Opportunistic omnivory impairs the use of the Atlantic blue crab *Callinectes sapidus* as a trace metal biomonitor in invaded Mediterranean coastal waters

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ABSTRACT

The contribution of non-indigenous species to the transfer of contaminants in invaded food webs represents an active research area. Here we measured trace metals and CN stable isotopes in five populations of the invasive Atlantic blue crab *Callinectes sapidus* and in baseline bivalve species from Spain, Italy and Greece. They were used to estimate trophic transfer effects and the trophic position and isotopic niche of *C. sapidus*. Maximum trophic transfer effects occurred where the crab showed the largest isotopic niches and highest trophic positions; furthermore, the consistency of trace metal profiles between bivalves and crabs co-varied with the trophic position of the latters. Omnivory may influence the success of an invasive species, but also limit its effectiveness for biomonitoring. However, our results indicated that stable isotopes analysis provides a clarifying background where to cast patterns of contamination of the blue crab as well as of other omnivorous biomonitor species.

1. Introduction

Natural ecosystems are seldom jeopardized by single perturbations; conversely, the superimposition of multiple biotic and abiotic stressors is almost the norm, with synergistic and only rarely additive responses from the biota (Galic et al., 2018; Pirotta et al., 2022). Chemical pollution and biological invasions are among the anthropogenic threats (listed in e.g., Salafsky et al., 2008; Gelcich et al., 2014) involved in the alteration of the functionality of coastal marine ecosystems (Johnston et al., 2015). Bioinvaders can affect biogeochemical pools and fluxes of energy and materials (including pollutants), and thereby alter the structure and functions of ecosystems (Ehrenfeld, 2010; Corrales et al., 2020). Contaminants such as trace metals can cause impairment of biological functions even at low concentrations, reducing the abundance of the most sensitive species while facilitating opportunistic or tolerant taxa (Johnston and Roberts, 2009; Mayer-Pinto et al., 2010). In addition, persistent pollutants and xenobiotics can bioaccumulate (i.e. when their uptake rate by an organism from the environment and/or from the diet exceeds excretion or elimination rates) or biomagnify (i.e., when their prey-to-consumer transfer rate exceed the excretion or elimination

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rates of the consumer) along food chains up to top predators, including economically valuable species (Gray, 2002; Romero-Romero et al., 2017).

Noticeably, the interaction between bioinvaders and chemical contaminants in aquatic environments has been primarily addressed focusing on the use of invasive species for biomonitoring purposes (e.g., Annabi et al., 2018; Mancinelli et al., 2018; Tzafriri-Milo et al., 2019; Squadrone et al., 2020) and on the role of contaminants in facilitating invasions (McKenzie et al., 2012; Kenworthy et al., 2018; Osborne and Poynton, 2019). In contrast, lesser efforts have been made to elucidate the complex and non-univocal effects of invaders on the mobilization of contaminants in aquatic food webs (Eagles-Smith et al., 2018; Flood et al., 2020; see also Schiesari et al., 2018). This is particularly evident for marine and transitional environments (Boyd et al., 2018; Ostrowski et al., 2021), while a number of freshwater investigations have provided contradictory evidence of how the re-wiring of food webs due to single- or multi-species invasions affects the transfer of contaminants across trophic levels (DeRoy and MacIsaac, 2020; Barst et al., 2020; Brown et al., 2022; Rennie et al., 2024 and literature cited).

In the present study, we focused on the Atlantic blue crab *Callinectes sapidus* Rathbun 1896 (blue crab hereafter), a portunid brachyuran originating from the western Atlantic coasts from the Gulf of Maine to Argentina (Millikin and Williams, 1984; Johnson, 2015; Cesar et al., 2003). The species was introduced in Europe in 1901 and appeared in 1947 in the Mediterranean Sea (but probably as early as 1935: Nehring, 2011). It is currently included in the list of the 100 most invasive species (Streftaris and Zenetos, 2006), and recognized as the cause of the consistent decline of several invertebrate and vertebrate species (Clavero et al., 2022).

During the last decade, the blue crab has spread almost ubiquitously in the Black Sea and in the eastern and central Mediterranean Sea, where it is currently expanding in the west and south of the basin (Mancinelli et al., 2017b, 2021; Bardelli et al., 2023; Castriota et al., 2024) as well as in neighbouring freshwater habitats (Scalici et al., 2022). Blue crabs are benthic feeders (Hines, 2007) and their life history and behavior place them in direct contact with environmental contaminants via the water column, sediments, and prey (Reichmuth et al., 2010; Parmar et al., 2016). Additionally, laboratory studies have repeatedly demonstrated extensive accumulation of water- and food-borne xenobiotics (Hale, 1988; Mothershead II et al., 1991; Reichmuth et al., 2010; Evans et al., 2000), and several metal-binding metallothioneins have been identified in the tissue of blue crabs (Brouwer and Lee, 2007). Hence, the species has been proposed as a biomonitor species (i.e., a species that, by accumulating pollutants in its tissues, may be used to assess the bioavailability of the pollutants themselves in the surrounding habitat; Rainbow, 1995) in estuaries and other transitional habitats (Weinstein et al., 1992; Mutlu et al., 2011; Salvat-Leal et al., 2020).

Here we assessed the concentration of 13 trace metals (B, Ba, Cd, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sr, V, and Zn) in blue crabs from five coastal systems in Spain, Italy, and Greece. In invaded European waters, most of the information on trace metal contamination are available mainly for blue crab populations from the Levantine sector of the Mediterranean Sea, and for a limited set of trace metals (e.g., Türkmen et al., 2006; Mutlu et al., 2011; Genc and Yilmaz, 2015; but see Zotti et al., 2016; Salvat-Leal et al., 2020; El Qoraychy et al., 2023 for recent exceptions). To provide a first evaluation of the effectiveness of the species for large-scale biomonitoring in Mediterranean waters, we compared its trace metal contents with bivalves. Since the early 1970s, bivalves have been used to monitor coastal ecosystems, as they accumulate inorganic and organic pollutants and result more informative of environmental contamination conditions than other non-living matrices (Zuykov et al., 2013; Beyer et al., 2017). In both native and invaded benthic habitats, the blue crab is a voracious predator of mussels, clams, and other bivalves (Seed, 1980; Micheli, 1997; Seitz et al., 2001; Prado et al., 2020; Meira et al., 2024), a trophic preference recently testified by the dramatic effects observed in Italian lagoons (ARPAV-ISPRA, 2023).

However, the diet of the species is far more diversified and omnivore, as it can opportunistically include a diverse set of vegetal and animal items such as plant material, crustaceans including conspecifics, gastropods, and fish (Hines, 2007; Mancinelli et al., 2017a; Rady et al., 2018; Kampouris et al., 2019; Prado et al., 2021).

Accordingly, in the present study we tested two alternative scenarios. The first assumed a direct trophic transfer of trace metals from bivalves to blue crabs, ultimately reflecting in bioaccumulation or even biomagnification phenomena in the latter. Alternatively, we hypothesized that the omnivory of the crab and thus its opportunistic consumption together with bivalves of multiple and differently-contaminated animal and vegetal resources would represent a confounding factor actually hampering its effectiveness as a biomonitor species.

To verify these hypotheses, along with the concentrations of trace metals we measured carbon and nitrogen stable isotopes in both *C. sapidus* and bivalves. Stable isotope analysis (SIA hereafter) has gained in the last decades a huge popularity for the study of invaded marine and coastal food webs (see Mancinelli and Vizzini, 2015 for a recent review) and, as it consents to relate contaminant concentrations in consumers to their trophic habits, it has long been acknowledged as a powerful tool to investigate accumulation and magnification phenomena along food chains (Jardine et al., 2006). In addition, SIA enables for robust, regional-scale analyses of species' dietary habits (e.g., Mancinelli, 2012a; Pethybridge et al., 2018; Figgener et al., 2019; Mancini et al., 2021, 2022). Specifically, here δ^{13}C and δ^{15}N values of blue crabs were used to estimate their isotopic niche and, taking bivalves as baseline species, their trophic position, to verify the occurrence of variations in feeding habits across the five locations and whether they related with changes in trace metal profiles.

2. Materials and methods

2.1. Study sites

The study was performed in 2016 in five Mediterranean coastal systems located in Spain, Italy, and Greece and characterized by established *Callinectes sapidus* populations (see Fig. 1 and Table S1 in the online supplementary material for further details).

In Italy, sampling operations were carried out in the Lesina and Acquatina lagoons (SE Italy, Fig. 1). The Lesina Lagoon is located on the northern coast of the Gargano Promontory (41°52′57.09″N, 15°26′34.71″E); it is approximately 22 km long and 3.5 to 1.5 km wide (52 km² total area), with depths ranging between 0.5 and 1.7 m (Mancinelli and Rossi, 2001; Spagnoli and Andresini, 2018). Two channels guarantee continuous water exchanges with the Adriatic Sea, while perennial and intermittent freshwater inputs occur in the southeastern sector of the lagoon. The Acquatina Lagoon is located in the Salento Peninsula (40°26′34.50″ N, 18°14′14.16″ E); The water body is approximately 1.9 km long with a surface area of 0.45 km²; depths range between 0.5 and 1.7 m (1.2 m average depth; Mancinelli, 2012b; Pagliara and Mancinelli, 2018). Freshwater inputs are located in the northward sector of the basin, which is connected with the Adriatic Sea by a mouth (Fig. 1).

In Spain, samples were collected in a coastal area in the southern sector of the Valencia Gulf between the towns of Cullera (0°15′26.29″W, 39° 8′6.18″N) and Oliva (0° 4′41.29″W, 38°55′36.24″N; Fig. 1). The coastal environment is open to the Balearic Sea with a number of river mouths, and is generally low and sandy (Sospedra et al., 2018). In Greece, samples were collected in the Pogonitsa Lagoon (NW Greece, 38°57′53.04″N, 20°48′52.15″E) and in Loudias Bay (NE Greece, 40°30′7.36″N, 22°40′51.33″E; Fig. 1). The Pogonitsa Lagoon is located on the Ionian Sea in the south-western sector of the Amvrakikos Gulf. The lagoon has a surface of approximately 0.45 km², an average depth of 1.2 m, with sandy vegetated bottoms, and it is connected to the Amvrakikos Gulf by two channels (Katselis and Koutsikopoulos, 2017). Loudias Bay is situated in the inner Thermaikos Gulf (northern Aegean
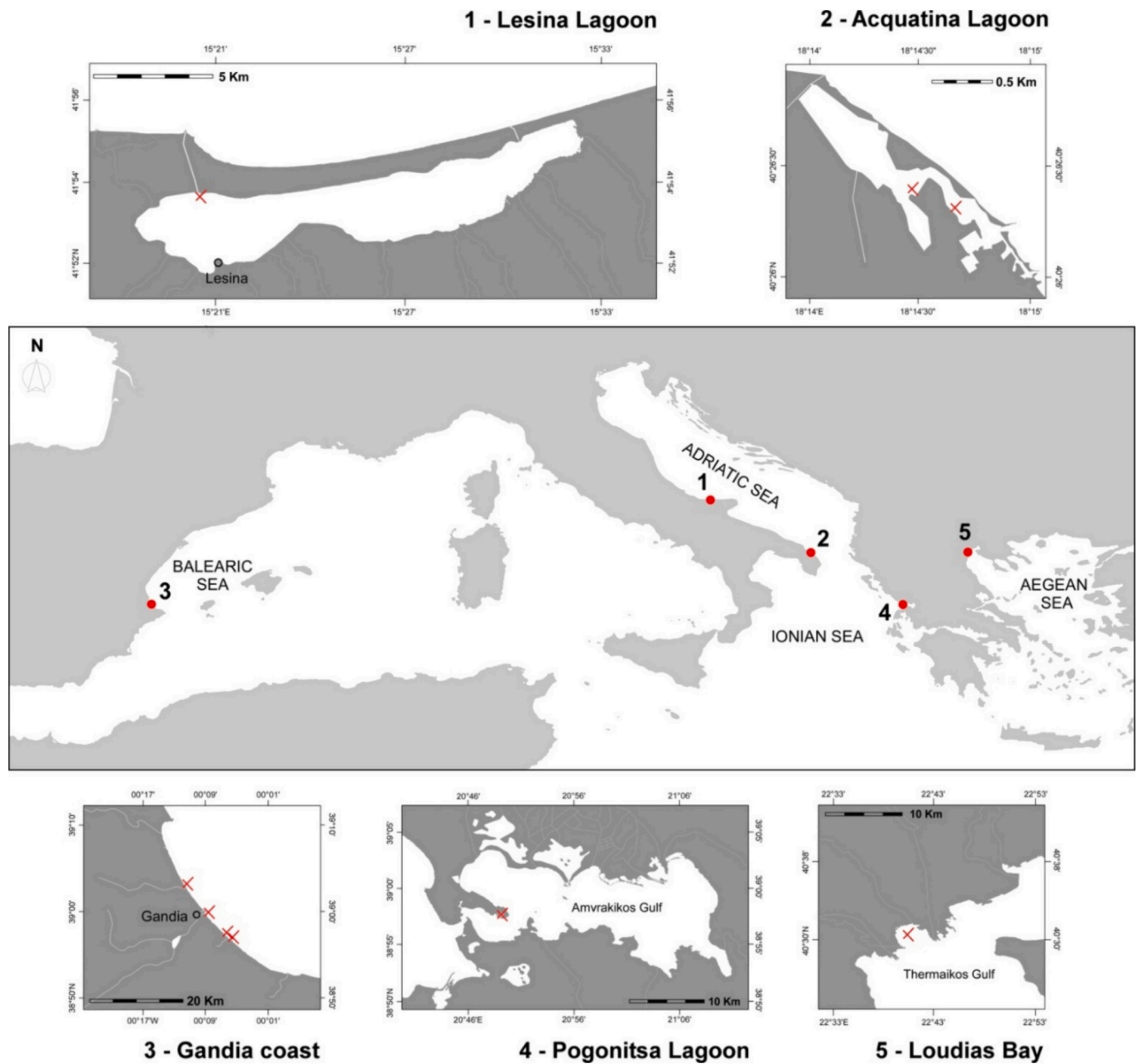
Fig. 1. The five coastal habitats included in the study. Red crosses in the inserts indicate the sampling locations where *Callinectes sapidus* and bivalves (i.e. *Arcuatula senhousia* for Lesina Lagoon and *Mytilus galloprovincialis* for the remaining locations; see text and Table 1 for further details) were collected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sea). The bay is open to the sea (Fig. 1) has a surface of approximately 15 km^2 and depths up to 20 m, and receives freshwater inputs from Aliakmon, Axios, and Loudias rivers (Poulos et al., 2000; Catsiki and Florou, 2006).

2.2. Sample collection

At each of the five locations 15 to 24 blue crabs were captured using crab traps or other netting devices in summer 2016 (Table 1; see Table S1 for additional details). After collection, specimens were transferred alive to the laboratory in refrigerated containers (4 °C), where they were sexed and had the cephalothorax width (i.e., the distance between the two outermost lateral spines; CW hereafter) measured with a caliper to the nearest 1 mm. Subsequently, crabs were euthanized by thermal shock (−80 °C for 10 min) to avoid artefacts on elemental

Table 1
Number of bivalve and *Callinectes sapidus* specimens collected at the five study locations. Taxonomic information on the bivalve species used as baseline for chemical analyses are included in parentheses.

Location	Bivalves	*Callinectes sapidus*
Lesina	10 (*Arcuatula senhousia*)	24
Acquatina	8 (*Mytilus galloprovincialis*)	20
Gandia	6 (*Mytilus galloprovincialis*)	19
Pogonitsa	6 (*Mytilus galloprovincialis*)	19
Loudias	6 (*Mytilus galloprovincialis*)	15

and isotopic determinations (Atwood, 2013). After crab collection, 6 to 10 individuals of locally dominant bivalve taxa were collected at each sampling location by hand (Table 1). *Mytilus galloprovincialis* was

sampled at all the locations except Lesina Lagoon, where the non-indigenous Asian date mussel *Arcuatula senhousia* has recently outnumbered other native bivalves in the area (Scirocco and Urbano, 2018). Active predation of the selected species by the blue crab was confirmed by multiple evidences including repeated visual observations (all locations), analysis of stomach contents (Gandia; Gil Fernández, 2018), and collection of crushed specimens showing unequivocal signs of predation (Acquatina and Lesina; see Blundon and Kennedy, 1982 for examples on mytilids).

After collection, bivalves were transferred alive to the laboratory where they had their shell length measured in mm using a caliper before being euthanized as already described.

2.3. Chemical analyses

Glassware and other equipment used to prepare samples for stable isotope and elemental analyses were preventively kept in diluted ultrapure HNO_3 65 % for 24 h, rinsed with Milli-Q water (Millipore Corp., Bedford, MA), and dried under a laminar flow hood.

For each crab, muscle tissues were removed from both claws using a ceramic scalpel, stored in falcon tubes, and randomly assigned to two groups. Samples were subsequently oven dried (40 °C until constant weight) and powdered using a mortar and pestle. The first group of samples was used for carbon (C) and nitrogen (N) total content and stable isotope analyses. To this end, subsamples of known dry weight were pressed into Ultra-Pure tin capsules (Costech Analytical Technologies) and analyzed using an Elemental Analyser connected with an Isotope Ratio Mass Spectrometer (Thermo Scientific Flash EA 1112 and IRMS Delta Plus XP). Concentrations of total C and N were reported as g kg^{-1} tissue dry weight. Isotopic values were expressed in conventional δ notation (as ‰) in relation to international standards (PeeDee Belemnite and atmospheric N_2 for C and N, respectively) using the formula

$$\delta X = \left[\left(R_{sample} / R_{standard} \right) - 1 \right] \times 10^3$$

where *X* is ^{13}C or ^{15}N and R is the corresponding $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio. Analytical precision based on the standard deviation of replicates of internal standards (International Atomic Energy Agency IAEA-NO-3 for $\delta^{15}N$ and IAEA-CH-6 for $\delta^{13}C$) was 0.1 ‰ for both $\delta^{13}C$ and $\delta^{15}N$.

The total concentration of the trace metals boron, barium, cadmium, chromium, copper, iron, lithium, manganese, nickel, lead, strontium, vanadium, and zinc (for brevity all are subsequently referred to as "metals") were determined on the second group of samples by wet digestion according to Raab et al. (2005). The procedure is described in detail elsewhere (Zotti et al., 2016); in brief, subsamples of known dry weight were mixed with 4 ml H_2O_2 and 6 ml HNO_3 at 180 °C for ten minutes using a microwave digestion system (Milestone START D). They were consequently cooled, diluted with ultrapure water to a final volume of 25 ml, filtered through Whatman No. 42 filter papers, and measured for metal contents using an inductively coupled plasma atomic emission spectrometer (ICP-AES; Thermo Scientific iCap 6000 Series). Results were expressed as mg kg^{-1} tissue dry weight; the minimum detection limit was 0.001 mg kg^{-1} for all metals.

For bivalves, the foot was dissected from each individual, dried, powdered, and analyzed for trace metals and stable isotopes adopting procedures identical to those described for *C. sapidus* tissues.

2.4. Data analysis

Values in the text are expressed as mean ± 1 SE if not otherwise specified. Morphometric and chemical data were preliminarily checked for normality (Shapiro-Wilk test) and homoscedasticity (Cochran's C-test); when necessary, they were square root- or log(x + 1)-transformed to meet the required assumptions.

We used Non-metric Multidimensional scaling (nMDS) ordination to explore multivariate patterns of variation in the metal contents of bivalves and crabs across locations. nMDS plots were produced using similarity matrices based on Euclidean distances estimated on log(x + 1)-transformed and *Z*-scaled concentration data. In general, a Type I (sequential sum of squares) permutational multivariate analysis of variance (PERMANOVA; Anderson, 2005) with 9999 unrestricted permutation of raw data was subsequently used to verify the effect on metal contents of the factor "location" (fixed, five levels), and the continuous covariate "size" as estimated by individual shell lengths in bivalves and individual cephalothorax widths in crabs. For the latter, the PERMANOVA design included the fixed factor "sex" to check for differences between males and females. *P* values for PERMANOVA tests were calculated using Monte Carlo permutations [*P*(MC) hereafter]. A Canonical Analysis of Principal Components (CAP) was further used to model changes in metal concentrations in individual crabs against their cephalothorax width across the five locations. Univariate comparisons were performed using Pearson product-moment correlation analyses and parametric ANOVAs followed by post-hoc Tukey's HSD tests. Statistical significance was evaluated at α = 0.05; when tests were reiterated, α values were always adjusted by performing sequential Benjamini-Hochberg corrections for multiple tests to reduce the risk of a type-I error (Benjamini and Hochberg, 1995).

Spearman rank correlation coefficients were eventually used to assess the congruence in metal contents between blue crabs and bivalves, as inter-specific comparisons performed using absolute metal concentrations may result biased by mechanistic, taxon-specific differences in the bioaccumulation of metals (Rainbow, 2018; McCue et al., 2020). Furthermore, to perform a general comparison of total metals content between bivalves and blue crabs at the different sampling locations we calculated the metal pollution index (MPI) according with the equation:

$$MPI = \left(Cf_1 \times Cf_2 \ldots \times Cf_k \right)^{1/k}$$

(Usero et al., 1997) where Cf_k = concentration value of the kth metal. When the measured contents were under the detection limits of the instrumentation (see previous paragraph), we assumed a concentration of 0.0005 mg kg^{-1}. Eventually, trophic transfer factors TTF_i were measured as the ratio of the concentration of each metal *i* in crabs to that in bivalves (DeForest et al., 2007). TTF_i were not calculated when metals in bivalves or in crabs were under the detection limits.

Univariate and multivariate procedures identical to those described for metals were adopted to analyse isotopic data. Noticeably, the majority of crabs showed C:N ratios <3.5; conversely, bivalves from Lesina and Acquatina, and to a minor extent from Loudias, had ratios exceeding 3.5 (Table S2, online supplementary material). Lipids are depleted in ^{13}C compared to proteins and carbohydrates and can significantly bias $\delta^{13}C$ estimations when samples have a lipid content >5 %, corresponding to a C:N ratio > 3.5 (Post et al., 2007). Hence, here the $\delta^{13}C$ of individuals characterized by a C:N value >3.5 was normalized using the mathematical procedure proposed by Post et al. (2007).

We used individual $\delta^{13}C$ and $\delta^{15}N$ values of crabs from each location to measure in $‰^2$ their isotopic niche in terms of *standard ellipse area* (SEA; Jackson et al., 2011). Given the different number of specimens per location (Table 1) a sample size-corrected version of SEA was calculated (SEA$_c$ hereafter), representing the core (40 %) isotopic niche area and allowing for robust comparisons across samples of varying numerosity (Jackson et al., 2011). For the sake of completeness, we also estimated four additional metrics originally described by Layman et al. (2007), i.e., *nitrogen range* (NR) and *carbon range* (CR) as indicators of the total nitrogen and carbon range exploited by each population, the *mean distance to centroid* (CD) as a measure of population trophic diversity, the *standard deviation of nearest neighbour distance* (SDNND) as an index of population trophic evenness. SEA$_c$ estimations were used for illustrative purposes; for statistical comparisons we calculated the Bayesian equivalent SEA$_B$ of SEA$_c$ (Jackson et al., 2011) using 100,000 posterior iterations of SEA$_c$ to compute credible intervals. Pairwise comparisons were

performed by calculating the probability that a population at location i had a SEA$_B$ posterior estimate differing from that calculated at location k with a probability of at least 95 % (Jackson et al., 2011). Since SEA estimations are independent from any inter-population difference in the isotopic baseline, we performed no correction for possible baseline shifts (e.g., Magioli et al., 2019).

The trophic position of crabs at the five locations (TP hereafter) was estimated implementing a Bayesian approach comparing the δ^{13}C and δ^{15}N values of the crabs themselves and those of bivalves, the latter adopted as isotopic baselines. This approach explicitly takes into consideration individual variability and propagation of sampling error (trophic enrichment factors, and measurements of baselines and consumers) in the modelling procedure and posterior estimates of parameters (Quezada-Romegialli et al., 2018). Specifically, here we used a single-baseline model with 5 parallel chains and 100,000 adaptive iterations. *M. galloprovincialis* and *A. senhousia* are filter feeders with diets mainly relying on phytoplankton and suspended particulate matter (Ezgeta-Balić et al., 2014); hence, a trophic position $\lambda = 2$ was assumed for both taxa. Given the omnivorous trophic habits of the blue crab, calculations were carried out adopting trophic enrichment factors of 0.27 ± 2.44 for δ^{13}C and 2.57 ± 1.72 for δ^{15}N (mean $\pm$ 1SD, $n = 27$ and 65, respectively). They were estimated as the average of published fractionation factors for aquatic crustaceans (including *C. sapidus*) feeding on both vegetal and animal items (Mancinelli and Bardelli, 2022; Mancinelli et al., 2022), and more consistent with other literature syntheses focused on crustaceans (Vanderklift and Ponsard, 2003; Mancinelli, 2012a; Carrozzo et al., 2014). Pairwise comparisons on posterior distributions of TP estimates were carried out as previously described for isotopic niche metrics.

All statistical procedures were implemented in the R package (R Development Core Team, 2023). Specifically, nMDS and PERMANOVA analyses were performed using the *metaMDS* and and *adonis* functions of the *vegan* library, respectively (Wood and Scheipl, 2020); *C. sapidus* isotopic niche metrics were calculated using the package *SIBER* (Jackson and Parnell, 2023), while *tRophicPosition* (Quezada-Romegialli et al., 2022) was adopted to estimate trophic positions.

3. Results

3.1. Trace metals in bivalves

The mean shell lengths of bivalves collected at each sampling location varied between 22.9 and 33.4 mm (Table 2), with significant differences in size observed only between *Arcuatula senhousia* from Lesina and *Mytilus galloprovincialis* from Gandia (HSD test after 1-way ANOVA, $P = 0.01$; $P > 0.05$ for the remaining comparisons).

Metal contents showed a marked pattern of spatial variation (Fig. 2; see Table S3 in the online supplementary material for mean concentrations $\pm$1SE), and significant, size-independent differences were confirmed by PERMANOVA (factor "location": Pseudo-$F_{4,31} = 16.5$, P (MC) $= 0.0001$; continuous covariate "size", Pseudo-$F_{1,31} = 3.3$, $P = 0.08$). Pairwise comparisons testing for inter-site differences were likewise significant [maximum P(MC) $= 0.03$ for the comparison Gandia vs. Loudias]. Similarly remarkable among-location variations were observed in MPI indices (Fig. 3; 1-way ANOVA, $F_{4,31} = 15.9$, $P < 0.0001$). Lesina showed the highest index (10.8), significantly different from those characterizing the remaining locations with the exception of Pogonitsa (HSD test, $P = 0.32$). Gandia, Pogonitsa, and Acquatina showed similar (HSD tests, $P > 0.05$ for all bivariate comparisons) intermediate values ranging between 4.1 (Acquatina) and 7.7 (Pogonitsa), while the MPI estimated for Loudias (0.9 ± 0.1) was significantly lower than those determined in other locations (Fig. 3; HSD tests, max $P = 0.003$ for the comparison Loudias vs. Acquatina).

At a single-metal level, significant differences were generally observed (1-way ANOVAs; P always <0.05); Cd, Fe, Li, Pb, V in *A. senhousia* from Lesina and Ba, Cr, Cu, Ni, Zn in *M. galloprovincialis*

Table 2

Summary of information on the sex (F = females, M = males), number (in square brackets), and size (CW = mean carapace width, in mm; SE in round brackets, range in italics) of *Callinectes sapidus* specimens collected at the five study locations. Taxonomic information on the bivalve species used as baseline for chemical analyses are included (Mg = *Mytilus galloprovincialis*. As = *Arcuatula senhousia*), together with details on the size (SL = shell length, in mm; SE in round brackets, range in italics) of the specimens.

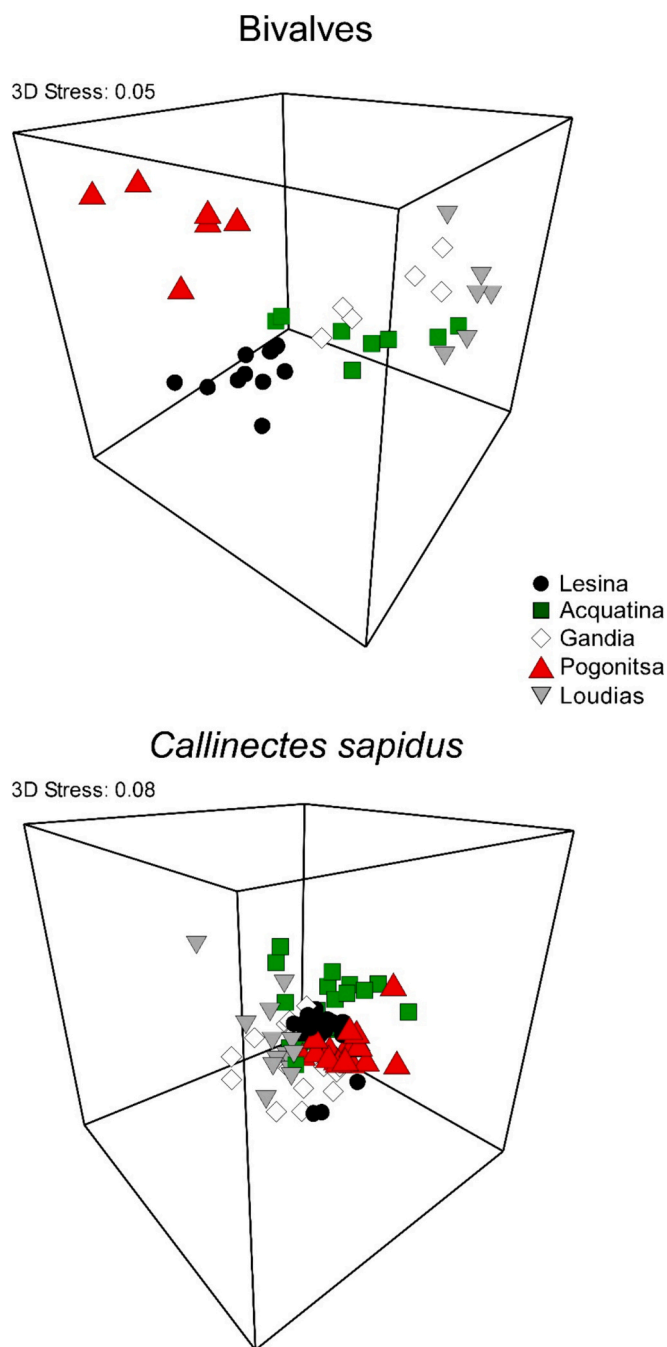| Location	Bivalves			*Callinectes sapidus*		
	Taxon	SL (mm)		Sex	CW (mm)	
Lesina	As	22.9 (1.5)	*18–32*	F [12]	152.2 (6.3)	*104–175*
				M [12]	131.7 (5.3)	*102–154*
				Total	141.9 (4.6)	
Acquatina	Mg	29.1 (3.1)	*18–39*	F [10]	153.6 (8.6)	*101–183*
				M [10]	170.2 (4.3)	*143–196*
				Total	161.9 (5.1)	
Gandia	Mg	33.4 (0.5)	*32–35*	F [12]	141.8 (9.2)	*99–186*
				M [7]	97.4 (6.9)	*70–128*
				Total	125.5 (8.1)	
Pogonitsa	Mg	26.7 (1.1)	*23–30*	F [10]	136.4 (4.7)	*122–167*
				M [9]	172.3 (3.8)	*158–192*
				Total	153.4 (5.2)	
Loudias	Mg	29.9 (3.1)	*19–35*	F [7]	132.1 (7.9)	*99–153*
				M [8]	116.9 (9.5)	*81–152*
				Total	124.1 (6.4)	

from Pogonitsa showed significantly higher contents than the remaining locations (HSD tests, P always <0.05; see also Table S3, online supplementary material). *M. galloprovincialis* from Acquatina showed peak concentrations only for B and Sr, with Cd and Li contents comparable to the maximum values observed in *A. senhousia* from Lesina (HSD tests, $P > 0.05$ for both comparisons). In contrast, Loudias generally showed the lowest metal contents with the exception of Cd, V, and Zn, followed by Gandia (HSD tests, P always <0.05); noticeably, Ni content was under the detection limits at both Loudias and Acquatina (Table S3).

3.2. Trace metals in blue crabs

A total of 97 adult crabs were analyzed, 51 females and 46 males (Table 2). Their carapace widths varied significantly among locations as well between sexes (2-way ANOVA, interaction factor "location×sex", $F_{4,87} = 7.6$, $P < 0.0001$). Males from Acquatina and Pogonitsa were significantly larger than those from the remaining locations (HSD tests, P always <0.05), while negligible differences were observed in the size of females (min $P = 0.8$ for Lesina vs. Loudias). Furthermore, males from Gandia were significantly smaller than females ($P = 0.001$), while negligible sex-related differences occurred for the remaining locations (min $P = 0.09$ for Pogonitsa).
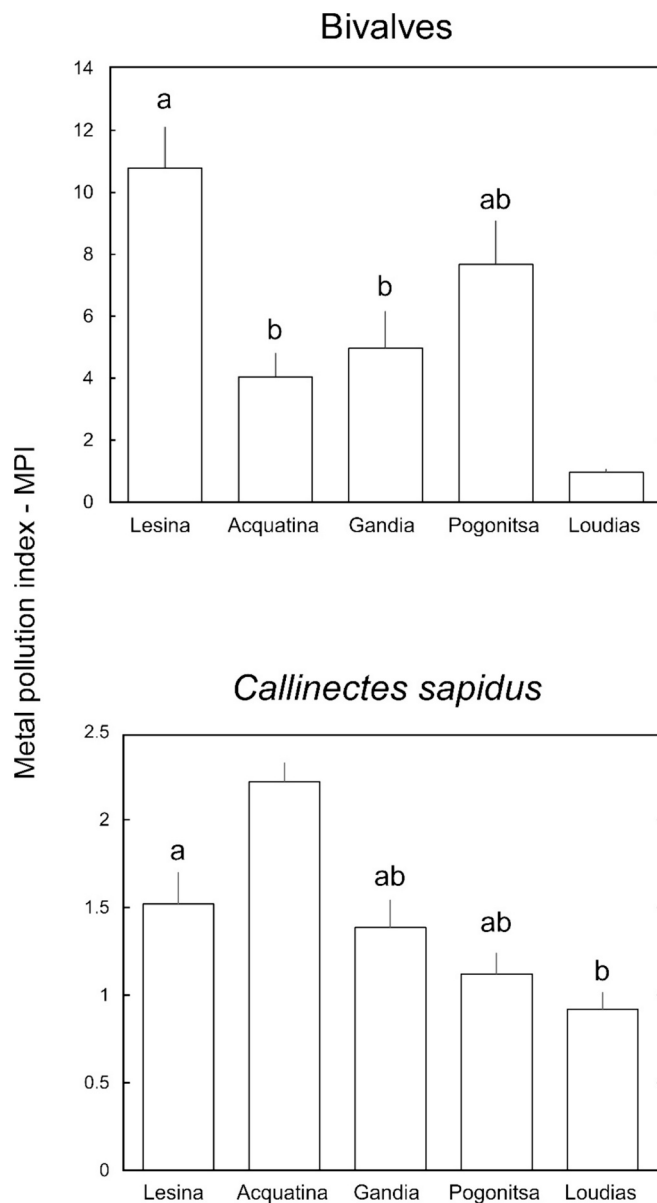
Metal contents in crabs varied significantly among locations [Fig. 2, Table 3; P(MC) always <0.05 for all pair-wise comparisons; see Table S4 in the online supplementary material for mean concentrations $\pm$1SE]. Since males and females showed no significant differences, the factor "sex" was not considered further. Conversely, a significant effect for the interaction term "location×size" was observed (Table 3); a CAP analysis confirmed a substantial co-variation between crabs' size and metal contents for all the locations except Pogonitsa (Fig. S1, online supplementary material). Additional univariate analyses showed significant among-location differences for all metals (1-way ANOVAs: P always <0.05) with the exception of Sr ($F_{4,92} = 1.6$, $P = 0.18$); they were related with crabs' size only for a negligible number of location/metal combinations (8 out of 65; Table S5, online supplementary material).

Significant location-dependent differences in metal contents were

Bivalves

3D Stress: 0.05

- ● Lesina
- ■ Acquatina
- ◇ Gandia
- ▲ Pogonitsa
- ▼ Loudias

Callinectes sapidus

3D Stress: 0.08

Fig. 2. 3d-nMDS plots based on Euclidean distance similarity matrices of trace metal concentrations in bivalves (top) and in *Callinectes sapidus* (bottom) from the five sampling locations.

Bivalves

Callinectes sapidus

Fig. 3. Metal pollution indices (MPI) in bivalves (top) and in *Callinectes sapidus* (bottom) from the five sampling locations. The results of post-hoc bivariate comparisons (Tukey HSD tests) are included; locations showing identical letters do not differ at $P < 0.05$. Please note the different scales in each plot.

Table 3
Summary of PERMANOVA results testing for the effects of sex and location as orthogonal fixed factors and size (carapace width, CW) as a continuous covariate on *Callinectes sapidus* trace metal contents and δ^{13}C and δ^{15}N values. Significant effects are reported in bold.

Source	d.f.	Trace metals		Stable isotopes	
		Pseudo-F	*P*(MC)	Pseudo-F	*P*(MC)
CW (1)	1	5.25	**<0.001**	1.2	0.09
Location (2)	4	9.22	**<0.001**	32.08	**0.001**
Sex (3)	1	1.59	0.14	1.92	0.15
1 × 2	4	1.82	**0.01**	0.88	0.54
1 × 3	1	0.74	0.61	2.77	0.09
2 × 3	4	1.21	0.21	1.61	0.14
1 × 2 × 3	4	0.87	0.63	0.51	0.86

observed between crabs and bivalves [two-way PERMANOVA with "location" and "taxon" as fixed factors: interaction factor "location×taxon": Pseudo-$F_{4,131}$ = 20.8, P(MC) = 0.001; bivariate inter-specific tests performed within each location: P(MC) always <0.001]. MPI values in crabs differed significantly among the five locations (1-way ANOVA, $F_{4,92}$ = 12.1, $P < 0.0001$). With the exception of Loudias, their MPI values were generally one order of magnitude lower than bivalves (Fig. 3) and showed a significantly different pattern of among-location variation (Pearson r = 0.13, P = 0.82, d.f. = 3). Acquatina showed the highest index (2.2 ± 0.1, mean ± 1SE; HSD tests, min P = 0.004 for the comparison Lesina vs. Acquatina); Loudias was characterized by the lowest MPI (0.9 ± 0.1), significantly different from that estimated for Lesina (Fig. 3B; HSD test, P = 0.04) but not from those

characterizing Gandia (1.4 ± 0.2) and Pogonitsa (1.1 ± 0.2; *P* always >0.05).

1-way ANOVAs performed at a single-metal level generally confirmed the lower metal concentrations in crabs as compared with bivalves, yet several exceptions were represented by Cd at Pogonitsa, Cu at Lesina, Acquatina, Gandia, and Loudias, Fe at Loudias, and Ni at Acquatina and Loudias (HSD tests, *P* always >0.05). Furthermore, while metal contents in crabs and bivalves from Pogonitsa showed a relatively low degree of correlation (Spearman $\rho = 0.53$, $P = 0.07$, d.f. = 11), positive relationships with different levels of significance were observed for the remaining locations (Fig. S2, online supplementary material).

The trophic transfer indices estimated for crabs (Table 4) showed relatively low values (overall TTF: 1.39 ± 0.38, mean ± SE) and a pronounced variability, ranging between 0.002 (Mn at Pogonitsa) and 15.8 (Cu at Gandia) with the majority of the indices <1 (72 %). In general, Cu and Zn were the metals showing the highest TTFs; values >1 were observed also for Cd, Sr, and V and, to a minor extent, for Ba, Fe, and Mn (Table 4).

3.3. Stable isotope analysis

A PERMANOVA performed on blue crabs δ^{13}C and δ^{15}N values (Table 5) with "sex" and "location" as orthogonal fixed factors and "body size" (CW) as a continuous covariate indicated a significant effect only for the factor "location" (Table 3). Thus, the effects of sex and body size were not considered in further isotopic analyses.

Bayesian estimations of standard ellipse areas (SEA$_B$) varied among the study locations independently from the number of analyzed crabs (Pearson $r = -0.68$, $P = 0.2$, d.f. = 3). SEA$_B$ values ranged between maxima of 13.9 and 11.9 ‰^{2} determined at Gandia and Loudias, respectively, to a minimum value of 1.3 ‰^{2} determined for Lesina, with Pogonitsa and Acquatina showing intermediate areas ranging between 6.1 and 6.2 ‰^{2} (Table 6; see also Fig. S3, online supplementary material). Bivariate tests indicated negligible differences in SEA$_B$ values between Gandia and Loudias, as well as between Pogonitsa and Acquatina (Table 6). With the exclusion of the carbon range CR, other Layman metrics showed a pattern of variation across the study locations similar to that verified for SEA$_B$ (Table 6; minimum Pearson $r = 0.94$, $P = 0.02$, d.f. = 3 for NR; for CR: $r = 0.76$, $P = 0.13$, d.f. = 3).

The trophic position of *C. sapidus* estimated using the δ^{13}C and δ^{15}N values of bivalves as baselines (Table 6) showed a remarkable among-location variability, with mean values ranging between 2.7 (Pogonitsa), indicating omnivorous trophic habits including both animal and vegetal items, and 4.1 (Loudias), suggesting a diet completely based on animal prey. Bivariate tests indicated that Gandia and Loudias had modal TP estimations significantly different from Lesina, the latter in turn significantly higher than Acquatina and Pogonitsa (Table 6).

Table 4

Trophic transfer factors (TTF) of metals in *Callinectes sapidus* calculated for each study location. Values >1 are reported in bold. For Acquatina, Gandia, and Loudias the TTF of Ni was not estimated as the metal content in bivalves or *C. sapidus* was under the detection limit of the instrumentation.

Metal/location	Lesina	Acquatina	Gandia	Pogonitsa	Loudias
B	0.08	0.02	0.26	0.09	0.13
Ba	0.06	0.46	1.03	0.01	**2.15**
Cd	0.35	0.27	0.74	**2.63**	**1.42**
Cr	0.02	0.08	0.83	0.01	0.47
Cu	**2.41**	**10.56**	**15.8**	0.92	**14.97**
Fe	0.01	0.13	0.07	0.02	**1.14**
Li	0.22	0.18	0.59	0.57	0.97
Mn	0.12	0.05	0.97	<0.01	**1.71**
Ni	0.03	–	–	0.05	–
Pb	0.03	0.23	0.2	0.08	0.33
Sr	0.8	0.31	**2.05**	0.39	**3.67**
V	0.3	0.7	**1.22**	**1.51**	0.6
Zn	**3.37**	**3.91**	**2.48**	0.52	0.66

Table 5

Mean δ^{15}N and δ^{13}C values (SD in parentheses) of bivalves and *Callinectes sapidus* from the five study locations.

	Bivalves		*Callinectes sapidus*	
Lesina[*]	−20.68 (0.15)	11.55 (0.17)	−16.82 (0.84)	14.01 (0.54)
Acquatina	−23.01 (0.49)	7.33 (0.47)	−19.74 (2.27)	10.69 (0.87)
Gandia	−21.52 (0.39)	7.03 (0.27)	−22.73 (2.88)	11.68 (1.6)
Pogonitsa	−19.87 (0.44)	7.61 (0.39)	−17.88 (1.49)	9.51 (1.28)
Loudias	−23.15 (0.58)	7.89 (0.21)	−20.73 (1.83)	13.16 (2.26)

[*] Bivalve species = *Arcuatula senhousia*; *Mytilus galloprovincialis* for the remaining locations.

Noticeably, for most of the metals the variation of crabs' TTF values across locations was related with their trophic position; the only exceptions were Zn and Cd, the latter showing a negative co-variation with TP values (Fig. 4). Furthermore, a significant positive relationship was observed between TP estimations and the degree of rank-correlation ρ between the metals content of crabs and bivalves (Fig. 4; Pearson $r = 0.89$, $P = 0.04$, d.f. = 3).

4. Discussion

4.1. Trace metals in bivalves

Independently from the sampling location, the mussel species included in the present study showed concentrations of hazardous metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) largely lower than the limits set by the European Union and the Food and Agriculture Organization (FAO, 1983; FAO/WHO, 1989; EC, 2006; see Table S6, online supplementary material). Accordingly, a low degree of metal contamination can be assumed for all the investigated systems. Furthermore, metal contents showed to vary significantly among locations independently from the size of the analyzed bivalves. Caution should be taken when considering these conclusions, as two methodological issues deserve to be explicitly considered. Firstly, a relatively low number of bivalve specimens (6 to 10, Table 1) were analyzed from each location. Indeed, this investigation was not specifically designed as a biomonitoring study exclusively focused on bivalves, thus requiring large sample sizes (Daskalakis, 1996; Saavedra et al., 2004a; Saavedra et al., 2009) and standardized approaches such as transplanting to cope with taxonomic differences (see further in this section; Cao et al., 2023). Since statistical analyses indicated for bivalves highly significant inter-location differences not related with size, we are confident of the robustness of our results within the specific context of the study; nevertheless, additional biomonitoring studies are necessarily required to confirm their generality. Secondly, we used *Mytilus galloprovincialis* across all the study locations with the exception of the Lesina Lagoon, where *Arcuatula senhousia* was analyzed.

Bivalves show significant inter-specific as well as intra-specific, size-related differences in the degree of metals accumulation (Saavedra et al., 2004b; Cai and Wang, 2019; but see Saavedra et al., 2004a). However, no single species of bivalve can be ubiquitously found, and in large-scale biomonitoring programs it is often necessary to collect multiple species; in this case, taxonomically-close species with similar life and trophic strategies should be chosen (Cao et al., 2023 and literature cited). We assumed *A. senhousia* as a surrogate of *M. galloprovincialis* since they belong to the same family (Mytilidae) and are epibenthic sessile filter-feeders (e.g., Inoue and Yamamuro, 2000); studies on coexisting populations of the two mussel species provided further evidence for the assumption, as negligible differences in C and N isotopic values (Feng et al., 2014) as well as in Hg, Cu, Pb, and Zn contents (Feng et al., 2016) have been observed. Finally, and most importantly, all the main conclusions of this study still holds if the data on Lesina Lagoon are not considered (see e.g., Fig. 4).

Table 6
Posterior Bayesian estimations of standard ellipse areas SEA$_B$ (A) and trophic positions (B) of *Callinectes sapidus* from the five study locations. SEA$_B$ estimations are expressed in ‰^{2}. For both metrics, modal values and 95 % confidence intervals are reported; identical letters in parentheses indicate that values do not differ with a probability of at least 95 %. In (A), for the sake of completeness sample size-corrected standard ellipse areas (SEA$_C$; see also Fig. S3 in the supplementary material C), *nitrogen ranges* (NR), *carbon ranges* (CR), *mean distances to centroid* (CD), and *standard deviations of nearest neighbour distance* (SDNND) are included.

	Lesina	Acquatina	Gandia	Pogonitsa	Loudias
A) Isotopic niche					
SEA$_B$ (mode)	1.28	6.22(a)	13.89(b)	6.11(a)	11.96(b)
SEA$_B$ (95 % CI)	0.88–2.01	3.83–8.53	8.75–18.33	3.63–9.33	8.54–19.03
SEA$_C$	1.4	6.52	15.14	6.32	12.17
NR	2.65	3.24	7.55	5.18	6.57
CR	3.31	8.63	9.22	4.78	6.97
CD	0.85	2.06	2.91	1.77	2.52
SDNND	0.25	0.4	0.78	0.51	0.83
B) Trophic position					
Mode	2.92(a)	3.36	3.78(b)	2.67(a)	4.16(b)
95 % CI	2.75–3.21	3.09–3.7	3.41–4.32	2.44–3.09	3.79–4.78
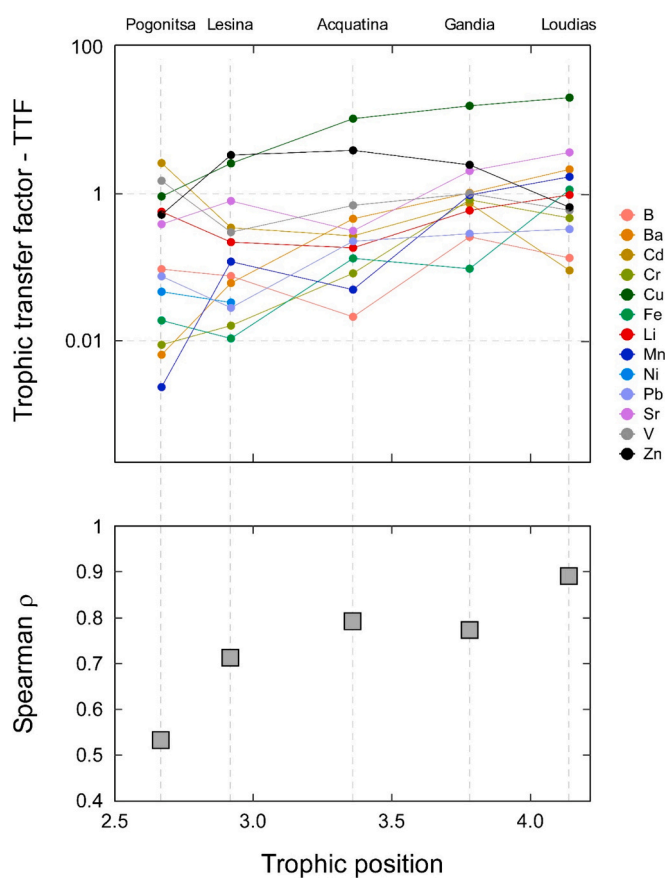
Fig. 4. Trophic position of *Callinectes sapidus* across the five study locations vs. metals trophic transfer factors (TTF; see Table 2 for means ±1SE; top) and Spearman rank correlation coefficients ρ estimating the consistency of metal profiles in bivalves and in *C. sapidus* (bottom). See text and Fig. S2 in the online supplementary material for details.

4.2. Trace metals in crabs

As for bivalves, metal contents in blue crabs were remarkably lower than international reference levels for human consumption (Table S6, online supplementary material). The blue crab represents an emergent target species as a shellfish product in invaded Mediterranean habitats (Mancinelli et al., 2017c; Kevrekidis and Antoniadou, 2018; Glamuzina et al., 2021; Kevrekidis et al., 2023). This is the first large-scale study providing a comparative assessment of metal contamination in

C. sapidus in Mediterranean waters, and even though additional, year-long studies are necessary to corroborate our results, they nonetheless suggest a low ecotoxicological risk for the crab.

Noticeably, our findings indicated that metal contents in crabs varied significantly across the five locations independently from sex; yet, body size exerted a significant effect. The actual influence of sex and size on the accumulation of contaminants in blue crabs remains debated. Depending on the geographical location and the metals analyzed, past investigations have highlighted either significant or negligible sex- and size-related effects (Sastre et al., 1999; Adams and Engel, 2014; Taylor and Calabrese, 2018; Salvat-Leal et al., 2020). Furthermore, when significant relationships occurred between the size of crabs and their content of a specific metal, they varied from negative to positive depending on location (e.g., for Pb: Genç and Yilmaz, 2017; Salvat-Leal et al., 2020). Here we observed a similar ambiguity: while CAP analysis showed in a multivariate context a generalized co-variation between size and metals content with the exclusion of Pogonitsa, at a single-metal scale the effect of crabs' size was limited and showed inconsistent patterns of variation across locations (Table S5, online materials). The factors governing the variability in metals accumulation in blue crabs are multiple, including the complex interplay of external inputs with physicochemical and biogeochemical conditions influencing, in turn, metal mobilization at a local scale (Taylor and Calabrese, 2018). Furthermore, differences in the morphometry of the analyzed crabs (Table 2), may have reflected in location-specific dilution or accumulation effects (Salvat-Leal et al., 2020): molting frequency in blue crab is inversely related with body size (Millikin and Williams, 1984), and contaminants sequestration in the exoskeleton during molting is an important mechanism for eliminating trace metals in brachyurans (Engel and Brouwer, 1987; Bergey and Weis, 2007).

This study showed a remarkable inconsistency in metals content between bivalves and crabs across the five investigated locations, with most metals showing concentrations higher in bivalves than in crabs, ultimately reflecting in generally low trophic transfer indices. Noticeably, the trophic position of *C. sapidus* varied by approximately one level among locations, scaling positively with the degree of trophic transfer of most of metals as well as with the consistency of crabs' metal profiles with those of bivalves.

Two mutually non-exclusive scenarios can explain these results. The first assumes an influence of metal contamination on the feeding habits of the crab. Heavy metals can determine significant behavioral alterations in crustaceans (Perez and Wallace, 2004; Weis et al., 2011). In particular, increasing habitat contamination was related with blue crabs showing an impaired predatory behavior, a lower trophic position, and bioaccumulation patterns more representative of low-level resources (e. g., aquatic plants or leaf detritus: Reichmuth et al., 2009, 2010). Here, a behaviorally-mediated trophic effect cannot be excluded, as Reichmuth

et al. (2010) found concentrations of Cr, Cu, and Pb (i.e., 1.1, 15.4, and 0.37 mg kg^{-1}, respectively) similar to or lower than those observed in this study (Table S4, online supplementary material). However, a pivotal yet improbable implication of this scenario is that the five locations investigated here, notwithstanding their considerable environmental differences (Table S1), offered to the crab similar trophic conditions in terms of e.g., resource diversity and availability, and that its trophic position was uniquely determined by behavioral mechanisms mediated by local contamination levels. A second hypothesis hinges on omnivory of the crab and how it opportunistically varies in response to local resource availability. Noticeably, at Acquatina, Lesina, and Pogonitsa crabs had TP values significantly lower than those determined at Gandia and Loudias. The first three locations are confined lagoons characterized by the occurrence of submerged vegetation represented by macroalgae or seagrasses, while Gandia and Loudias are open-sea habitats characterized by benthic environments with low or negligible vegetation cover (Table S1). Bivalves represent a major component of the crab's diet, yet conventional gut content analyses and stable isotope studies clearly confirmed that it feeds on multiple trophic levels, and that trophic positions may vary substantially depending on season, location, and ontogenetic stage (Hines, 2007; Mancinelli et al., 2013, 2016, 2017d; Rady et al., 2018; Prado et al., 2021). The occurrence of living and non-living plant material in the lagoons, together with their associated assemblages of primary consumers, implies that in these environments the blue crab may opportunistically feed on low-trophic level benthic resources characterized by contamination patterns differing from that of bivalves (among others, see Ghosn et al., 2020 for an example from a Mediterranean coastal area), ultimately obscuring any transfer effect. Conversely, in less-vegetated systems under open sea conditions the lack of primary producers and associated fauna may induce the blue crab, besides preying on bivalves, to focus on other animal prey from higher trophic levels and characterized by higher metal burdens. The generally higher TTF values, coupled with the higher trophic positions observed in blue crabs from Gandia and Loudias indirectly support this hypothesis, further confirmed by isotopic niche analyses. Indeed, compared with the remaining locations, crabs from Gandia and Loudias showed the largest isotopic niches (SEA$_B$), total nitrogen and carbon ranges (NR and CR) trophic diversity (CD) and evenness (SDNND), ultimately indicating a higher intra-specific heterogeneity in trophic habits. Resource-related differences in ingestion and assimilation rates, coupled with variations in uptake and excretion kinetics of contaminants cannot be excluded, as they may have further determined the observed differences (Jardine et al., 2006).

5. Conclusions

Regardless from the underlying causative mechanisms, our findings indicated that the opportunistic omnivory of the blue crab might hinder its use as a bioindicator, as locations-specific differences in trophic habits might ultimately reflect on metals content. Noticeably, besides *C. sapidus* a number of omnivorous crustaceans have been recommended for biomonitoring purposes, including other portunids (e.g., *Callinectes danae*: Bordon et al., 2016; *Portunus segnis*: Annabi et al., 2018) the green crab *Carcinus maenas* (Ben-Khedher et al., 2014) and the Louisiana crayfish *Procambarus clarkii* (Suárez-Serrano et al., 2010; Henriques et al., 2014). Our results emphasize the urgent need to identify biomonitor animal species - in marine as well as in other environments - after a thorough scrutiny of their trophic habits, as originally suggested by Rainbow (1995).

In conclusion, invasive omnivores are recognized to exert significant, hard-to-be predicted threats on ecosystem functions because of their ability to directly and indirectly impact multiple trophic levels (Shea and Chesson, 2002; Romanuk et al., 2009; Jackson and Britton, 2014; Pettitt-Wade et al., 2015; see also Médoc et al., 2018). Our study suggested that they might represent an open issue also in ecotoxicology. However, we indicated that an advanced assessment of their trophic

habits by stable isotope analysis could help in explaining the patterns of variation in contamination levels and, ultimately, provide more robust information for e.g., biomonitoring purposes.

Ethical statement

No ethical issues related with the use of animals in the performed analyses were involved.

CRediT authorship contribution statement

Raffaele De Giorgi: Investigation. **Roberta Bardelli:** Data curation. **Lucrezia Cilenti:** Writing – original draft. **Silvia Falco:** Investigation, Conceptualization. **Maria Teresa Guerra:** Data curation. **George Katselis:** Writing – original draft, Investigation, Data curation. **Kosmas Kevrekidis:** Writing – original draft, Investigation, Conceptualization. **Francesco Mancini:** Methodology, Investigation. **Lorenzo Doria:** Writing – original draft, Methodology. **Agnese Marchini:** Writing – original draft, Supervision. **Danilo Migoni:** Methodology. **Paride Papadia:** Writing – original draft, Methodology. **Salvatrice Vizzini:** Writing – original draft, Methodology, Investigation, Conceptualization. **Giorgio Mancinelli:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.116715.

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