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Bioaccessibility and cytotoxicity of arsenic and mercury in cooked seafood: Implications for food safety and human health risk

Marta Ventura [a,b,*], Ricardo Assunção [a,c], Beatriz Matos [b], Inês Coelho [a], Inês Delgado [a], Sandra Gueifão [a], Susana Santiago [a], Isabel Castanheira [b], Marta Martins [b,d,**]

[a] Food and Nutrition Department, National Institute of Health, Doutor Ricardo Jorge (INSA, IP), Av. Padre Cruz, Lisbon 1649-016, Portugal
[b] MARE - Marine and Environmental Sciences Centre, ARNET - Aquatic Research Network Associate Laboratory, NOVA School of Science and Technology, NOVA University Lisbon, Caparica, Portugal
[c] Egas Moniz Center for Interdisciplinary Research (CiiEM); Egas Moniz School of Health & Science, Caparica, Almada, Portugal
[d] Department of Environmental Sciences and Engineering, NOVA School of Science and Technology, NOVA University Lisbon (NOVA FCT), Caparica 2829-516, Portugal

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ABSTRACT

Seafood is an important component of healthy dietary patterns. However, it can also serve as a dietary source of toxic elements that bioaccumulate in aquatic environments and biomagnify through the food web. This study quantified arsenic (As) and mercury (Hg) in cooked seafood, assessed their bioaccessibility, and evaluated the cytotoxic potential of the bioaccessible fractions using the human intestinal HT-29 cell line. The highest As concentrations were found in bivalves (3.7 ± 0.2 mg kg^{-1}), and the highest Hg concentrations in scabbardfish (0.57 ± 0.01 mg kg^{-1}). The bioaccessibility of As was substantially higher (ranging from 91 ± 2% to 127 ± 6%) than that of Hg (ranging from 4.8 ± 0.3% to 24 ± 8%). The highest bioaccessibility was observed in fresh tuna, 127 ± 6% and 24 ± 8% for As and Hg, respectively. Cytotoxicity assays at 24 h, 48 h, and 72 h indicated that bioaccessible Hg concentrations remained below cytotoxic thresholds. These results highlight the importance of integrating bioaccessibility and toxicological indicators into the evaluation of seafood safety.

1. Introduction

Seafood, comprising fish and shellfish, is recognised for its nutritional value. This food group is a source of essential nutrients, including long-chain omega-3 polyunsaturated fatty acids, vitamins, and micronutrients such as iodine and selenium (Tilami and Sampels, 2018). Seafood is a key component of a healthy dietary pattern, including the Mediterranean diet, and is associated with well-established nutritional benefits (Hidalgo-Mora et al., 2020). Portugal has a highly developed fishing activity and is the leading consumer of fish and seafood in Europe (European Commission, 2020). In fact, seafood constituted over one-third of the overall total diet studies (TDS) samples in the first Portuguese harmonised TDS (Vasco et al., 2021).

However, seafood can also be a source of aquatic environmental contaminants, including chemical contaminants such as arsenic (As) and mercury (Hg) (Hellberg et al., 2012). These contaminants bioaccumulate through the food chain, particularly in predatory fish occupying higher trophic levels, ultimately reaching top consumers such as humans (Maulvault et al., 2015). Their occurrence raises public health concerns due to their environmental persistence and well-documented toxicological effects (Næss et al., 2020; Varol and Sünbül, 2019). As and Hg are recognized as neurotoxic and carcinogenic to humans even at low levels (Grandjean and Herz, 2015; IARC, 2012, 2024). These contaminants are also classified among the top ten chemicals of major public health concern, as defined by the Agency for Toxic Substances and Disease Registry (ATSDR., 2019).

Abbreviations: As, arsenic; EC, European Commission; EC_{50}, Effective Concentration at 50%; EFSA, European Food Safety Authority; GIT, Gastrointestinal Tract; Hg, mercury; HT-29, Human Colon Adenocarcinoma; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IVD, In Vitro Digestion; LOQ, limit of Quantification; MeHg, Methylmercury; SD, Standard Deviation; TDS, Total Diet Study; WHO, World Health Organization.

* Corresponding author at: Food and Nutrition Department, National Institute of Health, Doutor Ricardo Jorge (INSA, IP), Av. Padre Cruz, Lisbon 1649-016, Portugal.

** Corresponding author at: MARE - Marine and Environmental Sciences Centre, ARNET - Aquatic Research Network Associate Laboratory, NOVA School of Science and Technology, NOVA University Lisbon, Caparica, Portugal.

E-mail addresses: marta.ventura@insa.min-saude.pt (M. Ventura), marta.martins@fct.unl.pt (M. Martins).

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Seafood is primarily consumed in cooked form, a process that affects both its nutritional composition and contaminant levels. This underscores the importance of analysing foodstuffs in the form in which they are actually consumed (Liao et al., 2020; Tilami,and Sampels, 2018).

TDS are recognised by the World Health Organization (WHO) and the European Food Safety Authority (EFSA) as an important Public Health tool for assessing dietary exposure to beneficial and harmful substances (EFSA, FAO, 2011). These studies are based on food consumption surveys and consist of selecting, collecting at the retail level, and analysing commonly consumed foods representative of the overall diet of a population. TDS data are crucial for estimating dietary exposure as they integrate contaminant occurrence data with food consumption datasets (EFSA, FAO, 2011).

The gastrointestinal tract (GIT) serves as the primary interface between the external environment and the human body, facilitating nutrient absorption while providing a barrier against potentially hazardous substances. Therefore, diet plays an essential role in gut function, with intestinal cells being the first to encounter ingested compounds (Verhoeckx et al., 2015).

The amounts of substances in food do not necessarily correspond to the levels available to exert their biological functions in the human body. This discrepancy is explained by the concept of bioavailability, which is a complex process comprising two interrelated components: bioaccessibility and bioactivity. Bioaccessibility refers to the fraction of an ingested compound that is released from the food matrix within the GIT, making it available for absorption. Once absorbed, bioactivity encompasses the compound's transport through the systemic circulation to target tissues. Understanding these processes is essential for assessing the impact of dietary compounds on human health (Verhoeckx et al., 2015).

Different human *in vitro* digestion (IVD) methods are widely employed to simulate the human digestive process (Verhoeckx et al., 2015). A standardised static IVD model for food was developed by the INFOGEST COST Action (Brodkorb et al., 2019). This model was developed based on an international consensus regarding several key aspects, including fluid composition, enzymatic activities, and sample and fluid volumes. The harmonised IVD method considers the conditions of *in vivo* digestion and is well-suited for assessing the bioaccessibility of contaminants in food. This methodology offers several advantages over human nutritional studies, including faster results, increased efficiency, lower costs, the capacity to process a larger number of samples, and the avoidance of ethical concerns (Brodkorb et al., 2019).

Ma et al. demonstrated that the controlled physicochemical conditions of the INFOGEST method improve reproducibility and comparability across studies. This makes the method particularly valuable for food safety, risk assessment, and toxicological evaluations (Ma et al., 2024). This methodology is considered a reference ("gold-standard") static digestion model in food science (Minekus et al., 2014) and has been widely used to study the gastrointestinal fate of different foodstuffs, including seafood (Zhou et al., 2023). Luz et al. reported that the INFOGEST model was used in 65% of the reviewed studies and was considered one of the most effective approaches for simulating human gastrointestinal protein digestion (Luz et al., 2024). The INFOGEST method comprises three main steps: the oral, gastric, and intestinal phases (Brodkorb et al., 2019). Following ingestion and digestion, intestinal cells form the first biological barrier, interacting with food components as they are released from their matrix. Different cell lines are typically used to assess the cytotoxic effects of bioaccessible substances in the GIT (Verhoeckx et al., 2015). In particular, the human colon adenocarcinoma (HT-29) cell line is widely used as an *in vitro* model of the small intestine due to its ability to mimic key intestinal cell characteristics. This cell line is therefore a valuable model for evaluating the toxicity of contaminants across the intestinal epithelium (Verhoeckx et al., 2015), and has been extensively applied in food digestion and bioaccessibility studies (Ponce de León-Rodríguez et al., 2018).

The main goal of this study is to provide a comprehensive and integrated assessment of the potential toxicity of cooked seafood and its implications for human digestion. It combines occurrence data, gastrointestinal bioaccessibility, and *in vitro* toxicity within a single experimental framework. Specifically, this research aims to: 1) assess the concentrations of As and Hg in cooked seafood samples obtained from the Portuguese TDS study; 2) determine the concentrations of these contaminants in the bioaccessible fraction by applying the INFOGEST *in vitro* digestion (IVD) method to the same samples; and 3) evaluate the cytotoxicity of As and Hg using the HT-29 cell line model.

2. Materials and methods

2.1. Sampling collection

The seafood sampling strategy for the present study was established according to the TDS methodology carried out in Portugal, considering the foods most consumed by the Portuguese population, as previously described in detail by Vasco et al. (2021). The selection of seafood samples was based on results from the Portuguese TDS study (Ventura et al., 2020). Five seafood matrices were chosen for the present study: bivalves (clams and wedge clams), European sardine, fresh Atlantic cod, fresh tuna, and scabbardfish. These were selected due to their relevance to the Portuguese diet and their known content of contaminants of concern for human health, namely As and Hg. Briefly, the samples ($n = 60$) were purchased at the retail level from selected supermarkets in the Greater Lisbon region, representing the major food distribution chains in Portugal. Samples were cooked according to guidelines deriving from a Portuguese food consumption survey conducted in the framework of the Portuguese TDS pilot study (Vasco et al., 2021). To ensure the representativeness of TDS samples, each composite seafood sample was prepared by pooling equal portions (100 g) of 12 individual food items, in accordance with established statistical criteria.

In short, a total of five seafood pooled samples were analysed, each consisting of 12 subsamples prepared as consumed (edible portion only), thereby reflecting the common consumption habits of Portuguese households (Vasco et al., 2021).

2.2. In vitro bioaccessibility assay

The bioaccessibility assay was performed on the selected food items, including bivalves (i.e., clams and wedge clams), European sardine, fresh Atlantic cod, fresh tuna, and scabbardfish. The procedure followed the harmonised static IVD model developed under INFOGEST COST Action and recently revised by Brodkorb et al. (2019). Briefly, the IVD method consists of three sequential phases: oral phase, considering the simulated saliva fluid with amylase - 75 U/mL at the pH 7; gastric phase, considering the simulated gastric fluid with pepsin - 2000 U/mL at the pH 3; and intestinal phase, considering the simulated intestinal fluid containing a pancreatin-bile mixture - 100 U/mL and 10 mM, respectively, at the pH 7.

To determine the bioaccessibility of As and Hg, 1 g of each pooled sample was weighed, and the reagents and enzymes were added. Samples were then shaken at 37 ℃ for two minutes (oral phase) and for 120 min (gastric and intestinal phases). After the intestinal phase incubation, the reaction was stopped using 1 mM of Pefabloc® (Sigma-Aldrich, St. Louis, MO, USA) (Assunção et al., 2016; Brodkorb et al., 2019). Additionally, digests were immediately immersed in liquid nitrogen and stored at −80 °C until further analysis. All experiments were conducted in triplicate. A reagent blank was performed for each batch of samples and included in all bioaccessibility calculations to correct for background levels of contaminants in digestion fluids. The bioaccessibility assay is presented in Figure SF1. Bioaccessibility values were determined according to Eq. 1.

$$Bioaccessibility \quad (\%) \quad = \quad \frac{[Contaminants]\ in\ the\ digests}{[Contaminants]\ in\ the\ undigested\ samples} \times 100 \tag{1}$$

2.3. Concentrations of As and Hg in seafood samples

2.3.1. Determination of As content

The reagents used for As analysis were of high analytical grade, providing the highest level of purity for the experiments: ultrapure water was obtained using a Milli-Q Plus Millipore system (Q-POD Millipore, Interface, Portugal); HNO_3 pro analysis (65%) was distilled to ultrapure grade in an acid distillation system (Milestone SubPUR Unicam, Portugal). Samples were analysed in clean room facilities. A reagent blank was performed in each digestion cycle to control for potential contaminations.

Analysis of As in cooked samples was carried out as described by Coelho et al. (2013) with slight modifications in brief: 0.5 g of samples were weihged into PTFE (Poly tetra fluoroethylene) microwave vessels (Milestone Ethos 1 Series), 3 mL of ultrapure water, 4 mL of concentrated ultrapure HNO_3 and 1 mL of concentrated H_2O_2 were added and the the vessels were closed. The digestion proceded according to the following programme: in the first step, the temperature increased to 180 °C during 10 min; in the second step, the temperature was kept at 180 °C for 5 min; in the third step, the temperature increased to 210 °C during 12 min; in a fourth step the temperature kept at 210 °C during 5 min and in the final step the temperature decreased to 90 °C over 6 min. After cooling to room temperature, the contents of each vessel were transferred to a volumetric flask and made up to 25 mL with ultrapure water before analysis by ICP-MS (ICP-MS ThermoX Series II, Thermo Fisher Scientific, Waltham, MA, USA).

For analyzing samples obtained after the IVD assay (bioaccessible fractions) and the respective blank tests, a dilution of HNO_3 2% (v/v) was performed prior to analysis of As by ICP-MS.

The calibration curve and internal quality control solutions of As were prepared using high-purity ICP-MS stock standard solutions containing 1000 mg L^{-1} of As (Merck, Darmstadt, Germany). As internal standards, standard solutions of germanium (Ge) at 1000 mg L^{-1} (Inorganics Ventures, Christiansburg, VA, USA), yttrium (Y), and indium (In) at 1000 mg L^{-1} (Merck, Darmstadt, Germany) were used. The results were expressed by the average of three independent replicates (n = 3). The determination of As is presented in Figure SF1.

2.3.2. Determination of Hg content

The Hg content was determined in cooked samples (0.04–0.3 g) and bioaccessible fractions by atomic absorption spectrophotometry with thermal decomposition and amalgamation (TDA/AAS) using a Direct Mercury Analyzer DMA-80 (Milestone Srl, Sorisole, BG) with an automatic sampler, coupled to a terminal with easy DOC 2 software version 3.03. Briefly, the samples were placed in nickel boats in the automatic sampler and then entered into the combustion tube, where they were dried and thermally and chemically decomposed in the decomposition furnace at 650 °C. The Hg was selectively trapped in a gold amalgamator, carried by flowing oxygen through absorbance cells, and quantified by atomic absorption spectrophotometry at 253.7 nm (EPA, 2007). The determination of Hg is presented in Figure SF1.

2.4. Quality assurance

The analytical determinations of As and Hg followed a rigorous quality assurance programme according to the NP EN ISO/IEC 17025 standard requirement (ISO/IEC 17025:2017, 2017). Analytical procedures were carried out under rigorous metrological control and expressed as the average of three replicates. To analyse As by ICP-MS, a new calibration curve was prepared daily using a minimum of five standards at different concentrations. A Quality Control (QC) standard was included in each group of twelve test samples to validate the test run. Instrumental drift was monitored by the difference between the first and last QC samples (<5%). Recoveries ranging from 87% to 108% were obtained for As.

Regarding quality control in Hg quantification, samples were analysed at least in duplicate. Blanks and quality control samples were analysed in each assay, and Certified Reference Material NIST 1566b Oyster tissue was used to assess the method's accuracy and precision.

The Limits of Quantification (LOQ) were estimated to be ten times the standard deviation of the mean blank tests, after correction for sample weight and the appropriate dilution. The LOQs for As and Hg were 0.002 mg kg^{-1} and 0.004 mg kg^{-1}, respectively.

The figures of merit for this study are presented in Table ST1 and were obtained under rigorous quality control conditions. The working ranges established for each element align well with the characteristics of the matrices studied, ensuring precise and accurate quantification within specified limits. For both chemical elements, the repeatability, expressed as relative standard deviation, remained consistently below 10%, underlining the high precision of the analytical measurements. Uncertainties were calculated annually based on analytical internal quality control data from various matrices, demonstrating a robust and reliable performance. The laboratory also participated in proficiency testing schemes conducted by an accredited provider and achieved satisfactory results.

2.5. Cell culture

The HT-29 cells were cultured in a complete cell culture medium consisting of a 1:1 mixture of Dulbecco's Modified Eagle Medium (Merck) and F-12, supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL) mixture, and maintained in a humidified incubator at 37 ∘C and 5% CO_2. The culture medium was replaced every 2–3 days. Once the cells reached 80% confluency, the medium was aspirated, and the cell monolayer was washed three times with sterile phosphate-buffered saline. The monolayer was then treated with 1 mL of 0.25% (w/v) trypsin-EDTA and visualized microscopically to ensure complete cell detachment.

2.6. Cell viability assay

In vitro assays using HT-29 cell lines were performed to assess cell viability after 24, 48, and 72 h of exposure to As and Hg. Cell viability was analysed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described by Branco et al. (2021). The assay is based on the principle that, in metabolically active cells, mitochondrial dehydrogenases enzymes reduce MTT to formazan crystals. The resulting absorbance measured at 550 nm serves as an indicator of cell viability. Briefly, cells (5×10^3 cells/well) were cultured in 96-well plates and allowed to adhere for 24 h prior to contaminant exposure. To evaluate toxicity, the studied contaminants were added at the following concentrations, based on internal occurrence data: As (0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, 200 and 400 mg L^{-1}), and Hg (0.1, 0.25, 0.5, 1, 2, 5, 10, 15, 20 and 30 mg L^{-1}). Following exposure, 50 μL of MTT (400 μg mL^{-1} *per* well) was added to each well, followed by incubation at 37 °C for 2 h. Next, the supernatants were discarded, and the formazan crystals were dissolved in a 4:1 dimethyl sulfoxide/glycine buffer solution (pH 10.5). The 96-well plate was placed on a shaker platform and maintained in the dark to prevent light exposure, with gentle agitation for 20 min to ensure complete dissolution of the formazan crystals. Absorbance was subsequently measured at 550 nm using a microplate reader. The EC_{50} value was determined as the concentration of the compound that induced a 50% decrease in MTT relative to the control group. Figure SF1 illustrates the cell viability assay.

Table 1
Bioaccessibility (%) and occurrence levels (mean ± SD) of As and Hg in samples (fresh weight, n = 3).

Samples	As			Hg		
	Cooked samples x ± s (mg kg^{-1})	Bioaccessible fraction (mg kg^{-1})	Bioaccessibility (%)	Cooked samples x ± s (mg kg^{-1})	Bioaccessible fraction (mg kg^{-1})	Bioaccessibility (%)
Bivalves	3.7 ± 0.2*	3.5 ± 0.1	95 ± 9	0.024 ± 0.001	0.002 ± 0.001	8 ± 4
Cod Atlantic fresh	3.5 ± 0.1*	4.0 ± 0.1	114 ± 4	0.098 ± 0.003	0.008 ± 0.001	8.0 ± 0.4
European Sardine	2.7 ± 0.1*	2.45 ± 0.05	91 ± 2	0.036 ± 0.001	0.005 ± 0.001	14 ± 4
Fresh tuna	0.81 ± 0.02*	1.03 ± 0.02	127 ± 6	0.24 ± 0.04	0.06 ± 0.02	24 ± 8
Scabbardfish	1.27 ± 0.01*	1.3 ± 0.1	105 ± 5	0.57 ± 0.01	0.027 ± 0.001	4.8 ± 0.3

*published by Ventura et al. (2020)

2.7. Statistical analysis

Numerical values from the analysed samples were expressed throughout as the mean ± standard deviation (SD) of the mean.

The statistical analysis was conducted using IBM SPSS Statistics version 27. Significance levels were set at a p-value < 0.05 for all analyses. Spearman correlation coefficients were calculated to further explore connections within the dataset. Data normality was assessed using the Shapiro-Wilk test. For normally distributed data, independent-samples *t*-tests were performed to compare group means. For non-normally distributed data, the Mann-Whitney *U* test was used. The GraphPad version 10.0.2. was used for EC_{50} calculations.

3. Results and discussion

3.1. As and Hg levels in cooked seafood

The concentrations of As and Hg in the seafood samples are presented in Table 1.The levels of As in cooked seafood samples ranged from 0.81 ± 0.02 mg kg^{-1} in fresh tuna to 3.7 ± 0.2 mg kg^{-1} in bivalves (Table 1), these results were previously reported by Ventura et al. (2020).

The levels of Hg in cooked seafood samples ranged from 0.024 ± 0.001 mg kg^{-1} in bivalves to 0.57 ± 0.01 mg kg^{-1} in scabbardfish (Table 1). These concentrations are in the same order of magnitude as those reported for similar organisms in the literature (Millour et al., 2011; Okpala et al., 2017; Olmedo et al., 2013). The results of Hg in sardine (0.036 ± 0.001 mg kg^{-1}), and tuna (0.24 ± 0.04 mg kg^{-1}) (Table 1) corroborate those reported by Cano-Sancho et al. (2015) (sardine 0.014 ± 0.000 mg kg^{-1}, and tuna 0.185 ± 0.01 mg kg^{-1}) as well as the results reported by Mehouel et al. (2019) for sardine (0.04 ± 0.03 mg kg^{-1}). Additionaly, these are also consistent with the results published by Alves et al. (2018) for Hg in tuna (0.288 ± 0.004 mg kg^{-1}) (Alves et al., 2018; Cano-Sancho et al., 2015; Mehouel et al., 2019). In the present study, top predator fish presented higher Hg levels than the other analysed seafood samples, such as bivalves and sardines. This is in line with previous studies showing that Hg levels increase throughout the food web (EFSA, 2012). The European Commission (EC) has set a maximum level of 1.0 mg kg^{-1} for tuna and scabbardfish. The levels of Hg in these fish species, as well as in the remaining analysed seafood samples, were lower than those set out in legislation (European Union, 2023). In the present study, it's assumed that Hg is present as methylmercury (MeHg); this assumption is supported by Fioravanti et al. (2025). and the EFSA report (EFSA, 2012).

3.2. IVD fractions and bioaccessibility of As and Hg in cooked seafood

Regarding the bioaccessible fractions, the levels of As ranged from 1.03 ± 0.02 mg kg^{-1} in fresh tuna to 4.0 ± 0.1 mg kg^{-1} in fresh Atlantic cod (Table 1). The most analysed bioaccessible fractions for As align with the results reported by Cano-Sancho et al. (2015) (sardine 1.59 ± 0.13 mg kg^{-1}, and tuna 0.79 ± 0.03 mg kg^{-1}) (Cano-Sancho et al., 2015). In the case of cod, the result aligns with those published by Fu et al. (2021), specifically 2.86 ± 0.01 mg kg^{-1} and 3.20 ± 0.00 mg kg^{-1}. On the other hand, tuna levels are lower than those reported in the literature (2.16 ± 0.02 mg kg^{-1}) (Fu et al., 2021).

The amounts of Hg in the bioaccessible fractions ranged from 0.002 ± 0.001 mg kg^{-1} in bivalves to 0.06 ± 0.02 mg kg^{-1} in fresh tuna (Table 1); the results for tuna and sardine are similar to those reported in the literature (0.064 ± 0.008 mg kg^{-1}, 0.002 ± 0.000 mg kg^{-1}, respectively). When compared with results from a previous study, a similar tendency was observed for tuna (Torres-Escribano et al., 2011).

The bioaccessibility of As and Hg, expressed as the percentage of the contaminant content extracted from the food matrix at the end of the IVD assay relatively to the content in the same sample before the IVD assay, ranged from 91 ± 2% (European sardine) to 127 ± 6% (fresh tuna) for As, and from 4.8 ± 0.3% in scabbardfish to 24 ± 8% in fresh tuna for Hg (Table 1). The bioaccessibility of As in most of the analysed samples approached 100% (Table 1), which is in line with other results reported in the literature, where the bioaccessibility of As in seafood, including fish and shellfish, has been reported to range from 80% to 100% (Cano-Sancho et al., 2015; Moreda-Piñeiro et al., 2012). Lin et al. (2021) evidenced that the bioaccessibility of bivalves was 89%. Cano-Sancho et al. (2015) demonstrated that the As bioaccessibility for sardine was 89%. Cano-Sancho et al. (2015) reported, that tuna sample showed a As bioaccessibility of around 76%, which was lower than in the present study (Cano-Sancho et al., 2015; Fu et al., 2021; Lin et al., 2021). Another study corroborates the As bioaccessibility at around 100% across different fish species (Alves et al., 2018). Additionally, other studies reported bioaccessibility above 100%. Fu et al. (2021) reported that the As bioaccessibility was 128.97 ± 1.10% for tuna, and 101.74 ± 0.41% and 119.81 ± 0.29% for cod samples.

The higher bioaccessibility of As is generally attributed to the chemical forms present, which differ in solubility, as well as to the physical nature of seafood tissues (Chávez-Capilla et al., 2016). Additionally, the low pH during the gastric phase further enhances its bioaccessibility in the GIT (Alves et al., 2018; Maulvault et al., 2011). In seafood, arsenic is predominantly present as water-soluble species, particularly arsenobetaine (AsB) (EFSA, 2024).

Previous studies have concluded that organic As species are the predominant forms in fish (Afonso et al., 2018); these findings are consistent with other publications reporting AsB as the major species above 90% in fish (EFSA, 2009, 2024). Compared with the cereal (rice) and water groups, the fish group had low levels of inorganic arsenic, indicating it is a low-risk group (EFSA, 2024).

Contrary to the As, bioaccessibility of Hg was < 24%, varying from 4.8 ± 0.3% in scabbardfish to 24 ± 8% in fresh tuna.

The bioaccessibility of Hg in fresh tuna (24 ± 8%) (Table 1) is similar to that reported by Anacleto (2020) (30.8 ± 5.7%) (Anacleto et al., 2020), while it is higher when compared to the result reported by Torres-Escribano (2011) (9%) (Torres-Escribano et al., 2011). In the case of scabbardfish, the values were (4.8 ± 0.3%) (Table 1) lower than those published in the literature, i.e., 47% (Anacleto et al., 2020). The lower Hg bioaccessibility of cooked seafood may be attributed to the loss

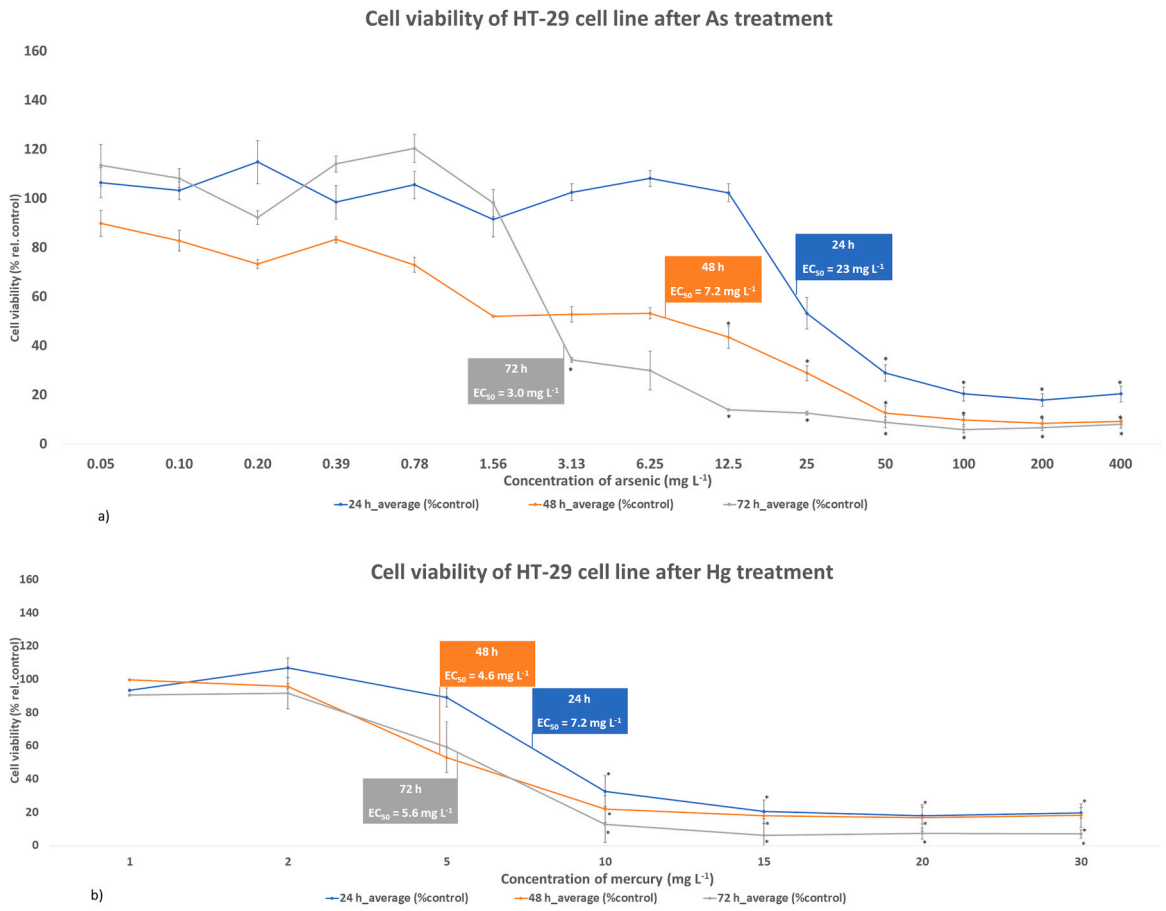

Fig. 1. Cell viability of HT-29 cells based upon the MTT assay, EC_{50} for each contaminant, and the respective exposure time. a) Effect of As treatment after 24, 48, and 72 h of exposure. b) Effect of Hg treatment after 24, 48, and 72 h of exposure. Each value represents three independent experiments (n = 3). $p < 0.05$ (*) represents a significant difference as compared to the negative control.

of native proteins, particularly cysteine groups, and structural alterations induced by heat exposure during the cooking process (Alves et al., 2018; Gong et al., 2025). Different forms of Hg bind to the proteins, which are denatured during cooking methods at high temperatures, making the active sites of the mercury-protein complex less accessible to digestive enzymes, thus limiting the activity of gastrointestinal enzymes that degrade protein structures bonded with Hg (Cano-Sancho et al., 2015; Gong et al., 2025; Mieiro et al., 2016). Additionally, some studies suggested that cooking can also promote the formation of insoluble Hg complexes or facilitate the loss of volatile Hg species, further decreasing its bioaccessibility. Overall, the heat-induced modifications in seafood muscle proteins play a crucial role in reducing Hg bioaccessibility after cooking (Anacleto et al., 2020; Costa et al., 2022; Ouédraogo & Amyot, 2011; Packull-McCormick et al., 2023).

3.3. Cell viability and cytotoxicity

The cytotoxicity of As and Hg on HT-29 intestinal cells was evaluated using the MTT assay. Fig. 1 illustrates the estimated toxic effective concentration at 50% (EC_{50}) for each contaminant, across the corresponding exposure time. The results showed a decrease in cell viability as both the contaminant concentration and exposure time increased, indicating a dose and time-dependent cytotoxic effect. The results obtained for HT-29 cell viability after As exposure demonstrated a statistically significant ($p < 0.05$) effect at concentrations of 50, 100, 200, and 400 mg L^{-1} after 24 h, relative to control cells. Furthermore, a statistically significant ($p < 0.05$) effect was observed at concentrations of 12.5, 25, 50, 100, 200, and 400 mg L^{-1} at 48 h. The results demonstrated statistically significant ($p < 0.05$) cell viability for concentrations of 3.13, 12.5, 25, 50, 100, 200, and 400 mg L^{-1} at 72 h (Fig. 1a). The cells were relatively resistant to As at lower concentrations during the first 24 h, with toxic effects becoming significant only at moderate to high doses over this short exposure time. After 72 h, a significant reduction in cell viability was observed even at lower tested concentrations. It should be emphasised that EC_{50} values derived from *in vitro* assays are intended as comparative indicators of relative cytotoxic potency and biological activity under the specific experimental conditions, and should not be directly interpreted as predictors of human health risk.

These results are consistent with those reported by Stevens et al. (2008), who analysed trioxide arsenic in HT-29 cells and found a dose-dependent relationship with cytotoxicity in these cells (Stevens et al., 2008). The results obtained for HT-29 cell viability after Hg exposure demonstrated a statistically significant ($p < 0.05$) effect at concentrations of 10, 15, 20, and 30 mg L^{-1} at 24, 48, and 72 h (Fig. 1b). Hg exhibits cytotoxic effects within just 24 h. Exposure to this inorganic contaminant induces significant, dose-dependent cytotoxicity in HT-29 cells, which becomes evident as early as 24 h and remains consistent for up to 72 h. These findings suggest that Hg cytotoxic effects are consistent across other cell lines (Sutton et al., 2002). The results obtained for cytotoxicity of Hg in HT-29 cells contrast with those of As, for which longer exposure times or higher doses were required to reach similar levels of toxicity.

The EC_{50} concentrations for As over 24, 48, and 72 h periods were 23, 7.2, and 3.0 mg L^{-1}, respectively (Fig. 1a). These results were lower after 24 h and higher at the remaining time points compared to those reported by Stevens et al. (2008), who found EC_{50} values of 9.8, 9.4, and 9.0 mg L^{-1} at 24, 48, and 72 h, respectively (Stevens et al., 2008).

Table 2
The concentration of As and Hg in bioaccessible fraction after in vitro digestion (mean ± SD) for each sample and their EC_{50} values after different exposure times in HT-29 cells. All the results are expressed in mg kg^{-1}.

Samples	As				Hg			
	Bioaccessible fraction	EC_{50} 24 h	EC_{50} 48 h	EC_{50} 72 h	Bioaccessible fraction	EC_{50} 24 h	EC_{50} 48 h	EC_{50} 72 h
Bivalves	3.5 ± 0.1	23	7.2	3.0	0.002 ± 0.001	7.2	4.6	5.6
Cod Atlantic fresh	4.0 ± 0.1				0.008 ± 0.001			
European Sardine	2.45 ± 0.05				0.005 ± 0.001			
Fresh tuna	1.03 ± 0.02				0.06 ± 0.02			
Scabbardfish	1.3 ± 0.1				0.027 ± 0.001			

Concerning EC_{50} values for HT-29 cells, exposure to Hg for 24, 48, and 72 h, resulted in 7.2, 4.6, and 5.6 mg L^{-1}, respectively (Fig. 1b).

Studies on inorganic contaminants such as As and Hg in intestinal cells remain scarce; however, some studies are available for liver cells. In hepatic cells (HepG2) Cordier et al. (2021) reported an EC_{50} of 6.71 mg L^{-1} for As and 26.23 mg L^{-1} for Hg after 24 h of exposure. The EC_{50} value observed by Cordier et al. (2021) for As was lower than that reported in the present study, whereas the value for Hg was higher (Cordier et al., 2021). These findings suggest that HepG2 cells are more sensitive to As than HT-29 cells, as a lower concentration of As is required to induce 50% toxicity in liver cells compared to the HT-29 cells, consistent with the liver being a primary target organ for As toxicity, and therefore more sensitive. Otherwise, HepG2 cells appear to be less sensitive to Hg than the HT-29 cells used in the present study.

Table 2 presents the concentrations of As and Hg in the bioaccessible fractions after *in vitro* digestion of each seafood sample, with the EC_{50} values for these compounds obtained using HT-29 cells as a model. Regarding As, the EC_{50} at 72 h was lower than the concentrations detected in bioaccessible fractions obtained after the digestion of fresh Atlantic cod and bivalves. These findings suggest that the digested fractions of these two seafood items, i.e., the bioaccessible amounts of As, could be associated with cytotoxic effectsin the analysed cells, indicating a potential risk of local intestinal toxicity (Stevens et al., 2008).

In contrast, the results obtained for Hg in the bioaccessible fractions of seafood samples were significantly lower than the EC_{50} at 24, 48, or 72 h of HT-29 cell exposure, indicating that the bioaccessible fractions of these seafood items will not pose cytotoxicity at the intestinal level.

3.4. *Significance for the risk assessment*

The findings of this study are relevant to the overall risk assessment. Bioaccessibility data represent the maximum potential dose that can induce toxic effects. The lower Hg bioaccessibility levels suggest a negligible risk (Table 2). As well as the Hg concentrations in the bioaccessible fraction were significantly below the EC_{50} threshold, suggesting an insignificant risk (Table 2). It should be noted that variability in dietary habits or cooking methods could be considered important sources of uncertainty that should be considered in the health risk assessment context.

The As species in fish does not constitute an risk, once the major species in this group is AsB that is non toxic species (EFSA, 2024).

According to EFSA, Hg in fish, including seafood, is essential to provide data on risk assessment (EFSA, 2012).

4. Conclusions

The present study was conducted under representative conditions and in accordance with the recommendations issued by international organisations. Integrating compositional data with bioaccessibility measurements provides a more robust and realistic estimation of dietary exposure. This study assessed the concentrations and bioaccessibility of As and Hg in cooked seafood samples, as well as their cytotoxic potential effects on HT-29 cells.

The concentrations of As in the analysed samples were consistent with results previously reported in the scientific literature. The levels of Hg detected were below the limits established in the current legislation and are not considered to pose a health risk to the Portuguese population.

The findings demonstrated that both the concentrations in the bioaccessible fractions and the overall bioaccessibility of As were significantly higher than those of Hg. The concentrations of As in the bioaccessible fraction of most analysed samples were comparable to the EC_{50} values observed after 72 h of exposure in HT-29 cells. The bioaccessible fractions of Hg were significantly lower than the EC_{50} values at 24, 48, or 72 h of HT-29 cell exposure, indicating that the Hg concentrations in these fractions are not expected to cause cytotoxicity in the intestinal epithelium. The bioaccessible fraction is a relevant dose metric for linking dietary exposure to *in vitro* toxicological responses.

AsB is recognised as the predominant form of arsenic in fish and most seafood and is generally regarded as posing negligible toxicological concern. The MeHg is the principal mercury species in marine fish. Nevertheless, the mechanisms governing the accumulation of AsB and MeHg in marine fish remain insufficiently understood and warrant further investigation, which will constitute one of the objectives of future research.

Overall, the results presented provide valuable contributions to assessing the associated human health risks from dietary exposure to these contaminants.

CRediT authorship contribution statement

Marta Ventura: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Ricardo Assunção:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Beatriz Matos:** Methodology. **Inês Coelho:** Writing – review & editing, Methodology. **Inês Delgado:** Writing – review & editing, Methodology. **Sandra Gueifão:** Methodology. **Susana Santiago:** Writing – review & editing, Writing – original draft, Methodology. **Isabel Castanheira:** Writing – review & editing, Validation, Supervision, Investigation. **Marta Martins:** Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2026.109229.

Data availability

Data will be made available on request.

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