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Trace metals in six fish by-products of two farmed fishes, the gilthead sea bream (*Sparus aurata*) and the meager (*Argyrosomus regius*): Interactions with the environment and feed

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ABSTRACT

For the large producers of farmed fish, the utilization of fish by-products as ingredients of food is important and thus their characterization, in terms of chemical composition and safety, becomes necessary. In this study, eleven trace metal concentrations in by-products (head, gills, guts, trimmings, bones and skin) of two Mediterranean farmed fishes, the gilthead sea bream (*Sparus aurata*) and the meager (*Argyrosomus regius*) were determined by ICP-MS. According to overall concentrations, the sequence of trace metal levels in decreasing order was Fe > Zn > Mn > Cu > As > Cr > Pb > Ni > Cd > V>Co. Higher median concentrations ($\mu g\ g^{-1}$ d.w.) of As were determined in bones and guts (2.16 and 2.04), Cd, Co, Cu, Pb in guts (0.175, 0.058, 16.3, 0.381), Cr in skin and bones (1.51 and 1.45), Ni in guts and skin (0.231, 0,218), Fe and Mn in gills (50.1, 16.8) and V in trimmings (0.052). Trace metal levels determined in fish feed were considerably lower compared to EC limits (Directive 2002/32/EC). The highest bioconcentration factor (BCF) values were calculated for Cd, Ni, Pb in muscle, for Fe, Mn, Zn in gills, for Cd, Co, Cu, Zn in guts and for Cr in bones. Exploitation of fish by-products as additives in food industry appears feasible, based on the concentrations of metals examined.

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Aquaculture; farmed fishes; fish by-products; trace metals; accumulation

Introduction

Fish and fish products constitute a great source of, among others, quality proteins, omega-3 fatty acids, carbohydrates and liposoluble vitamins (Alasalvar *et al.* 2002), important for balanced nutrition and optimal health (Oken *et al.* 2012). It has been stated that fish consumption is related with the prevention of cardiovascular diseases (Maehre *et al.* 2015), hypertension (Morris *et al.* 1993), iron deficiency (Navas-Carretero *et al.* 2008), various types of cancer (Fernandez *et al.* 1999; Wu *et al.* 2015), depression (Li *et al.* 2016) and Alzheimer's disease (Kröger and Laforce 2016).

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However, fish may simultaneously contain toxic substances including trace metals, PAHs and PCBs. Since fish remain at a relatively high level within the aquatic food chain, there is a greater risk for trace metal bioaccumulation particularly in larger fishes (Zhang and Wang 2007; Banerjee *et al.* 2015; Kumari and Maiti 2019). Trace metals may enter the fish chain through the aquatic environment and/or feeding, in case of farmed fish (Streit 1998; Eneji *et al.* 2011; Pal and Maiti 2018), with the mineral content of fish being also affected by culinary treatment (Kalogeropoulos *et al.* 2012; Costa *et al.* 2013). The concern on the trace metal content of our diet and its adverse effect on human health (Jarup 2003; Zheng *et al.* 2013; Afonso *et al.* 2015) are hence extended to all fish food products retailed in the market, particularly those promoted according to the dietary advice and suggested to be consumed 2–3 times a week (Willett *et al.* 1995; FAO 2010; USDA 2015; D'Alessandro *et al.* 2019).

The rapidly increasing demand for fish subject to human consumption is leading to the gradual exploitation of several wild stocks (Christensen *et al.* 2014). Under this perspective, aquaculture is expected to provide effective solutions for the constantly increasing global population (Duarte *et al.* 2009), being one of the fastest growing food sectors in the world (FAO 2014). Toward this direction, Greece is a pioneer in marine aquaculture, with a significant expansion observed after 1985, to which a series of factors have contributed, such as the prevailing geomorphological and hydrobiological conditions and its climate. In the production of Mediterranean fish species Greece is classified second in the production of gilthead sea bream (*S. aurata*) and of the European sea bass (*Dicentrarchus labrax*), corresponding respectively to 29 and 31% of the global production, with approximately 80% of the production being exported (Chavelas 2016; FAO 2018).

In the aquaculture sector approximately 10,000 persons are employed, together with 10,000 more occupied in processing, services etc. The specific sector corresponds to only 0.2% of the domestic product, whereas its exports correspond to 3% of total national exports and 12% of exports of the primary sector (data from Hellenic Statistical Authority). In 2016 316 fish farms and 29 nurseries were operating throughout the Greek territory and the total annual production of Mediterranean fishes exceed 100,000 tons (Hellenic Aquaculture 2018). The principal farming method comprises floating cages and to a much smaller extent land-based farming installations.

The positive health effects related to fish, in combination with the need for sustainable food production, have led to a growing interest on the exploitation of fish by-products according to the principles of circular economy (Olsen *et al.* 2014). For a large-scale farmed fish producer, such as Greece, the exploitation of fish by-products is of particular significance. In a previous study, fish by-products have been studied in terms of their peptides and fatty acids content, as a potential source of bioactive compounds (Kim and Mendis 2006). Thus, their utilization as ingredients of food subject to human consumption supports the sustainability of aquaculture and of human diet in general. However, it is important that these by-products are fully characterized, in terms of their chemical composition and safety. The nutritive content of 6 fish by-products was evaluated in a recent study (Kandyliari *et al.* 2020), while in the present one their safe potential exploitation is examined.

Several data have been reported so far pertaining with the trace metal content of farmed fish (Carvalho *et al.* 2005; Erkan and Özden 2007; Costa *et al.* 2013; Kalantzi *et al.* 2013; Iamiceli *et al.* 2015; Kalantzi *et al.* 2016; Marengo *et al.* 2018; Milenkovic

et al. 2019; Pal and Maiti 2019; Renieri *et al.* 2019). However, a lack of bibliographic data characterizes trace metal concentrations in different fish by-products, information which would be necessary in order to ensure their safety. The objective of the present study was the determination of 11 trace metals in the muscle and in the 6 different by-products (bones, gills, guts, head, skin, trimmings) from two species, the gilthead sea bream and the meager (*A. regius*). The gilthead sea bream is one of the two most widely farmed marine species in the Mediterranean (Alasalvar *et al.* 2002) while meager represents an emerging species for the industry. Processing of these fish species generates great amounts of by-products. This study fills the existing research gap on trace metal content of these fish by-products, in order to ensure the safety of their reuse. In parallel, the study aims to evaluate a) the relation between fish size and metal concentration, b) the impact of the environment and feed to trace metals concentrations in the fish by-products, c) the respective bioconcentration factors (BCF) and d) the relation of study results with previously published literature and legislation limits.

Materials and methods

Fish by-products sampling and preparation

Samples were obtained from the pilot scale cage farm of the Hellenic Center for Marine Research (HCMR), located within the Gulf of Souda, Crete island, Greece (Figure 1). The farm is certified as an aquaculture facility from the national veterinary authority (code GR94FISH0001) and is also registered as breeding and experimental facility for marine fish with approval codes EL91-BIObr-03 and EL91-BIOexp-04, respectively. Several Mediterranean species have been reared in the farm such E. sea bass, gilthead sea bream as well as emerging species such as meager and greater amberjack (*S. dumerili*). Rearing is performed in polyethylene cages (rectangular and/or circular) and standard rearing methodologies are applied.

Fish samples were collected from groups reared under normal conditions and were euthanized, following harvesting, with the use of ice water. Sampling was performed by personnel accredited by the Federation of European Laboratory Animal Science Associations (FELASA) on the "Care and use of laboratory animals".

Two different fish species of two different age/size classes were collected in June 2017. Specifically, 60 individuals of gilthead sea bream (n = 60; 16 aged 1^+, of mean body weight 403.47 ± 72.92 g, mean length 21.4 ± 1.5 cm and 44 aged 0^+, of mean body weight 160.16 ± 30.79 g, mean length 28.3 ± 1.7 g) and 36 individuals of meager (n = 36; 6 aged 1^+, of mean body weight 1256.45 ± 232.32 g, mean length 53.8 ± 3.9 cm and 30, aged 0^+, of mean body weight of 235.76 ± 38.45 g, mean length 26.2 ± 1.8 cm) were used in the study. The size class (small and large) was defined by the fish age, which for; small fishes were assumed to have a body weight lower than 300 g for meager and 200 g for gilthead sea bream. The mean age of the big sea breams was 22 months and 10 months for the small ones, while for the meager was 23 and 10 months for the big and small ones respectively.

Fish feed in the form of pellets was provided with automatic feeders to the different groups. Feed distributed in different amounts, ranged between 0.7 and 1.2% of fish body weight depending on the age of the fish. Three feed types, noted as F1, F2 and F3 were used for the feeding of gilthead sea bream and meager. F1, having a pellet size of 4.5 μm,

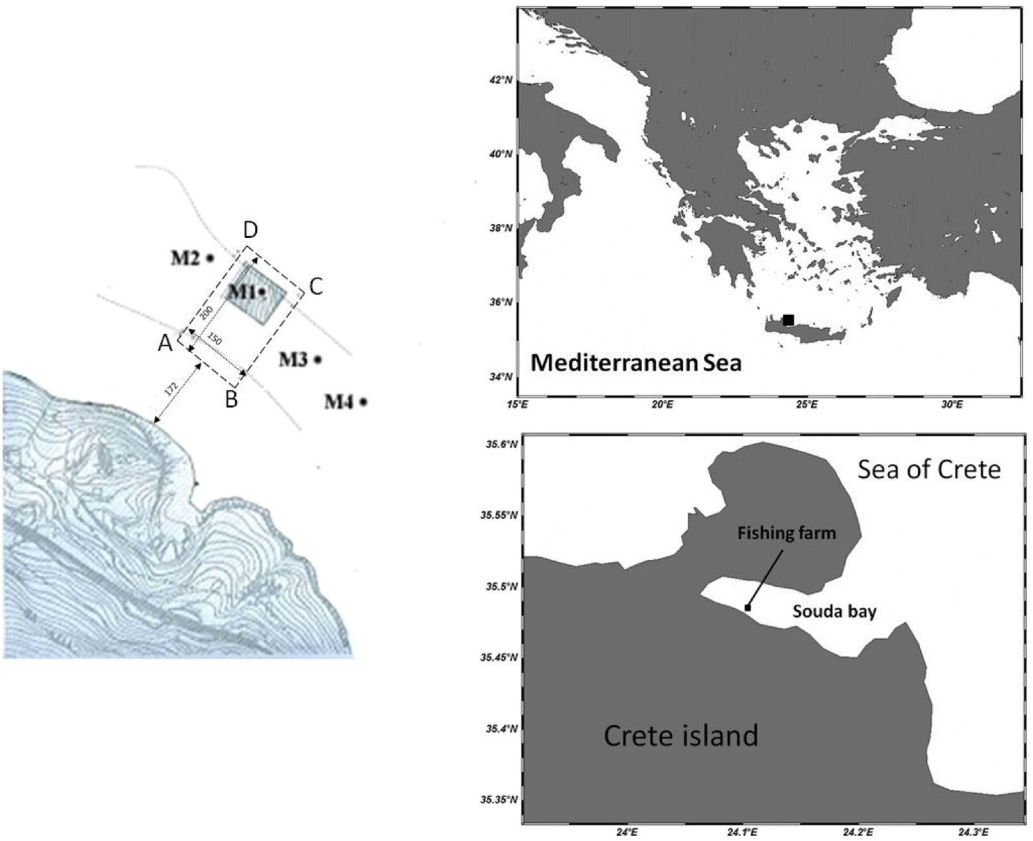

Figure 1. Sampling sites. Points A, B, C, D define the aquaculture location.

was used to feed small fishes between 100 and 300 g, F2, having a pellet size of 6.0 μm, for fishes with size between 300 and 800 g and F3, having a pellet size of 8.0 μm, for fishes with sizes bigger than 800 g. The pellets composed of fishmeal, maize gluten, fish oil, sunflower flour and soy flour with a nutrient composition of 43.0% crude protein, 17.0% crude fat, 2.7% crude fiber, 8.2% ash, 8.5% moisture, 1.15% total P, 1.5% total Ca, Vit. A 10.000 IU, Vit. D3 2.000 IU, Vit. E 400 mg, Vit. C 500 mg, choline 750 mg.

Each fish group was sampled from different cages, on the same day. After collection, samples were rinsed with deionized water and cut, in order to obtain six different by-products, namely trimmings, skin, guts, bones, head and gills, which were kept separately in tightly packaged polypropylene bags at −80 °C. Pooling of by-products samples together with their lyophilization and subsequent homogenization are described in detail in Kandyliari *et al.* (2020). In brief, more than 72 pooled samples of fish by-products were obtained (2 fish species, 2 sizes of each species, 6 fish by-products, 3 individual pool replicates).

Trace metals determination in fish samples and feeds

All materials that came into contact with the samples were previously washed thoroughly, soaked in dilute HNO_3 (Merck, Darmstadt, Germany) and rinsed with ultrapure

Table 1. Certified and determined values ($\mu g\ g^{-1}$) of trace elements concentrations in reference materials.

Trace metal	DORM-4 (fish protein)			IAEA-436 (fish flesh)		
	Certified value	Determined value	% Recovery	Certified value	Determined value	% Recovery
As	6.87 ± 0.44	6.89 ± 0.56	100	1.98 ± 0.17	2.00 ± 0.15	101
Cd	0.299 ± 0.018	0.298 ± 0.038	100	0.052 ± 0.007	0.054 ± 0.006	104
Co				0.042 ± 0.006	0.044 ± 0.004	105
Cr	1.87 ± 0.18	1.82 ± 0.84	97	0.194 ± 0.058	0.204 ± 0.016	105
Cu	15.7 ± 0.5	15.6 ± 0.5	99	1.73 ± 0.19	1.76 ± 0.09	102
Fe	343 ± 20	346 ± 29	101	89.3 ± 4.2	86.5 ± 4.4	97
Mn	3.17 ± 0.26	3.30 ± 0.25	104	0.238 ± 0.042	0.244 ± 0.036	102
Ni	1.34 ± 0.14	1.31 ± 0.21	98	0.069 ± 0.041	0.071 ± 0.051	103
Pb	0.404 ± 0.062	0.417 ± 0.070	103	0.068 ± 0.057	0.064 ± 0.009	94
V	1.57 ± 0.14	1.51 ± 0.13	96	0.014 ± 0.020	0.014 ± 0.005	100
Zn	51.6 ± 2.8	49.0 ± 2.6	95	19.0 ± 1.3	19.5 ± 1.2	103

water of 18.2 MΩ cm (Millipore, Bedford, MA, USA). For the preparation of all required solutions, class A volumetric glassware was used.

For the determination of As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V and Zn samples were digested with HNO_3 65% supra pure (Merck) with subsequent addition of H_2O_2 30% (Merck), according to the procedures described in de Macêdo *et al.* (2015), Islam *et al.* (2015) and Milanov *et al.* (2016).

The determination of trace metals in the digested samples was performed by inductively coupled plasma mass spectrometry (ICP-MS), employing a Thermo Scientific ICAP Qc (Waltham, MA, USA) instrument. Measurements were carried out in a single collision cell mode, with kinetic energy determination (KED) using pure He. Matrix induced signal suppressions and instrumental drift were corrected by internal standardization (^{45}Sc, ^{103}Rh).

The limits of detection (LODs) were calculated (in $ng\ g^{-1}$ referring to dry weight) according to USEPA (1997) equal to 75.0 for Fe, 25.0 for Zn and below 10.0 for all the other metals examined. For the statistical treatment of the data, values lower than the LOD were assigned the value of LOD divided by 2.

The verification of the accuracy and precision of the method was provided by the analysis of the certified reference materials (CRM) IAEA-436 tuna fish flesh homogenized (IAEA Reference Materials) and DORM-4 fish protein for trace metals (National Research Council Canada). The recoveries of the trace metals measured were in the range 94 to 105% (Table 1). Recovery tests were also performed, through the analysis of metal-spiked samples, with the recovery efficiency for spiking sample analysis being ±15% for all elements. The precision was calculated ranging from 2.9% to 18%.

Seawater sampling

Seawater samples were collected in PP containers soaked for 1 week in 10% HNO_3 supra pure (Merck, Darmstadt, Germany), rinsed with ultrapure water of 18.2 MΩ cm (Millipore, Bedford, MA, USA) and double bagged.

Seawater samples were collected twice, during October 2017 and May 2018 at the sea area of the cage facilities (Figure 1). A total of 16 seawater samples (2 L each) were collected during both samplings, from four different locations and at two different depths.

A set of samples was obtained from each location, 0.5 m above the sea bottom (B samples) and 0.5 m below the sea surface (S samples). Sampling points are located: i) within the aquaculture unit (65 m sea depth; samples $M1_B$ and $M1_S$), ii) at a distance of 65 m west of the unit (55 m sea depth; samples $M2_B$ and $M2_S$), iii) at 125 m east of the unit (77 m sea depth; samples $M3_B$ and $M3_S$) and iv) at 250 m east of the unit (86 m sea depth; samples $M4_B$ and $M4_S$).

Trace metals determination in seawater

Dissolved trace metals

The concentrations of total dissolved trace metals were determined using the rapid solvent extraction technique described by Danielsson *et al.* (1982). The procedure was carried out inside a clean room in order to minimize the risk of metal contamination. Trace metals determination in the preconcentrated samples was performed by inductively coupled plasma mass spectrometry (ICP-MS) using a Thermo Scientific ICAP Qc instrument (Waltham, MA USA). Measurements were carried out in a single collision cell mode, with kinetic energy discrimination (KED) using pure He. The limits of detection (LOD) of trace metals in sea water (ng L^{-1}) were equal to 0.60 for As, 0.36 for Cd, 0.24 for Co, 1.2 for Cr, 4.8 for Cu, 36 for Fe, 2.4 for Mn, 4.8 for Ni, 0.60 for Pb and V and 12 for Zn. Quality control measures included the use of the certified reference material (CRM) "NASS-6, Seawater reference material for trace metals" (National Research Council Canada), which was analyzed for the evaluation of measurement accuracy. Obtained recoveries were calculated in the range ±20%.

Particulate trace metals

Total trace metals concentrations in suspended particulate matter (SPM) were determined following filtration of 2 L of seawater through previously weighed 0.45 μm filters of nitric cellulose (Millipore, Bedford, MA, USA), further digestion of the filters with the addition of HNO_3 65% supra pure (Merck, Darmstadt, Germany) and dilution to a final volume of 25 mL. Trace metals concentrations in the digested solutions obtained were determined using ICP-MS as described above.

Statistical analysis

For the statistical analysis SPSS package, version 24 (SPSS Inc, Chicago, IL, USA) was used. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess the normality of the data. Both tests provided p values less than 0.05, and the null hypothesis (that the data are normally distributed) was rejected. Therefore, the Mann-Whitney U and Kruskall-Wallis non-parametric tests were used to statistically compare values between two groups and among more than two groups, respectively. The tests were two-sided, and p values less than 0.05 were considered statistically significant. Principal component analysis (PCA) was used in order to obtain an overview of the potential relationships existing between metals in the fish by-products studied.

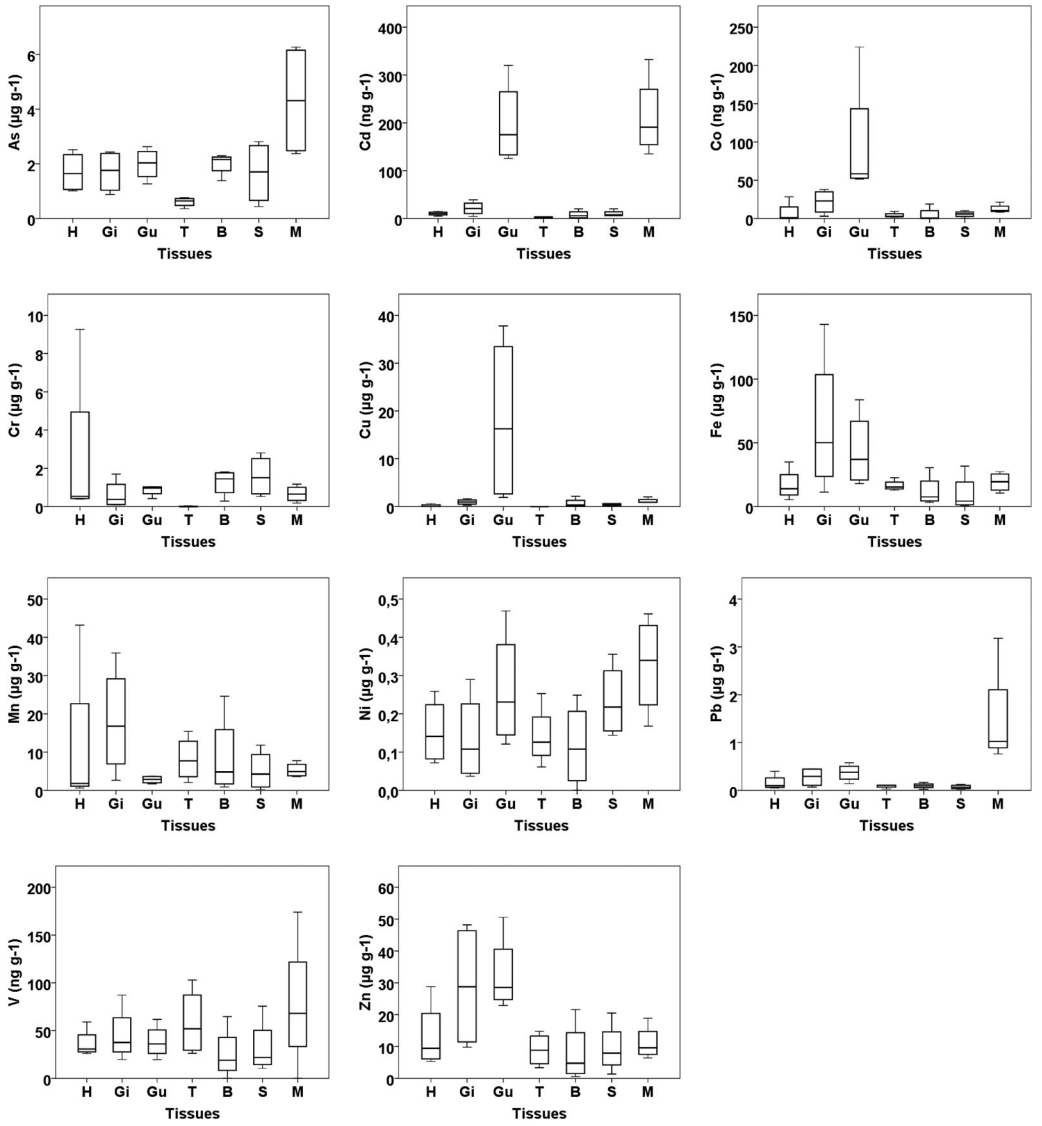

Figure 2. Boxplots of trace metal concentrations of fish by-products and muscle samples (H: Head; Gi: Gills; Gu: Guts; T: Trimmings; B: Bones; S: Skin; M: Muscle).

Results and discussion

Metals in fish samples

The concentrations of the 11 trace metals examined in farmed fish muscles (M) and by-products (bones/B, Gills/Gi, Guts/Gu, head/H, skin/S, trimmings/T) examined are summarized in Figure 2. According to the overall mean d.w. (dry weight) concentrations, the sequence of trace metal levels in decreasing order was Fe > Zn > Mn > Cu > As > Cr > Pb > Ni > Cd > V > Co, indicating that essential trace metals such as Fe, Zn, Mn and Cu are characterized by comparatively higher levels, as shown also by Aydin-Önen and Öztürk (2017). From the trace metal distribution among the

different fish tissues, guts were found to accumulate As, Cd, Co, Cr, Cu, Fe, Ni, Pb, Zn, gills Cd, Co, Fe, Mn, Zn, bones As, Cr, skin Cr, Ni and trimmings V (Figure 2).

Median concentrations of As in fish samples studied were found to follow the decreasing order M > B, Gu > Gi, S, H > T; for Cd M, Gu ≫ Gi > H, S, B, T; for Co: Gu > Gi ≫ M > S > T, H > B; for Cr S, B > Gu > M, H > Gi > T; for Cu Gu ≫ Gi, M > S, B > H > T; for Fe Gi, Gu > M, T, H > B, S; for Mn Gi ≫ T, M, B, S > Gu, H; for Ni M > Gu, S > H, T, Gi, B; for Pb M ≫ Gu, Gi > T, H, B, S; for V M, T > Gi, Gu, H > S, B and for Zn Gi, Gu ≫ M, H, T, S > B (Figure 2). Both principal element uptake pathways and tissues functioning as elements' targets are regulated by the concentration of the contaminant as well as by the age, size, physiology, habitat, feeding and growth rate of the organism (Alam *et al.* 2002). Absorption and adsorption through active and passive exchanges between fish and water constitute the main mechanisms of metal uptake by fish gills (Dural *et al.* 2007). Although the muscle demonstrates a relatively low metabolic activity (Matasin *et al.* 2011; Tapia *et al.* 2012), it might accumulate metals due to a long-lasting exposure and metal transport from other tissues, despite the fact that this consideration mainly refers to large fish species with a life span of several years (Alhashemi *et al.* 2012). Similarly, accumulated metals in guts, in which intestines are included, might contain digested and/or undigested material related to food borne contaminants (Kalantzi *et al.* 2016). In accordance, since relatively high median concentrations for many metals were determined in guts and gills, it seems that both fish feeding and metal concentrations in seawater constitute significant metal sources in fishes. Cr appears to differentiate compared to the rest of the metals examined, with its higher concentrations being measured in bones and skin. Bone is considered as a major storage site (Kelly *et al.* 2008). Outridge and Scheuhammer (1993) reported that bone tissue often accumulates higher concentrations than other tissues in animals chronically exposed to Cr. Similarly, relatively elevated As concentrations were determined in bones. Tokar *et al.* (2015) report that As may be deposited in bones, mostly in cases of chronic exposure.

In order to evaluate any data trends and gain an overview of the relationships among the trace metals examined, principal component analysis (PCA) was performed individually for the fish by-products studied. Quite interesting results were obtained for gills, for which two principal components (PCs) with eigenvalues > 1 are extracted (PC1 explained up to 53.4% of the total variance and PC2 up to 28.5%). According to the component values of metals obtained for gills, Mn, Co, Pb and Ni in PC1 and Cd and Zn in PC2 are clustered well (loadings > 0.8). Particularly elevated Mn concentrations were determined in gills in comparison to the rest of the fish by-products examined (Figure 2), whereas according to the evaluation of environmental concentrations (described in detail below), relatively high levels of dissolved Pb were found within the aquaculture unit. Dissolved Cd and Zn in seawater are principally labile and consequently bioactive, since they occur either in free ionic forms or bound to inorganic or organic ligands of low molecular weight, detected often in quite elevated concentrations in the gills of marine organisms such as marine bivalves (Sakellari *et al.* 2013).

Correlation between fish species and different fish sizes

Statistically higher concentrations were determined for As (muscle, head, gills, skin) and V (gills, trimmings, skin) in gilthead sea bream in comparison to meager (Kruskall-Wallis test; $p < 0.05$), without considering the fish size. Higher trace metal

concentrations were measured in meager regarding Cr (muscle, bones), Cu (guts) and Mn (guts, trimmings, bones) (Table S1, supplemental material; Figure 2). Excluding the aforementioned trends, no significant differences were observed between the two fish species examined, considering the rest of the trace elements studied.

It is noted that in the present study no robust correlation was calculated between trace metal concentrations and fish size and age (small, large). The total sum of the concentrations of the trace metals studied was calculated comparatively higher for small meagres than large ones in five out of the six by-products examined (head, gills, trimmings, bones and skin), whereas in gilthead sea bream in three fish by-products (gills, sking, guts) (Table S2). Based on fish size, higher concentrations in small meagres ($p < 0.05$) were determined for Cr, Mn and Co in head, Cr in gills, Cu in guts, Mn, Fe and Co in bones, while in small size gilthead sea breams for Fe in gills and skin. Similarly, higher concentrations in large size meagres were determined for Co and Cu in guts and Cu in bones (Table S1). It is indicated that in several fish by-products, trace metals concentrations are higher in small size fish (mainly regarding meager). Accordingly, Canli and Atli (2003) have reported several negative correlations between trace metals levels and the age in different Mediterranean fish species they investigated. It is well known that one of the most important factors playing a significant role in metal accumulation in marine animals is metabolic activity (Langston 1990), since the decreasing trend in the metal load of organs with age of mature stages is the result of daily ration of fish with age (Marmulla and Rosch 1990). Metabolic activity in young fishes is normally higher than that of older ones, thus metal accumulation was shown to be higher in younger fishes (Canli and Furness 1993).

Environmental concentrations

Table 2 presents the trace elements concentrations measured in samples of seawater and suspended particulate matter (SPM) from the two samplings carried out. Statistically significant seasonal differences (Mann-Whitney; $p < 0.05$) were observed in the trace elements' seawater concentrations for the cases of Cr ($p = 0.001$), Fe ($p = 0.021$), Mn, ($p = 0.001$), Pb ($p = 0.046$), V ($p = 0.003$) and Zn ($p = 0.012$). No statistically important differences in trace metals' concentrations were found between the two different depths examined (0.5 m above the sea bottom and 0.5 m below the sea surface). Seawater samples collected from the different sampling sites studied showed no particular differences in terms of trace metals levels (Table 2).

The seawater trace metal levels determined in the present study are considerably lower compared to those reported in other marine aquaculture areas. The results reported by Dolores Galindo *et al.* (2012) for Cd, Cu, Pb and Zn in seawater in the Bay of Cadiz, Spain, exceed those of the present study. Liu *et al.* (2019) have found significantly higher concentrations of Cd, Pb Cr, Zn and Cu in Beibu gulf in South China, trend which is followed also by the results reported for Fe and Mn by Steckert *et al.* (2019) in Santa Catarina, southern Brazil.

Compared to typical seawater concentrations (referred to in parenthesis and calculated using the average density for surface seawater; data from Bruland and Lohan (2003)) Cd, Cr, Mn, Ni and V levels measured herewith in seawater samples were found

Table 2. Trace metal concentrations in samples of seawater and suspended particulate matter (SPM) (B: 0.5 m above the sea bottom; S: 0.5 m below the sea surface).

Sample Seawater	Cd (ng L^{-1})	Co (ng L^{-1})	Cr (ng L^{-1})	Cu (μg L^{-1})	Fe (μg/L) (μg L^{-1})	Mn (μg L^{-1})	Ni (μg L^{-1})	Pb (ng L^{-1})	V (ng L^{-1})	Zn (μg L^{-1})
October 2017										
$M1_B$	9.85	9.39	73.1	0.102	0.867	0.110	0.264	18.2	122	0.239
$M1_S$	4.57	10.0	68.3	0.226	0.852	0.092	0.215	12.1	150	0.222
$M2_B$	6.28	10.6	35.1	0.237	0.258	0.093	0.262	6.77	170	0.233
$M2_S$	6.47	13.3	54.9	0.295	0.371	0.097	0.270	10.1	149	0.380
$M3_B$	6.05	5.67	49.2	0.191	0.291	0.077	0.209	5.30	192	0.148
$M3_S$	4.65	10.9	56.8	0.243	0.308	0.085	0.240	8.08	190	0.185
$M4_B$	3.43	5.78	66.0	0.128	0.469	0.162	0.103	5.84	186	0.186
$M4_S$	4.61	9.64	54.2	0.116	0.319	0.070	0.186	7.76	155	0.283
May 2018										
$M1_B$	23.8	11.6	25.4	0.315	1.09	0.053	0.239	28.6	40.3	0.461
$M1_S$	25.6	11.7	24.2	0.354	0.887	0.028	0.273	21.8	156	0.591
$M2_B$	5.57	10.6	18.9	0.189	0.660	0.029	0.179	10.8	77.0	0.270
$M2_S$	5.57	8.89	17.0	0.208	0.920	0.041	0.162	14.9	32.2	0.454
$M3_B$	6.78	7.38	14.4	0.191	0.691	0.038	0.194	9.65	22.3	0.286
$M3_S$	5.06	8.68	13.1	0.372	0.638	0.038	0.161	12.3	20.9	0.399
$M4_B$	8.55	9.67	15.9	0.211	0.828	0.032	0.164	7.91	16.8	0.204
$M4_S$	13.6	11.6	14.2	0.224	0.693	0.045	0.186	11.6	27.3	0.383
SPM	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})	(μg g^{-1})
October 2017										
$M1_B$	0.245	0.564	13.7	10.0	2268	42.5	11.2	8.50	9.74	8.81
$M1_S$	0.480	0.244	18.9	9.38	500	7.46	11.9	3.24	4.21	7.97
$M2_B$	0.106	0.508	10.2	7.05	1838	41.4	8.88	4.76	7.99	8.98
$M2_S$	0.079	0.136	8.33	7.36	394	6.37	5.55	2.30	3.30	7.96
$M3_B$	0.063	0.431	10.8	5.52	1599	37.1	5.16	4.38	6.60	8.56
$M3_S$	0.069	0.141	9.38	6.82	527	6.79	5.05	2.24	2.79	8.35
$M4_B$	0.056	0.171	12.7	4.98	420	7.21	6.59	2.24	6.06	7.95
$M4_S$	0.003	0.022	3.89	2.11	79.3	1.25	1.00	2.63	0.668	8.36
May 2018										
$M1_B$	0.099	0.411	10.6	4.74	1303	30.1	3.05	7.05	5.29	16.6
$M1_S$	0.099	0.229	9.09	9.42	534	7.78	4.13	6.20	1.87	12.9
$M2_B$	0.038	0.385	8.83	4.52	1248	30.0	2.43	4.57	4.79	16.1
$M2_S$	0.038	0.091	6.43	4.82	350	5.93	1.64	1.82	1.41	9.82
$M3_B$	0.036	0.447	7.75	3.24	1008	33.8	2.03	2.99	3.55	8.49
$M3_S$	0.043	0.156	8.83	17.4	396	6.73	1.54	1.89	1.80	12.3
$M4_B$	0.059	0.451	8.89	4.77	1321	33.0	2.38	5.02	4.78	18.1
$M4_S$	0.111	0.178	7.31	5.10	397	7.35	1.88	1.95	1.74	11.2

lower (0.115–115 ng L^{-1}; 0.160–0.266 ng L^{-1}; 0.004–0.282 μg L^{-1}; 0.123–0.738 μg L^{-1}; 1.57–1.88 μg L^{-1}), Co, Cu, Pb and Zn levels were comparable (0.241–18.1 ng L^{-1}; 0.032–0.293 μg L^{-1}; 1.08–32.3 ng L^{-1}; 0.033–0.603 μg L^{-1}), whereas Fe levels were higher (0.001–0.115 μg L^{-1}). The results of the present study are also comparable to those reported by Cotte-Krief *et al.* (2002) for the Celtic Sea (Cd: 0.122–33.7 ng L^{-1}; Cu: 0.064–0.127 μg L^{-1}; Ni: 0.118–0.252 μg L^{-1}) and by Morley *et al.* (1997) for western Mediterranean Sea (Cd: 0–33.7 ng L^{-1}; Cu: 0.064–0.127 μg L^{-1}; Ni: 0.120–3.60 μg L^{-1}; Fe: 0.045–0.196 μg L^{-1}; Mn: 0.017–0.302), whereas lower than those reported by Tang *et al.* (2002) for Galveston Bay, Texas (Cd: 0.122–7.87 ng L^{-1}; Cu: 0.064–0.508 μg L^{-1}; Ni: 0.240–1.20 μg L^{-1}; Fe: 1.68–3.36 μg L^{-1}).

Regarding the suspended particulate matter (SPM) measured in seawater samples collected in the present work its concentrations demonstrated a small variation, ranging between 10.3 mg L^{-1} at station $M4_S$ and 12.0 mg L^{-1} at $M1_B$ and $M4_B$ sampling sites. Statistically significant differences characterize the SPM of the different depths sampled, in terms of its concentrations in Co ($p = 0.002$), Fe ($p = 0.002$), Mn ($p = 0.002$), Pb

($p = 0.024$) and V ($p = 0.001$). However, no statistically significant differences were observed among the four sampling locations, whereas seasonal differences characterize only the Ni ($p = 0.012$) and Zn ($p = 0.002$) SPM concentrations. Similar Cd, Fe, Mn, Ni and Zn concentrations in SPM were reported in the present study in comparison to those recorded by Dolores Galindo *et al.* (2012), whereas lower for Co and higher for Cu and Pb.

The bioconcentration factor (BCF) may be used in order to evaluate the bioconcentration ability of a species in an ecosystem (Chong and Wang 2001). For estimating potential sources together with the enrichment of fish in trace metals originating from the abiotic environment, the bioconcentration factors (BCF) defined as fish tissue/water and fish tissue/particulate matter were calculated. The BCFs are expressed as the ratio between median metal concentrations in each tissue to median metal concentrations in seawater and suspended particulate matter, respectively. Although BCFs express metal enrichment accounting separately for each abiotic compartment considered, other compartments obviously participate as well in trace metal bioaccumulation, particularly those related with fish feeding (Dolores Galindo *et al.* 2012). However, BCFs may serve as a useful tool in the evaluation of the effect exerted by the abiotic environment. In case a metal has a BCF (referring to water) ranging between 1000 and 5000 it is considered as 'bioaccumulative', whereas in case the BCF is lower than 1000 the metal may be considered as 'not bioaccumulative' (Costanza *et al.* 2012).

The calculated mean values of the BCFs referring to bioaccumulation from seawater were lower to 1000 for V and Ni, ranged between 1000 and 5000 for Co and were higher than 5000 for the rest of the trace metals studied (Table 3). Regarding trace metal bioaccumulation of fish tissues from suspended particulate matter expressed per mass unit of it, BCFs are lower than 1000 for all trace metals studied excluding Zn (Table 3). Among the different fish tissues examined, the highest BCF values of the studied trace metals were calculated for Fe, Mn, Zn in gills, for Cd, Co, Cu, Zn in guts, for Cr in bones and for Cd, Ni, Pb in muscle. Metal bioconcentration trends followed the order $Mn > Zn > Fe > Pb > Cr > Cu > Cd > Cd > Co > Ni > V$ for dissolved trace metals in seawater and $Zn > Cd > Mn > Cu \gg Pb > Cr$, $Co > Ni > Fe > V$ for trace metals associated with particulate matter. Zn and Mn exhibited hence the highest BCFs in the compartments examined and despite the lower Zn and Mn levels measured in suspended particulate matter in comparison to those of Fe, their accumulation from the specific environmental compartment is several times higher than that of Fe. Cd appears to be mainly bioconcentrated from suspended particulate matter, despite the lower Cd concentrations, compared to the rest of the trace metals examined, detected in both fish tissues and the environmental compartments studied. Similar results were reported by Dolores Galindo *et al.* (2012), who showed that Cd accumulates mainly in the liver, kidney, gills and intestines of fishes.

Fish feed

Table 4 summarizes the mean data of trace metal concentrations measured in the different fish feeds used. No statistically significant differences were found among the different feeds, which do not actually differ in terms of their metal content. This may be

Table 3. BCF values of trace metals referring to seawater and suspended particulate matter (H: head, Gi: gills, Gu: guts, T: trimmings, B: bones, S: skin, M: muscle).

	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Fish tissue / Seawater (μg kg^{-1} / μg L^{-1})										
H	1687	118	17521	285	20651	29756	700	9665	229	34268
Gi	3423	2339	12264	2947	74093	273171	536	28134	280	104159
Gu	28386	5938	32165	74713	54700	46423	1146	36459	266	103255
T	425	288	33	78	22576	125366	625	10144	384	31971
B	955	95	47934	1632	11244	78618	536	8995	140	17269
S	1215	616	4959	2294	6292	69187	1082	6411	162	28734
M	30981	1078	21455	4202	28793	80244	1687	98086	502	34702
Fish tissue / SPM (μg kg^{-1} /mg kg^{-1})										
H	158	5	59	12	26	240	39	32	8	1065
Gi	320	97	41	121	94	2205	30	94	10	3238
Gu	2652	247	108	3060	70	375	64	122	9	3210
T	40	12	0	3	29	1012	35	34	13	994
B	89	4	161	67	14	635	30	30	5	537
S	113	26	17	94	8	558	61	22	6	893
M	2894	45	72	172	37	648	95	329	18	1079

attributed to the fact that their main difference relates to their respective pellet size and not to the ingredients used by the manufacturer. Aiming at protecting consumers' health, the European Commission has set maximum levels for specific heavy metals in animal feed. EU regulation limits for As, Cd and Pb are equal to 10, 2 and 5 mg kg^{-1} respectively (EC 2017, 2015, 2002). The concentrations of the trace metals measured in the present study not only met these regulation limits, but also they are well below. Additionally, the average concentrations of trace metals in fish feed were considerably higher compared to the calculated concentrations in wet weight of the different fish muscle tissues, which is in accordance with the previously published work of Kalantzi *et al.* (2016). Pb was the only exception, since its concentration measured in fish feed was lower compared to Pb concentration in fish muscle. This may suggest that although fish feed constitutes the main source of metals detected in fish muscles, other sources may also exist contributing to the total metal content of fish, such as sea water and sediments (Ferreira *et al.* 2008; Alhashemi *et al.* 2012; Kalantzi *et al.* 2016).

Legislation

The present study aims at the investigation of the potential safe use of fish by-products as additives in the food industry, in terms of their metals' content. Since there are no available legislation limits of metals contained in fish by-products, the assessment is carried out with the use of limits referring to fish muscle. For As, Cd, Cr, Cu, Fe, Ni, Pb and Zn there have been set maximum permitted levels, from several national bodies, which are equal to 13.5, 0.05, 4, 20, 10.2, 80, 0.3 and 50 mg kg^{-1} of wet tissue, respectively (FAO 1983; MAFF 2000; IEIC 2002; EC 2006; Marengo *et al.* 2018). Co, Mn and V maximum concentrations in fish products have not been addressed by regulatory agencies so far. In order to compare our results with the above limits we calculated the mean concentrations of the different metals based on the corresponding moisture content of the different fish tissues. Our measurements demonstrated that As concentrations in all fish by-products examined were lower compared to the regulation limits, case which is similar to that of Cr, the concentrations of which were in accordance with

Table 4. Trace metal concentrations (μg g^{-1}) in fish feed samples (mean ± standard deviation).

Feed	As	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
F1	1.83 ± 0.04	0.285 ± 0.030	0.252 ± 0.010	0.824 ± 0.060	27.0 ± 1.2	446 ± 12	171 ± 40	3.22 ± 0.12	0.773 ± 0.280	1.73 ± 0.24	118 ± 6
F2	2.60 ± 0.03	0.242 ± 0.001	0.262 ± 0.001	1.45 ± 0.01	25.7 ± 0.4	361 ± 56	112 ± 10	3.19 ± 0.14	0.337 ± 0.070	1.68 ± 0.04	140 ± 5
F3	1.69 ± 0.10	0.232 ± 0.001	0.208 ± 0.010	0.456 ± 0.050	27.0 ± 1.6	255 ± 49	103 ± 6	2.61 ± 0.02	0.287 ± 0.001	1.36 ± 0.06	146 ± 5

Table 5. Concentrations of trace metals (mg kg^{-1}) in different fish by-products of gilthead sea bream and meager reported in literature.

Area	Tissue	Parameter		As	Cd	Co	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Sparus aurata														
Greece, Aegean Sea[a]	gills	range	w.w.	0.93–1.15	<0.006	0.05–0.06	0.09–0.13	<10.75	23.57–27.02		0.33	<0.04	0.06–0.07	17.86–18.38
	bones	range	w.w.	1.10–1.98	<0.006	0.10–0.14	0.08–0.12	<10.75	6.71–12.76		0.64–0.82	<0.04–0.05	0.04–0.06	14.81–16.76
	intestines	range	w.w.	1.17–1.931.38	0.07–0.08	0.01	0.07–0.08	0.55–0.56	3.06–16.11		<0.43	0.02–0.03	0.02–0.04	14.82–16.95
	gills	mean	w.w.	1.21	<0.006	0.06	0.04	<10.75	18.38		0.24	<0.04	0.05	19.24
	bones	mean	w.w.	1.62	<0.006	0.08	0.07	<10.75	<6.99		0.50	<0.04	0.03	13.77
	intestines	mean	w.w.	1.25	0.04	0.00	0.06	0.77	<6.99		<0.43	0.01	0.02	14.91
Greece, Aegean Sea[b]	gills	mean	d.w.				0.25	0.64						23.97
Italy, Rovigo lagoon[c]	gills	mean	w.w.					1.26						23.72
Tunisia, Monastir[d]	gills	mean	w.w.		0.29			2.35						
Turkey, Iskenderun Bay (wild)[e]	skin	mean	w.w		2.044		1.534	19.583	63.194	3.946		3.052		133.38
	gills	mean	w.w		0.188		3.920	8.264	33.349	20.433		2.428		141.807
	intestines	mean	w.w		0.433		0.456	16.905	124.743	12.728		2.032		38.165
Kuwait, Auha (wild)[f]	gills	range	w.w.	1.92–2.16			1.21–4.6	3.64–4.99			0.37–1.01		1.57–3.21	9.25–12.44
Kuwait, Kuwait Bay (wild)[f]	gills	range	w.w.	<0.25–2.30			0.46–1.30	4.49–5.56			0.37–0.62		1.38–2.54	11.44–13.97
Italy, farmed stock[g]	gills	mean	d.w.					1.5						27.9
	intestines	mean	d.w.					2.8						19.5
Greece, Crete island[h]	gills	median	w.w.	0.838	0.008	0.006	0.133	0.247	27.2	4.41	0.035	0.090	0.022	10.2
	guts	median	w.w.	0.928	0.066	0.022	0.374	1.00	12.9	0.748	0.121	0.109	0.018	9.38
	bones	median	w.w.	0.813	0.004	0.000	0.264	0.045	2.33	0.621	0.009	0.021	0.007	1.38
	skin	median	w.w.	1.15	0.005	0.003	0.587	0.214	7.01	2.58	0.134	0.037	0.022	5.93
Greece, Crete island[h]	gills	median	d.w.	2.38	0.022	0.018	0.371	0.703	77.2	12.5	0.100	0.255	0.063	29.0
	guts	median	d.w.	2.45	0.175	0.057	0.998	2.65	34.0	1.98	0.319	0.287	0.047	24.8
	bones	median	d.w.	2.25	0.011	0.001	0.730	0.125	6.45	1.72	0.026	0.058	0.019	3.83
	skin	median	d.w.	2.67	0.013	0.008	1.37	0.499	16.3	6.02	0.313	0.087	0.050	13.8
Argyrosomus regius														
Greece, Crete island[h]	gills	median	w.w.	0.398	0.006	0.007	0.258	0.345	14.4	6.76	0.049	0.085	0.008	8.27
	guts	median	w.w.	0.520	0.076	0.047	0.242	11.3	18.2	1.23	0.070	0.154	0.010	13.8
	bones	median	w.w.	0.841	0.003	0.005	0.848	0.630	8.64	7.63	0.099	0.064	0.016	5.79
	skin	median	w.w.	0.252	0.003	0.001	0.688	0.131	1.62	1.62	0.059	0.022	0.006	1.93
Greece, Crete island[h]	gills	median	d.w.	1.04	0.021	0.026	0.899	1.20	50.1	23.6	0.172	0.297	0.028	28.8
	guts	median	d.w.	1.54	0.223	0.139	0.715	33.5	53.8	3.62	0.207	0.454	0.030	40.6
	bones	median	d.w.	1.75	0.006	0.010	1.77	1.31	18.0	15.9	0.207	0.134	0.033	12.1
	skin	median	d.w.	0.661	0.007	0.003	1.80	0.344	4.25	4.26	0.156	0.057	0.015	5.06

[a]Kalantzi et al. (2016);
[b]Castritsi-Catharios et al. (2015);
[c]Cirillo et al. (2012);
[d]Ghedira et al. (2010);
[e]Dural et al. (2011);
[f]Beg et al. (2015);
[g]Minghetti et al. (2008);
[h]Present study.

the regulation limits set by Hong Kong government (FAO 1983) and of Cu which met the regulations limits set by MAFF and CEFAS (FAO 1983; MAFF 2000). Cd concentrations in guts of both gilthead sea bream and meager exceeded the EC regulation limit set for Cd muscle meat of fish (0.05 mg kg^{-1}w.w.). In the study of Marengo *et al.* (2018), the limit of Fe in fish is set at 10.2 mg kg^{-1}. However, in our study the samples of gills and guts of meager and gilthead sea bream lie above this limit. All of our samples also met India's regulations for maximum levels for Ni in fish (IEIC 2002). The content of Pb was in accordance with EU regulation (EC 2006) excluding the cases of meager and gilthead sea bream guts. Slightly elevated concentrations of Pb in seawater, as already described, were found in the sampling point located inside the aquaculture. Zn concentrations in all fish by-products samples met MAFF regulation limits (MAFF 2000).

Comparison to literature

As summarized in Table 5, there is a limited number of studies in literature referring to trace metal concentrations in different fish by-products/tissues of *S. aurata*, whereas no relevant data are available for *A. regius*. The results of the present study for gilthead sea bream generally lie within the range reported in previously published works for farmed fishes (Minghetti *et al.* 2008; Kalantzi *et al.* 2016). In the case of wild gilthead sea bream the concentrations of metals in fish by-products reported were significantly higher (Table 5).

Conclusions

The present study aimed at the evaluation of 11 trace metals' levels, focusing on the safety of using six fish by-products as food additives subject to human consumption. Under the perspective of circular economy, exploitation of fish by-products is particularly challenging for Greece, which constitutes one of the most significant producers of farmed fishes such as gilthead and sea bream. The pathways of metal uptake in the different fish tissues with emphasis to environmental concentrations and fish feed were also investigated.

Trace metal levels in seawater samples collected from the marine area of the pilot fish farm, from which fish samples were obtained, were determined similar to the corresponding of non-polluted marine areas. It is noteworthy that in the by-products of large size fishes lower metal concentrations are frequently found, compared to those of smaller size ones, trend which seems to be related with the increased metabolic activity of small fishes. The use of fish by-products as additives in food industry appears to become feasible, employing the concentrations of the 11 trace elements studied as a criterion.

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All animals used in the research were treated humanely according to institutional guidelines. The farm is certified as an aquaculture facility from the national veterinary authority (code GR94FISH0001) and is also registered as breeding and experimental facility for marine fish with

approval codes EL91-BIObr-03 and EL91-BIOexp-04 respectively. Sampling was performed by personnel accredited by the Federation of European Laboratory Animal Science Associations (FELASA) on the "Care and use of laboratory animals".

Disclosure statement

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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