Cadmium, lead, and mercury in two commercial squid species from the north Adriatic Sea (central Mediterranean): contamination levels and health risk assessment

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Abstract

In this study, lead (Pb), cadmium (Cd), and total mercury (Hg) concentrations in European squids (*Loligo vulgaris*) and flying squids (*Todarodes sagittatus*) from the northern Adriatic Sea (Italy) were analyzed. The risk of the Italian population being exposed to potentially hazardous metal concentrations through the consumption of these products was also assessed. Compared to European squids, flying squids showed three times higher total Hg concentrations and one hundred times higher Cd concentrations to the point that more than 6 and 25% of the samples exceeded the maximum Hg and Cd limits established by the current legislation. From the evaluation of dietary exposure levels, it emerged that the consumption of flying squids was associated with the highest Pb intake by children and, consequently, with the lower margin of exposure values in relation to the risk of neurotoxicity (margin of exposure=33). Consumption of flying squids, especially by children, was also associated with higher intakes of Cd, inorganic, and methyl-Hg, which, respectively, accounted for 156, 113, and 23% of the tolerable weekly intakes established for these contaminants at European level. The obtained results raise concern and it may be necessary to provide specific dietary advice on the moderate dietary consumption of some cephalopod species, especially to the youngest and most vulnerable segment of the population. However, besides the highly conservative deterministic method adopted in this study, a refined consumer exposure assessment should be performed through the probabilistic methodology, which is more suitable to represent the real exposure scenario.

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Introduction

Squids have significant economic and nutritional value for nations bordering the Mediterranean Sea. The dietary consumption of fresh squids is particularly appreciated in Italy, which is one of the leading EU economies in this production sector. With a volume of 460 tons captured in 2020, European squids (*Loligo vulgaris*) and flying squids (*Todarodes sagittatus*) are the main squid species caught in Italian waters, especially in the Adriatic Sea which, alone, accounts for 30% of the total national production (FAO, 2020). Squids are of optimal nutritional value for human consumption but, on the other hand, they may transfer chemical contaminants to humans, such as heavy metals (Sangiuliano *et al.*, 2017). Indeed, compared to other fishery products, cephalopods have been reported to bioaccumulate and bioconcentrate metals into their tissues at higher levels (Penicaud *et al.*, 2017). Contamination of cephalopods tissues depends on taxonomy, life cycle, feeding habits, and trophic status, and it is highly influenced by both natural and anthropogenic factors affecting the characteristics of the marine environment, *i.e.*, the geological characteristics of the area and the leakage of debris and pollution from the coast (Bustamante *et al.*, 2006; Raimundo *et al.*, 2014).

Due to the toxicity even at low amounts and the long persistence in the environment, cadmium (Cd), lead (Pb), and mercury (Hg) are of particular concern from a public health perspective (Zrelli *et al.*, 2021). Non-cancer effects such as neurological, endocrine, immunological, cardiovascular, and nephrological dysfunctions have been reported following chronic dietary exposure

(EFSA, 2009, 2010, 2012). Simultaneously, evidence for mutagenicity and carcinogenicity in humans has been also clearly provided (EFSA, 2009, 2010, 2012). To limit the exposure of the population to these metals, maximum limits in several food categories have been established by the European Union and toxicological reference values for safe intake were defined by the European Food Safety Authority (EFSA) (European Commission, 2006; EFSA 2009, 2010, 2012).

Data concerning concentrations of metals in cephalopods originating from the Adriatic Sea were mainly focused on the analysis of products fished in the southern and central Adriatic Sea, among which different species of octopus (Storelli and Marcotrigiano, 2004; Storelli *et al.*, 2006; Storelli, 2008), cuttlefish (Storelli *et al.*, 2006), and squids, including European squids (Storelli *et al.*, 2006; Storelli, 2008, 2009). To the best of our knowledge, only two studies dealt with the quantification of toxic metals in samples of flying squid originating from the central and the southern Adriatic Sea (Perugini *et al.*, 2009; Miedico *et al.*, 2015). On the contrary, there is no data in the literature on the quantification of metals in different squid species from the northern Adriatic Sea, which could favor the accumulation of metals in fisheries due to the low water depth, limited water exchange, greater abundance of fish, and consequent higher trophic transfer rates. These factors, along with the traditional high consumption of both European and flying squid by Italian consumers, may expose the local population to hazardous amounts of metals and, therefore, to long-term toxic effects.

The present work has three main goals: i) to investigate the occurrence of Cd, Hg, and Pb in the edible muscle tissues of European and flying squids caught in the northern Adriatic Sea; ii) to compare the concentration levels of metals within the two squid species; iii) to evaluate the exposure of local squid consumers to these toxic metals and verify whether risk for public health exists.

Materials and Methods

Sample collection, preparation, and processing

A set of 50 European squids (*Loligo vulgaris,* Cephalopoda: Loliginidae, 13-15 cm dorsal mantle length) and a set of 50 flying squids (*Todarodes sagittatus*, Cephalopoda: Ommastrephidae, 18-22 cm dorsal mantle length), each from three different batches, were fished by trawl nets during the autumn-winter season in the Italian northern Adriatic Sea (central Mediterranean, FAO fishing area 37.2.1). During the collection period, the samples were transported fresh on ice to our laboratories, where they were immediately stored at -21±2°C. At the time of analysis, samples were thawed overnight at refrigeration temperature (4±2°C) and then rinsed with deionized water. For each specimen, the skin, gladius, head, arms, tentacles, and viscera were separated without damage, while the whole mantle and fins were manually ground and homogenized. A sub-portion of each ground sample (~30 g) was stored in borosilicate glass vials at -80°C for 24 hours and destined to lyophilization (-55°C, 0.001–0.002 mbar pressure) for 24 hours (LyoQuest -55 Plus freeze-dryer, Telstar Co., Terrassa, Barcelona, Spain). Lyophilized samples were then powdered and stored in plastic bags.

Moisture analysis

A sub-portion of approximately 5 g of homogenized fresh (non-lyophilized) squid samples was destined for moisture content determination, following a slightly modified AOAC 950.46 B method (AOAC, 1990). Minced samples were air-dried in an oven at 85°C for about 18 hours and the water content was calculated as the percentage difference between wet and dry weights. Average moisture contents were 84.1±4.1% in European squid samples and 77.0±4.4% in flying squid samples.

Determination of total mercury concentrations

Single-purpose atomic absorption spectroscopy (AMA-254 Advanced Mercury Analyzer, Altec Ltd., Prague, Czech Republic) was used to directly measure total Hg content in lyophilized samples (~50 mg, 3 replicates), as previously reported (Varrà *et al.*, 2021). Briefly, the analysis consisted of i) a sample drying step (60 seconds at 120°C); ii) a sample decomposition step (150 seconds at 750°C); iii) a Hg release step (45 seconds at 900°C); iv) a Hg reading step (60 seconds at 253.6 nm). Method detection limits (MLOD) was 0.2 μg/kg.

Determination of lead and cadmium concentrations

Inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7900 Agilent Technologies, Inc., Santa Clara, CA, USA) was used to measure both Cd and Pb contents, as previously reported (Varrà *et al.*, 2021). Before ICP-MS analysis, samples were fully mineralized by treatment with 1 mL of 30% H_2O_2 (Fluka Chemie AG, Buchs, Switzerland) and 4 mL of 16% HNO_3 (Lach-Ner, Neratovice, Czech Republic) in a microwave oven (Speedwave MWS3$^+$, Berghof, Eningen, Germany). Digested samples were finally diluted to 25 mL with ultrapure water.

The operating conditions of the Agilent 7900 ICP-MS as well as the validation of the sample preparation method, instrument calibration, and validation were performed by following the methodology previously prosed by Varrà *et al.* (2021). Calibration curves at 5 concentration levels (1, 5, 10, 50, 100 μg/L) and with coefficients of determination (r^2)>0.998 were used for the quantification of the analytes.

Six certified reference materials were used to evaluate the performances of the analytical method: BCR® 184 Bovine Muscle and BCR® 185 Bovine Liver (IRMM, Geel, Belgium); CRM 12-02-01 Bovine Liver and CRM 12-2-03 Lucerne (pb-anal, Kosice, Slovakia); NIST SRM 1577 Bovine Liver and NIST SRM 1566 Oyster Tissue (NIST, Gaithersburg, MD, USA). Recovery values were in the range of 91-101% and 96-109% for Cd and Pb, respectively. Intraday and interday precision values for both elements were below 10%. MLODs were 0.24 and 0.70 μg/kg for Cd and Pb respectively.

Data elaboration and statistics

Before data elaboration, metal concentrations measured on dry samples were transformed into wet concentrations (μg/kg wet weight) based on the average moisture contents previously measured (*moisture analysis* section). Since Hg speciation into organic Hg (methylmercury, *Me*Hg) and inorganic Hg (*i*Hg) was not directly performed, these were mathematically calculated starting from total Hg concentrations measured in samples. Conservative conversion factors for mollusks adopted by EFSA were used for this purpose, according to which *Me*Hg and *i*Hg were presumed to be 80 and 50% of total Hg concentrations (EFSA, 2012). The equality of variance and the normal distribution of data were evaluated by performing Levene's and Shapiro–Wilk's tests, respectively. Due to the non-normal distribution and heteroscedasticity of most of the data, the Mann-Whitney U test was used to compare ranks between the two squid species. Potential correlations in accumulation rates between pairs of metals in European or in flying squid tissues were assessed by performing a Spearman correlation analysis (positive and negative bivariate correlation were defined at $r \geq 0.6$ and $r \leq 0.6$). Statistically significant differences for all tests were identified at $P \leq 0.05$. The statistical software OriginPro 2021 (v. 9.8.0.200, OriginLab Corporation, Northampton, MA, USA) was employed to perform analyses and plot the results.

Dietary exposure assessment

The dietary exposure to metals of different age groups of the Italian population was calculated by considering both median (P50) and 95th percentile (P95) chronic consumption data of squids. Data on food consumption of children, adolescents, adults, the elderly, and the very elderly which are listed in the EFSA Comprehensive European Food Consumption Database were employed (EFSA, 2022a).

For Pb, the daily intakes by the 5 age groups were hence calculated by multiplying Pb concentrations by the P50 or P95 mean daily consumption data. For Cd and Hg, the weekly intakes were calculated in the same way and the final outcome was multiplied by 7. The necessity of calculating the daily intake for Pb and the weekly intakes for Cd and Hg was driven by the different approaches used to characterize the risk associated with their dietary exposure, as suggested by EFSA (EFSA, 2005). Indeed, the risk related to Cd and Hg (which exert threshold toxic effects) was evaluated by the percentage ratio of the estimated weekly intakes (EWIs) to the relative tolerable weekly intakes (TWIs). The risk related to Pb (which exerts non-threshold genotoxic and cancerogenic effects), was instead evaluated using the margin of exposure (MOE) approach, *i.e.*, by calculating the ratio of the daily benchmark dose lower limits (BMDLs) of Pb to the relative estimated daily intakes (EDIs). Usually, MOEs<10,000 are considered of health concern when the BMDLs are derived from animal studies (due to the inclusion of safety factors deriving from uncertainty). Since in the case of Pb BMDLs have been calculated directly from human studies, EFSA concluded that, for this substance, MOEs<1 (indicating that the intake of a toxic substance is higher than the dose responsible for adverse health effects) should be considered of health concern (EFSA, 2005, 2010).

The following TWIs were used: Cd (2.5 µg/kg bw/week), *Me*Hg (2.5 µg/kg bw/week), and *i*Hg (4 µg/kg bw/week). The following BMDLs were used for Pb: $BMDL_{01}$ (0.5 µg/kg bw/day) in relation to the 1% increased risk of developmental neurotoxicity; $BMDL_{01}$ (1.5 µg/kg bw/day) in relation to the 1% increased risk of cardiovascular effects; $BMDL_{10}$ (0.63 µg/kg bw/day) in relation to 10% increased risk of nephrotoxicity (EFSA, 2022b).

Results

Concentrations of heavy metals in squids

Concentrations of Cd, Hg (*Me*Hg, *i*Hg), and Pb (all on a wet weight basis) of the 50 European squids and the 50 flying squids are illustrated in Figure 1, where mean, median, standard deviation, and interquartile range (25 and 75% percentiles) values are reported. Among the tested metals, Cd showed the maximum variation in terms of median concentrations between the two squid species, being 0.0050 mg/kg in European squids and 0.33 mg/kg in flying squids. Hence, median concentrations were approximately 100 times lower in European squids than in flying squids ($P \leq 0.05$).

Total Hg median concentrations were 0.069 mg/kg and 0.31 mg/kg in European and flying squid, respectively. Starting from these amounts, a three-time higher degree of contamination of flying squid with *Me*Hg and *i*Hg ($P \leq 0.05$) was extrapolated: concentrations of *Me*Hg were in the 0.015-0.072 range, while those of *i*Hg in the 0.014-0.65 mg/kg range (Figure 1). As for Pb, it reached the highest concentration level of 0.032 mg/kg in one flying squid sample, but the median concentrations in this species (0.011 mg/kg) were not found to be statistically different from concentrations measured in European squids (0.014 mg/kg, P>0.05).

When performing correlation analyses between pairs of metals (Cd-Hg, Cd-Pb, Pb-Hg), no significant positive or negative patterns of covariation in both squid species were detected (r<0.6 and P>0.05) and, therefore, no specific coaccumulation trends were identified among the investigated metals.

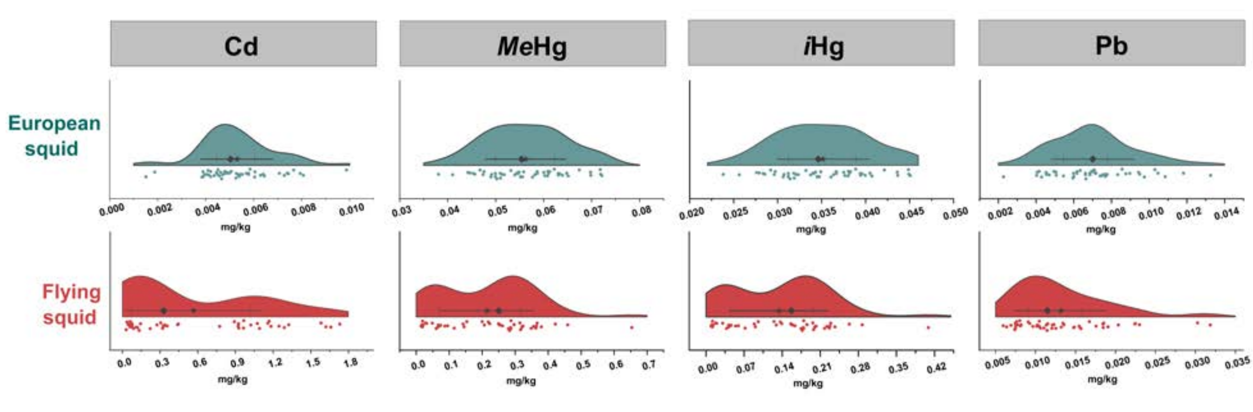

Figure 1. Raincloud plot showing data point concentrations (rain) and distribution (cloud) of cadmium, methylmercury, inorganic mercury, and lead (mg/kg wet weight) in European squids (N=50, blue) and flying squids (N=50, red). Within the cloud: stars, mean values; diamonds, median values; endcaps of horizontal line whiskers, standard deviations; vertical lines intersecting horizontal line whiskers, the lower 25% and the upper 75% quartiles.

Compliance of squids with European limits for heavy metals

By comparing the contamination levels with the maximum limits established at the European level for cephalopods (European Commission, 2006), all the samples of both squid species were largely below the maximum limits of Pb of 0.3 mg/kg. In flying squids, the maximum limit of total Hg of 0.5 mg/kg was exceeded by 3 out of 50 samples, resulting in 6% of non-conformity. On the contrary, the higher contamination levels of Cd found in flying squids resulted in more than 25% of the samples analyzed exceeding the maximum limit of 1 mg/kg (*i.e.*, 13 out of 50 specimens being non-compliant with legislation and unsafe for human consumption). From a deeper analysis of data, the exceedance of the maximum limits of Cd by flying squids was found to be randomly distributed within the whole dataset. Indeed, a potential correlation with the characteristics of individual samples or sample batches was not identified.

Dietary intakes of heavy metals via squid consumption and health risk

The EWIs of Cd and Hg and the EDI of Pb by the different Italian population age classes are summarized in Table 1. These data were calculated from chronic consumption rates of squids by Italian children, adolescents, adults, elderly, and very elderly, which were, respectively, 0.98, 0.65, 0.45, 0.47, and 0.37 g/kg bw/day for P50 consumers and 3.62, 2.02, 1.51, 1.58, and 0.72 g/kg bw/day for P95 consumers (EFSA, 2022a).

The critical concentrations of Cd found in flying squids coupled with the higher consumption rates have meant that the younger population was highly exposed to this contaminant through the consumption of this fishery product (Table 1). Indeed, an intake of 3.9 µg/kg bw/week was calculated for P50 children, which rose up to 14 µg/kg bw/week in the case of P95 children. On the contrary, the very elderly population was the least exposed to all the tested metals, mainly because of physiological difficulties in consuming cephalopods related to advancing age. In particular,

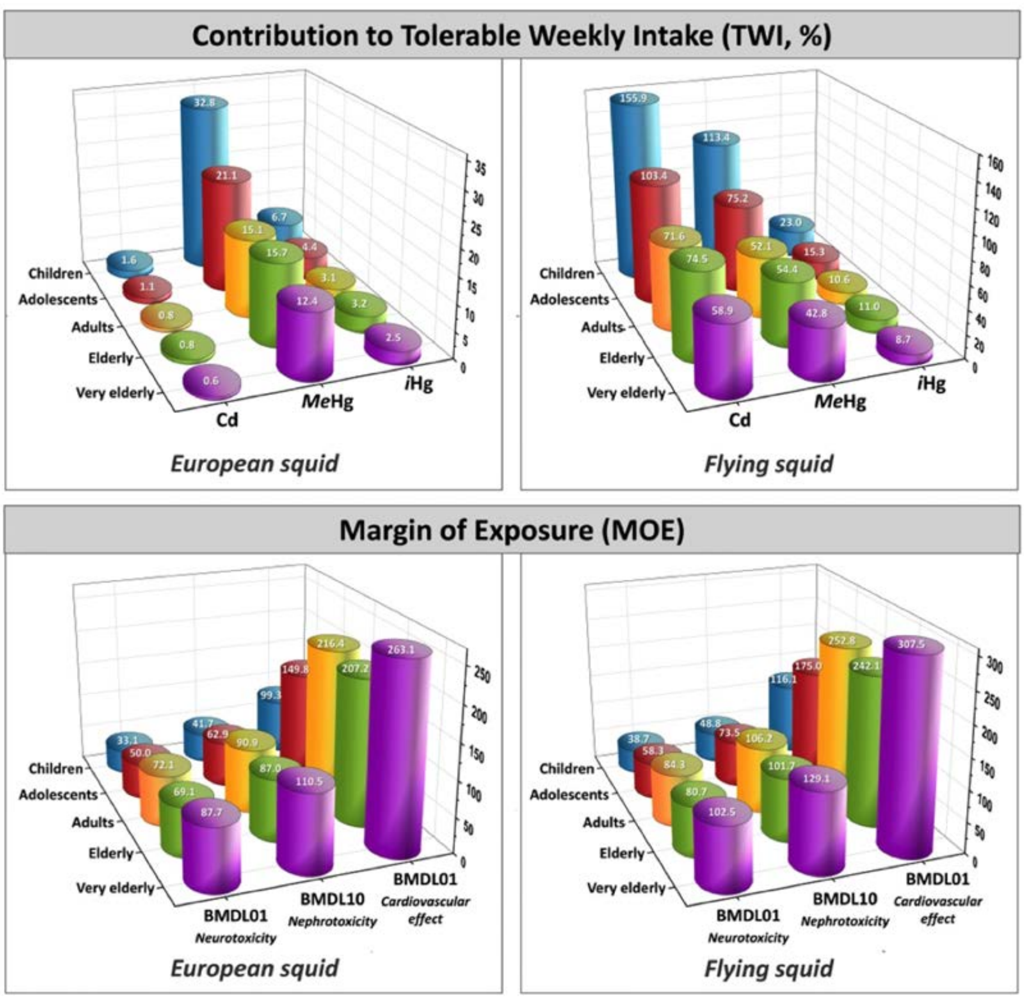

Figure 2. Contribution percentage of European and flying squid consumption (median percentile consumers) to tolerable weekly intakes of cadmium, methylmercury, and inorganic mercury (upper plots) and margin of exposure values to lead in relation to neurotoxic, nephrotoxic, and cardiovascular effects (lower plots).

intakes of Pb by the P50 population were 0.0057 µg/kg bw/day when considering European squid consumption and 0.0049 µg/kg bw/day when considering flying squid consumption (Table 1).

Globally, the EWIs of Cd, *Me*Hg, and *i*Hg when consuming European squids (P50 estimates) were all far below the TWIs recommended by EFSA in all the population groups (Figure 2). Similarly, no exceedance of the TWI of these metals was observed in P50 adults, elderly, and very elderly consuming flying squids, although the contributions of this product to the Cd, *Me*Hg, and *i*Hg TWIs were 5 times higher than those of European squids (Figure 2). On the contrary, the EWIs of Cd and *Me*Hg through flying squid consumption by children accounted respectively for 156 and 113% of the established TWIs, suggesting a potential public health concern (Figure 2).

The risk characterization related to the presence of Pb did not reveal a particular risk for health in any population group. The resultant MOEs were all >1, despite lower (and most worrying) levels of 33 and 39 related to neurotoxic effects in P50 children consuming European and flying squids, respectively (Figure 1). Although MOEs to Pb referring to P95 consumers were also >1, they were significantly lower than those related to P50 consumers. Values in the 11-53, 13-66, and 31-158 ranges related to neurotoxic, nephrotoxic, and cardiovascular side effects, respectively, were calculated for all the P95 population age classes (*data not shown*).

Discussion

Because of the ability of cephalopods to accumulate significant amounts of contaminants in their tissues, the determination of toxic metals in different cephalopod species is extremely important from a sanitary point of view. Generally, fish and seafood from the Mediterranean Sea were ascribed as more contaminated with *Me*Hg due to the high temperature of the water and the low lighting of the seabed of this area, which favor methylation of *i*Hg (Bustamante *et al.*, 2006). In this study, concentrations of total Hg (which are directly proportional to those of *Me*Hg) were found to be largely compliant with legal limits and highly comparable with those reported by other authors who analyzed the same cephalopod species originating from different geographical areas (Storelli *et al.*, 2006; Storelli, 2008; Lourenço *et al.*, 2009; Perugini *et al.*, 2009; Barone *et al.*, 2015; Ariano *et al.*, 2019). The exposure scenario of children consuming flying squids in relation to *Me*Hg is of particular concern since its intake account for 113% of the TWI. EFSA reported a mean *Me*Hg intake of up to 1.45 and 7.48 µg/kg bw/week in P50 and P95 European children, respectively (EFSA, 2012). These exposure values were similar to those calculated in this study, but it should be remarked that our estimates refer to the sole consumption of cephalopods, while those reported by EFSA refer to the consumption of a wider fish and seafood food category.

Compared to many other metals, it was demonstrated that 60-90% of the total Hg amount in cephalopods is preferentially stored in the muscular tissue rather than the digestive glands, due to its higher affinity to specific sulfhydryl groups of protein rather than to lipids of the digestive gland (Bustamante *et al.*, 2006). This could suggest that evisceration may only partially represent a valid strategy to increase the safety of consumption of cephalopods in relation to contamination with Hg. Moreover, as found by Storelli *et al*. (2010), pelagic cephalopods (such as squids) tend to be contaminated with Hg to a lesser extent compared to benthic cephalopods (such as cuttlefish and octopuses). These results were different from Bustamante's research results, which attributed to squids a higher degree of contamination with Hg due to their pelagic diet (constituted by fish and other cephalopod preys), which, naturally, contains more Hg than the benthic diet (mainly

Table 1. Estimated weekly intakes for cadmium (Cd), methylmercury (*Me*Hg), and inorganic mercury (*i*Hg) and estimated daily intakes of lead (Pb) through the consumption of European and flying squids by mean (P50) and high (P95) children, adolescents, adults, elderly, and very elderly consumers. Data are expressed as µg/kg bw/week (Cd, *Me*Hg, iHg) µg/kg bw/day (Pb).

		European squid		Flying squid	
		P50	P95	P50	P95
Population group	Metal				
Children	Cd	0.041	0.15	3.9	14
	*Me*Hg	0.43	1.58	1.47	5.4
	*i*Hg	0.27	0.98	0.92	3.4
	Pb	0.015	0.056	0.013	0.048
Adolescents	Cd	0.027	0.085	2.6	8.0
	*Me*Hg	0.28	0.88	0.98	1.9
	*i*Hg	0.18	0.55	0.61	3.0
	Pb	0.010	0.031	0.0086	0.027
Adults	Cd	0.019	0.063	1.79	6.0
	*Me*Hg	0.20	0.66	0.68	2.3
	*i*Hg	0.12	0.41	0.42	1.4
	Pb	0.0069	0.023	0.0059	0.020
Elderly	Cd	0.020	0.066	1.87	6.3
	*Me*Hg	0.20	0.69	0.71	2.4
	*i*Hg	0.13	0.43	0.44	1.5
	Pb	0.0072	0.024	0.0062	0.021
Very elderly	Cd	0.015	0.030	1.47	2.9
	*Me*Hg	0.16	0.31	0.56	1.1
	*i*Hg	0.10	0.20	0.35	0.68
	Pb	0.0057	0.011	0.0049	0.0095

P50, median percentile; P95, 95th percentile; Cd, cadmium; *Me*Hg, methylmercury; *i*Hg, inorganic mercury; Pb, lead.

constituted by crustacean, mollusk, echinoderm, and polychaete preys) (Bustamante *et al.*, 2006).

As for Pb, the degree of contamination observed in all the samples closely matched concentration levels reported in different surveys performed across Europe, being Pb globally present at so low concentrations that it was unlikely to be considered a food safety concern (Storelli, 2008; Yusá *et al.*, 2008; Lourenço *et al.*, 2009; Sangiuliano *et al.*, 2017; Zrelli *et al.*, 2021). Consequently, no severe issues emerged from the dietary exposure assessment to Pb, since MOEs were all >1. Nevertheless, some considerations can be made about the MOEs of children in relation to the risk of neurotoxicity. Considering that infants and children are the most vulnerable category to neurodevelopmental disorders induced by Pb exposure and that the dietary sources of Pb are many and varied (EFSA, 2010), MOEs<1 can be likely achieved by the Italian younger population throughout the whole diet.

The most distinctive feature that become evident from this study was the significantly higher bioaccumulation of Cd in flying squids compared to European squids. The amount of Cd found was in the same order of magnitude as that reported by Piras *et al.* (2013), but, contrary to our results, these authors did find equal concentrations also in muscular tissues of *Loligo vulgaris* samples. Conversely, higher concentrations (up to 8.5 mg/kg) were measured in *Todarodes Sagittatus* than in *Loligo vulgaris* samples by Miedico *et al.* (2015). With equal prey type, ontogenetic stage, and environmental conditions, variations of Cd concentrations in the muscular tissues of different cephalopods were supposed to be related to species-specific digestive physiologies (Penicaud *et al.*, 2017). As a matter of fact, the digestive gland of Loliginidae differs from that of Ommastrephidae since it lacks specific typical yellow-brown vacuoles (heterolysosomes and heterophagosomes), also known as boules (Bustamante *et al.*, 2002; Rodrigo and Costa, 2017). These structures participate in enzymatic intracellular digestion and are involved in the storage, detoxification, and mobilization of Cd and other metallic contaminants; therefore, their absence in European squids may be associated with a reduced accumulation tendency of Cd and, hence, justify the lower Cd concentrations compared to flying squids (Bustamante *et al.*, 2002). Given that the concentration of Cd in flying squids reported in this study alone contributed to 156 and 103% of the Cd TWI in P50 children and adolescents and considering that cereals, vegetables, nuts, and meat contribute more than fish and seafood to Cd exposure (EFSA, 2009), it is clear that the chronic consumption of this food products may be, in the long-term, problematic for children health.

In summary, these results raise new questions on the safety of certain cephalopod species from local fisheries which are traditionally demanded by inhabitants of the place and call for the need to adopt proper risk management actions to reduce the exposure of the population, and, especially, of the youngest segment.

Conclusions

The results of this study show that European and flying squids from the Italian northern Adriatic Sea can be considered safe for consumption in relation to their contamination with Pb. However, significantly higher concentrations of Cd and Hg found in flying squids, led to estimate dietary exposure levels above the established toxicological reference values in case of chronic consumption by children and adolescents.

Since the contamination levels of flying squids are likely to be attributed to species-specific biological characteristics rather than marine pollution problems, the establishment of maximum permitted limits differentiated by species (and not unique for all cephalopod species) could help adequately protect consumers. In addition, specific dietary an advice concerning restriction on overall ration intakes of cephalopods or the preferential choice of species that are naturally less contaminated, to be addressed to the most vulnerable groups of the population, would represent a valid strategy to minimize the potential health risks associated with metallic contaminants. Finally, it is worth mentioning that the estimates provided by the deterministic approach, traditionally adopted for dietary exposure assessments to chemicals, should be refined through the more recent and less consolidated probabilistic methods that, taking into account the range of variation in consumption, residues, and other relevant parameters rather than using point estimates as in deter as well as allowing quantification of uncertainties affecting the assessment, provide scenarios closer to reality.

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