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Inorganic contaminants in fish of the Mediterranean Sea: biomonitoring and toxicologic analysis

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Abstract

Aquatic systems throughout the world are increasingly under a wide array of anthropogenic stressors, including the release of a myriad of toxic substances into the environment. The presence of twenty-seven heavy metals, vitamins, and minerals was investigated in muscle tissues collected from 46 wild fishes distributed among *Thunnus thynnus* (TT), *Pagellus bogaraveo*, and *Dentex dentex* (DD) from the Mediterranean Sea. The mean concentration of each element was calculated and compared to the provisional tolerable daily or weekly intake (PTWI) or to the established tolerable upper intake when possible. Specie-specific distribution was documented for the following chemical elements: aluminum (Al), potassium (K), cobalt (Co), molybdenum (Mo), silver (Ag), cadmium (Cd), tin (Sn), and thorium (Th). For the elements without regulated maximum dietary limits, iron (Fe) was highest in TT, while Th was significantly predominant in DD. Several metals were found, often simultaneously, in quantities above the acceptable levels. Specifically, the median concentrations of mercury (Hg) and Cd in the pooled species were significantly higher than their relative PTWI. Significant differences among species were reported for selenium (Se), inorganic arsenic (As), nickel (Ni), and zinc (Zn). Other elements [Al, chromium (Cr), copper (Cu), Sn, lead (Pb), and manganese (Mn)] were found to be at or below the corresponding acceptable levels. The maximum safe consumption calculated for mercury (Hg) leads to advising a limited recommended weekly intake for all the tested Mediterranean Sea fish species.

Introduction

Fish meat is considered essential in a balanced diet, being an important source of proteins and lipids of high biological value, as fish accumulate minerals originating from their diet in skeletal tissues and other organs (Carvalho *et al.*, 2005). Although nutrient composition data for fish is widely available, specific information on the presence and concentration of potentially harmful chemical elements in fish meat is confined to selected minerals, and trace metals concentrations are available for a few seafood species only (Carvalho *et al.*, 2005). The oceanic burden of both essential and non-essential trace elements discharged to coastal ecosystems has been of serious environmental and health concerns (Cohen *et al.*, 2001; Raimundo *et al.*, 2011). The concept of environmental status takes into account the structure and functioning of the marine ecosystems together with natural physiographic, geographic, and climatic factors, as well as physical and chemical conditions, including those resulting from human activities in the area (Borj *et al.*, 2011; Korpinen *et al.*, 2021). In recent years, the Water Framework Directive and the Marine Strategy Framework Directive (Commission of the European Communities, 2008) have been implemented to improve the quality status of transitional and coastal waters (Borj *et al.*, 2011; Raimundo *et al.*, 2011). Several field studies and laboratory experiments have evidenced that accumulation of metals in tissues is mainly dependent upon water conditions such as salinity, pH, hardness, and temperature (Canli *et al.*, 2003; Ansari *et al.*, 2004; Álvarez *et al.*, 2009). Furthermore, anatomical and physiological aspects such as the sizes of marine animals have been shown to play an important role in metal contents of tissues (Canli *et al.*, 2003). In order to ensure the protection of human health, the maximum authorized concentrations of "heavy metals" in waters and fishery products have been set by the European legislation and commission regulation (Commission of the European Communities, 2006, 2008; European Commission, 2021). Metals can be categorized as biologically essential and non-essential. The non-essential metals (*i.e.*, Al, Cd, Hg, Sn, and Pb) do not bear recognized biological functions, and their toxicity rises with increasing concentrations. On the other hand, essential metals [*i.e.*, Cu, Zn, Cr, Ni, Co, Mo, Fe, Se, vanadium (V), and Mn] have a known biological role, and toxicity occurs either for metabolic deficiencies or at high concentrations (Kennedy, 2011). The most bioavailable form of metals resulting in toxicity is represented by the dissolved ionic form. The function of multiple physiologic drainage/filter systems of fishes can be affected by metals (*e.g.*, gills, liver, and gastrointestinal tract); toxicity depends on metal form and speciation, bioavailability, toxicokinetics, and toxicodynamics (Kennedy, 2011; Chen and Liao, 2012)

The research that is the subject of this article was generated from a chapter of a PhD thesis concerning aquatic pollution and biological monitoring of the marine environment (Santagostino, 2016).

The aim of this study was to evaluate the role of selected pelagic (*Thunnus thynnus*) and benthopelagic (*Dentex dentex* and *Pagellus bogaraveo*) fish species as ecological biomonitors of Mediterranean Sea pollution by quantifying 27 inorganic elements in skeletal muscle. The selection of pelagic and benthopelagic species allowed the assessment of distinct ecological niches, which are differently influenced by sediment–water contaminant exchanges and trophic transfer mechanisms (Suedel *et al*., 1994; Hilgendag *et al*., 2022).

Furthermore, the toxicological relevance of the detected contaminants was assessed through comparison with established tolerable dietary intake limits, providing a risk-oriented interpretation for human consumers.

Material and Methods

Case selection and collection

Atlantic bluefin tunas (*i.e.*, *Thunnus thynnus*, n=20), blackspot seabreams (*i.e.*, *Pagellus bogaraveo*, n=13), and common dentex (*i.e.*, *Dentex dentex,* n=13), were legally captured from the FAO catch areas 37.1.1 (Western Mediterranean Sea along the coastal regions of Spain and the eastern end of France) with a commercial license. All fish included in this study were homogeneous in size (considered as a measure of age), grossly inspected, individually labeled, and weighed.

Analytical chemistry analysis

The presence and level of 27 metallic elements were investigated in dorsal skeletal muscle (50-70 g) from all fish samples. Based on the last electron subshell in the atom to be occupied, metallic elements were divided into four broad categories: s-block [sodium (Na), magnesium (Mg), K, calcium (Ca), strontium (Sr), barium (Ba)], p- block [Al, As, antimony (Sb), Se, Sn, thallium (Tl), Pb], d-block transition (V, Cr, Mo, Mn, Zn, Co, Cd, Hg, Ag, Ni, Cu, Fe), and f-block (U, Th), as previously reported by Duffus (2002). This scheme is based on a consideration of general reactivity, and provides the basis for a rational consideration of the chemical and biological behavior of metallic elements and their compounds. All samples were individually placed in plastic bags, freeze-dried for 24 hours, and then ground into a fine powder using a porcelain mortar and pestle. Powdered samples were diluted with concentrated HNO3 (Suprapur HNO3 65%, Merck, Darmstadt, Germany), placed in a graphite heating block (DigiPREP digestion system, SCP Science), and digested at 75°C for 12 hours. Digested samples were diluted 1:10 with double-deionized water (ddH2O) and then further diluted 1:5 with a 2 % HNO3. Double-deionized water was used as a blank and included in each batch to determine contamination during sample preparation. All solutions were prepared with analytical reagent grade chemicals and Milli-Q® Ultrapure Water Solutions Type 1 (Millipore S.A., St Quentin en Yvelines, France). Working standards were prepared in 6% (v/v) nitric acid solution without further purification. Internal standard solutions (100 mg L-1) of bismuth (Bi), germanium (Ge), indium (In), lithium (Li), lutetium (Lu), rhodium (Rh), scandium (Sc), terbium (Tb) were purchased from Agilent Technologies (Waldbronn, Germany), and were diluted to a 1 mg L-1. A multi-element standard stock solution (10 µg mL-1) composed of 26 elements was also purchased from Agilent Technologies. This stock solution was used throughout for the preparation of calibration standards. Hg was added from its 10 µg mL-1 stock solution because of its incompatibility with other elements. The measurement of individual peaks of standard materials with their retention times and peak heights was determined prior to the analysis of the samples of interest. Precision and accuracy of analysis were also ensured through repeated analysis of samples against certified reference materials for all metals. In the method validation, the certified reference material IAEA-407 fish homogenate was purchased from the International Atomic Energy Agency, Analytical Quality Control Service (Vienna, Austria), and was used as provided without further grinding. Muscular samples were analyzed with inductively coupled plasma mass spectrometry (ICP/MS, Agilent 7700X ICP-MS (Agilent Technologies, Waldbronn, Germany) equipped with a CETAC ASX 500 Model 510 autosampler (CETAC, Omaha, NE), as previously reported [16, 17, 18]. The Agilent 7700 ICP-MS was equipped with the following: concentric quartz nebulizer with quartz spray chamber and connecting pipe (Agilent Technologies, Waldbronn, Germany); 0.25 mm ID pump tubing for internal standards; 1.02 mm ID pump tubing for sample uptake; 1.52 mm ID pump tubing for draining; and nickel-tipped sampling and skimmer cones. The sample solutions were pumped by a peristaltic pump from tubes arranged on the autosampler, and aspirated into the argon plasma. For better operating conditions, the instrument was operated with standard 1,550 W radio frequency (RF) power and 14.9 L/min argon plasma gas flow. Carrier gas (helium) was optimized at 1.00 L/min. Electrostatic lens voltage was set in the range of 190-195 V. Typical optimized nebulizer gas and auxiliary gas flows were 0.9 L/min helium. Results were expressed in µg/g on a wet weight basis, as required by EU Directive (Commission of the European Communities, 2006). The limit of quantification was 0.005 µg/g. Levels of Hg, Al, Cr, Mn, Ni, Cu, Zn, As, Se, Cd, Sn, Sb, Pb, uranium (U) were compared to the corresponding provisional tolerable weekly intake (PTWI) or tolerable daily intake established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) (EFSA, 2009; WHO, 1989, 2022).

Statistical analysis

To evaluate the spread of environmental metallic elements in fish, the association between the presence of contamination in the species was assessed for each metallic element through logistic regression models. For each model, the presence/absence of metallic element was considered the response variable, with the presence defined as any concentration of metal higher than the limit of quantification (0.005 µg/g), and the absence otherwise. Fish species were included as categorical covariates in regression models, codified through dummy variables. Model results were reported as estimated proportions of contaminated fish for each species. The association was evaluated through tests of hypothesis with a two-step procedure. In the first step, the null hypothesis of no overall difference among the species was assessed using the Likelihood Ratio test. In each case, when the null hypothesis was rejected ($p<0.05$), comparisons between each couple of fish species (*Dentex vs. Pagellus*; *Dentex vs. Thunnus*; and *Pagellus vs. Thunnus*) were performed. Since the proportions of contaminated fish were often equal or near to 100% (or 0% in some cases), we used the Fisher exact test, which is adequate in such cases. The no difference between species was considered the null hypothesis. The p-values were corrected for test multiplicity by Bonferroni's rule. The distribution of the concentration of metallic elements was compared among fish species. The following criteria were adopted: i) only those fish in which the metal was present (defined above) were considered; ii) species with few contaminated fish (fewer than 5) were excluded. Due to the relatively low dimension of the sample and the non-symmetric (non-Gaussian) distribution of metal concentrations, robust methods of analysis were needed. Therefore, we compared median concentrations among fish species through quantile regression models (Cade and Noon, 2003; Koenker, 2005). For each metallic element, the model included metal concentration as response variable and fish species as covariate. Results were reported as median concentrations for each species and compared using the two-step approach previously described. The variance-covariance matrix of estimates was calculated by the xy bootstrap method, and then used for performing Wald tests. The median concentrations were further compared with the acceptable levels defined by JECFA (WHO, 2022), where available. For each metallic element, when the test of no overall difference of median concentrations was rejected in the analysis described above, a test of hypothesis comparing the median concentration with the acceptable level of the metal was performed for each species. Otherwise, fish species were pooled, and the overall median concentration was compared with the acceptable level. In each case, the null hypothesis was that the median concentration was equal to the acceptable level.

Results

Caseload selection and groups

A total of 46 wild fish from the Mediterranean Sea were collected in the winter season. The medium weight of the three fish categories was 37.96 kg (range 31-45kg) for Atlantic bluefin tunas, 1.23 kg (range 0.8-2.5 kg) for blackspot seabreams, and 1.89 kg (range 0.9-3.2) for common dentex. The medium length of blackspot seabreams was 21.71 cm (range 18-29 cm), 43.30 cm (range 38.6-50 cm) for common dentex, and 133.15 cm (range 118-141 cm) for Atlantic bluefin tunas. Based on length and weight, all fish were considered sexually mature. For all fish, samples were collected from skeletal muscles.

Heavy metals and minerals

The estimated proportions of contaminated fish for each substance are reported in Table 1. For several elements, the proportions were equal to 100%; in such cases, the p-values were not reported, as they were equal to one. A different spread in distinct species was documented for the following chemical elements: Al, K, Co, Mo, Ag, Cd, Sn, and Th. Major differences were found between *Pagellus* on one side and *Thunnus* and *Dentex* on the other side. The blackspot seabreams (*Pagellus*) were characterized by a higher frequency of Al and Sn (100.0% for both metals; significantly different with respect to *Thunnus* and *Dentex*), whereas tunas and dentexshowed a higher frequency of Cd and Th. The frequencies of Cd and Th in the latter species were significantly different with respect to the *Pagellus*, although not significantly different from one another. Ag was predominant in tunas (85.0%, significantly different from seabreams and dentex). Mo had a higher frequency in tunas. Other differences were found for Co and K, with a higher frequency in seabreams and tunas than in dentex. No significant differences were found for Pb. The mean concentrations of heavy metals and minerals (μg/g) in the dorsal skeletal muscle of the 3 analyzed species are listed in Table 2. The mean levels of U, Tl, Sb in all fish tested, Ag in blackspot seabreams, and Co and Sn in common dentex were below or close to the limit of quantification (0.005 μg/g). For this purpose, the concentrations of Sb, Tl, and U in all fish tested, Ag in blackspot seabreams and Co and Sn in common dentex were excluded. For the elements without maximum dietary limits, the concentration of Fe (37.634 μg/g) was highest in tunas, with a significant difference compared to the other species. Na (1348.823 μg/g), Sr (1.718 μg/g) and Ba (0.046 μg/g) were significantly higher in seabreams. Also, higher median concentrations of Mg, Ca, and V were evidenced in seabreams, although a significant difference at a level of 0.05 μg/g did not emerge when compared to the other species. In dentex, Th (0.093 μg/g) was significantly predominant. Although Mo (0.031 μg/g) was also higher in dentex, no statistically significant differences were noted. Several metals were found in quantities above the acceptable levels as defined by JECFA (WHO, 2022) (Table 3). Specifically, no significant differences among fish species were found for Hg and Cd. However, the median concentrations of Hg (0.780 μg/g) and Cd (0.04 μg/g) in the pooled species were significantly higher than their respective PTWI. Significant differences among species were reported for Se, inorganic As, Ni, and Zn. Furthermore, their median concentrations were significantly higher than the tolerable weekly intakes. Specifically, Se inorganic As and were excessively present in all species (Se: 0.682 μg/g, 0.821 μg/g, and 3.196 μg/g for *Dentex*, *Pagellus* and *Thunnus*, respectively; inorganic As: 17.25 μg/g, 4.085 μg/g, 2.371 μg/g for *Dentex*, *Pagellus* and *Thunnus*, respectively). An excessive amount of Ni was noted for *Pagellus* (0.058 μg/g) and *Thunnus* (0.043 μg/g), whereas Zn (11.464 μg/g) was increased above tolerable levels only in tunas. Other elements (Al, Cr, Cu, Sn, Pb, and Mn) were found to be at or below the corresponding acceptable levels.

Discussion

Research on fish contamination and human exposure is often framed in terms of a single contaminant. Given the need to define an appropriate baseline for cumulative exposure to environmental contaminants, this approach might not be the most appropriate. In the current study, benthopelagic and pelagic species from the Mediterranean Sea were examined as possible biomonitors of distinct environmental pollution niches. In this view, the integration between ecological biomonitoring and toxicological interpretation based on provisional tolerable dietary intakes represents a complementary approach to evaluate the potential health implications linked to fish consumption and environmental exposure.

The choice of different fish species is derived from the knowledge that biological and ecological factors have a significant influence on metal bioaccumulation, bioavailability, and transference (Hosseini *et al.*, 2013). Bioavailability is influenced by a complex variety of interrelated factors, such as antagonism or synergism of toxic compounds, water temperature, pH, salinity, and dissolved oxygen, and the sensitivity of the organisms depending on age and reproductive status (Ansari *et al.*, 2004). Several studies have also indicated that the concentration of trace minerals in fish muscle may be influenced not only by external factors (food source, environment) but also by anatomical and physiological aspects (Álvarez *et al.*, 2009). Some unexpected findings were represented by the high simultaneous levels of multiple metallic elements in the same group. Unlike routine analytical surveys, the present study applies a multi-element assessment across ecologically distinct fish species and interprets contamination profiles through both environmental (biomonitoring) and regulatory toxicological frameworks. This dual perspective provides contextual information on sediment-water exposure pathways and potential consumer risk, thereby strengthening the ecological and public health relevance of the findings. Despite the large body of analytical reports on selected toxic metals, comprehensive multi-element profiles integrating ecological and toxicological interpretation remain scarce for Mediterranean species.

Due to the homogeneously chosen small size and young age of the examined fish, the relatively high level of metals in skeletal muscle was considered a matter of concern. An immediate comparison with other caseloads was difficult due to the paucity of data regarding benthopelagic fishes as bioindicators of environmental pollution. Our results partially paralleled previous findings in *Thunnus thynnus* from the Mediterranean Sea (Storelli *et al.*, 2010). However, our levels of Ca, Fe, Cu, Zn, Se, Sr, Cr, Ni, Hg, Pb, and Cd were lower than previous reports (Carvalho *et al.*, 2005). Marine sediments are generally considered basins for pollutants and a possible source of contamination for aquatic species (Ansari *et al.*, 2004; Sapozhnikova *et al.*, 2004; Cousin and Cachot, 2014; Rahmanpour *et al.*, 2016; Sheikh Fakhradini *et al.*, 2021). According to the different ecology, different levels of some metallic elements were observed in the three selected groups. Specifically, elements with a defined PTWI, such as Hg, Cd, Se, inorganic As, Ni, and Zn, were determined to be higher with significant differences among species. This is in contrast with recent reports (Di Bella *et al.*, 2006). Excessive levels of Se and inorganic As were present in all species. Selenium is an essential micronutrient and cofactor for important antioxidant enzymes (Arnold *et al.*, 2014). In humans, epidemiological studies have indicated a link between neurotoxicity and acute Se exposure, although such an effect could also arise secondarily to a low-level chronic Se overexposure (Vinceti *et al.*, 2014). Great progress on selenium toxicity in the aquatic species has been made during the last two decades. In freshwater fish, prolonged exposure to high toxic levels has been linked to increased mortality, growth depression, reproduction impairment, and migration (Hamilton, 2004). Se has a narrow margin between dietary requirement and toxicity (Arnold *et al.*, 2014), and may represent a potential cause of gonadal lesions and consequent reproductive impairment in wild fish populations. Arsenic enters rivers and is transported downstream, moving from water to sediment (Williams *et al.*, 2006), and it tends to concentrate and persist in the upper seasoil, where benthopelagic fish species (such as dentex) live and feed. In this study, the median concentration of total As (organic and inorganic) was higher than the PTWI set for inorganic As by WHO (1989). Despite being present almost exclusively as an organic substance within marine organisms, arsenical compounds are not uniformly distributed throughout the tissues, and inorganic As may still represent 1-3.5% of the total metal (Borak and Hosgood, 2007; Storelli *et al.*, 2010). Inorganic arsenic, found mainly as arsenate and to a lesser extent as arsenite, represents the predominant toxic form of arsenic in seawater (Borak and Hosgood, 2007). Due to its toxicity and health risk for humans, the PTWI set for inorganic As is no longer considered appropriate (EFSA, 2009); however, no new tolerable intake levels have been established for food (EFSA, 2009). An overload of Ni was recorded for *Pagellus* and *Thunnus*, with its median concentration being significantly higher than the tolerable weekly intake. Nickel has been classified as a Group I carcinogen (IARC, 1990). In rainbow trouts, Ni toxicity has been shown to induce contractions of vascular smooth muscle (Brix *et al.*, 2004), similar to those described in our group of tunas. Zinc has been included in the list of essential elements for its probable beneficial role at low doses; however, toxicity occurs either at metabolic deficiencies or at high concentrations (Kennedy, 2011). Gills represent the main target for Zn toxicity, with consequent impairment of branchial physiology, Ca2+ uptake imbalance, hypocalcemia, death (Niyogi and Wood, 2006; Kori-Siakpere and Ubogu, 2008). In our study, Zn was increased above tolerable levels only in tunas. Furthermore, a recent study showed that bioaccumulation of zinc in fish is inversely related to the aqueous exposure (McGeer *et al.*, 2011). For Hg and Cd, our study failed to evidence significant differences among fish species from different ecosystems. However, their median concentrations in the pooled species were significantly higher than their relative PTWI. This is of great importance from the human perspective, as the main source of Hg exposure for humans is represented by the ingestion of contaminated fish, especially predators, where Hg is almost entirely present as methylmercury (MeHg). The developing human nervous system is a sensitive target for MeHg exposure (Clarkson *et al.*, 2003). Although MeHg at low levels is generally considered safe, an association with subclinical autoimmunity among reproductive-age females has been discovered (Somers *et al.*, 2015). Current research suggests that MeHg production in coastal marine sediments is one of the most important sources (Sunderland *et al.*, 2006), where methylation is largely controlled by bacterial activity and the bioavailability of inorganic Hg (Chen *et al.*, 2008). Recent studies indicate that MeHg concentrations are higher in pelagic than in benthic fauna, suggesting that chemical flux into the water column may be more important than biotransfer mechanisms (Chen *et al.*, 2008; Chen *et al.*, 2014). However, MeHg burdens in similar trophic-level fish have been reported to be higher in demersal than in pelagic species (Garcia-Hernandez *et al.*, 2007). Cadmium has been defined as a human carcinogen (Group 1) by IARC (2012); however, there is little evidence of associations between oral exposure and increased cancer rates. Cadmium remains an important determinant of cardiovascular diseases and mortality in U.S. adults (Tellez-Plaza *et al.*, 2012). In fish, Cd toxicity has been reported as a possible cause of decreased innate immunity (Ghiasi *et al.*, 2010). Our finding of several infectious organisms could possibly be attributed to increased levels of this element. For the elements without maximum dietary limits, an increased concentration of Fe, Na, Sr, Ba, and Th was documented. Although these elements are considered less important than human toxicants, their increment could still be responsible for metabolic and organic perturbations for marine organisms with detrimental osmoregulatory failure. Sodium chloride represents about 77% of the total dissolved solids in seawater, with calcium, magnesium, and carbonate comprising the largest proportion of the remaining solutes (NRC, 2005). Marine animals are constantly trying to retain water within their bodies. Given the high number of sodium-potassium ATP-ase pumps, gills are responsible for the excretion of salts in seawater and retention of water in order to osmoregulate (NRC, 2005). In our caseload, the extension and severity of the branchial lesions were more prominent in seabreams, where a Na imbalance was noted. Furthermore, the increasing Na concentration in dentexand seabreams considered herein has been significantly correlated with the increasing median value of muscular H-indices. Other elements (Al, Cr, Cu, Sn, Pb, and Mn) were found to be at or below the corresponding acceptable levels.

Conclusions

The potential health risks related to fish consumption because of their content of environmental toxic contaminants are recognized worldwide. Fish meat currently represents the main source of human intake of persistent inorganic contaminants. Although the effects of persistent inorganic contaminant exposure on humans are not completely understood, it is known that the immune system, reproductive tract, and endocrine system could be affected, along with the onset of neoplastic diseases. Moreover, despite the simultaneous finding of multiple inorganic contaminants, little is still known regarding the effect of persistent or repeated exposure to these combinations on human health. The global assessment of marine status through the use of fish from different hydrological settings as bio-indicators represents a complementary choice to evaluate the responses of marine organisms to pollutants, and to assess the maximum amount of residues without adverse effects. For these reasons, experimental and observational studies addressing combined toxic effects of high levels of multiple contaminants should be implemented in different marine ecosystems, in order to provide valuable insights in their cumulative effects upon chronic exposure.

Taken together, these findings highlight the relevance of multi-element approaches for both marine biomonitoring strategies and consumer exposure assessment.

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Table 1. Presence of metallic elements in fish. For each metallic element, the following are reported: the estimated percentage of contaminated fish (columns 2-4 for *Dentex*, *Pagellus* and *Thunnus* respectively); likelihood ratio tests to compare the percentages of contaminated fish among the three species; Fisher exact tests, for specific comparisons between fish species, corrected for test multiplicity (multiple comparisons). *Statistically significant differences.

Elements	***Dentex* (n=13)**	***Pagellus* (n=13)**	***Thunnus* (n=20)**	**Likelihood Ratio**	**Multiple comparisons**		
	%	**%**	**%**	**Chi-square p-value**	**D *vs.* P**	**D *vs.* T**	**P *vs.* T**
Hg	100.0	100.0	100.0				
Na	100.0	100.0	100.0				
Mg	100.0	100.0	100.0				
Al[a]	23.1	100.0	5.0	38.62, p<0.0001*	p=0.00032*	p=0.82698	p<0.0001*
K[a]	23.1	84.6	65.0	11.27, p=0.00358*	p=0.00405*	p=0.02408*	p=0.22788
Ca	100.0	100.0	100.0				
V	100.0	100.0	100.0				
Cr	100.0	100.0	100.0				
Mn	100.0	100.0	100.0				
Fe	100.0	100.0	100.0				
Co[a]	7.7	69.2	85.0	22.36, p<0.0001*	p=0.010082*	p<0.0001*	p>0.999
Ni	100.0	100.0	100.0				
Cu	100.0	100.0	100.0				
Zn	100.0	100.0	100.0				
As	100.0	100.0	100.0				
Se	100.0	100.0	100.0				
Sr	100.0	100.0	100.0				
Mo	53.85	69.25	100.0	14.18, p=0.00083*	p>0.999	p=0.00465*	p=0.05242
Ag	15.4	15.4	85.0	24.19, p<0.0001*	p>0.999	p=0.00048*	p=0.00048*
Cd	84.6	38.5	100.0	19.68, p<0.0001*	p=0.12422	p=0.44318	p=0.00028*
Sn	7.7	100.0	35.0	30.47, p<0.0001*	p<0.0001*	p=0.32311	p=0.00048*
Ba	100.0	100.0	100.0				
Ti	0.0	0.0	0.0				
Pb	100.0	100.0	95.0	1.69, p=0.42853			
Th	100.0	30.8	100.0	29,43, p<0.0001*	p=0.00137*	p>0.999	p<0.0001*
U	0.0	0.0	0.0				
Sb	0.0	0.0	0.0				

Table 2. Median concentrations of metallic elements. For each metallic element, the following are reported: estimated median concentrations for each fish species (columns 2-4); Wald tests for a global comparison of median concentrations among species; Wald tests for specific comparisons, corrected for test multiplicity (multiple comparisons). df = degrees of freedom. A fish species with fewer than 5 contaminated fish was excluded from the analysis. *Statistically significant differences. For Al and Ag, only one species was included; thus, only the median concentrations were reported. For K, Co, Sn, and Th, two species were included; thus, only median concentrations and Wald test were reported.

Elements	***Dentex* (n=13)**	***Pagellus* (n=13)**	***Thunnus* (n=20)**	**Wald test**	**Multiple comparisons**		
	%	**%**	**%**	**F (df) p-value**	**D vs P**	**D vs T**	**P vs T**
Hg	0.955	0.445	0.941	2.05 (2,43) p=0.14137			
Na	758.312	1348.823	841.891	5.63 (2,43) p=0.00673*	p=0.00665*	p=0.49762	p=0.02267*
Mg	280.168	336.515	284.116	1.17 (2,43) p=0.12654			
Al[a]		0.210					
K[a]		3056.123	2525.207	4.21(1,22), p=0.05222			
Ca	98.068	362.687	72.623	3.07 (2,43) p=056959			
V	0.042	0.056	0.037	2.84 (2,43) p=0.06941			
Cr	0.031	0.044	0.030	3.41 (2,43) p=0.04228*	p=0.18606	p=0.87391	p=0.06231
Mn	0.072	0.126	0.167	7.99 (2,43) p=0.00112	p=0.00450*	p=0.00435	p=0.23079
Fe	5.626	5.662	37.634	14.75 (2,43) p<0.0001	p=0.98187	p<0.0001*	p<0.0001*
Co[a]		0.007	0.011	3.93 (1,24) p=0.05907			
Ni	0.039	0.058	0.043	10.04 (2,43) p=0.00026*	p=0.00145*	p=0.44256	p=0.00113*
Cu	0.178	0.265	1.354	8.05 (2,43) p=0.00107*	p=0.18687	p=0.00310*	p=0.00264*
Zn	3.407	4.165	11.464	30.43 (2,43) p<0.0001*	p==.05219	p<0.0001*	p<0.0001*
As	17.253	4.085	2.371	16.4 (2,43) p<0.0001*	p<0.0001*	p<0.0001*	p=0.02711
Se	0.682	0.821	3.196	55.45 (2,43) p=0.0001*	p=0.02358*	p<0.0001*	p<0.0001*
Sr	0.384	1.718	0.306	5.69 (2,43) p=0.00643*	p=0.06495	p=0.76411	p=0.01662*
Mo	0.031	0.012	0.020	0.76 (2,33) p=0.47397			
Ag[a]			0.014				
Cd	0.030	0.011	0.079	1.16, (2.33) p=0.32666			
Sn[a]		0.038	0.008	1.22 (1,18) p=0.28384			
Ba	0.022	0.046	0.024	3.52 (2,43) p=0.03835*	p=0.03224*	p=0.62987	p=0.03224*
Pb	0.007	0.014	0.008	4.36 (2,42) p=0.01904*	p=0.03094*	p=019648	p=0.04222*
Th[a]	0.093		0.059	16.67, (1,31) p=0.00029*			

Table 3. Acceptable concentration levels. For each metallic element, the following data are reported: acceptable concentration level (PTWI), Wald tests for the comparison between the median concentrations and the acceptable level, corrected for test multiplicity, except in the following cases: [a]since no significant difference between species emerged in previous analysis (see: Table 2), the comparison was performed for pooled species, [b]for Al, only one specie was included in the analysis. *Statistically significant differences.

Elements	**PTWI**	**Wald test**
Hg[a]	0.0016	pooled: t=5.48 p<0.0001*
Al[b]	2	*Pagellus*: t=-10.15 p<0.0001*
Cr	1.4	*Dentex*: t=-194.08 p<0.0001* *Pagellus*: t=-229.99 p<0.0001* *Thunnus*: t=-755.14 p<0.0001*
Mn	1.17	*Dentex*: t=-63.68 p<0.0001* *Pagellus*: t=-82.88 p<0.0001* *Thunnus*: t=-44.32 p<0.0001*
Ni	0.035	*Dentex*: t=-0.86 p<0.999* Pagellus: t=8.94 p<0.0001* *Thunnus*: t=2.89 p=0.01801*
Cu	3.5	*Dentex*: t=-68.44 p<0.0001* *Pagellus*: t=-69.46 p<0.0001* *Thunnus*: t=-6.38 p<0.0001*
Zn	7	*Dentex*: t=-10.21 p<0.0001* *Pagellus*: t=-13.07 p<0.0001* *Thunnus*: t= 5.36 p<0.0001*
As	0.015	*Dentex*: t= 5.33 p<0.0001* *Pagellus*: t= 5.93 p<0.0001* *Thunnus*: t= 7.10 p<0.0001*
Se	0.007	*Dentex*: t=18.16 p<0.0001* *Pagellus*: t=19.11 p<0.0001* *Thunnus*: t=13.09 p<0.0001*
Cd[a]	0.007	pooled: 2.72 p=0.01015*
Sn[a]	14	pooled: t=-1180.24 p<0.0001*
Pb	0.025	*Dentex*: t=-26.45 p<0.0001* *Pagellus*: t=-3.82 p<0.0001* *Thunnus*: t=-27.24 p<0.0001*

PTWI, provisional tolerable weekly intake.