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The isopod *Eurydice spinigera* and the chaetognath *Flaccisagitta enflata:* how the habitat affects bioaccumulation of metals in predaceous zooplankton

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Bioaccumulation processes result in a high enrichment of metals in zooplankton communities. In terms of trace element concentrations, most information involves copepods, which are often the dominant forms in the community, but are not the only common zooplankton species.
We analyzed the concentrations of 20 trace elements in *Eurydice spinigera* (Isopoda) and *Flaccisagitta enflata* (Chaetognata), which represent two different species of marine zooplankton that share the same feeding predaceous strategy, from a highly productive coastal region (Ligurian Sea, Northwestern Mediterranean).
Our results demonstrated that metal transfer was deeply influenced by the different habitats, as the carnivorous isopod *E. spinigera,* which spends most of its lifetime on the seabed, had the highest concentrations of most of the analyzed trace elements (Al, As, Cd, Ce, Cr, Fe, La, Mn, Ni, Pb, Se, V and Zn) and consequently the highest bioaccumulation factors (BAFs). Conversely, in the carnivorous Chaetognatha *F. enflata,* which is not a benthonic species, the highest levels of copper and tin were found. Moreover, arsenic speciation analysis confirmed the presence of inorganic As (III+V) in *E. spinigera*. In the perspective of utilizing a marine organism as a bio-indicator of metal transfer, it is crucial to consider both feeding behavior and feeding habitat.

KEYWORDS: trace elements, zooplankton, hyperbenthos, chaetognaths, isopods, Mediterranean Sea.

INTRODUCTION

In the last decade, there has been a considerable interest in understanding metal accumulation in marine organisms as they can greatly influence the cycling, fluxes, and residence times of metals in marine systems. Aquatic invertebrates are exposed to chemicals from both the particulate and dissolved phases, and play a key role in the trophic transfer of metals in aquatic food chains as food uptake has been increasingly recognized as an important source for accumulation of contaminants (Fisher and Reinfelder, 1995). Zooplankton is particularly critical to the functioning of ocean food webs due to their abundance and their vital ecosystem roles. In assessing environmental quality with respect to trace elements in seawater, the bioavailable fraction is of major importance as toxicity depends on the bioavailable exposure concentration (Kahle and Zauke, 2003). This bioavailable fraction can be assessed by determining the amount of metals incorporated into organisms, which is the main goal in biomonitoring (Rainbow, 1993). Bioaccumulation, along with persistence and acute toxicity, can be used for identifying aquatic environmental hazards in order to determine the potential for adverse effects to biota (McGeer *et al.,* 2003). The BAF (Bioaccumulation Factor) is a model for bioaccumulation that predicts partitioning between an exposure medium (marine water) and biota (zooplankton species), and is calculated as the ratio of internal biota concentration to exposure concentration (McGeer *et al*., 2003).

The order Isopoda is a ubiquitous monophyletic *taxon* that includes around 10,131 species (Boyko *et al*., 2008) found in all ecosystems from the deepest oceans to the montane terrestrial habitats and deep underground in caves or aquifers. The most significant feature of the group is the diversification into a number of different ecological roles or modes of life (Argano and Campanaro, 2010), and isopod representatives occur in the marine environment from the littoral to abyssal zones (Naylor, 1972).

Isopods of the family Cirolanidae dominate the upper shore of sandy beaches in most temperate and tropical regions (Bruce, 1986). Isopods belonging to the genus *Eurydice,* which are highly predaceous carnivores, particularly including copepods and cladocerans (Macquart-Moulin, 1998), have pelagic phases during the night (Macquart-Moulin, 1992; Macquart-Moulin and Patriti, 1996). The circadian rhythm stimulates them to emerge from the sediment at dusk (Macquart-Moulin, 1973, 1976), i.e. an endogenous light-controlled vertical migration occurs (Macquart-Moulin, 1972, 1985) and the animals gather at the sea surface (Champalbert and Macquart-Moulin, 1970; Tully and O'Ceidigh, 1986, 1987; Macquart-Moulin, 1992; Macquart-Moulin and Patriti, 1996). The hyponeustonic pattern of distribution is observed throughout the night, and at dawn the animals return to the bottom and burrow into the sediment (Macquart-Moulin, 1998). The upward evening migration and the downward morning migration are both very fast, and only a few specimens have been recorded in the deep or intermediate layers during the night (Macquart-Moulin, 1998). This nocturnal migratory behavior occurs close inshore along the whole continental shelf. The migratory behavior of several *Eurydice* spp., included *E spinigera*, may constitute a mechanism for directly ensuring active vertical transfer of organic matter and trace elements between the bottom, the surface and the various water masses along the whole continental margin, including the shelf and the slope regions.

The relatively small isolated *phylum* Chaetognatha, also known as arrow worms, includes a total of 209 species that have been recorded in the world's oceans, of which 20 (16 planktonic and 4 benthic,) have been reported in the Mediterranean Sea (Furnestin, 1979; Bieri, 1991; Kehayias *et al*., 1999; Ghirardelli, 2010). Exclusively predaceous, chaetognaths are found in marine habitats including estuaries, open oceans, tide pools, polar waters, marine caves, coastal lagoons and deep sea waters (Bone *et al*., 1991). Moreover, chaetognaths are distributed from the surface to great depths, while some species exclusively live close to the sea floor (Pierrot-Bults and Nair, 1991). The abundance of chaetognaths is often second only to copepods in the zooplankton of many marine environments (Feigenbaum and Maris, 1984; Shannon and Pillar, 1986; Gibbons, 1992). The biomass of chaetognaths is estimated to be 10-30% of that of copepods in the pelagic realm; thus, they play a significant role in the transfer of energy from copepods to higher trophic levels (Bone *et al.*, 1991; Feigenbaum, 1991; Froneman *et al*., 1998; Giesecke and González, 2004). The diet of chaetognaths includes a variety of pelagic organisms, consisting mainly of copepods, but they may also prey on larvaceans, cladocerans and fish larvae, thus strongly influencing the zooplankton and ichtyoplankton communities (Faigenbaum, 1991; Casanova, 1999; De Souza *et al*., 2014). Inter- and intra-specific predation has been reported among various species of chaetognaths (Pearre, 1982). Prey selectivity is most often attributed to the prey size (Pearre, 1982), but factors such as prey swimming behavior, conspicuousness and availability, may also be significant (Duró and Saiz, 2000; Coston-Clements *et al*., 2009). Moreover, chaetognaths are prey to many larger organisms including fishes, whales, other marine invertebrates and molluscs. Diel vertical migration (DVM), is common among chaetognaths (Terazaki, 1996; Giesecke and González, 2004; Johnson *et al*., 2006; Kehayias and Kourouvakalis, 2010). However, most studies on chaetognath DVM have been conducted in areas with water depths exceeding 50 m, while studies in shallow waters are scarce (Sweatt and Forward, 1985).

Chaetognaths have another characteristic that makes them particularly interesting from an oceanographic point of view. They have been shown to be good indicators of water masses (Pierrot-Bults, 1982; Ulloa *et al*., 2000; Kehayias *et al.,* 2004) and, consequently, appear to be very suitable for studying the effects of physical processes- acting at the mesoscale- on the dynamics and variability of zooplankton populations (Duró and Saiz, 2000). Ecologically, *Flaccisagitta enflata* predominates in the tropical-subtropical epipelagic waters (Pierrot-Bults and Nair 1991; Duró and Saiz, 2000). *F. enflata* is adapted to the uppermost layers of the warm-water sphere, which has the lowest density and a vertical range of only 100 m to 200 m (Kapp 1991); numerically, it is the most important chaetognath neritic species of the Mediterranean Sea (Batistic, 2003; Ghirardelli and Gamulin, 2004). In the Northwestern Mediterranean Sea, the main copepod prey of *F. enflata* was reported to be *Centropages typicus* and *Temora stylifera* (Duró and Saiz, 2000). Recently, bioaccumulation of trace elements in chaetognaths belonging to the family Sagittidae, was investigated in the coastal regions of India (Bhattacharya *et al*., 2014), and in the White Sea (Budko *et al*., 2015).

Secondary consumers in zooplankton communities, Isopoda and Chaetognata could be suitable "indicators" of the presence and transfer of metals in a marine environment. Bioindicators are organisms- a particular species or communities of species- used to assess the quality of an environment or changes in the environment due to anthropogenic disturbances or natural stressors. In a previous study, we analyzed the potentiality of marine zooplankton to be bioindicators of trace elements in coastal ecosystems (Battuello *et al*., 2016). We found that the examined zooplankton showed a great ability to accumulate concentrations of metals that were several thousand times more than concentrations detected in marine water, in particular the essential elements iron, copper, zinc, cobalt and manganese and the nonessential element cadmium. We then focused on the influence of the different feeding modes (herbivorous, omnivorous and carnivorous) in metal bioaccumulation in Calanoida copepods (Battuello *et al.*, 2017), and we found that there was a reduced metal accumulation in carnivores compared to herbivores. In fact, the herbivorous species showed the highest concentrations and BAFs for most of the analyzed metals, in particular for the nonessential elements aluminum and cadmium, and for the essential trace elements copper, iron, manganese and zinc. Nevertheless, not all species or communities can serve as successful bioindicators, and expanding on this topic, our study focused on the influence of different habitats in metal bioaccumulation in two carnivorous zooplankton species: *Eurydice spinigera* (Isopoda) and *Flaccisagitta enflata* (Chatognata). In fact, both these species have the potential to be sentinel species of a marine environment, being able to accumulate and concentrate metals to measurable levels above those in the surrounding waters; in addition, they share the same feeding strategies as they are both carnivores, and they are both at the top of the zooplankton food web. However, they have different habitat requirements, and we therefore postulated that the habitat could be significant in the bioaccumulation of metals through the marine food chain.

The main objectives of the present study were:

i) to analyze, for the first time, the concentrations of 20 trace elements in two zooplanktonic marine species, *E. spinigera* (Isopoda) and *F. enflata* (Chaetognata)

ii) to evaluate the relevance of these two predaceous species in the bioaccumulation and transfer of trace elements through the marine food chain

iii) to establish the suitability of *E. spinigera* and *F. enflata* as bioindicators of different compartments of a marine coastal environment.

METHODS

Study area and sampling site

The study area is a highly productive Italian coastal region characterized by heavy commercial maritime traffic and numerous industrial plants. Indeed, the area currently has one of the highest levels of shipping in the whole Mediterranean basin, and is a recipient of pollutants coming from the highly developed coastline of Italy. Furthermore, this coastal area also experiences summer tourism, which leads to a substantial increase in inhabitants and consequently to elevated risks of pollution (Barrier, 2016). As a result, municipal wastewater treatment plants show effluents characterized by a lower water quality and an increase in the nutrient concentration of marine water (Renzi *et al.*, 2010). The sampling site was situated off the Italian coast, in the transition zone between the Northern Tyrrhenian Sea and the Southern Ligurian Sea (Fig. 1). The sampling station (43°28’10” N, 10°01’55” E) was located at 12.5 nm off the Tuscan coast, above the continental shelf. The sector under investigation is characterized by a large extension of the continental shelf and limited depth (100 m), even at considerable distances from the coast (18 miles) (Chiocci and La Monica, 1996). The Ligurian Sea lies at the north-east edge of the Western Mediterranean and is connected to the southern basin (Tyrrhenian Sea) via the Corsica Channel. The general circulation of the Ligurian Basin is characterized by a permanent basin-wide cyclonic circulation involving both the surface Modified Atlantic Water (MAW) and the lower Levantine Intermediate Water (LIW) (Millot, 1999; Bozzano *et al.*, 2014). The Northern Current is generally weaker in the summer than during the winter and the contribution from the Tyrrhenian Sea is strongly reduced in summertime (Aliani *et al.*, 2003). The flow originates before the Ligurian Sea due to the merging of the Western and Eastern Corsican Current through the Corsica Channel (Artale *et al.*, 1994).

Climatic forcing can greatly change the intensity of currents, but the general pattern can be considered permanent (Molinero *et al.*, 2005; Birol *et al.*, 2010). Moreover, due to the interplay of these particular oceanographic, climatic and physiographic factors, the area is highly productive and hosts a rich and complex ecosystem. This is also sustained by vertical mixing and coastal upwelling, generated by the prevailing northwesterly wind, which pumps nutrients and other organic substances contributed by rivers into the euphotic zone where they fertilize growing phytoplankton populations (Bozzano *et al.,* 2014). Hence, the area attracts several cetacean species and is part of the "Cetacean Sanctuary" protected area.

Sampling

Zooplankton samples were collected during September 2015 (summer). The sampling station was located on the continental shelf above a bottom depth of 111 m (Fig.1). Surface zooplankton samples were caught with a WP-2 standard net, having a mesh size of 200 μm and a diameter of 57 cm. The net was towed horizontally at the water surface and the sampling time was approximately 15 min at a vessel cruising speed of 2 knots. Each net was fitted with a flow meter (KC Denmark model 23.090) to measure the volume of water filtered, which ranged from 251.63 to 329.2 m^3. Net hauls were consistently carried out at night to allow surface sampling of isopods and chaetognaths, involved in nictemeral migrations.

The entire sample from each net was divided into two aliquots immediately after sampling, using a Folsom splitter. One aliquot was fixed in 4% neutralized formaldehyde buffered with borax and kept in the dark, for analyzing the zooplankton composition, with particular attention to the identification and quantification of dominant isopod and chaetognath species (Boltovskoy, 1981). The second aliquot was also immediately fixed, in the same manner, for subsequent analysis of trace element concentrations of target species (Fang *et al*., 2014; Fernandez de Puelles *et al*., 2014). To avoid possible contaminations on the surface of the zooplanktonic organisms, each sample was washed four times with distilled water for elimination of fine particulates and kept frozen for trace metals analyses. In order to quantitatively analyze the trace metals contained in the isopods and chaetognaths, and compare the differences in metal content relative to their different distribution and feeding behavior within the water column, samples were sorted, and the selected species were analyzed for trace metal determination. Regarding the chetognaths, only adult specimens with empty guts were taken.

Depending on the size and abundance of the different target species, about 300 – 600 specimens of the two target species were selected separately for each of the four samples. Shallow seawater samples for total dissolved trace metal analysis were collected at a depth of 1 m using 5 L Niskin bottles and stored in a cool box until being subjected to filtration. All samples were kept under refrigerated conditions before analysis.

Detection of trace elements

E. spinigera and *F. enflata* samples (n=4 for each species) were accurately rinsed with Milli-Q water to remove the formaldehyde buffer before trace elements quantification.

Determination of aluminum (Al), arsenic (As), beryllium (Be), cadmium (Cd), cerium (Ce), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), lanthanum (La), lead (Pb), nickel (Ni), antimony (Sb), selenium (Se), tin (Sn), (thallium) Tl, vanadium (V) and zinc (Zn) was performed after wet digestion using acids and oxidants (HNO_3 and H_2O_2) of the highest quality grade (Suprapure). Samples were subjected to microwave digestion (microwave oven ETHOS 1 from Milestone, Shelton, CT, USA) with 7 mL of HNO_3 (70% v/v) and 1.5 mL of H_2O_2 (30% v/v). Ultrapure water was added to samples to reach a final weight of 50 g (Arium611VF system from Sartorius Stedim Italy S.p.A., Antella - Bagno a Ripoli, FI, Italy). All metals were quantified by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany). Multi-elemental determination was performed after daily optimization of instrumental parameters, and use of an external standard calibration curve; rhodium and germanium were used as internal standards. Analytical performances were verified by processing Certified Reference Materials (Oyster Tissue -SRM 1566b from the National Institute of Standard and Technology), along with blank reagents in each analytical session. The limit of quantification (LOQ) for each element, the reference material values and the percentages of recovery obtained are shown in Table S1.

A chelating polymer resin, the SPR-IDA Reagent (Suspended Particulate Reagent – Iminodiacetate, by Cetac Technologies, Omaha, USA) was used for pre-concentration/ matrix elimination of seawaters. A volume of 15 mL of seawater was directly added to a pre-cleaned 15 mL volume polypropylene centrifuge tube. A 100-µL aliquot of a 10% suspension of SPR-IDA reagent beads was then pipetted directly onto the sample. Tubes were covered with parafilm and contents were mixed thoroughly. Samples were then spiked with 0.5 µg L^{-1} yttrium, which functions as an internal standard, in order to correct for any volume differences in the blanks, samples, and spiked samples. High-purity ammonium hydroxide (NH_4OH, 29%) was added in two steps (25 µL + 20 µL) to adjust the pH to approximately 8. The SPR-IDA beads were then allowed to settle for approximately 1 h. Samples were then placed in a centrifuge and spun at 2000 rpm for 10 min. The supernatant liquid was carefully poured off to minimize any loss of beads, which were mostly compacted at the bottom of the tube. A solution of deionized water, adjusted to pH 8 with high purity NH_4OH, was then added to the 15 mL mark of the sample tube and the contents were mixed. The beads were again allowed to settle, centrifuged, and the resulting supernatant liquid was carefully poured off and discarded. A 0.5 mL aliquot of 7% v/v absolute high-purity nitric acid (Suprapure) was then added to the bead residue to extract any bound metal ions. The resulting extract was diluted to 3 mL with deionized water and analyzed by ICP-MS. The following metals were then quantified in seawater: Al, Cd, Co, Cu, Fe, Mn, Ni, Pb, Zn.

Arsenic speciation

Arsenic speciation analysis was conducted by high performance liquid chromatography coupled to an inductively coupled plasma mass spectrometer (HPLC-ICP-MS); ICP-MS Xseries II, Thermo Scientific, Bremen, Germany and HPLC Spectra System MCS 1000, Thermo Scientific, Bremen, Germany) following the Thermo Scientific application note n° 40741.

The following As species were investigated: DMA (Dimethylarsinic acid), MMA (Monomethylarsonic acid), AsB (Arsenobetaine), iAs (sum of As III, arsenite, and As V, arsenate). The limit of quantitation of the method (LOQ) was 0.020 mg $Kg^{-1}$for all the arsenical species.

Bioaccumulation factors (BAFs)

The bioaccumulation factor (BAF) is the ratio of the concentration of a chemical in an organism compared to the concentration in water. BAFs were estimated for Al, Cd, Co, Cu, Fe, Mn, Ni, Pb and Zn, the same elements that were quantified in both seawater and in the analyzed species.

For estimating BAFs, the metal levels were expressed as μg kg^{-1} in *E. spinigera* and *F. enflata* as μg L^{-1} in seawater.

Statistical analysis

All statistical analyses were performed within the R statistical framework (R Core Team, 2015). Normality of data and equality of variance were assessed. The abundance of *E. spinigera* and *F. enflata*, as well as the whole mesozooplanktonic assemblages from each replicate sample was compared using the Kruskal-Wallis test ($p < 0.05$ was considered as statistically significant), to determine if it was acceptable to combine these datasets. There were no significant differences between the four replicates (Kruskal-Wallis $X^2 = 1.550$, d$f = 3$, $p = 0.671$).

Before analyzing metal concentrations, we performed the D'Agostino-Pearson normality test to determine the distribution of the values. The unpaired t test was used to test differences in metal concentrations between the isopod and the chetognath. Results were considered statistically significant with p values of < 0.05. Statistical calculations were performed using Graph Pad Statistics Software Version 6.0 (GraphPad Software, Inc., USA).

RESULTS AND DISCUSSION

Zooplankton communities

The mesozooplankton communities of the replicates showed comparable compositions, so we presented their mean values to facilitate data discussion. Copepods made up the bulk of the zooplankton biomass (74.36%), with 61.21% of the total biomass comprised of calanids (Calanoida). Cladocerans were the second largest group (9.47%), followed by chaethognaths (3.21%), pteropods (3.03%), larvaceans (2.42%), siphonophores (2.11%), euphausiids (2.05%), isopods (1.84%), mysidaceans (1.30%), and ostracods (0.21%). Overall, the zooplankton collected in summer 2015 presented a mean biomass value of 2.57 mg m^{-3} (expressed as dry weight, Lovegrove, 1966). Chaetognaths were represented by three species, namely the dominant neritic *Flaccisagitta enflata* (3.622 ind.m^{-3} ± 2.417), the epiplanktonic *Mesosagitta minima* (1.763 ind.m^{-3} ± 0.335) and *Parasagitta friderici* (0.134 ind.m^{-3} ± 0.018), a neritic species. Only two isopod species were recorded: the hyperbenthic *Eurydice spinigera* (Cirolanidae, 1.030 ind.m^{-3} ± 0.011) and the neustonic *Idotea metallica* (Idoteidae, 0.031 ind.m^{-3} ± 0.007).

Trace elements in seawaters

The concentrations of dissolved Mn, Fe, Cu, Zn, Al, Ni Co, Cd and Pb are shown in Fig. S1 and are reported as μg L^{-1}. In surface water, metal concentrations were found in the following order: Zn>Ni>Fe>Al>Pb>Co>Mn>Cu>Cd; i.e. Zn and Ni were the nutrient trace elements with the highest concentrations, 11.43 and 10.60 μg L^{-1}, respectively. The level of the nonessential element Al was relatively low (1.50 μg L^{-1}) and in line with previous findings in the Mediterranean Sea (Caschetto and Wollast, 1979; Battuello *et al.*, 2016). Trace metals in coastal waters are usually higher than concentrations in the open ocean, owing to metal influx from continental sources, such as ground water and coastal sediments (Sunda, 2012). The concentrations that were detected in seawater were comparable or lower than those recently detected in the Mediterranean Sea (Safaa, 2015; Ebling and Landing 2015; Battuello *et al.,* 2016). The metal concentrations in water does not provide information on metal bioaccumulation or biomagnification in biota (Ricart *et al.*, 2010; Maceda-Veiga *et al.,* 2013) but it is necessary to estimate the bioaccumulation factors.

Trace elements in *E. spinigera* and *F. enflata*

The concentration of trace elements (Fig. 2 and 3) was in the following order: Zn>Cu>Al>Fe>Mn>Pb>Ni>Se>Cd>Cr>As>Co>V>Ce>Sn>Mo>La>Sb in the isopod *E. spinigera* and Zn>Cu>Fe>Al>Ni> Mn>Pb>Se>Cr>Sn>As>Co>V>Cd>Mo=Sb>Ce>La in the chaetognath *F.*

enflata. In Table 1 descriptive statistics were shown for each species samples. The statistical evaluation results are shown in Table 2.

Essential trace elements

Isopods had the highest values for all the essential trace elements (cobalt, chromium, iron, manganese, molybdenum, nickel, selenium and zinc), with the exception of copper, which was higher in chaetognaths, and molybdenum, which was the same concentration in both species.

Statistically significant differences were found between the two species for all the essential elements (Table 2).

Manganese and zinc values were much higher in isopods than in chaetognaths (Table 1, Table 2, Fig. 2). Mn is a naturally occurring metal in seawater, and it is well known that it can be significantly bioconcentrated by aquatic biota at lower trophic levels (WHO, 2004); Mn concentration was an order of magnitude higher in *E. spinigera* (4.40 mg Kg^{-1}) than in *F. enflata* (0.49 mg Kg^{-1}).

Zinc is essential for the biological requirements of marine plankton, and its concentration usually greatly exceeds that required for normal metabolism in tissues of aquatic organisms because it concentrates more effectively than others elements. Accordingly, Zn was the most represented element in both species, but its concentration was much higher in *E. spinigera* (234.02 mg Kg^{-1}) than in *F. enflata* (98.12 mg Kg^{-1}). These essential elements, Mn and Zn, showed nutrient-like vertical distributions, being depleted in surface waters due to uptake by the biota, and increased in concentration with increasing depths, because of the remineralization of sinking organic matter (Sunda, 2012). This is consistent with the higher concentrations of Mn and Zn observed in the benthonic species *E. spinigera.*

Copper was higher in chaetognaths (84.60 mg Kg^{-1}) than in isopods (53.15 mg Kg^{-1}) possibly indicating a higher availability of this element in the upper water column. It is well known that marine organisms are able to concentrate significant amounts of copper in seawater, which is required as this element is a component of enzymes and hemocyanin (Paimpillil *et al*., 2010). Regarding isopods, and crustaceans in general, the hepatopancreas is the most important storage organ of heavy metals, containing more than 50% of the total copper in the body (Hopkin *et al*., 1985; Góral *et al*., 2009).

Iron is an essential element for zooplankton, due to its role in mitochondria of catalyzing redox reactions during respiration. Marine mesozooplankton can be affected by Fe deficiency in food, and, due to the role that zooplankton plays in the cycling of Fe and C, these results could have implications for biogeochemical cycles (Chen, 2011). Moreover, a low Fe content in Fe-limited phytoplankton seems to cause physiological stress in crustacean zooplankton (Chen, 2011). We found a slightly higher iron content in *E. spinigera* than in *F. enflata* (34.31 and 29.601 mg Kg^{-1}, respectively, Table 1).

Chromium, cobalt and selenium concentrations were almost twice as much in *E. spinigera* than in *F. enflata* (Fig. 2, Table 1). In the open sea, Cr is involved in biogeochemical cycles, with biologically mediated Cr removal in the surface layers, and elevated Cr levels in deeper waters because of mobilization of Cr upon breakdown of sinking biogenic particles (Campbell and Yeats, 1981). Nickel is another essential metal for aquatic organisms but it is toxic at elevated concentrations. There is a relatively high assimilation efficiency and bioavailability of Ni to marine planktonic organisms (Hutchins and Bruland, 1994); accordingly, we found 1.01 mg kg^{-1} of nickel in *F. enflata* and 1.66 mg kg^{-1} in *E. spinigera*.

Nonessential trace elements and rare earth elements

The concentrations of the nonessential trace elements aluminum, arsenic, antimony, cadmium, lead, tin and vanadium and of the two rare earth elements lanthanum and cerium are shown in Fig. 3.

Beryllium and thallium concentrations were undetectable (< LOQ).

Differences in nonessential metal levels related to species were statistically significant (Table 2), apart from antimony.

The highest values for all these elements were registered in *E. spinigera,* with the exception of Sn, which was higher in *F. enflata* and Sb, which differed slightly in the two species.

Aluminum was the most represented nonessential trace element in both species, reflecting its ubiquity in the aquatic environment. The concentration of Al in *E. spinigera* was twice as much as that in *F. enflata* (38.80 and 15.52 mg kg^{-1}, respectively); this agreed with a previous report in marine zooplankton (Battuello *et al.,* 2016), where higher Al levels were found with increasing water depths.

Seawater naturally contains 1–5 μg L^{-1} of total arsenic, which is mainly arsenate and arsenite (Caunette *et al.,* 2012). Arsenic tends to shows a nutrient-like vertical profile in the water column, indicating biological uptake of arsenic by marine phytoplankton along the phosphate transport pathway. In addition to inorganic arsenic (iAs), the methylated arsenic species monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are present in water. Phytoplankton accumulates and methylates the inorganic arsenic (Karadjova *et al.,* 2008), after which phytoplankton organisms are ingested by zooplankton organisms, which also have other arsenic compounds, such as arsenobetaine (AsB) (Caumette *et al.,* 2011). We found a total arsenic level of 0.07 mg Kg^{-1} in

Flaccisagitta enflata, and the all arsenical species investigated by As speciation analysis were undetectable (< LOQ), due to this low content. Interestingly, the isopod *E spinigera* showed a total As content that was more than three times greater, 0.28 mg Kg^{-1}, and a concentration of 0.03 mg Kg^{-1} for the sum of inorganic As species (III+V) was found, while the organic species AsB, DMA and MMA were < LOQ.

The two toxic elements cadmium and lead were an order of magnitude higher in *E spinigera* than in *F. enflata* (Table 1, Fig.3). Cd has no significant physiological role and it is mainly adsorbed on the surface of zooplanktonic debris or fecal pellets during its transportation to bottom waters (Kremling and Pohl, 1989). Pb is known to form colloids in seawater, which can be adsorbed onto planktonic debris (Paimpillil *et al.,* 2010). We previously observed an increase in Pb concentrations in marine organisms with increasing water depths (Battuello *et al.,* 2016), and as such, it is not unexpected to find the highest Pb level (3.07 mg Kg^{-1}) in the benthonic species *E spinigera,* which is a level comparable or lower than previous findings in Mediterranean coastal areas (Rossi and Jamet, 2008). Similarly, vanadium concentrations were higher in *E. spinigera* in this study and in planktons from deep waters (Battuello *et al.,* 2016), while tin, which is usually present as organotin compounds in proximity to harbor areas, as well as to industrial and domestic points of effluent discharge, was higher in *F. enflata* (Table 1, Fig. 3).

Dissolved rare earth elements such as cerium and lanthanum are reported to be present in very low concentrations in open seawater, typically in the order of pg L^{-1} (Wang and Yamada, 2007), but can be bioaccumulated by marine invertebrates, such as zooplankton, and enter the food chain (Palmer *et al.*, 2006). Accordingly, we found low Ce and La concentrations in both species (Fig. 3), but the highest values were registered in the benthonic species *E. spinigera* (0.06 and 0.03 mg Kg^{-1} respectively).

Bioaccumulation factors (BAFs)

Given the wide range of concentrations present in the BAF dataset, values were converted to a log scale to aid visual comparisons (Fig. 4). Our results confirmed the high potential of both species, particularly *E. spinigera*, to be bioaccumulators of metals.

The estimated BAFs were in the following order:

E. spinigera Cu>Cd>Al> Zn> Fe>Mn> Pb>Ni>Co

F. enflata Cu>Al> Fe>Zn> Cd> Mn> Pb>Ni>Co

BAF trends were fairly similar between the two species, but the BAF order of magnitude was quite high in the benthonic species, reflecting a greater availability of metals in the deeper waters and in the seabed sediments. The mechanisms of metal bioaccumulation have been studied in terrestrial isopods, which showed a great capacity to bioaccumulate metals from the environment, especially copper, which concentrated in the hepatopancreas (Wieser *et al.*, 1977). Accordingly, copper was the most accumulated trace element in both species, while cadmium was bioaccumulated at different levels in isopods and chetognaths, being more concentrated in *E. spinigera,* probably reflecting the vertical distribution of dissolved Cd in ocean waters, characterized by a surface depletion and deep water enrichment (Boyle *et al.,* 1976).

Bioaccumulation of a chemical is affected by rates of uptake, metabolism, and elimination, as well as the storage capacity of an organism, and several abiotic and biotic factors affect the bioavailability of metal compounds, e.g. metal speciation, physicochemical parameters of the environment, and biological–physiological properties of the exposed organism (McGeer *et al.*, 2003). Bioaccumulation itself is not an indicator for a toxic response, since only a certain proportion of the total internally-accumulated metal concentration- the body burden - may be metabolically available (Herrmann *et al.,* 2016). However, the bioaccumulation factors clearly reflect the presence and availability of metals in a determined ecosystem and in different habitats, confirmed by our study.

Arsenic

Seawater naturally contains 1–5 µg L^{-1} of total arsenic, which is mainly arsenate and arsenite; in addition to inorganic arsenic (iAs), the methylated arsenic species monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) are present in seawater (Caumette *et al.,* 2012). Arsenic tends to shows a nutrient-like vertical profile in the water column, indicating biological uptake of arsenic by marine phytoplankton along the phosphate transport pathway. Phytoplankton is able to accumulate and methylate the inorganic arsenic (Karadjova *et al.,* 2008), and it contains iAs as the majority of identified arsenic, with methylated arsenic MMA and DMA and arsenosugars as organoarsenic compounds (Caumette *et al.,* 2012). Zooplankton organisms ingest phytoplankton, and other arsenic compounds are found in zooplankton, such as arsenobetaine (AsB) (Caumette *et al.,* 2012). Marine zooplankton contains AsB as a minor compound in herbivorous zooplankton and as a major compound in carnivorous zooplankton (Shibata *et al*., 1996). We found a very low total arsenic level in *Flaccisagitta enflata* (0.07 mg Kg^{-1}) and the arsenical species that could be detected by As speciation analysis (water soluble species) were undetectable (< LOQ). Only one study has investigated the presence of As in Sagittoidea (Shibata *et* al., 1996, Japan Sea) and found arsenobetaine to be the dominant arsenic species. The authors suggested that the arsenic compounds in zooplankton reflect their feeding habit; carnivorous species accumulate arsenobetaine, while herbivorous species accumulate arsenosugars.

Other studies performed on carnivorous zooplankton, such as amphipods and Antarctic krill collected in the ocean always found arsenobetaine as a major compound (Caumette *et al.,* 2012). Arsenobetaine is described as the only non-toxic arsenic compound and its presence in organisms is assumed to be the result of a detoxification process, but recent studies seem to support the function of AsB in an osmolytic role, suggesting a relationship between salinity and AsB accumulation in marine organisms (Clowes *et al*., 2004; Larsen and Francesconi, 2003).
Interestingly, in our findings, *E. spinigera* showed a total As content (0.28 mg Kg^{-1}) more than three times higher than *Flaccisagitta enflata (*0,07 mg Kg^{-1}), and 0.03 mg Kg^{-1} of iAs (III+V) was found in the isopod. Among the organic As compounds, traces of MMA were found (0.01 mg Kg^{-1}), while AsB and DMA levels were < LOQ.
Experimental studies have shown that arsenic can be accumulated from water, food or sediment (Maher and Butler, 1988). The isopod *E. spinigera* is a benthic species living mostly on marine sediments, which are the largest geochemical reservoir of arsenic, containing in excess of 99.9% of the element (Maher and Butler, 1988). Strong correlations of the concentration of As in tissues of benthic organisms and in sediments has demonstrated the ability of organisms to use a fraction of particulate-bound arsenic; both arsenic (V) and arsenic (III) are found in the interstitial waters of sediments and bacterial reduction may mediate the redox chemistry of arsenic in sediments (Maher and Butler, 1988). We therefore suggest that the different As levels between the two carnivorous invertebrates, *Flaccisagitta enflata* and *Euridyce spinigera* are due to a different exposure to different habitats, i.e. water columns and sediments, and the presence of iAs in isopods may be related to its benthic habit, living in close association with sediments.

CONCLUSIONS

The widespread development and application of bioindicators has been in place since the 1960s, and bioindicators are commonly used because environmental practitioners need cost-effective tools that are easy to measure and which provide results that can be clearly communicated to decision makers.
The effectiveness of isopods as excellent bioindicators and bioaccumulators of heavy metals in biomonitoring programs is supported by scientific literature (Longo *et al.,* 2013; García-Hernández *et al.*, 2015), since they are abundant and widely distributed. Soft-bodied forms such as chaetognaths are also important members of the zooplankton for which comparable information is largely lacking. The analyzed species share the same feeding behavior as they are exclusively predaceous, and are at the top of the zooplanktonic food web. However, the fact that they have

different habitats- one is hyperbenthic and the other neritic- greatly affects metal bioaccumulation, as shown in this investigation.

The overall objective of bioindicators is to assess the quality of an environment and how it changes over time, but the use of a single species may represent an oversimplification of a complex system. Nonetheless, as recently pointed out by Siddig and coauthors (2016), a considerable number of studies used only a single species to monitor ecosystem changes and quality, and this proportion is increasing over time. Our results support the consideration that no single species can adequately indicate the presence of metals or other contaminants in an ecosystem. Depending upon the specific environment, appropriate bioindicator species or groups of species must be selected. Moreover, in the perspective of utilizing marine organisms as bio-indicators of metal transfer through the marine web chain, it is crucial to consider both their habitat and feeding behavior.

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LEGENDS

Fig. 1

Ligurian Sea (Western Mediterranean): sampling site

Fig. S1

Trace elements in marine seawater (log scale)

Fig. 2

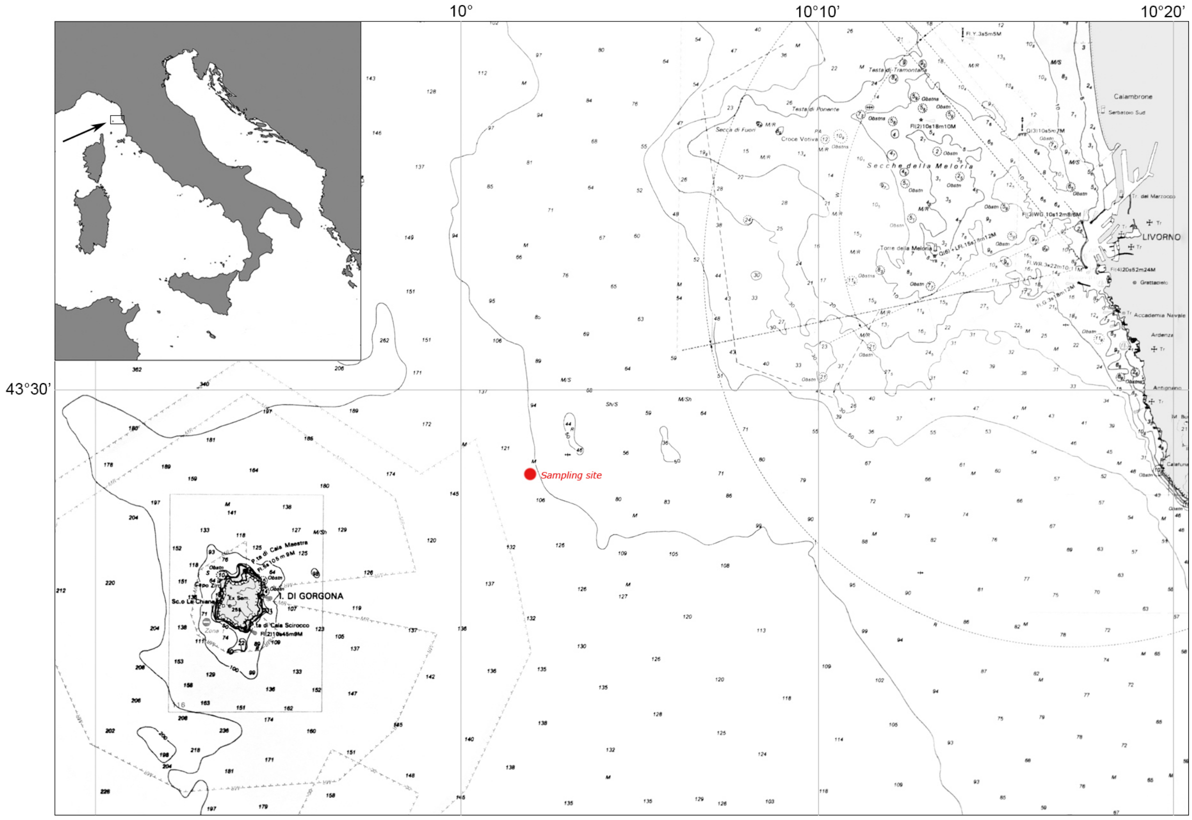
Box-plot diagrams of essential trace elements (mean concentrations ± SD) in *F. enflata* and *E. spinigera*. Metal levels are expressed in mg kg^{-1} wet weight (Y axis).

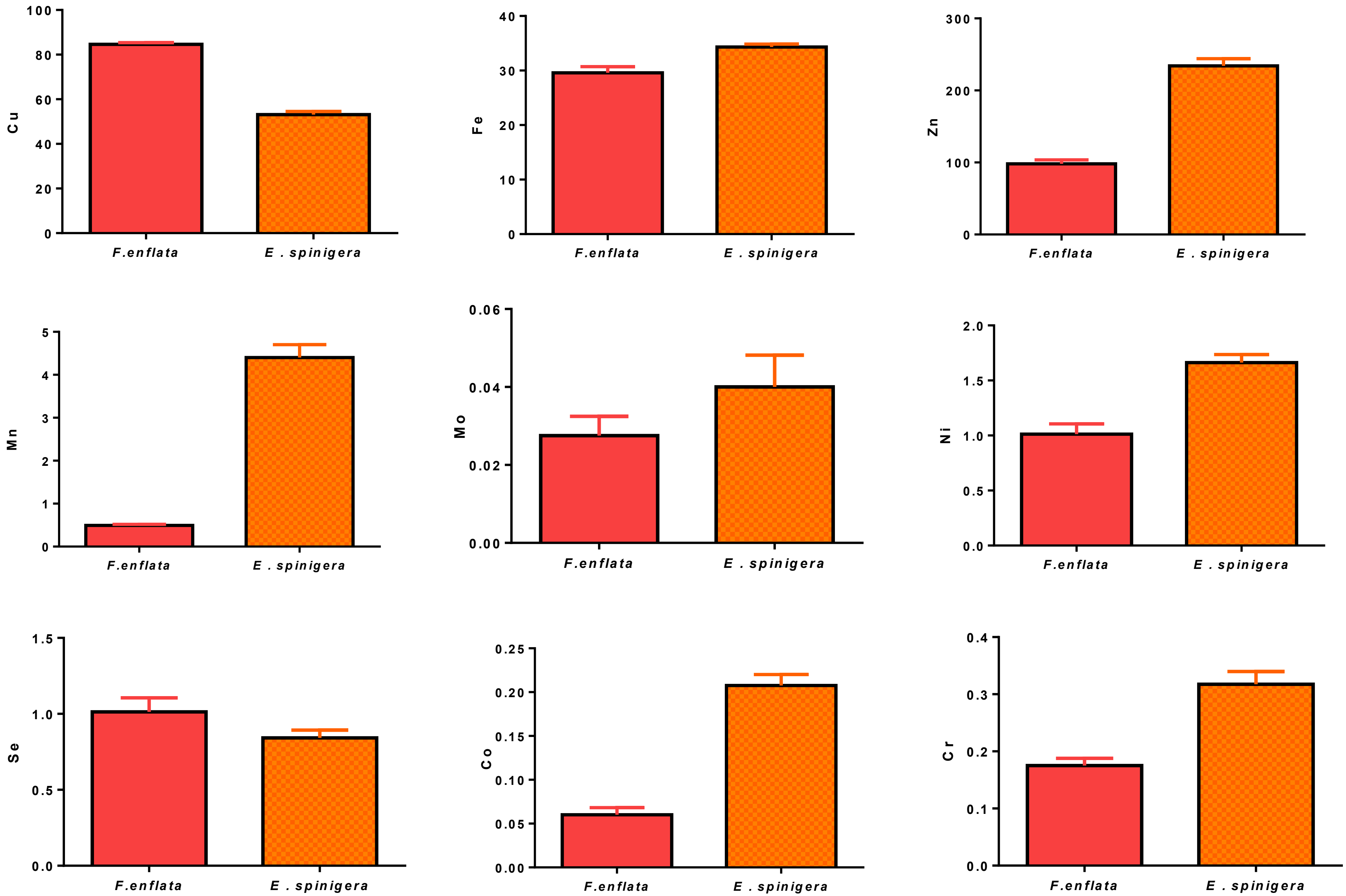
Fig. 3

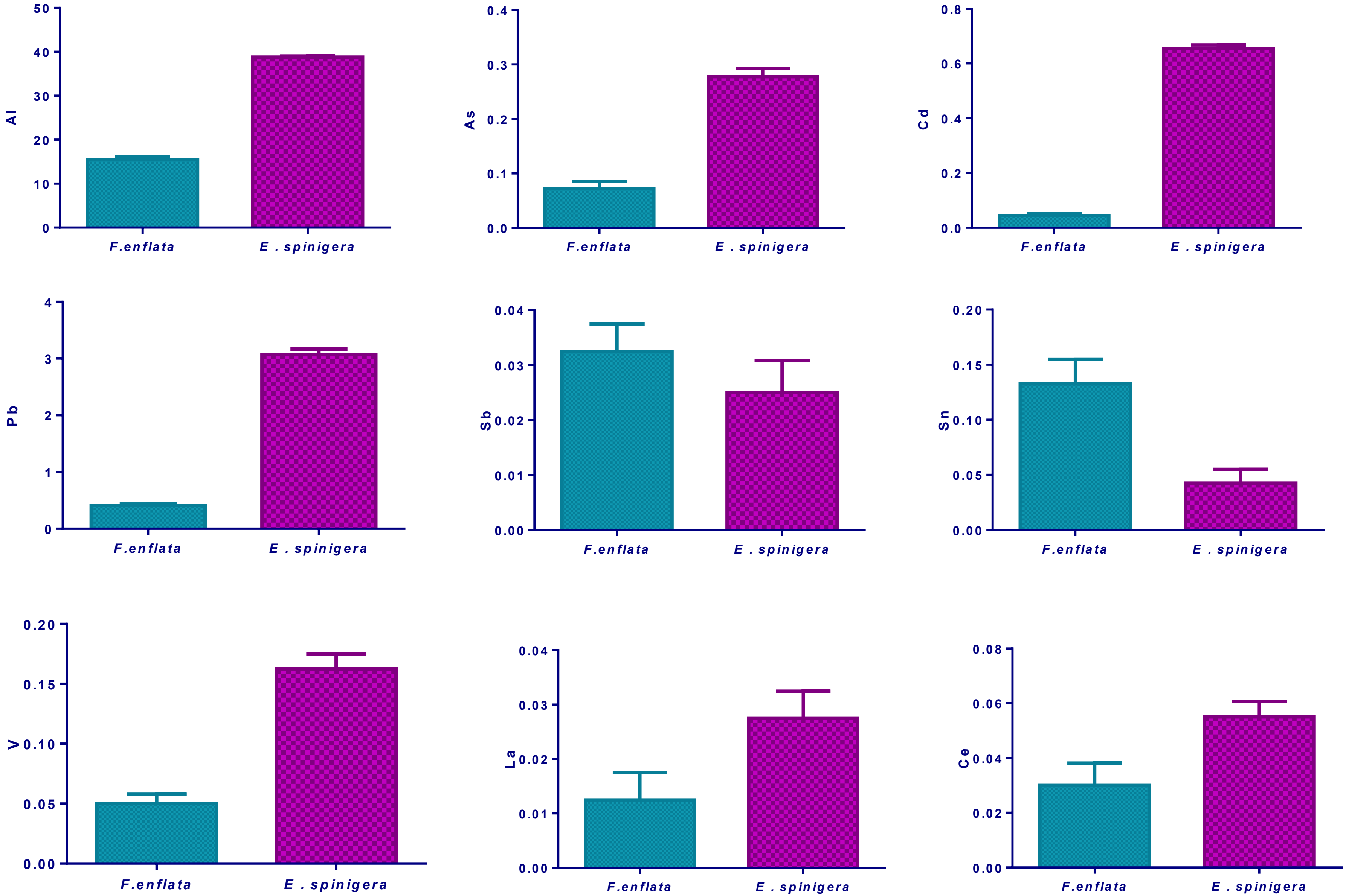
Box-plot diagrams of nonessential trace elements (mean concentrations ± SD) in *F. enflata* and *E. spinigera*. Metal levels are expressed in mg kg^{-1} wet weight (Y axis).

Fig. 4

Bioaccumulation factors (BAFs) in in *F. enflata* and *E. spinigera*.


10°
10°10'
10°20'
43°30'
Sampling site
LIVORNO
I. DI GORGONA
Secche della Meloria
Torre della Meloria
Calambrone
Accademia Navale
Ardenza
Antignano


Cu
Fe
Zn
Mn
Mo
Ni
Se
Co
Cr
F.enflata
E . spinigera


Al
50
40
30
20
10
0
F.enflata
E . spinigera
As
0.4
0.3
0.2
0.1
0.0
F.enflata
E . spinigera
Cd
0.8
0.6
0.4
0.2
0.0
F.enflata
E . spinigera
Pb
4
3
2
1
0
F.enflata
E . spinigera
Sb
0.04
0.03
0.02
0.01
0.00
F.enflata
E . spinigera
Sn
0.20
0.15
0.10
0.05
0.00
F.enflata
E . spinigera
V
0.20
0.15
0.10
0.05
0.00
F.enflata
E . spinigera
La
0.04
0.03
0.02
0.01
0.00
F.enflata
E . spinigera
Ce
0.08
0.06
0.04
0.02
0.00
F.enflata
E . spinigera

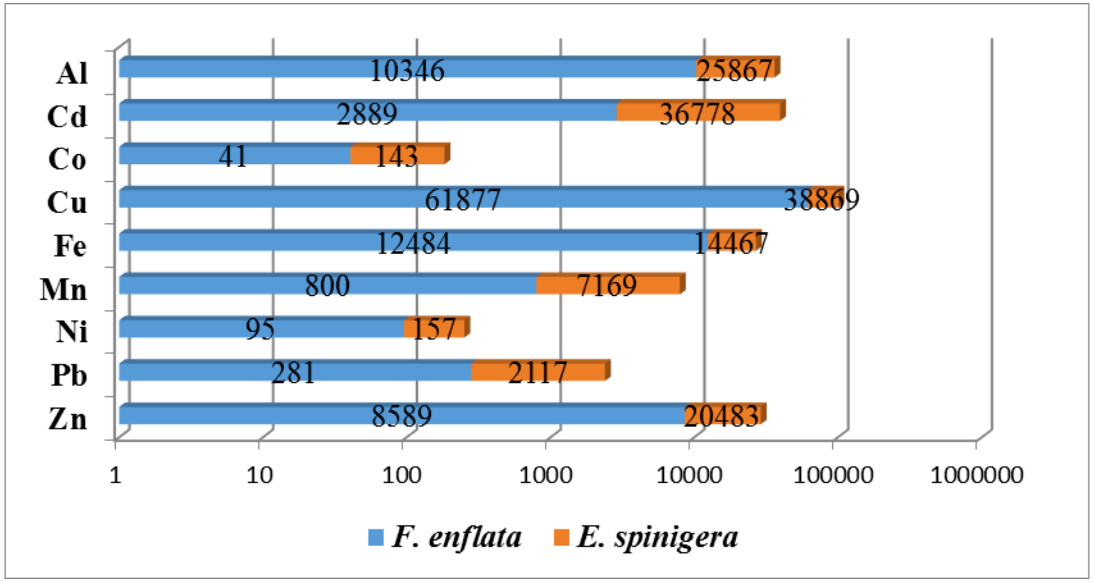

Al
Cd
Co
Cu
Fe
Mn
Ni
Pb
Zn
10346
25867
2889
36778
41
143
61877
38869
12484
14467
800
7169
95
157
281
2117
8589
20483
1
10
100
1000
10000
100000
1000000
F. enflata
E. spinigera

Table 1
Trace elements (mg Kg^{-1} w.w.) in the chetognat *F. enflata* and in the isopod *E. spinigera*

Flaccisagitta enflata

	sample 1 (n= 618 individuals)	sample 2 (n= 602 individuals)	sample 3 (n= 576 individuals)	sample 4 (n= 603 individuals)	mean	SD	min	max
Al	14.53	15.65	15.87	16.02	15.52	0.72	14.53	15.87
As	0.09	0.07	0.07	0.06	0.07	0.01	0.07	0.09
Cd	0.04	005	0.04	0.05	0.05	0.01	0.04	0.05
Ce	003	0.02	0.03	0.04	0.03	0.01	0.02	0.03
Co	0.05	0.06	0.07	0.06	0.06	0.01	0.05	0.07
Cr	0.16	0.19	0.17	0.18	0.18	0.02	0.16	0.19
Cu	84.81	85.64	83.95	83.98	84.60	0.85	83.95	85.64
Fe	30.91	30.02	28.33	29.12	29.60	1.31	28.33	30.91
La	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02
Mn	0.52	0.51	0.48	0.46	0.49	0.02	0.48	0.52
Mo	0.03	0.03	0.02	0.03	0.03	0.01	0.02	0.03
Ni	0.98	1.13	1.03	0.91	1.01	0.08	0.98	1.13
Pb	0.39	0.42	0.44	0.39	0.41	0.03	0.39	0.44
Sb	0.04	0.03	0.03	0.03	0.03	0.01	0.03	0.04
Se	0.39	0.38	0.36	0.35	0.37	0.02	0.36	0.39
Sn	0.11	0.12	0.14	0.16	0.13	0.02	0.11	0.14
V	0.06	0.04	0.05	0.05	0.05	0.01	0.04	0.06
Zn	100.02	99.97	8.99	102.51	98.12	5.78	89.99	100.02

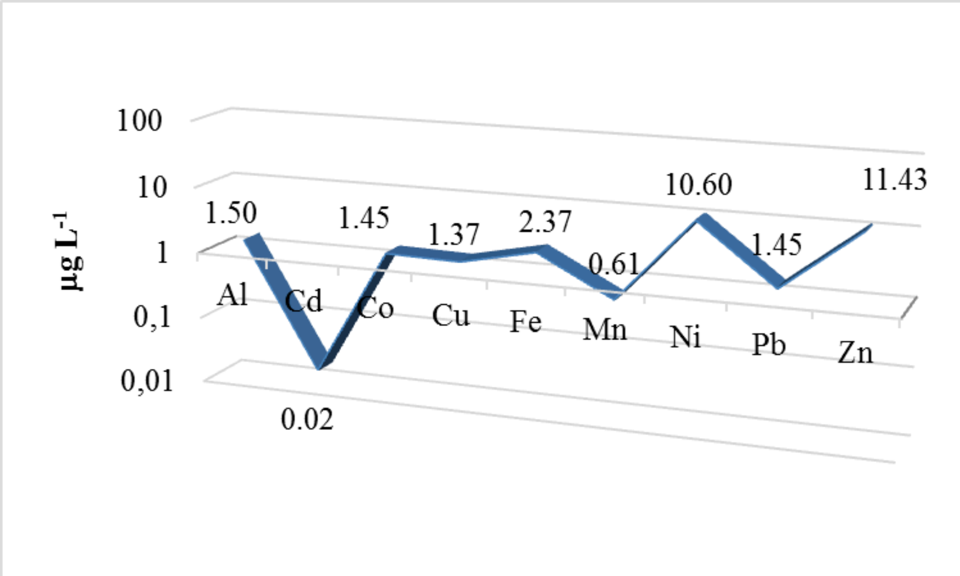
Eurydice spinigera

	sample 1 (n= 297 individuals)	sample 2 (n= 303 individuals)	sample 3 (n= 305 individuals)	sample 4 (n= 328 individuals)	mean	SD	min	max
Al	39.06	38.44	38.95	38.76	38.80	0.33	38.44	39.06
As	0.26	0.29	0.29	0.27	0.28	0.02	0.26	0.29
Cd	0.64	0.67	0.65	0.66	0.66	0.02	0.64	0.67
Ce	0.05	0.05	0.06	0.06	0.06	0.01	0.05	0.06
Co	0.19	0.22	0.21	0.21	0.21	0.02	0.19	0.22
Cr	0.34	0.33	0.29	0.31	0.32	0.03	0.29	0.34
Cu	53.25	55.08	51.89	52.38	53.15	1.60	51.89	55.08
Fe	33.69	33.98	34.67	34.87	34.30	0.50	33.69	34.67
La	0.02	0.03	0.03	0.03	0.03	0.01	0.02	0.03
Mn	4.41	4.69	3.99	4.52	4.40	0.35	3.99	4.69
Mo	0.03	0.04	0.05	0.04	0.04	0.01	0.03	0.05
Ni	1.63	1.75	1.69	1.58	1.66	0.06	1.63	1.75
Pb	2.99	3.21	3.01	3.06	3.07	0.12	2.99	3.21
Sb	0,02	0.03	0.02	0.03	0.03	0.01	0.02	0.03
Se	0.90	0.87	0.79	0.81	0.84	0.06	0.79	0.90
Sn	0.06	0.03	0.04	0.04	0.04	0.02	0.03	0.06
V	0.18	0.15	0.16	0.16	0.16	0.02	0.15	0.18
Zn	245.71	235.08	221.32	233.98	234.02	12.23	221.32	245.71

Table 2. Unpaired t test, comparison between *F.enflata* and *E.spinigera*

Trace element	P value	Summary of P values
Al	P < 0.0001	****
As	P < 0.0001	****
Cd	P < 0.0001	****
Ce	P = 0.0025 (P < 0.01)	**
Co	P < 0.0001	****
Cr	P < 0.0001	****
Cu	P < 0.0001	****
Fe	P < 0.0001	****
La	P = 0.0054 (P < 0.05)	**
Mn	P < 0.0001	****
Mo	P < 0.0041 (P < 0.05)	*
Ni	P < 0.0001	****
Pb	P < 0.0001	****
Sb	P = 0.0972 (P > 0.05)	NS
Se	P = 0.0259 (P < 0.05)	*
Sn	P = 0.0011 (P < 0.05)	**
V	P < 0.0001	****
Zn	P < 0.0001	****

* Significant at the 0.05 probability level
NS not statistically significant



µg L-1
100
10
1
0,1
0,01
1.50
0.02
1.45
1.37
2.37
0.61
10.60
1.45
11.43
Al
Cd
Co
Cu
Fe
Mn
Ni
Pb
Zn

Figure captions

Figure 1

Ligurian Sea (Western Mediterranean): sampling station

Figure 2

Box-plot diagrams of essential trace elements (mean concentrations ± SD) in *F. enflata* and *E. spinigera*. Metal levels are expressed in mg kg^{-1} wet weight (Y axis).

Figure 3

Box-plot diagrams of nonessential trace elements (mean concentrations ± SD) in *F. enflata* and *E. spinigera*. Metal levels are expressed in mg kg^{-1} wet weight (Y axis).

Figure 4

Bioaccumulation factors (BAFs) in in *F. enflata* and *E. spinigera*.