5. 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 tuna, Mediterranean scaldfish, red mullet, European anchovy, Atlantic hourse mackerel, and grey-eel catfish (Afonso et al., 2015; Copat et al., 2014; Looi et al., 2016). This ratio increased with bioaccessibility, due to the reduction of MeHg bioaccessibility after steaming. Cabañero and co-authors (2004) 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 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 (Copat et al., 2014; Kaneko and Ralston, 2007; Looi et al., 2016; Ordiano-Flores et al., 2012; Ralston et al., 2007; Ralston and Raymond, 2014).

4.6. 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). In this sense, considering seafood's elemental profiles, 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 parsimoniously 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 mussels and for seaweeds due to the remarkably high levels of I.

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. Nevertheless, in agreement with previous studies (e.g. Maulvault et al., 2011, 2013), results clearly

showed that, on the one hand, cooking procedures may induce notorious changes in seafood elemental profiles and, on the other hand, only a fraction of the initial element content may be available to be absorbed by the human intestinal epithelia after digestion (i.e. bioaccessible).

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 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. Steamed mussels, shrimp, octopus and tuna are an added value to human health. 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_{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. This study clearly reveals that food risk and benefit assessment should take 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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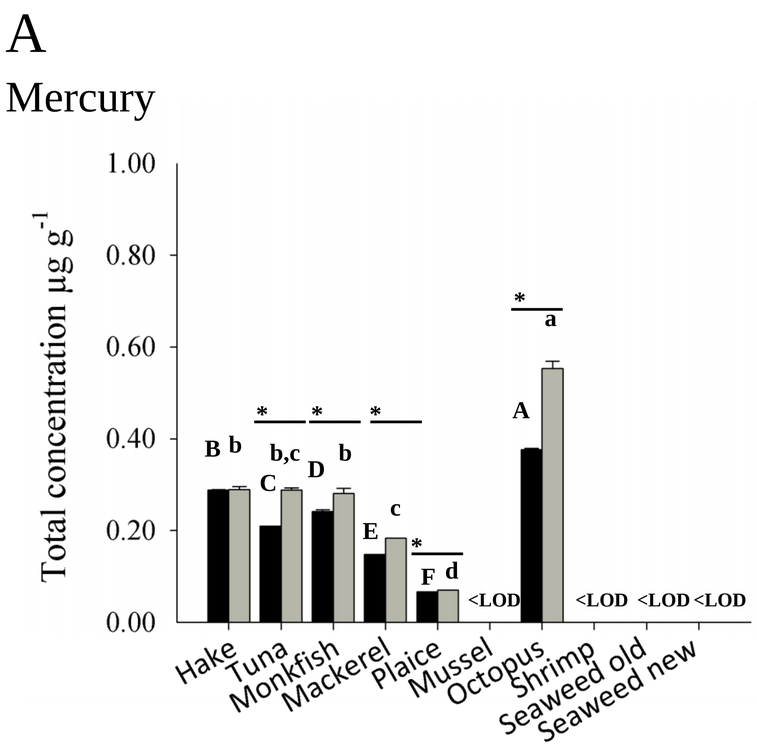
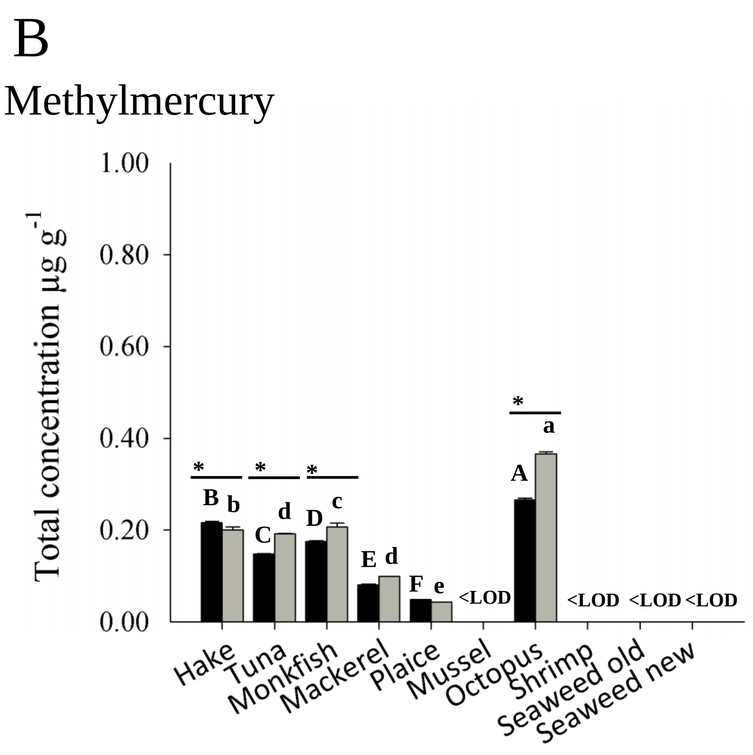
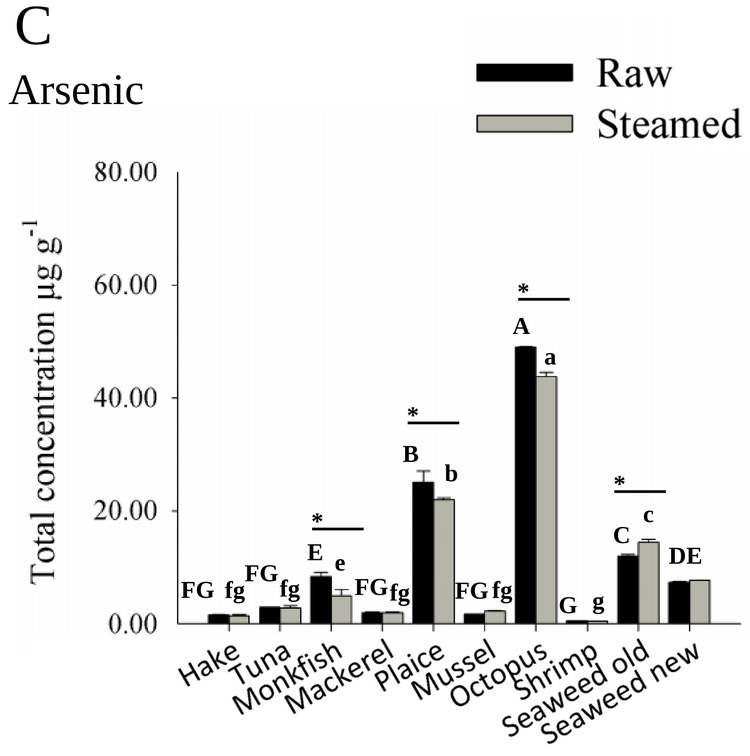
Legend to supplementary figures:

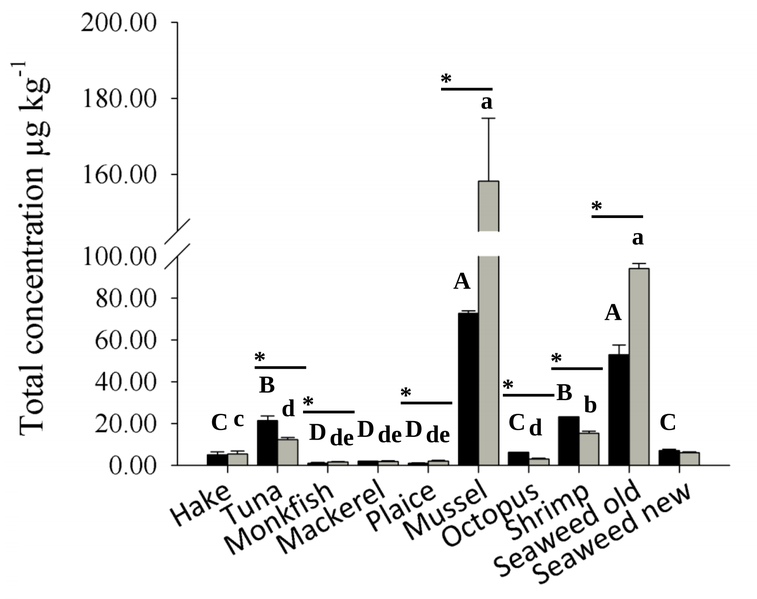
Supplementary Fig. 1 – Scheme of the *in vitro* digestion protocol used to access elements bioaccessibility.

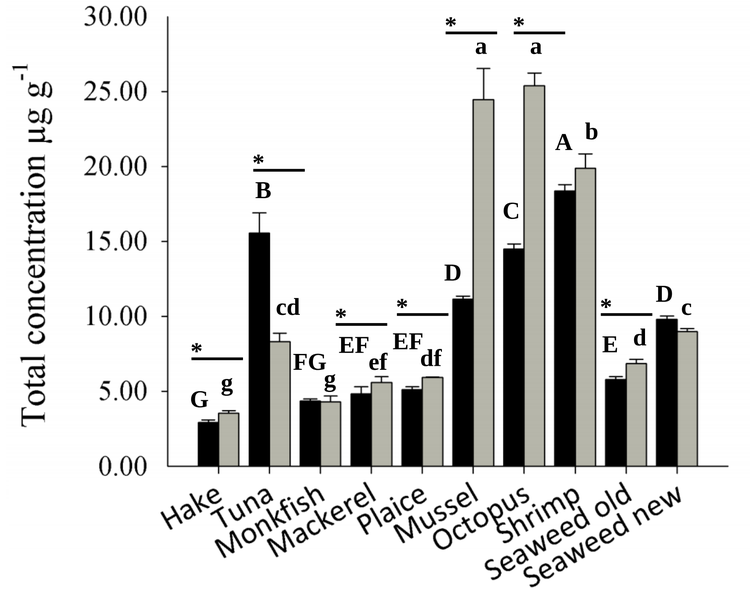
Figure1

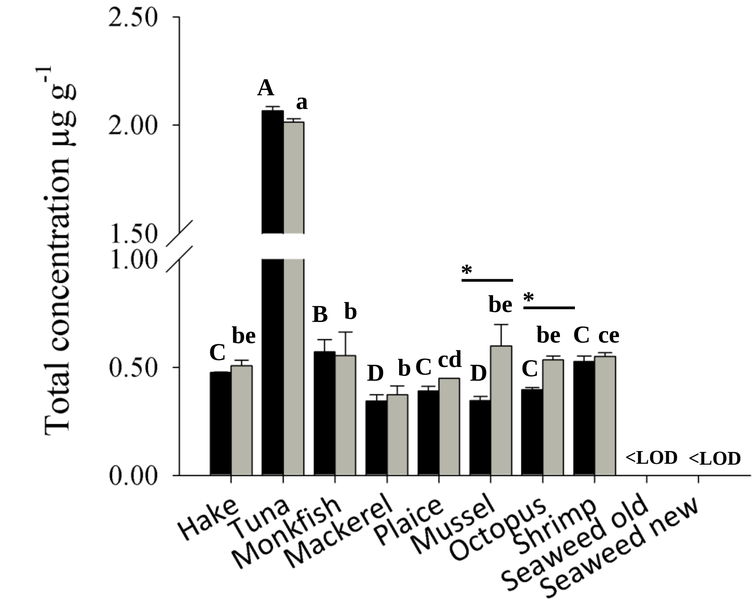

A
Mercury
Total concentration µg g-1
1.00
0.80
0.60
0.40
0.20
0.00
B b
C
b,c
D
b
E
c
F
d
<LOD
A
a
<LOD
<LOD
<LOD
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new

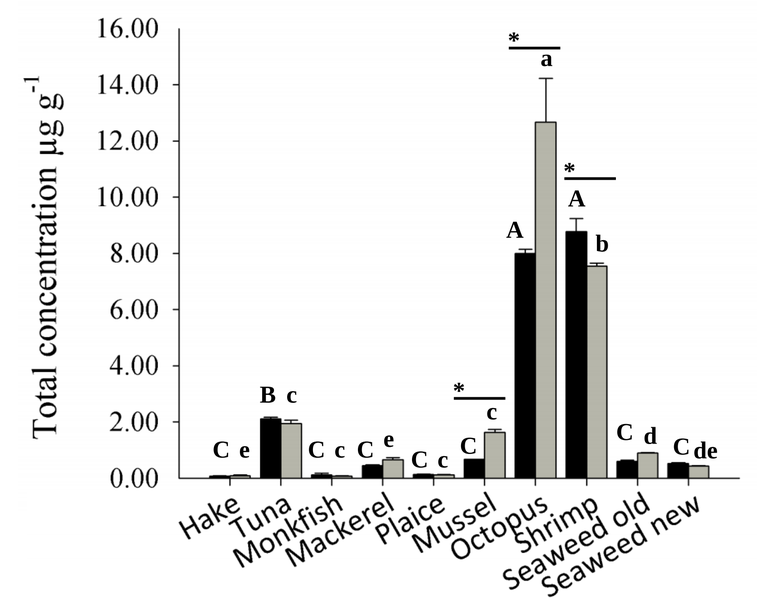

B
Methylmercury
Total concentration µg g-1
1.00
0.80
0.60
0.40
0.20
0.00
B b
C
d
D
c
E
d
F
e
<LOD
A
a
<LOD
<LOD
<LOD
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new


C
Arsenic
Raw
Steamed
Total concentration µg g-1
80.00
60.00
40.00
20.00
0.00
FG fg
FG
fg
E
e
FG
fg
B
b
FG
fg
A
a
G
g
C
c
DE
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new


D
Cadmium
Total concentration µg kg-1
200.00
180.00
160.00
100.00
80.00
60.00
40.00
20.00
0.00
C c
B
d
D de
D de
D de
A
a
C d
B
b
A
C
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new

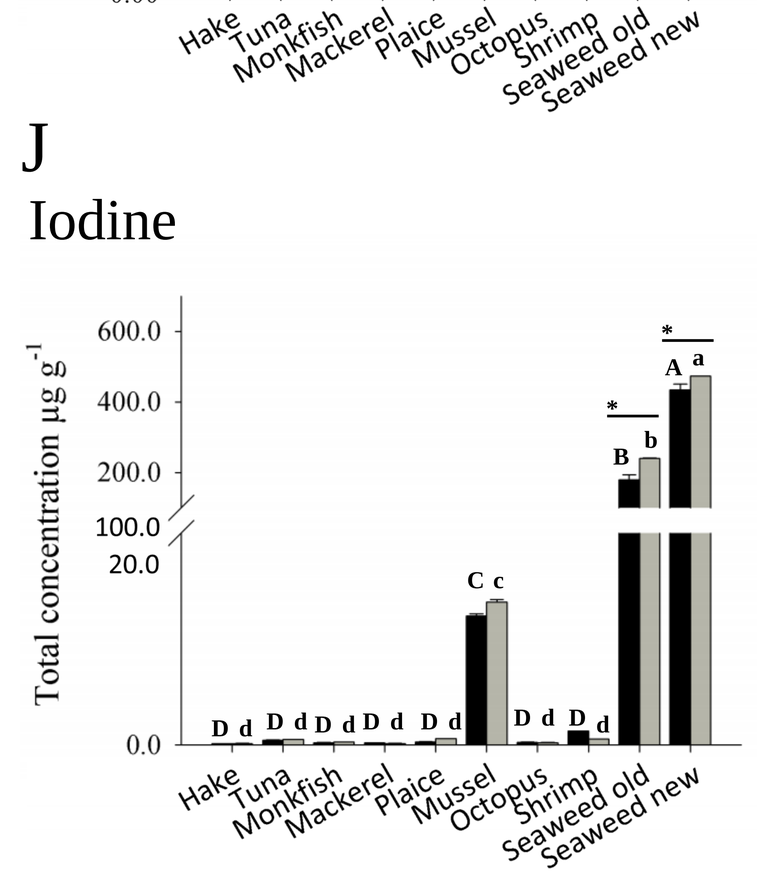

E
Zinc
Total concentration µg g-1
30.00
25.00
20.00
15.00
10.00
5.00
0.00
G
g
B
cd
FG
g
EF
ef
EF
df
D
A
a
C
A
a
A
b
E
d
D
c
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new


F
Selenium
Total concentration µg g-1
2.50
2.00
1.50
1.00
0.50
0.00
C
be
A
a
B
b
D
b
C
cd
D
be
C
be
C
ce
<LOD
<LOD
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new


G
Copper
Total concentration µg g-1
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
C e
B c
C c
C
e
C c
C
c
A
a
A
b
C
d
C
de
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new

H
Manganese
Total concentration µg g-1
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
<LOD
d
D
d
D
d
C
d
D
d
A
a
BC
bc
B
b
BC
bc
C
c
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new

I
Strontium
Total concentration µg g-1
300.0
200.0
100.0
35.0
20.0
0.0
C
c
B
b
A
a
D
d
D
d
D
d
D
d
CD
d
d
CD
d
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new


J
Iodine
Total concentration µg g-1
600.0
400.0
200.0
100.0
20.0
0.0
D d
D d
D d
D d
D d
C c
D d
D d
B
b
A
a
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new

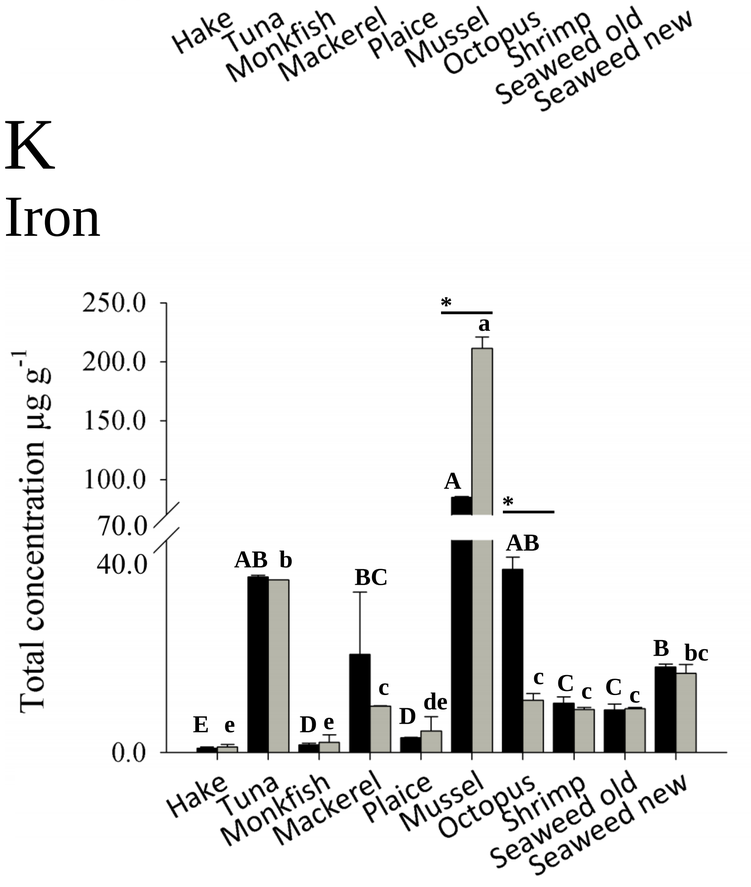
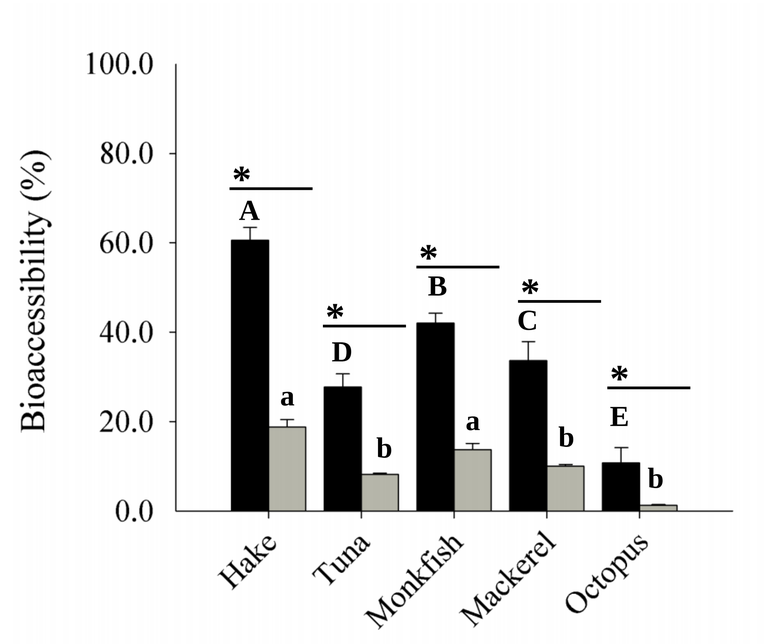

K
Iron
Total concentration µg g-1
250.0
200.0
150.0
100.0
70.0
40.0
0.0
E e
AB
b
D e
BC
c
D
de
A
a
AB
c
C
c
C
c
B
bc
Hake
Tuna
Monkfish
Mackerel
Plaice
Mussel
Octopus
Shrimp
Seaweed old
Seaweed new

Figure2

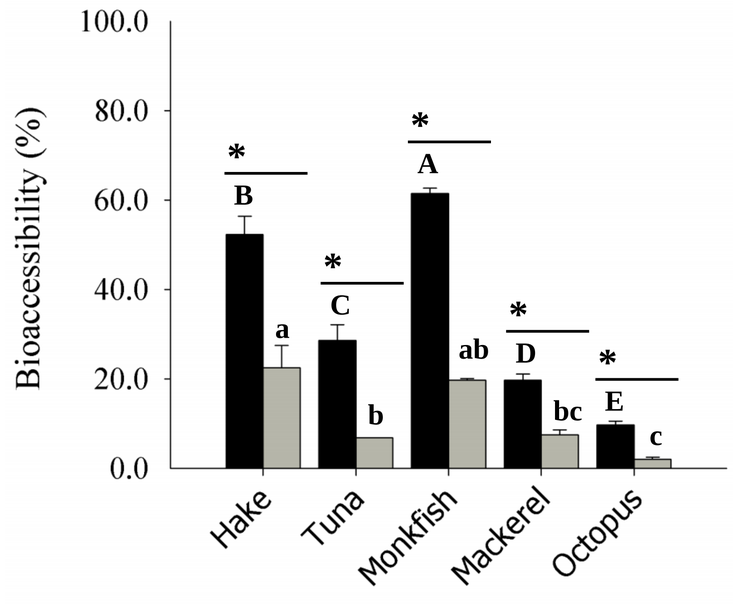
A

Mercury



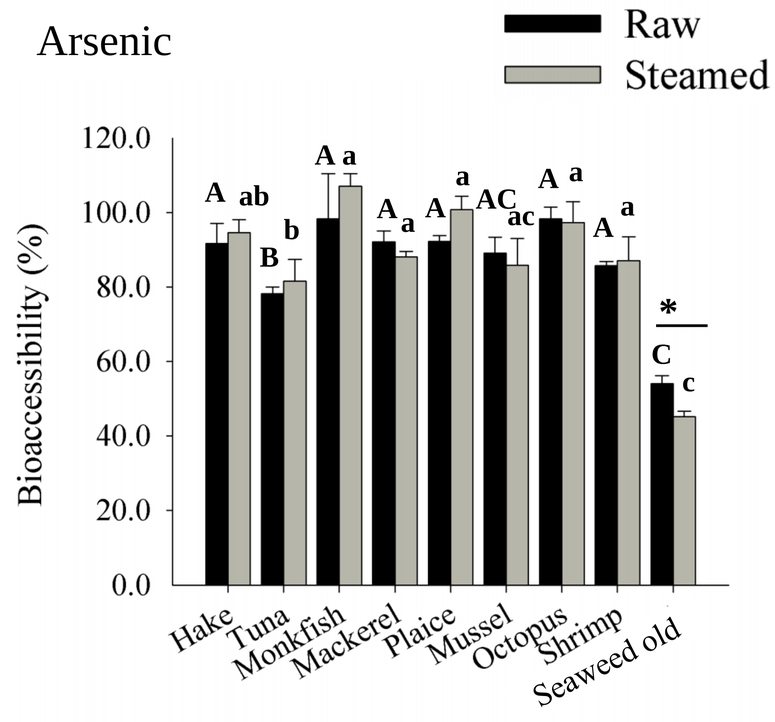
B

Methylmercury



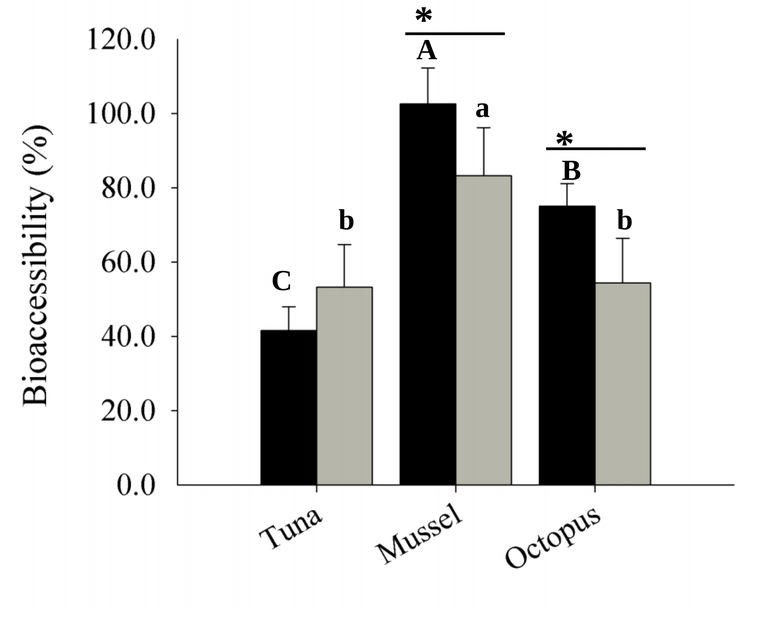
C

Arsenic



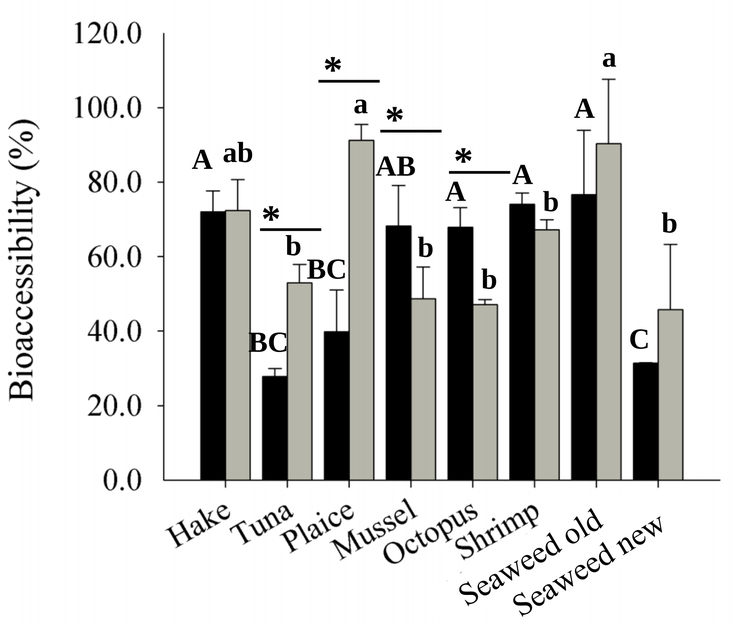
D

Cadmium



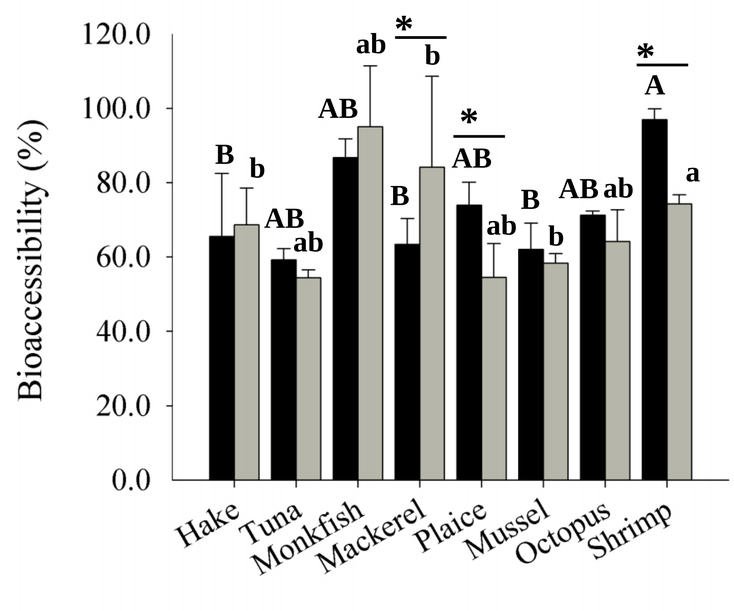
E

Zinc



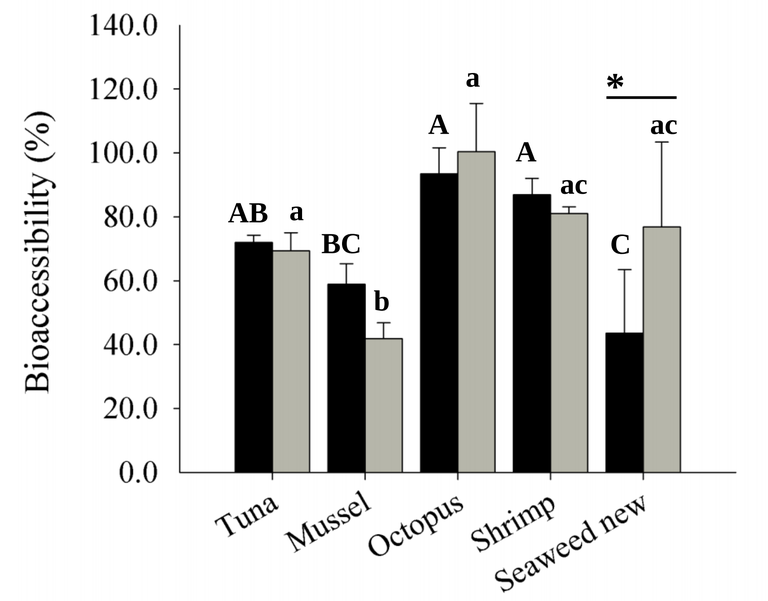
F

Selenium



G

Copper



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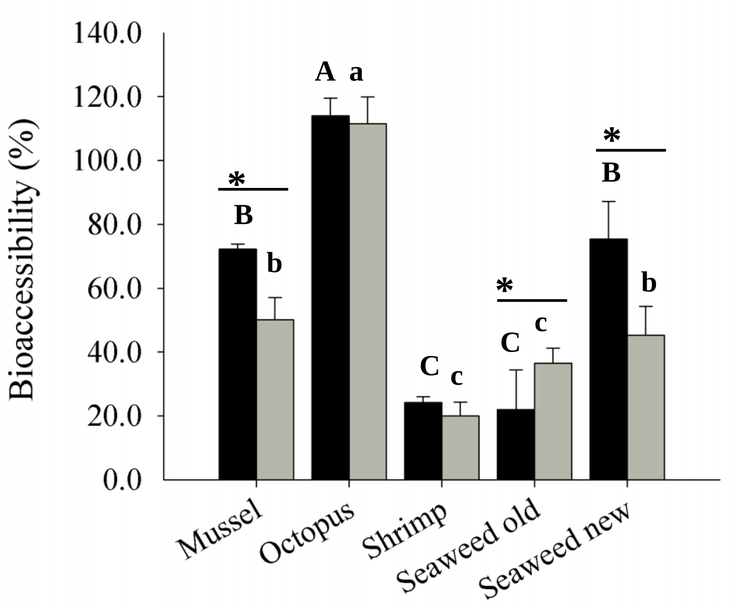
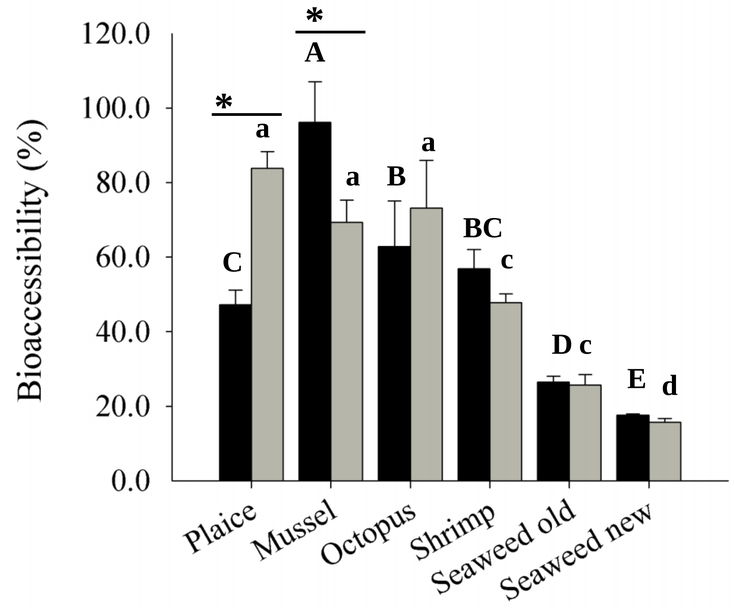
Manganese



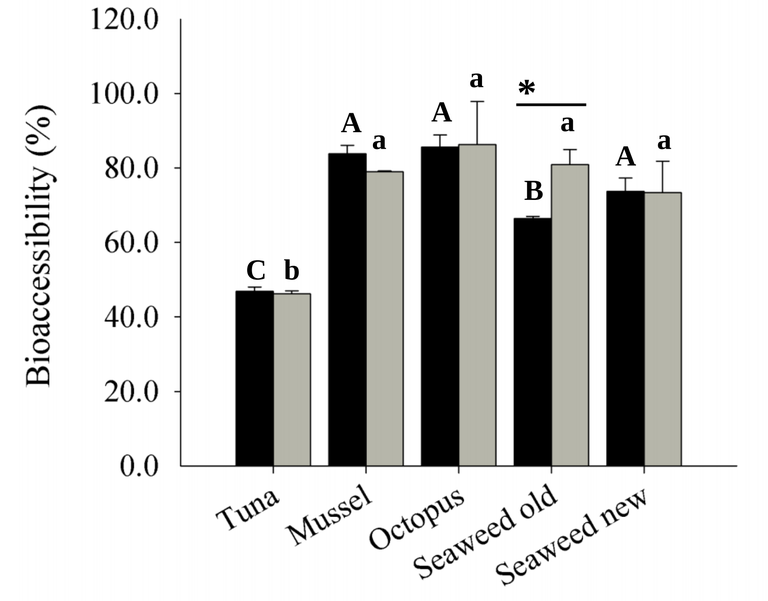
I

Strontium



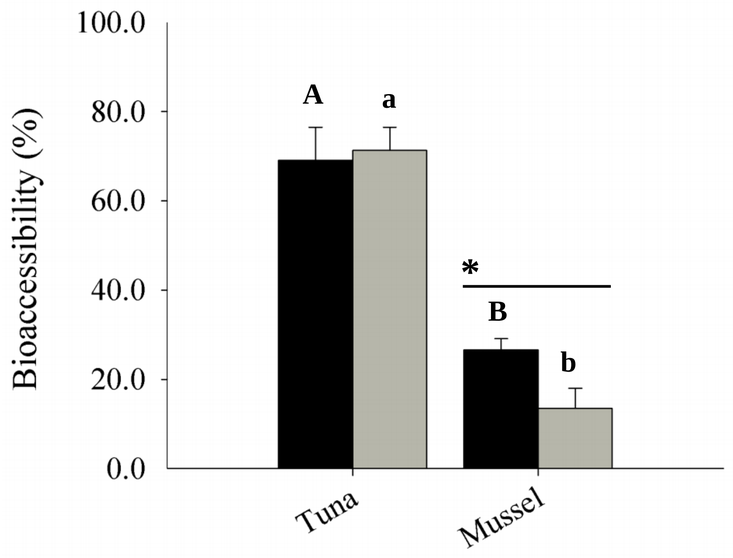
J

Iodine



K

Iron



Supplemental Figure 1

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Supplemental Table 1

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Supplemental Table 2

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Supplemental Table 3

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Supplemental Table 4

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Supplemental Table 5

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Supplemental Table 6

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