Combining metal and stable isotope analyses to disentangle contaminant transfer in a freshwater community dominated by alien species

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ABSTRACT

Freshwater ecosystems are negatively impacted by various pollutants, from agricultural, urban and industrial wastewater, with metals being one of the largest concerns. Moreover, freshwater ecosystems are often affected by alien species introductions that can modify habitats and trophic relationships. Accordingly, the threat posed by metals interacts with those by alien species, since the latter can accumulate and transfer these substances across the food web to higher trophic levels. How metals transfer within such communities is little studied. We analysed the concentration of 14 metals/metalloids (Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Ni, Pb, Se, Zn, hereafter 'metal(s)') of eight fish and three crustacean species co-existing in the Arno River (Central Italy), most of which were alien. To assess the pathway of contaminants within the community, we coupled metal analysis with carbon and nitrogen stable isotope analysis derived from the same specimens. Crustaceans showed higher metal concentration than fish, except for Cd, Hg and Se that were higher in fish. We found evidence of trophic transfer for six metals (Cd, Cr, Hg, Mg, Se, Zn). Additionally, ontogenetic differences and differences among various fish tissues (muscle, liver, and gills) were found in metals concentration. Considerable biomagnification along the trophic chain was found for Hg, while other metals were found to biodilute. Using stable isotopes and Hg as a third diet tracer, we refined the estimations of consumed preys in the diet previously reconstructed with stable isotope mixing models. Alien species reach high biomass and can both survive to and accumulate high pollutants concentrations, potentially posing a risk for their predators and humans. A combined effect of environmental filtering and increased competition may potentially contribute to the disappearance of native species with lower tolerances.

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1. Introduction

Metals/metalloids (hereafter referred to as 'metals') pollution is one of the most serious environmental hazards (Huber et al., 2016; Hou et al., 2017; Yang et al., 2018), as these substances are persistent in the environment and accumulate in organisms, thus representing both an ecological and human health risk (Alhashemi et al., 2012; Varol and Sünbül, 2017; Liu et al., 2018). They accumulate in animal tissues over time (bioaccumulation) and they transfer along the trophic web through their diet (biomagnification) which leads to high concentrations in higher trophic positions (Markert et al., 2003). As such, even elements occurring at low concentrations can represent a considerable risk for predators and humans (Ballutaud et al., 2019). For instance, mercury (Hg) is very toxic and well known for its biomagnification properties (e.g. Carrasco et al., 2011; Kehrig et al., 2013; Subotić et al., 2013).

In recent years, metals accumulation and transfer among

organisms has attracted increasing attention in the literature (Gall et al., 2015; Sun et al., 2020), especially for freshwater ecosystems impacted by anthropogenic activity collecting pollutants from multiple sources (Obiakor et al., 2017; Dubois et al., 2018; Amoatey and Baawain, 2019). In freshwater ecosystems, metals can naturally originate from geological sources, making comparisons among different sites difficult, but may also be introduced through anthropogenic activity such as urban, agricultural or industrial activity (Maceda-Veiga et al., 2012; Giri and Singh, 2015; Zhaoyong et al., 2018).

Aquatic organisms can accumulate metals from their local environment (e.g. water or sediment) through their gills and skin, or through the digestive system after consumption of contaminated food sources (Squadrone et al., 2013; Has-Schön et al., 2015). Within species, metals distributions depend on the allocation and affinity of specific metals to different tissues/organs, and their concentrations rely on a trade-off between intake and excretion rate (Kuklina et al., 2014; Jovičić et al., 2015; Vilizzi and Tarkan, 2016). Several abiotic and biotic factors further affect metals concentrations: the degree of environmental contamination, the duration of exposure, and their availability ("bioavailability") which is influenced by pH, temperature, chemical form and the interactions between those (Kouba et al., 2010; Has-Schön et al., 2015). Among biotic factors, species, age, size, tissue type, sex, nutritional and reproductive status, functional and feeding habitat (i.e. benthic vs. pelagic zone) as well as the functional feeding group (i.e. herbivorous, omnivorous and predatory) have a great effect on the accumulation of metals (Szarek-Gwiazda and Amirowicz, 2006; Alhashemi et al., 2012; Squadrone et al., 2013; Wei et al., 2014; Jovanović et al., 2017; Signa et al., 2017; Yi et al., 2017).

Metal biomagnification is strongly dependent on the community composition (Ali and Khan, 2019). For instance, alien species, especially the invasive ones that are particularly adaptable (e.g. Dörr et al., 2020), are known to be favoured by environmental disturbances, facilitating their establishment (Karatayev et al., 2009; Strayer, 2010; Crooks et al., 2011). They are known for their capability to bioaccumulate higher metal concentrations compared to native species, due to their greater tolerance towards pollutants (McKenzie et al., 2012; Bielen et al., 2016; El Haj et al., 2019). Indeed, physiological tolerance has been described as a determinant for both the establishment and the achievement of high abundances in alien fish (Marchetti et al., 2004) and alien crustacean species revealed higher concentrations than the native ones, probably linked to different physiology (Gherardi et al., 2002). On the other hand, evidence suggests that native fish and amphibian populations may decline due to high metals concentrations (e.g. Robinet and Feunteun, 2002; Affandi and Ishak, 2019; Sierra-Marquez et al., 2019; Weir et al., 2019). Moreover, alien species can modify the communities' trophic structure, leading to new interactions such as competition with (e.g. Balzani et al., 2016; Haubrock et al., 2020) and predation on both native and alien species (e.g. Haubrock et al., 2018). The result is a complex system where energy, biomass and even metals transfer, follow new pathways (e.g. Haubrock et al., 2019a,b). These pathways are usually determined using an experimental approach, with dietary uptake being the dominant pathway compared to water uptake for many metals (Zhang et al., 2011; Wang, 2013; DeForest and Meyer, 2015; Erickson et al., 2011, 2019), but remain hard to disentangle in the field. Given the characteristics and abundance of alien species, they play an important role in metals transfer in the food web. Additionally, invaded communities not only are likely to accumulate metals at higher concentrations but may also present high biomagnification rates.

Coupling stable isotope analysis (SIA) with the analysis of metals can help shed light on these questions. Indeed, SIA is used to study trophic interactions and reconstruct communities' structures, providing long-term and time-mediated information on consumed resources, owing to predictable changes in isotopic ratios between the prey and its consumer (Boecklen et al., 2011). Nitrogen isotopic signature ($\delta^{15}N$) is related to the consumer's trophic level, while the carbon isotopic signature ($\delta^{13}C$) indicates the main carbon source (Post, 2002; Layman et al., 2012). Moreover, stable isotope mixing models can provide estimates of the proportion of consumed prey species contributing to the consumer's diet (e.g. Parnell et al., 2013). The information on biomagnification processes and food pathways within the community derived from SIA can help estimate metals transfer along the trophic chain with high accuracy (Watanabe et al., 2008). Furthermore, we hypothesize that metals analysis (and the information provided about energy pathways, Metian et al., 2009) coupled with SIA can possibly refine the resolution of trophic linkages compared to SIA alone. The analysis of other biomagnifying pollutants has been shown to more robustly refine dietary reconstructions derived from SIA only (Ballutaud et al., 2019). We suggest that the combination of metals analysis with SIA will increase the accuracy of information obtained from complex communities with intense multifaceted interactions that arise from species introductions. Indeed, despite SIA results potentially being affected by species that were not sampled (Phillips et al., 2014), inclusion of biased diet priors (Franco-Trecu et al., 2013), or general misinterpretation, this technique has proved especially useful for better understanding alien species impacts (e.g. Vander Zanden et al., 1999). Hence, regarding invaded communities, we hypothesize that metals transfer and bioaccumulation will be substantial among alien species due to life history traits, predation and habitat use. In particular, we hypothesize that benthic and predator species show the highest concentrations due to metals deposition in detritus (that could be enriched compared to water; Luoma and Rainbow, 2008) and biomagnification along the food chain, respectively.

The overall aim of this study was to investigate a community invaded by multiple alien species that was previously analysed with SIA and stable isotope mixing models to couple the obtained results with an analysis of metals. Specifically, we analysed ten alien and one native species in order to (i) assess if concentrations of metals for which maximum permitted levels for human consumption were determined by the European Commission (2008; i.e. Cd, Hg and Pb) exceeded those thresholds, (ii) assess and compare metals accumulations between different species, (iii) identify possible biomagnification processes, and (iv) refine diet reconstruction by using stable isotopes and metals pathways along the trophic web. To this end, we attempted to disentangle complex feeding interactions within an invaded community by investigating inter- and intraspecific differences in metal concentrations among different tissues/organs and age classes to consequentially identify if changes in life history related habitat use and feeding behaviour affect metals concentration of alien species. Last, we related metal concentrations with the trophic level and carbon pathways as estimated by stable isotopes analyses to quantify species bioaccumulation and community biomagnification. Metals that were found to significantly biomagnify were coupled with stable isotopes as additional tracers to investigate trophic relationships potentially not detected by SIA alone.

2. Materials and methods

2.1. Study site and sampling

For the purposes of this study we used the community of the Arno River in Tuscany (Italy). The Arno River is the second biggest

river in Central Italy. Particularly in Florence, the Arno River is anthropogenically and hydromorphologically altered (i.e. divided by weirs slowing its flow). Its historical use led to repeated introductions of species from different areas mainly for sport fishing, which has significantly altered its species composition through time (Haubrock et al., 2019a). As such, it represents a suitable study site, because the inner-Florence section of the Arno is composed of almost entirely alien species. Additionally, feeding interactions of this community were recently reconstructed using a combined approach of diet analysis and stable isotope mixing models (Haubrock et al., 2019a), which allows for a precise reconstruction of the dietary origin of metals.

The aquatic vegetation is dominated by the native pondweed *Potamogeton* sp. and two alien plants, the common reed *Phragmites australis* and the watermilfoil *Myriophyllum* sp. The aquatic fauna is mainly composed by alien species, introduced in different times in the last decades (Nocita, 2007). The community is structured as a typical pyramidal trophic web (Haubrock et al., 2019a). European catfish *Silurus glanis* and channel catfish *Ictalurus punctatus* occupy the top predator position, followed by the opportunistic predatory pumpkinseed (*Lepomis gibbosus*), and several omnivorous (the common bleak *Alburnus alburnus*, topmouth gudgeon *Pseudorasbora parva*, goby *Padogobius* sp.) and benthivorous (the common carp *Cyprinus carpio*, common barbel *Barbus barbus*, tench *Tinca tinca*) fish. Macroinvertebrates are represented by crustaceans (the killer shrimp *Dikerogammarus villosus*, grass shrimp *Palaemon antennarius*, red swamp crayfish *Procambarus clarkii*), molluscs (the white ramshorn *Gyraulus chinensis*) and few aquatic insects (Ephemeroptera and Odonata) (Haubrock et al., 2019a). All the aforementioned species are alien, except for *P. antennarius*. Other present native species include the green frog *Pelophylax* kl. *esculentus* (Haubrock et al., 2019a) and a population of the endangered river crab *Potamon fluviatile* (Cianferoni et al., 2019). The presence of some species is particularly important because they are either consumed by humans (e.g. the invasive species *I. punctatus* and *S. glanis*; Squadrone et al., 2013), serve as keystone species, or because of their ability to tolerate high environmental stress including metal pollution (e.g. *P. clarkii*: Bini et al., 2015; Bissattini et al., 2018).

Community sampling was conducted at three locations of the inner-Florence section of the Arno River (43.7 N, 11.3 E, ~2.4 km length), that is delimited by weirs, from April to June 2018. Several different methods were used to sample the different species: fish were caught with standard fishing rods and the help of local fishers using a variety of baits, while crustaceans were collected using funnel traps, hand nets and through kick sampling. Fish specimens were immediately euthanized via stunning followed by gill cutting with a clean ceramic blade, in compliance with the authorization ("Autorizzazione alla pesca scientifica Regione Toscana"), while crustaceans were killed via freezing. Samples were stored in ice during transport and then preserved in the freezer at $-20\,°C$ until further processing to not alter either stable isotopes or metal values (Gibbs et al., 1974; Arrington and Winemiller, 2002). Metals samples were taken from the same specimens used for stable isotope analysis in Haubrock et al., 2019a. Overall, 112 individuals from eight fish species, and 45 individuals from three crustacean species were sampled. For each specimen, we measured weight and length (total length for fish and cephalothorax length for crayfish). Individuals of the same species were similar in size and age (Table 1). Only three individuals of *C. carpio* and only the juvenile age class of *S. glanis* (<80 cm) were sampled. Only one individual of *T. tinca* and one of *B. barbus* were caught due to their rarity, so they were included in the dataset to roughly indicate their metals concentration, but not used in further statistical analysis. Life stages of *I. punctatus* were distinguished following the methodology suggested in Haubrock et al. (2018), with specimens with a total length > 30 cm being considered as adults. To have a proxy of the environmental metals baseline three water samples of 50 ml each, were collected 50 cm under the water surface to avoid the collection of floating organic material using acid-washed glass containers and then stored at $-20\,°C$.

2.2. Metal sample preparation and analyses

For the analysis of metals, a sample of dorsal muscle tissue without skin was taken from each fish specimen. For larger fish species, additional tissues were sampled: gills and liver for *S. glanis* and *I. punctatus* (including both juveniles and adults) and gills for *C. carpio*. Muscle tissue was taken from the abdomen of *P. clarkii*, while *D. villosus* and *P. antennarius* were processed as a whole (when dry weight was >50 mg) and pooled when specimens were too small for the analyses (2–4 individuals per pool). We did not clean the gut of *D. villosus* and *P. antennarius* before the analyses due to difficulties during the field sampling. More specifically, specimens should have been kept at specific environmental conditions, with isolated individuals due to frequent cannibalism. Additionally, faeces consumption is also a common phenomenon, especially during starvation. However, the relative proportion of faeces in gammarid crustaceans in the wild was found to be low compared with the body mass (e.g. Worischka et al., 2018). Samples

Table 1
Species sampled from the Arno River: their stable isotope (from Haubrock et al., 2019a) and morphological data (mean ± standard error), status and sample number. Length was measured as total length for fish and cephalo-thorax length for crayfish. Length of *D. villosus* and *P. antennarius* was not recorded.

Species	δ^{15}N (‰)	δ^{13}C (‰)	Length (cm)	Weight (g)	Alien	n
Fish						
Alburnus alburnus	15.6 ± 0.4	−26.2 ± 0.1	9.8 ± 0.4	7.6 ± 0.7	Yes	7
Barbus barbus	14.4	−26.1	17	36	Yes	1
Cyprinus carpio	14.1 ± 1.0	−25.2 ± 0.4	31.2 ± 5.7	584.3 ± 92.6	Yes	3
Ictalurus punctatus (juveniles)	16.2 ± 0.2	−26.2 ± 0.2	22.4 ± 1.4	117.8 ± 21.5	Yes	19
I. punctatus (adults)	15.6 ± 0.3	−27.0 ± 0.1	45.2 ± 2.1	959.2 ± 165.1	Yes	20
I. punctatus (overall)	15.9 ± 0.2	−26.6 ± 0.1	33.8 ± 2.2	538.45 ± 106.3	Yes	39
Lepomis gibbosus	15.7 ± 0.4	−25.8 ± 0.1	9.0 ± 0.6	20.0 ± 4.2	Yes	16
Pseudorasbora parva	14.8 ± 0.3	−26.0 ± 0.1	8.4 ± 0.2	4.8 ± 0.5	Yes	4
Tinca tinca	9.1	−26.9	8.3	11	Yes	1
Silurus glanis (juveniles)	17.1 ± 0.1	−25.5 ± 0.1	27.5 ± 1.1	127.1 ± 15.0	Yes	37
Crustaceans						
Dikerogammarus villosus	9.3 ± 0.4	−26.1 ± 0.2	−	0.10 ± 0.02	Yes	6
Palaemon antennarius	12.7 ± 0.2	−26.7 ± 0.2	−	0.11 ± 0.02	No	11
Procambarus clarkii	13.7 ± 0.2	−23.1 ± 0.5	6.5 ± 0.7	26.2 ± 1.7	Yes	10

were weighed wet, dried in an oven at 60 °C for 48 h, and weighed again dry. In total, 235 organic matrices were prepared, weighing 50−500 mg (dry weight). Each sample was diluted in 10 ml of nitric acid, then mineralized with microwave radiation (1600 W, 210 °C; Olesik, 1991; Low et al., 2009; Ghanthimathi et al., 2012) to homogenize them and subsequently analysed through Inductively Coupled Plasma − Optical Emission Spectrometry (ICP-OES). Water samples were filtered to remove any organic or sediment particles, diluted 1:10 with hydrochloric ultrapure acid and then mineralized as the other samples. A total of 11 blanks (one every approximately 20 samples) were also prepared to control for contamination. Before running the analyses and at the end of each measurement session, certified standards of known metals concentration (multistandard concentrations: 0.1, 1 and 10 ppm; Hg standard concentrations: 0.01 and 0.05 ppm) were used to calibrate the instruments and to be sure that any possible instrumental bias occurred. In compliance with quality assurance and quality control (QA/QC), three replicates for each sample (from the same digestion solution) were run and their relative standard deviations (RSD) calculated. The respective metal mean concentration was used for further analyses. For each sample, the following metal concentrations were determined: aluminium (Al), arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), magnesium (Mg), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn). The analytical detection limit for all metals was 0.01 ppm on dry weight basis. Data on dietary as well as stable isotopes analysis conducted on the same samples were retrieved from Haubrock et al., 2019a. Concentrations in blanks were <1% of the samples and all the RSD were <10%.

2.3. Statistical analyses

The metals concentration in water was converted from mass/l to mass/mass of water for comparison with the samples from fish and invertebrates. Before statistical analyses, each value of samples that presented metal concentrations below the detection limit (0.01) was substituted with the value of the detection limit itself (see Soto et al., 2016) and metal concentrations were $\log_{10}$ transformed to account for multiplicative effects.

To compare the concentration of Cd, Hg and Pb in fish muscle and crustaceans with the EU thresholds for human consumption (2008; fish: 0.05, 0.5, 0.3 ppm wet weight; crustaceans: 0.5, 0.5, 0.5 ppm wet weight for Cd, Hg and Pb, respectively), we converted the metals concentration referred to dry weight in metals concentration referred to wet weight, using the formula from Cresson et al. (2017):

$$M_{iw} = M_{id} * (1 - (W_w - W_d) / W_w)$$

where M_{iw} is the concentration of metal i referred to wet weight, M_{id} is the concentration of metal i referred to dry weight, W_w is the wet weight of the sample and W_d is the dry weight of the sample. For the other analyses, ppm referred to dry weight was used as measure unit.

Ordination diagrams were produced using Canoco software (5.12 version). A Principal Component Analysis (PCA) with species as supplementary variables was used because response data (metal) had a gradient of 1.9 standard deviation units. This analysis was complemented with a constrained ordination (Redundancy Analysis, RDA) with species as explanatory variables. Muscle was chosen for this analysis in the case that multiple tissues were analysed for a specific species.

All other statistical analyses were performed using R software (3.6 version, R Core Team). To test for differences in metal occurrence among different species (considering different life stages of

I. punctatus as individual groups), we performed a non-parametric MANOVA (permutations = 9999, method = "bray") using the function 'adonis' of the package "vegan" (Oksanen et al., 2019), followed by t-tests with Benjamini-Hochberg p value correction (Benjamini and Hochberg, 1995), allowing multiple comparisons using the wrapper function "pairwiseAdonis" (Arbizu, 2019). Again, muscle tissue was chosen for this analysis as it was comparable among all species. Then, univariate analyses (ANOVA, Welch ANOVA and Kruskal-Wallis tests) were performed for each metal to test for differences in concentrations among species, followed by post-hoc tests (Tukey and Mann-Whitney). Subsequently, we tested for intraspecific differences of each metal and tissue between juveniles and adults of I. punctatus, as this species is highly abundant and shows ontogenetic differences in habitat use and feeding behaviour (Haubrock et al., 2018). Further, we tested for concentration differences of each metal among tissues in those species with multiple tissue collected (i.e. I. punctatus, S. glanis and C. carpio) using univariate (Kruskal-Wallis, ANOVA and Welch ANOVA), followed by post-hoc (Mann-Whitney and Tukey) tests.

Assuming a dietary origin for metals, for five fish species (A. alburnus, P. parva, L. gibbosus, S. glanis, I. punctatus juveniles and adults) the Biotransfer factor (BTF), i.e. transfer of metals across trophic levels for each fish, was estimated using the formula:

$$BTF = M_{ij} \bigg/ \sum_{ik} (pk * M_{ik}) \qquad [2]$$

with M_{ij} being the mean concentration of metal i in the muscle tissue of predatory fish j, M_{ik} as the mean concentration of metal i in each prey k and p being the proportion of the prey k in the diet of the predator j, as estimated from previously run isotope mixing models (Haubrock et al., 2019a; Table S1). Indeed, instead of assuming an equal contribution of each prey belonging to the lower trophic level, we used diet priors in this calculation. For those predators for which stomach contents were available, we used proportions derived from Bayesian mixing models as this allowed us to include the prey proportion as a prior in these models. In the absence of stomach content data, we used proportions estimated by non-Bayesian mixing models (Haubrock et al., 2019a). If BTF >1, biotransfer can be assumed, meaning that trophic transfer occurs for that specific consumer (Kehrig et al., 2013). For species with multiple tissues collected, we used only muscle for BTF calculation.

At the community level, we estimated the biomagnification factor (BMF), an indicator of the increase in accumulated HMs across the trophic levels as well as the food web magnification factor (FWMF), an indicator of accumulation magnitude. The BMF was calculated for each metal as the slope of the linear regression model:

$$\log_{10} [M_{ij}] = \alpha + \beta * \left(\delta^{15} N_j \right) \qquad [3]$$

where M_i is the concentration of metal i in the individual j, β is the angular coefficient of the linear regression, α the intercept, and $\delta^{15}N_j$ the nitrogen signature of the individual j (Cui et al., 2011; Kehrig et al., 2013), for which we used the values provided in Haubrock et al., 2019a. Last, the food web magnification (FWMF) factor was estimated as following:

$$FWMF = 10^{\beta} \qquad [4]$$

under the assumption that an FWMF > 1 indicates the occurrence of biomagnification, while a FWMF < 1 indicates trophic dilution (Kehrig et al., 2013).

However, these two latter metrics commonly used in the

literature do not account for the significance of the linear relationships. Therefore, to identify relationships between metal concentrations in the community and stable isotope signatures along the food web, we built a linear model using the 'step' function for each log_{10} transformed metal concentration as response variable and isotope data ($\delta^{15}N$ and $\delta^{13}C$; Haubrock et al., 2019a) as predictor. For species with multiple tissues sampled, only muscle was used for these analyses. Finally, we coupled dietary priors (from Haubrock et al., 2019a; Table S2), stable isotope signatures and Hg concentrations as a third tracer in a Bayesian model to refine diet reconstruction (i.e. the proportion of preys consumed) using the package "ESCROC" (Ballutaud et al., 2019). For crustaceans, for which diet priors were not available, we used a 0 (predation not likely) and 1 (predation possible) scale, as suggested for the model (Ballutaud et al., 2019). Among metals, Hg was selected for its significant biomagnification in the present and other communities. In the model, both trophic enrichment factors (TEF; Post, 2002) and trophic magnification factor (TMF; Lavoie et al., 2013) were used. For predatory species that are frequently consumed, namely *S. glanis* and *I. punctatus*, we displayed differences in the estimated prey proportion between stable isotopes mixing models (retrieved from Haubrock et al., 2019a) and the ESCROC model.

3. Results

Metal concentrations of Cd, Hg and Pb were found to be lower than the maximum permitted levels for human consumption as determined by the European Commission (2008), with Cd being < 0.01 ppm for both fish and crustaceans, Hg < 0.04 ppm in fish and <0.01 ppm in crustaceans and Pb < 0.02 ppm in fish and <0.48 ppm in crustaceans. Other metal concentrations showed high variability. Metal concentrations in water were generally found to be low (range 0.01–0.07 ppm) except for Mg, naturally occurring in the local geological substrate (Table S3).

Fish and crustaceans were clearly separated in their metal concentrations (adjusted explained variation: 58.75%, for RDA test on all axes: pseudo-F = 21.4, p < 0.001). Higher concentrations were found in crustaceans for almost all metals, especially in *D. villosus*, except for Cd, Hg, and Se, which showed higher concentrations in fish. The only metal that did not display such clear patterns was Zn (Figs. 1 and 2). Overall, metal concentrations were significantly different among species (npMANOVA: $F_{9,120} = 14.99$, p < 0.001; Table S4). Univariate analysis showed a significant difference among species for each metal (p < 0.001 in each test except for Cd: p < 0.05; Table S5, Fig. 2). It should be noted that Cd, Co and Se showed a high number of values below the detection limit. However, despite a high variability in concentrations in species with samples above the detection limit, it can be seen that high concentrations occurred only in some species (Fig. 2).

Metal concentrations were found to be different in different age classes of *I. punctatus*. Overall, juveniles accumulated more Mg ($\chi^2 = 6.73$, df = 1, p < 0.01) and Zn ($\chi^2 = 4.62$, df = 1, p < 0.05) in muscle, and Cu ($\chi^2 = 7.58$, df = 1, p < 0.01), Mg ($\chi^2 = 5.57$, df = 1, p < 0.05) and Zn ($\chi^2 = 14.82$, df = 1, p < 0.001) in gills, while adults had higher concentration of Cr in muscle ($F_{1,34} = 10.15$, p < 0.01) and of Fe in gills ($\chi^2 = 6.83$, df = 1, p < 0.01). Cd and Hg were below the detection limit in all gill samples, while no ontogenetic differences were recorded for any metal in the liver. No clear general pattern was identified for tissue differences in *I. punctatus*, *S. glanis* or *C. carpio* (Table S6). Cd and Co in *C. carpio* and Cd in *S. glanis* were below the detection limit.

At the community level, linear models identified varying effects of $\delta^{15}N$ and $\delta^{13}C$ on metal concentrations except for Cd, Mg, Se, and Zn, on which they showed no significant effect (Table 2). The effect of $\delta^{15}N$ was always negative except for Hg, while the effect of $\delta^{13}C$ was more variable depending on the metal. The estimated Biotransfer factor (BTF) was >1 in all fish species for Hg, Se, Cd and Zn, with the only exceptions (BTF < 1) for these two latter metals found
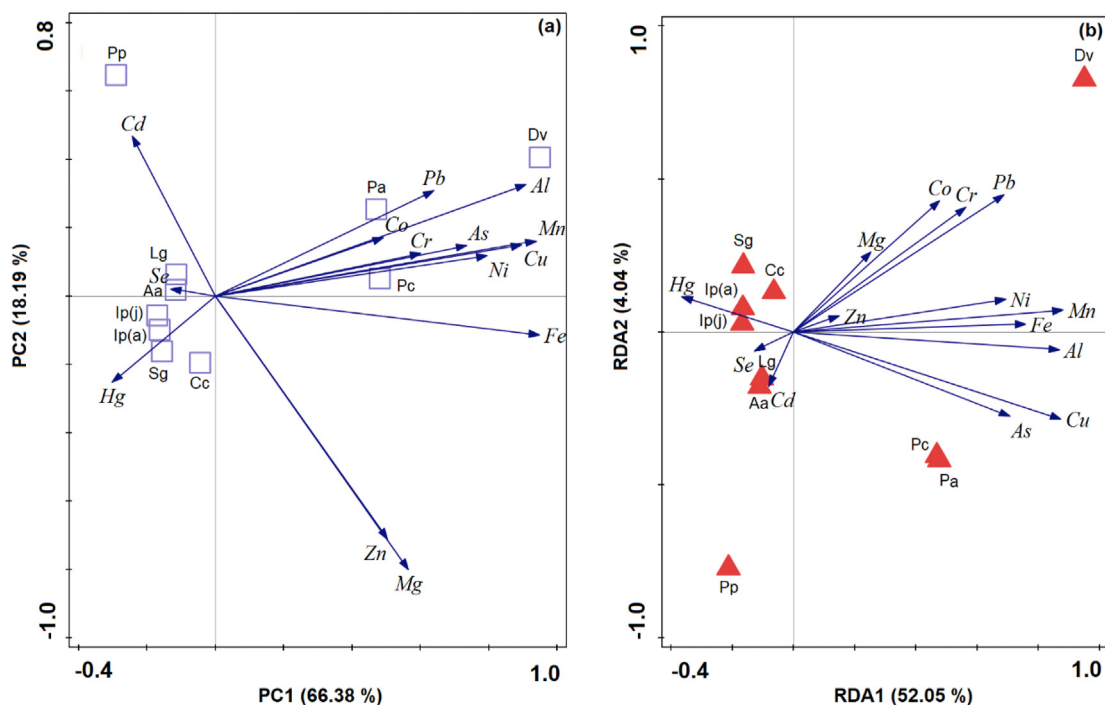

Fig. 1. Principal Component Analysis plot (a) with metals as response data and species as supplementary variables; and Redundancy Analysis plot (b) with metals as response data and species as explanatory variables. Aa = *Alburnus alburnus*, Dv = *Dikerogammarus villosus*, Sg= *Silurus glanis* juveniles, Pa= *Palaemon antennarius*, Pc = *Procambarus clarkii*, Pp = *Pseudorasbora parva*, Lg = *Lepomis gibbosus*, Cc = *Cyprinus carpio*, Ip(a) = *Ictalurus punctatus* adults, Ip(j) = *I. punctatus* juveniles.
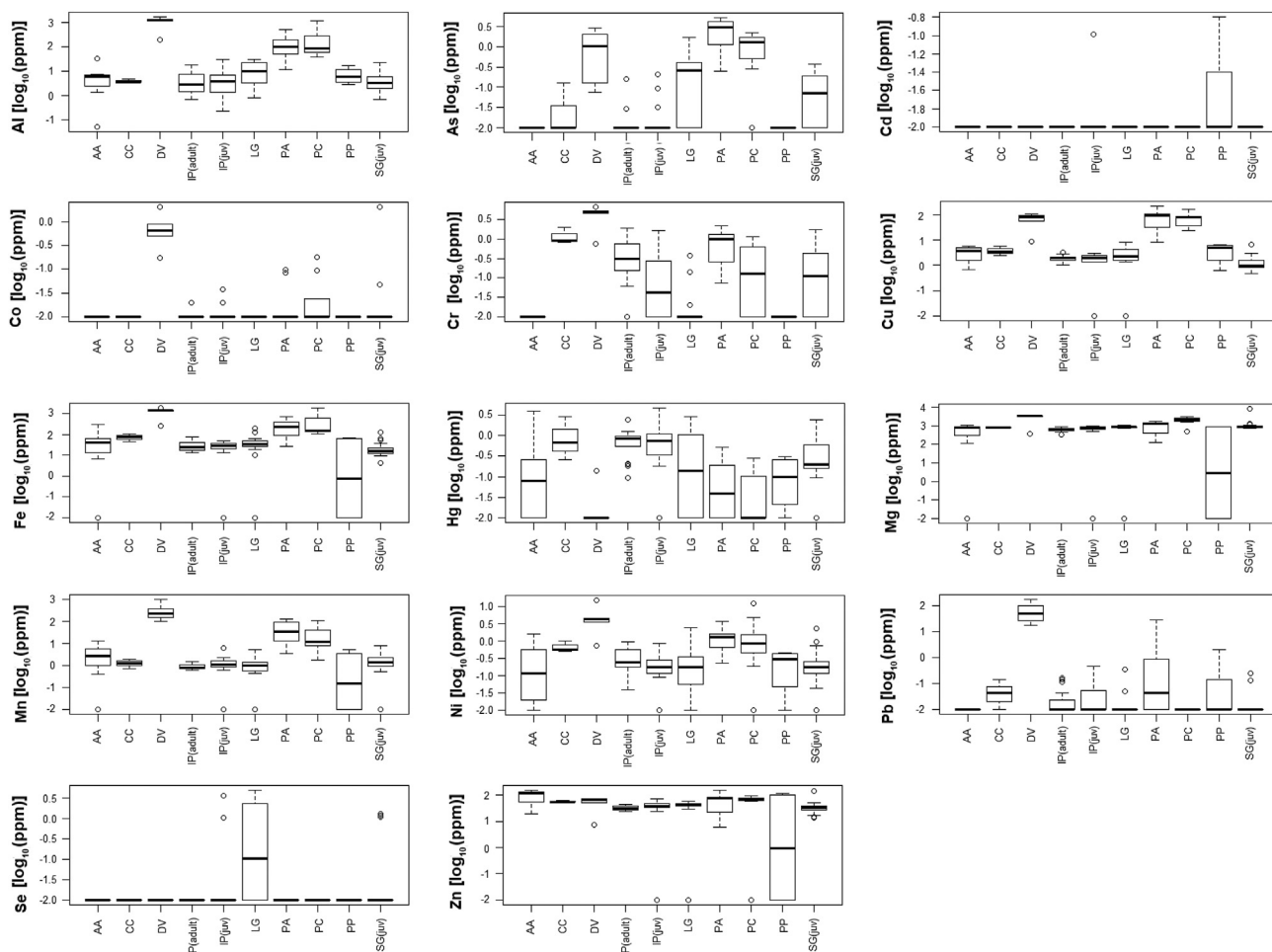
Fig. 2. Boxplot for metals concentrations (log transformed) in all species. AA = *Alburnus alburnus*, DV = *Dikerogammarus villosus*, SG= *Silurus glanis* juveniles, PA= *Palaemon antennarius*, PC= *Procambarus clarkii*, PP= *Pseudorasbora parva*, LG = *Lepomis gibbosus*, CC= *Cyprinus carpio*, IP (adult) = *Ictalurus punctatus* adults, IP (juv) = *I. punctatus* juveniles. Boxes represent the second and third quartiles, whiskers the first and fourth quartiles and dots represent the outliers of data distribution. The black line represents the median.

in *S. glanis* (for Cd) and *I. punctatus* juveniles and *S. glanis* (for Zn). BTF >1 were also found in *I. punctatus* adults for Cr and in *I. punctatus* adults and *S. glanis* for Mg (Table 3). BMF >0 and FWMF >1, suggesting biomagnification, were found for Se, Zn, and Hg, with the latter having a high FWMF (1.62). Other metals showed BMF <0 and FWMF <1, suggesting biodilution (Table 3) (). However, the significance of linear models clearly shows that only Hg biomagnified along the food chain.

The ESCROC model revealed a significant reliance of *S. glanis* on small crustaceans, cyprinids and juveniles of *I. punctatus*. The diet of *I. punctatus* adults was estimated to be primarily based on small fish (*A. alburnus*, *P. parva* and *L. gibbosus*), while *I. punctatus* juveniles were found to prey extensively on *P. antennarius*. The diet of *L. gibbosus* and *C. carpio* was almost entirely constituted by *D. villosus* and *P. clarkii*. The cyprinid *A. alburnus* was found to prey on both *D. villosus* and *P. antennarius*, while *P. parva* was found to prey only on *P. antennarius*. Similarly, *D. villosus* was found to rely only on *P. antennarius*, while *P. clarkii* was estimated to prey extensively on *D. villosus* (Fig. 3). These results showed some differences compared to those derived from SIA-only (Fig. S1). In particular, ESCROC estimated a lower consumption of crustaceans and a greater consumption of fish for *I. punctatus* and *S. glanis.* On the contrary, a higher predation on crustaceans was found for both *L. gibbosus* and *C. carpio*, although the low number of specimens caught for the latter should lead to a precautious approach. The results for *P. parva* and *A. alburnus* reflected the previous results of stable isotope mixing models (Haubrock et al., 2019a), with a high proportion of *P. antennarius* in the diet of both. Additionally, *D. villosus* was found to significantly prey on *P. antennarius* while being an important prey for *P. clarkii*.

4. Discussion

Rivers are complex and dynamic systems. The continuous flow makes it difficult to accurately assess metal concentrations, because only a fraction of metals settles onto the bottom while the majority are transported downstream by the water. Hence, it is preferable to use animals as an indicator of environmental pollution in these ecosystems (Maceda-Veiga et al., 2012; Jovanović et al., 2017). Alien species which tend to reach high abundances and compete with native ones, often have high tolerance towards environmental pollution (including metals contamination). This makes them suitable bioindicators as they can accumulate high concentrations without dying, proxying high environmental levels (Alomar et al., 2016).

Our results show significantly different levels of bioaccumulation among species, possibly linked to different species behaviour and feeding activity. Previous literature (Cui et al., 2011; Giri and Singh, 2015; Zuo et al., 2018) has shown that crustaceans tend to accumulate higher metals concentrations compared to fish,

Table 2
Linear models for each metal concentration as response variable and isotope data (δ^{15}N and δ^{13}C) as predictors. Asterisks show significance levels: * <0.05, ** <0.01, *** <0.001.

| Metal	Covariate	Estimate	Standard error	t-value	p	F	Adjusted R^2
Al	Intercept	8.62	1.18	7.33	<0.001 ***	$F_{2,127} = 73.92$	0.53
	δ^{15}N	−0.29	0.02	−11.78	<0.001 ***		
	δ^{13}C	0.13	0.04	2.90	<0.01 **		
As	Intercept	6.51	1.60	4.07	<0.001 ***	$F_{2,127} = 22.36$	0.25
	δ^{15}N	−0.20	0.03	−5.89	<0.001 ***		
	δ^{13}C	0.18	0.06	3.11	<0.01 **		
Cd	Intercept	−1.98	0.01	−164.30	<0.001 ***	−	−
Co	Intercept	0.08	0.25	0.31	0.76	$F_{1,128} = 58.33$	0.31
	δ^{15}N	−0.13	0.02	−7.64	<0.001 ***		
Cr	Intercept	−1.46	1.66	−0.88	0.38	$F_{2,127} = 15.02$	0.18
	δ^{15}N	−0.18	0.03	−5.12	<0.001 ***		
	δ^{13}C	−0.12	0.06	−2.01	<0.05 *		
Cu	Intercept	7.11	1.23	5.76	<0.001 ***	$F_{2,127} = 44.16$	0.40
	δ^{15}N	−0.23	0.03	−9.01	<0.001 ***		
	δ^{13}C	0.12	0.05	2.59	<0.05 *		
Fe	Intercept	4.55	0.53	8.64	<0.001 ***	$F_{1,128} = 33.85$	0.20
	δ^{15}N	−0.20	0.03	−5.82	<0.001 ***		
Hg	Intercept	−9.04	1.32	−6.84	<0.001 ***	$F_{2,127} = 36.76$	0.36
	δ^{15}N	0.21	0.03	7.51	<0.001 ***		
	δ^{13}C	−0.20	0.05	−4.06	<0.001 ***		
Mg	Intercept	2.72	0.09	28.91	<0.001 ***	−	−
Mn	Intercept	4.09	0.49	8.43	<0.001 ***	$F_{1,128} = 61.64$	0.32
	δ^{15}N	−0.25	0.03	−7.85	<0.001 ***		
Ni	Intercept	1.80	0.37	4.92	<0.001 ***	$F_{1,128} = 42.37$	0.24
	δ^{15}N	−0.15	0.02	−6.51	<0.001 ***		
Pb	Intercept	0.02	1.49	0.01	0.99	$F_{2,127} = 42.31$	0.39
	δ^{15}N	−0.28	0.03	−9.02	<0.001 ***		
	δ^{13}C	−0.10	0.05	−1.91	0.06		
Se	Intercept	−1.77	0.06	−29.32	<0.01 **	−	−
Zn	Intercept	1.44	0.07	20.58	<0.001 ***	−	−

Table 3
Biotranfer factor (BTF) calculated for each species with data on diet and metal, and Biomagnification factor (BMF) and Food web magnification factor (FWMF) calculated for the community for each metal.

Species	BTF													
	Al	As	Cd	Co	Cr	Cu	Fe	Hg	Mg	Mn	Ni	Pb	Se	Zn
Alburnus alburnus	0.04	0.00	1.25	0.14	0.01	0.05	0.24	6.55	0.60	0.07	0.28	0.00	1.25	1.89
Ictalurus punctatus (juveniles)	0.02	0.02	1.53	0.05	0.14	0.03	0.06	7.10	0.57	0.01	0.12	0.00	39.35	0.77
I. punctatus (adults)	0.05	0.05	1.06	0.18	1.40	0.11	0.20	6.26	1.14	0.03	0.52	0.01	2.18	1.07
Lepomis gibbosus	0.04	0.32	1.74	0.06	0.04	0.07	0.12	13.46	0.68	0.02	0.21	0.00	212.69	1.11
Pseudorasbora parva	0.04	0.01	6.81	0.12	0.01	0.08	0.10	1.78	0.41	0.03	0.19	0.07	1.43	1.18
Silurus glanis (juveniles)	0.05	0.53	0.45	0.77	0.62	0.12	0.14	1.25	1.53	0.05	0.37	0.00	3.08	0.61
	BMF													
Whole community	−0.29	−0.20	0.00	−0.13	−0.18	−0.23	−0.20	0.21	−0.01	−0.25	−0.15	−0.28	0.02	0.01
	FWMF													
Whole community	0.51	0.63	1.00	0.74	0.66	0.59	0.63	1.62	0.98	0.56	0.71	0.52	1.05	1.02

except for some metals like Hg (Kuklina et al., 2014). That is because crustaceans commonly feed on detritus and dead matter and occupy the benthos in lentic microhabitats, that are potential sinks of pollutants. Indeed, benthic organisms are known to show high metal concentrations (Kehrig et al., 2013), as they are in direct contact with deposited substances. Additionally, in organisms without liver but hepatopancreas, such as crustaceans and insects, metals transport and detoxification take considerably longer (Ahearn, 2010). Further, crustaceans such as *P. clarkii* are prey for many, both native and alien, animals (Bissattini et al., 2018) which increases the chances of transferring metals to higher trophic levels and posing an ecotoxicological risk to the entire community.

Overall, fish species consuming crustaceans as a significant proportion of their diet (as determined by stomach content and stable isotope analyses on the Arno community, Haubrock et al., 2019a; e.g. *I. punctatus*, *S. glanis*) did not show higher metal concentration than crustaceans themselves, as indicated by the species-specific Biotransfer factor (BTF) estimations. However,

trophic transfer was found to occur in all the considered species for Hg and Se, while in *I. punctatus* and *S. glanis* occurred only for Cr and Mg but not for Cd and Zn that were transferred in other species. This could be attributable to different uptake/excretion rates that may differ across species and trophic levels, resulting in biodilution (Watanabe et al., 2008; Adams et al., 2020). Moreover, the diet estimated using both stable isotopes and Hg concentrations indicates a lower consumption rate of crustaceans than previously found by SIA-only results (Haubrock et al., 2019a). In particular, a lesser reliance on the heavily contaminated *D. villosus* and an important consumption of small fish were found for *I. punctatus* and *S. glanis*. Indeed, these fish may feed upon other fish species (mainly cyprinids: Carol et al., 2009; Syväranta et al., 2010) but also plants and algae (Haubrock et al., 2019a). Diet estimations derived from the combined analysis of stable isotopes and metal indicate a high reliance on *D. villosus* and *P. clarkii* for both *L. gibbosus* and *C. carpio*. An important consumption of *P. clarkii* was found in other introduced populations of *L. gibbosus* (e.g. Haubrock et al., 2019b).
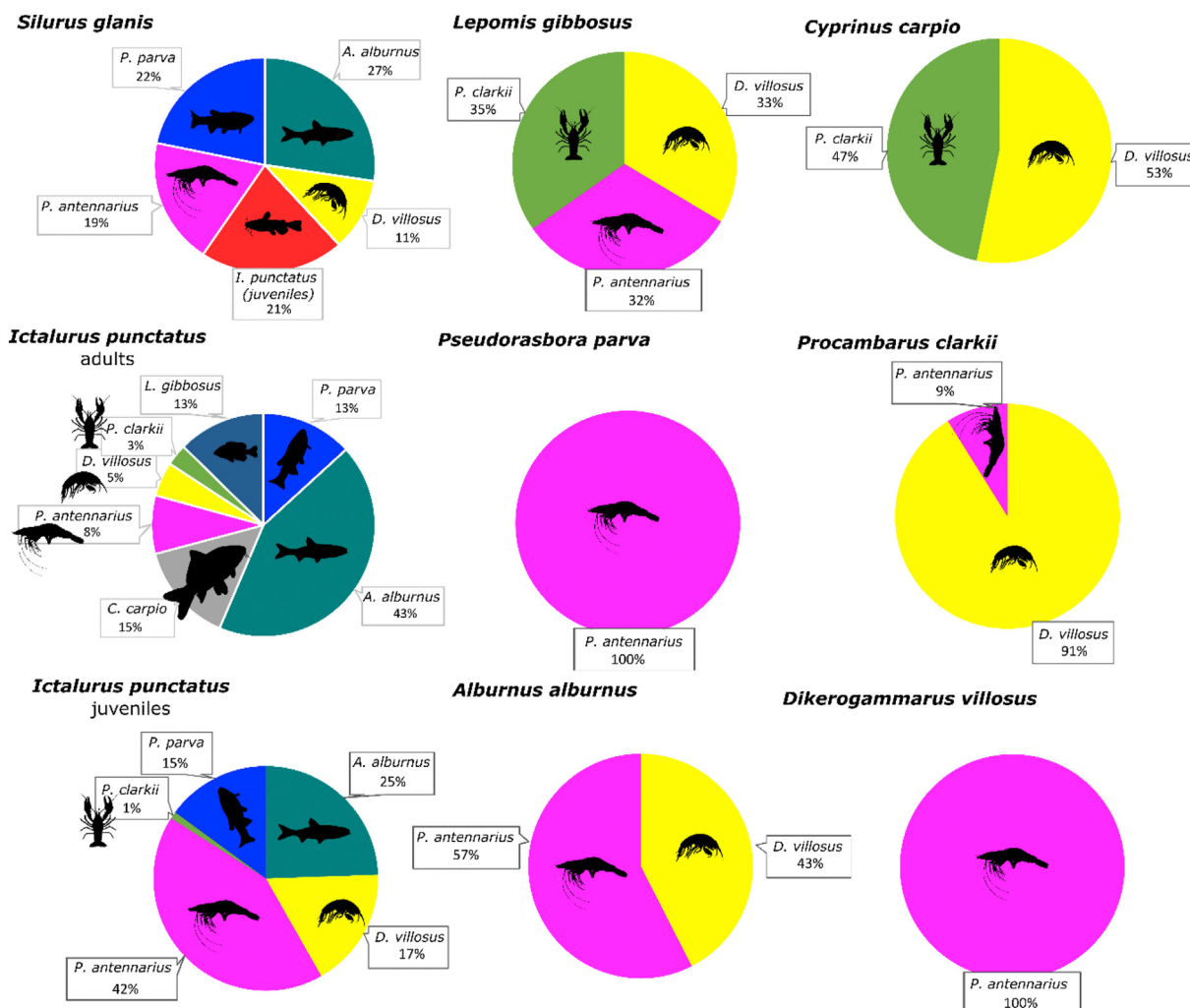
Fig. 3. Relative prey proportions [%] in the diet of each consumer considering diet priors, carbon and nitrogen stable isotope signatures and Hg concentrations. *S. glanis* = *Silurus glanis*; *I. punctatus* = *Ictalurus punctatus*; *P. parva* = *Pseudorasbora parva*; *A. alburnus* = *Alburnus alburnus*; *P. antennarius* = *Palaemon antennarius*; *D. villosus* = *Dikerogammarus villosus*; *P. clarkii* = *Procambarus clarkii*; *C. carpio* = *Cyprinus carpio*.

Interestingly, the model estimated a considerable consumption of *P. antennarius* by the killer shrimp *D. villosus* and an extensive predation on this latter by *P. clarkii*. More studies on the feeding interactions among these crustacean alien species are needed.

Intraspecific differences among fish tissues or organs can be explained by their different role, biodistribution of metals within the organism and trade-off between uptake and excretion rates (Adams et al., 2020). Given human consumption and subsequent risks to human health, fish muscle is the most commonly analysed tissue in biomonitoring studies. However, in the present study, a generally low metal accumulation capacity of muscles was found. While metal concentration in muscle reflects a diet origin, gills are usually an indicator for contaminants in aquatic environments. Indeed, gills are the main site where exchange between circulatory system and the environment occurs, thus they accumulate metal suspended in the water. The liver, instead, is an active excretion organ, thus prone to accumulate metals bound with metallothioneins with the aim of detoxification (Subotić et al., 2013; Vilizzi and Tarkan, 2016; Jia et al., 2017). Both environment and diet are known as important uptake routes for metals (Van Campenhout et al., 2009; DeForest and Meyer, 2015), potentially reflecting the habitat use and feeding habitat. Some sampled species (i.e. *S. glanis*, *I. punctatus*, and *C. carpio*) typically occupy the benthic zone and the

difference in bioaccumulation between age classes in *I. punctatus* could be explained by different ontogenetic feeding habits. Indeed, juveniles feed more on detritus (Haubrock et al., 2018, 2020) compared to adults, and detritus could be enriched relative to other prey with these metals (Luoma and Rainbow, 2008; DeForest and Meyer, 2015). The different habitat use of adults and juveniles (juveniles closer to the riverbank and on the bottom and adults more pelagic; Endo et al., 2015; Haubrock et al., 2020) suggests that these metal concentrations are lower in the open water column while accumulating in the riparian zone. This explanation fits well with the higher metal concentrations in crustaceans, that usually occupy the riparian benthic zone. These observations were also mirrored by the linear model, which highlighted differences in δ^{15}N (indicating the consumption of nitrogen enriched detritus) and in δ^{13}C (a proxy of spatial feeding habitat, i.e. pelagic vs benthic/littoral zone) that significantly affect Cr and Cu concentrations, respectively (Haubrock et al., 2019a).

Overall, the linear models and the FWMF show a negative relationship between δ^{15}N and metals concentration, indicating that they biodilute across trophic levels in line with findings of previous studies (e.g. Cui et al., 2011). The only notable exception is Hg, that is well known to biomagnify (e.g. Signa et al., 2017) and to be toxic even at low concentrations, due to its non-essential metal

nature (i.e. the lack of specific regulatory systems in organisms, Kaus et al., 2017). Although no data were used on metal concentrations of native species in the Arno community (which consists primarily of birds, amphibians and other invertebrates), Alomar et al. (2016) found similar contaminant levels in alien and native predators (catfish, otter and cormorants) within the Loire river, suggesting that invasive predators could be good indicators of native predators' pollutants accumulation. Moreover, native species might present sublethal detrimental effects due to prolonged metals exposure, affecting long-term individual features like health, growth, survival or reproduction (Barbee et al., 2013; Kumar et al., 2017; Sadeghi et al., 2018; Javed and Usmani, 2019). Further studies applying biodynamic modelling to alien species are required to better understand their metal tolerance. In particular, a comparison in metals uptake and excretion rate between alien and native species counterparts could provide evidence for their physiological tolerance facilitating their establishment.

5. Conclusions

The combination of stable isotope and metal analyses can help to disentangle how co-occurring alien species accumulate and transfer metals along the food web. Alien species usually reach high biomass and are known to tolerate and accumulate high pollutant concentrations. As expected for a community dominated by alien species, the metal accumulation in the Arno River community was substantial, even if high biomagnification was found only for Hg. Our study suggests that metal pollution together with competition for resources with alien species as well as habitat changes might have caused the disappearance of native predatory fish. Although beyond the scope of this paper, it is possible that threats from some metals may exist for native predators (e.g. aquatic birds) as well as for humans. To develop a more comprehensive picture, however, further research is needed to investigate possible sublethal effects on the native fauna and alien species tolerance towards metals.

Authors statement

Paride Balzani: Conceptualization, Formal Analysis, Investigation, Data Curation, Writing-Original Draft; **Phillip J. Haubrock**: Conceptualization, Investigation, Writing-Review & Editing; **Francesco Russo**: Investigation, Writing-Review & Editing; **Antonín Kouba**: Methodology, Validation, Writing-Review & Editing; **Peter Haase**: Writing-Review & Editing; **Lukáš Veselý**: Methodology, Visualization, Writing-Review & Editing; **Alberto Masoni**: Data Curation, Writing-Review & Editing; **Elena Tricarico**: Conceptualization, Writing-Review & Editing, Resources, Supervision.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115781.

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