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Beyond the mean:
a comparison of trace- and macroelement correlation profiles of two lacustrine populations of the crayfish *Procambarus clarkii*

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Abstract

In invertebrate biomonitors of chemical pollution, emphasis has been generally given to mean accumulation patterns and how they reflect varying environmental levels of contamination. Intra-population variability, and how it is related with individual phenotypic traits, has received less attention. Here, a set of analytes including trace elements (B, Ba, Cd, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sr, V, and Zn), macroelements (C, Ca, K, Mg, N, Na), and carbon and nitrogen stable isotopes (standardized $\delta^{13}C$ and $\delta^{15}N$) was measured in two populations of the crayfish *Procambarus clarkii* from Lake Trasimeno and Lake Bolsena (Central Italy). The influence of location, sex, body size, and condition factor was assessed; in addition, the analyte correlation profiles of the two populations were compared to verify their congruence. In general, only significant inter-lake differences were observed in the concentration of both trace- and macroelements in crayfish tissues, generally reflecting the local chemistry of water and of benthic non-living matrices (sediment and plant detritus). CN isotopic signatures excluded for the two crayfish populations the occurrence of variations in their omnivorous trophic habits. Correlation profiles varied considerably between the two populations in the nature and strength of the bivariate relationships. However, Mantel tests and procrustean analyses indicated a general, significant congruence; C, N, and, to a lesser extent K, Li, Ni, Pb, and $\delta^{13}C$ were the analytes showing the highest procrustean residuals, indicating that their associations with other analytes may be partially influenced by inter-population differences in growing phases. Our study indicates that the local geochemistry of the lacustrine environment influences the elemental fingerprint of *Procambarus clarkii*; the considerable inter-individual variability in the concentration of analytes, however, does not significantly reflect on their association, thus corroborating its effectiveness as an indicator species.

Keywords: red swamp crayfish, elemental fingerprint, stable isotopes, inter-individual variability, biomonitoring

1. Introduction

The use of invertebrate species is to date recognized as an effective way of monitoring the quality of freshwater environments, due to their general capacity to accumulate pollutants, either from water or from food sources (Colin et al., 2016). Among others, crayfish have long been acknowledged as effective biomonitors of chemical contamination (*sensu* Caro, 2010), given that they live in contact with polluted sediments, resist to environmental stress, and have a relatively long life span (Reynolds and Souty-Grosset, 2011). Furthermore, the accumulation of contaminants in their tissues is dose- and time-dependent (Kouba et al., 2010 and literature cited). Hence, a number of investigations have focused on pollutant accumulation patterns in populations of these decapods, and on how they reflect environmental levels of contamination (Goretti et al., 2016; Gedik et al., 2017 among the most recent on *Procambarus clarkii*). Noticeably, ecotoxicological studies have generally focused on changes in the central value of a biomonitor response, considering variance among individuals as a nuisance ("the tyranny of the golden mean" in Bennett, 1987; Devin et al., 2014). Inter-individual variability has been generally overlooked (but see Lobel et al., 1992; Saavedra et al., 2009 for exceptions), notwithstanding the recognition that it influences the effect of pollutants and the performance of an impacted population (Forbes and Depledge, 1996; Calow, 1996), and limits the effectiveness of sampling efforts and protocol design (Berthet et al., 2011; Devin et al., 2014; Crespo et al., 2015). Current approaches focus on standardization, by limiting genetic and phenotypic variability among organisms (Barrick et al., 2016). However, this may limit the generality of results, and lead to inadequate conclusions (Devin et al., 2014). Alternatively, correlational and variance partitioning procedures are increasingly used to model the contribution of phenotypic traits to the range of response variation within a population, and provide more ecologically-sound evidence of impacts (among others, McKinley et al., 2012; Drouhot et al., 2014; Chouvelon et al., 2017).

This approach has an important implication. If the contribution of individual response variability related with e.g., age, sex, growth, or reproduction is invariant in space or time, the reliability of the species as a biomonitor is corroborated, since other unpredictable intra-population sources of variation, in turn related with genotypes adaptation to local environmental conditions, are demonstrated to play only a secondary role (Berthet et al., 2011; Marchand et al., 2013; Devin et al., 2014). This line of reasoning can be extended to carbon, nitrogen, and other elements. Some recent studies have indicated extensive inter-individual variation in organismal elemental composition, due to the interplay of environmental factors with e.g., ontogeny, sex, and genetics (Goos et al., 2017 and literature cited; see also Leal et al., 2017). As for trace elements, the actual contribution of phenotypic traits in influencing the stoichiometry of freshwater invertebrate and vertebrate consumers is still widely debated (Atkinson et al., 2016).

Here, the first purpose was to assess the influence of sex, body size, and condition factor on the concentration of heavy metals and metalloids (B, Ba, Cd, Cr, Cu, Fe, Li, Mn, Ni, Pb, Sr, V, Zn; trace elements hereafter) as well as of macronutrients and major cations (C, N, Ca, Mg, K, Na; macrolements hereafter) in two populations of the swamp crayfish *Procambarus clarkii*. To this end, adult male and female specimens of different sizes were collected in Lake Bolsena and Lake Trasimeno, two basins located in central Italy. Element concentrations were measured in crayfish abdominal muscle, and compared with data on trace elements in bottom sediments and leaf detritus, as well as on cations concentration in lake water obtained from literature sources and monitoring campaigns. Nitrogen stable isotope signature was included among the explanatory variables to consider the potential effect of changes in trophic habits of the two populations. *P. clarkii* is an omnivore, adapting its diet - in terms of the relative contribution of animal and vegetal matter - to local trophic conditions (Gherardi and Barbaresi, 2007), and several studies have shown that heavy metal concentrations co-vary with nitrogen isotopic signatures (Alcorlo and Baltanás, 2013; Verburg et al., 2014).

Once tested the effects of phenotypic traits, the second aim of the study was to verify the consistency of elemental correlation profiles between Bolsena and Trasimeno crayfish. A core question was addressed: as the mean elemental fingerprint of crayfish populations changes responding to local environmental conditions, does the nature and strength of element bivariate correlations across specimens of the two populations remain unaffected? Bioindicator species are generally exposed to multiple metals, and site-independent interactions may influence metals uptake (Vellinger et al., 2013; Cedergreen, 2014). Specifically, metal-metal interactions are recognized to influence the accumulation kinetics of crayfish tissues, due to similarities in metabolic behaviour ultimately related to the sizes, valences, and functions of the interacting elements (e.g., Tunca et al., 2013; Bellante et al., 2015; Fikirdeşici Ergen et al., 2015). However, Liu and Wang, (2015), have recently highlighted the need to focus on interactions between trace- and macroelements to provide a more advanced interpretation of biomonitoring data, as macroelements may vary considerably due to local environmental conditions and affect trace elements concentration patterns (see also Yin and Wang, 2017). To date the nature and strength of interactions between trace- and macroelements have received relatively limited attention in crustaceans (e.g., heavy metals vs. calcium: Rainbow and Black, 2005; Tan and Wang, 2008) and are virtually unexplored in crayfish.

Classical univariate and multivariate non-parametric procedures where used to compare the two populations; Mantel tests and procrustean superimposition analyses were used to verify the consistency of their correlation profiles. Recently, procrustean analysis has been proven useful for multivariate environmental data (Sergeant et al., 2016); here, the procedure was used to estimate the concordance of the elemental multidimensional correlation structure of crayfish specimens from Lake Bolsena and Lake Trasimeno.

2. Materials and methods

2.1. Study sites

The investigation was performed in Lake Bolsena and Lake Trasimeno (central Italy; Fig. 1); in Table 1 the most important characteristics of the lakes are summarized. Lake Bolsena is located in a volcano-tectonic depression 305 m above sea level. It is the fifth lake in size in Italy and the largest volcanic basin in Europe; it has a single effluent (River Marta), a number of ephemeral tributaries, and receives most of its water from rainfall (Mosello et al., 2004). The lake is characterized by a low level of anthropogenic impact; it is used for recreational purposes and as a supply of water for drinking and agriculture (Di Francesco et al., 2016). Only during the summer months the population within the lake watershed rises from 22,000 up to 39,000 units, determining a considerable increase in wastewater discharges; other sources of pollution are represented by fertilisers derived from agricultural runoff (Di Francesco et al., 2016).

Lake Trasimeno is the largest laminar lake in the Italian peninsula. It is of tectonic origin, and is situated 258 m above sea level. The basin has a single artificial effluent and is fed by a tributary (Fosso Anguillara) and several ephemeral creeks; its hydrological regime is driven by precipitations and is characterized by considerable seasonal and multiannual oscillations in water level and chemistry (Ludovisi and Gaino, 2010). The watershed of the basin has diffuse anthropogenic sources of pollutants of agricultural, livestock, industrial, and urban origin; together with its hydrological features and shallowness (Tab. 1; average depth of 4.7 m) they make it highly vulnerable to contamination (Goretti et al., 2016).

Native to the southern United States, the red swamp crayfish *Procambarus clarkii* (Girard, 1852) was recorded in Italian waters in 1989 (Morpurgo et al., 2010) and to date is considered invasive, progressively extending its range from northern to southern regions of the peninsula (Cilenti et al., 2017). The species was observed in Lake Trasimeno in 2000 (Dörr et al., 2001) and in Lake Bolsena between 2004 and 2005 (Chiesa et al., 2006); to date, the basins are characterized by abundant wild populations, exploited as a fishery product (Scalici et al., 2009).

2.2. Samples collection

With the support of local fishermen, in early November 2014 crayfish were captured using fyke nets in shallow embayments (approximate depth = 2 m) in the localities of Marta and Sant'Arcangelo (Lake Bolsena and Lake Trasimeno, respectively; Fig 1). At both study sites, lake bottoms and shores were characterized by accumulations of decaying plant material. In Bolsena, *Chara* spp. detritus dominated; in Trasimeno reed (*Phragmites australis* Trin. ex Steudel) leaf litter and detritus of the genera *Myriophyllum*, *Potamogeton*, and *Vallisneria* were abundant (Fig. A, supplementary online material).

After collection, intact crayfish were transferred alive in refrigerated containers (4°C) to the laboratory, where they were sexed, wet weighed to the nearest 0.1 g, and had their carapace length measured in mm from the tip of the rostrum to the edge of the carapace using a caliper. After measurements, crayfish were euthanized by thermal shock (-80°C for 10 min) to avoid artifacts on elemental and isotopic determinations (Atwood, 2013). At each sampling location, after crayfish collection several individuals (min n = 20 per site) of the detritivorous amphipod *Echinogammarus* sp. were sampled using a hand net. Amphipods were placed alive in sterile falcon tubes and transferred to the laboratory, where they were kept in distilled water for 12 h to clear gut contents, and subsequently euthanized as already described. In addition, samples of the surficial sediment layer (3 replicates per site) were randomly collected by driving a methacrylate core (400 mm length, 114 mm ∅) into the sediment to a depth of approximately 5 cm. The core was extracted after sealing its upper end; the overlying water was then removed by aspiration and the sediment was transferred in sterilized plastic bags to refrigerated containers (4°C).

2.3. Chemical analyses

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

The abdominal muscle tissue was removed from each crayfish using a ceramic scalpel avoiding contamination from gut contents, stored in falcon tubes, frozen (-20°C, 48 h), freeze-dried for 48 h, and subsequently powdered using a mortar and pestle. Each sample was subsequently subdivided into two aliquots. The total concentrations of boron, barium, calcium, cadmium, chromium, copper, iron, potassium, lithium, magnesium, manganese, sodium, nickel, lead, strontium, vanadium, and zinc were determined on the first aliquot by wet digestion according to Raab et al., (2005). The procedure is described in detail in Zotti et al., (2016); in brief, subsamples of known dry weight (0.232 ± 0.005 g, mean ± 1SE; 0.119-0.321 g min-max) 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 elements content using an inductively coupled plasma atomic emission spectrometer (ICP-AES; Thermo Scientific iCap 6000 Series). Results were expressed as mg or g Kg^{-1} tissue dry weight.

The second aliquot was used for C and N total content and stable isotope analyses. To this end, subsamples were pressed into Ultra-Pure tin capsules (Costech Analytical Technologies) and analysed using an Elemental Analyser (Thermo Scientific Flash EA 1112) connected with an Isotope Ratio Mass Spectrometer (Thermo Scientific Delta Plus XP). Concentrations of total carbon (C) and nitrogen (N) were expressed as g Kg^{-1} tissue dry weight; isotopic signatures were expressed in conventional δ notation (as ‰) in relation to international standards (PeeDee Belemnite for carbon and atmospheric N_2 for nitrogen). 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 0.2 ‰ for both $\delta^{13}C$ and $\delta^{15}N$.

Amphipods were frozen, freeze-dried and powdered in composite samples obtained by combining 2-4 conspecifics of similar size. 5 composite samples per location were analyzed for C and N total content and stable isotope signature according to the aforementioned procedures. Since preliminary tests showed for both crayfish and amphipod tissues C:N ratios generally < 3.5 - 4, lipids were not removed following Skinner et al., (2016).

Sediment samples were frozen, freeze-dried, and sieved through a 2 mm plastic mesh. Plant detritus retained in the sieve was collected and powdered. Subsequently, subsamples of sediment and detritus (approximately 1 g and 0.25 g dry weight, respectively) were wet digested and measured for the concentration of B, Cd, Cr, Cu, Li, Mn, Ni, Pb, V, and Zn according to the aforementioned procedures. Results were expressed as mg Kg^{-1} dry weight.

2.4. Statistical analysis

Values in the text are expressed as mean ± 1 SE if not otherwise specified. Prior to analyses, all data were checked for normality (Shapiro-Wilks test) and homoscedasticity (Levene's test). Individual wet weight, carapace length and condition factor data met the assumptions after log-transformation. Comparisons were performed using a Student *t*-test for unpaired samples and separate variance estimation. Conversely, isotopic signatures and elemental concentrations were both non-normal and heteroscedastic; non-parametric univariate procedures (i.e., Mann-Whitney U test and Spearman rank correlation) were generally adopted.

Crayfish WW values were regressed against the respective carapace lengths (CL) according to the allometric equation $WW = a \times CL^b$, where a = initial growth coefficient and b = growth constant (Froese, 2006). Condition factors (CF hereafter) were calculated for each crayfish specimen i according to the equation (Streissl and Hödl, 2002):

$$CF_i = WW_i / a \times CL_i^b$$

C and N isotopic signatures of crayfish specimens were standardized, as $\delta^{15}N$ and $\delta^{13}C$ of basal resources can vary considerably across sampling locations (e.g., Layman et al., 2007), and influence consumer signatures without reflecting real differences in trophic habit. To this end, $\delta^{15}N$ signatures were converted into trophic position values (TP hereafter) according to the equation (Mancinelli et al., 2016, after Jepsen and Winemiller, 2002):

$$TP = \left(\delta^{15}N_{cf} - \delta^{15}N_{bas}\right)/\Delta^{15}N + \lambda$$

where $\delta^{15}N_{cf}$ is the nitrogen isotopic signature of the crayfish, $\Delta^{15}N$ is the trophic level fractionation of $\delta^{15}N$, while $\delta^{15}N_{bas}$ and λ are the nitrogen isotopic signature and the trophic level of the baseline indicator, respectively. $\delta^{15}N_{bas}$ was calculated using the mean isotopic signature of *Echinogammarus* sp. sampled at the two study locations (see Olsson et al., 2009 for assumptions). Accordingly, a $\lambda = 2$ was adopted (assuming basal resources = trophic level 1, primary consumers = trophic level 2, etc.); a mean trophic fractionation $\Delta^{15}N$ of 3.4‰ was derived from the literature (Glon et al., 2016). Carbon isotopic signatures were standardized using the equation in Olsson et al. (2009):

$$\delta^{13}C_{st} = \left(\delta^{13}C_{cf} - \delta^{13}C_{bas}\right)/\Delta^{13}C_{bas}$$

where $\delta^{13}C_{cf}$ is the carbon isotope signature of crayfish, while $\delta^{13}C_{bas}$ and $\Delta^{13}C_{bas}$ are the mean carbon isotope signature and the carbon range ($\delta^{13}C_{max} - \delta^{13}C_{min}$) of the baseline indicator, respectively.

For both crayfish populations, Euclidean distance similarity matrices were constructed with log-transformed and Z-scaled trace- and macroelement concentration data. Exploratory 3d non-metric multi-dimensional scaling (nMDS) was subsequently performed, while permutational multivariate analysis of variance (PERMANOVA; Anderson, 2005) was used to test the null hypothesis of no difference in elemental concentrations between the two lakes (fixed factor

"location", two levels) as influenced by sex (fixed factor "sex", two levels), and the continuous covariates "body size", "condition factor", and "trophic position" (log-transformed and Z-scaled). The carbon isotopic signature $\delta^{13}C_{st}$ was not included among the covariates as a preliminary screening indicated a significant negative correlation with TP values (Spearman correlation r = -0.29, $P < 0.05$, n =47; see also Fig. 3 in Results). *P* values were calculated using 999 unrestricted permutations.

For each population, Spearman rank correlation matrices were constructed using trace-, macrolements, and standardized isotopic signatures; their similarity was subsequently tested using a Mantel test with 999 permutations. Spearman coefficients were subsequently used as distance measures to construct lake-specific 3d nMDS plots of analytes; procrustean shape analysis was used to compare the similarity of the two plots, corroborate Mantel test results and to identify the analytes characterized by the lowest degree of inter-lake consistency. In brief, procrustean analysis fits one *n*-dimensional configuration to another using a combination of origin translation, rigid rotation and reflection of reference axes and uniform central dilation or contraction of scaling (Legendre and Legendre, 2012). The combination of transformations is found analytically, so as to minimize the sum of the squared distances between each point in the fitted configuration and its corresponding point in the target configuration (residuals). Compared with other tests of similarity between two matrices (e.g., Mantel test), procrustes analysis produces a correlation-like statistic (m^2), providing an overall measure of the concordance between the data sets, and estimates residual values that allow for the comparison of individual scores (Sergeant et al., 2016). In addition, a randomization test (PROTEST), can be used to test m^2 significance between the two compared matrices (Peres-Neto and Jackson, 2001).

All statistical procedures were implemented using the R package (R Development Core Team, 2016). Specifically, nMDS, PERMANOVA, and procrustes analyses were performed using the *metaMDS*, *adonis*, and *procrustes/protest* functions of the *vegan* package, respectively (Oksanen et al., 2016).

3. Results

3.1. Trace elements in sediments and plant detritus

Co, Cr, Cu, Mn, Ni, V, and Zn concentrations were significantly higher in sediments from Lake Trasimeno than in those from Lake Bolsena, while only Li was higher in Bolsena than in Trasimeno; B and Pb, conversely, showed negligible inter-lake differences (Tab. 2A). Element concentration patterns in plant detritus generally reflected those in sediments ($r = 0.72$, $P = 0.01$ and $r = 0.78$, $P = 0.005$, Lake Bolsena and Lake Trasimeno, respectively; 10 d.f.); Zn and V, however, showed negligible inter-lake variations (Tab. 2B). Noticeably, Cd, below the detection limits in sediments, in detritus was found at concentrations ten times higher in Trasimeno than in Bolsena. Additionally, detritus Mn content in Trasimeno was two orders of magnitude higher than in Bolsena (Tab. 2B).

3.2. Body size and condition factor of P. clarkii

On average, *Procambarus clarkii* individuals were characterized by a wet weight of 33.3 ± 1.8 g and a carapace length of 53.1 ± 0.9 mm. WW and CL values of Trasimeno specimens were significantly smaller than those from Bolsena, and negligible differences were generally observed between sexes (Tab. 3). The power regressions between the carapace length and wet weight data were highly significant for both lakes (Bolsena: $WW = 0.0001 \times CL^{3.1627}$, $r = 0.96$, $P < 0.0001$, 23 d.f.; Trasimeno: $WW = 0.0003 \times CL^{2.9386}$, $r = 0.92$, $P < 0.0001$, 22 d.f.). The condition factors estimated using the parameters derived from the allometric relationships differed significantly between lakes, with Trasimeno specimens showing higher values compared with Bolsena; no sex-related effects were detected (Tab. 3).

3.3. Elemental and isotopic fingerprint of P. clarkii

The nMDS analysis performed on trace elements indicated a separation of the two populations (Fig. 2A). A significant effect was detected only for the factor "location" (PERMANOVA, Pperm = 0.0001), while negligible effects were observed for all other factors, alone or interacting with each other (min Pperm = 0.09 for the interaction body size×location×sex). For each combination location-analyte, negligible inter-sex differences were generally observed (Mann-Whitney U tests, always $P > 0.05$). However, the mean concentration of analytes varied significantly between the two populations, with the exceptions of Co, Pb, V, and Zn (Tab. 4); specifically, Bolsena crayfish were characterized by significantly higher concentrations of B, Sr, and Li, the latter showing concentrations one order of magnitude higher than in Trasimeno crayfish. All the other trace elements, conversely, showed significantly lower concentrations.

The concentrations of B, Cd, Co, Cr, Cu, Li, Mn, Ni, Pb, V, Zn in crayfish tissues were significantly related with those determined in sediments and leaf plant detritus in both lakes (Fig. 3). Noticeably, Cu and Zn were characterized by an apparent incongruity (Fig. 3), and their exclusion determined a considerable increase in the significance of the positive covariation patterns of generally observed (Fig. 3).

Macronutrients (C, N, Ca, Mg, K, Na) confirmed what determined for trace elements. The nMDS plot showed for the two populations a clear separation (Fig. 2B). A PERMANOVA analysis indicated a significant effect only for the factor "location" (Pperm = 0.001); the factor "sex" and the covariates were characterized by negligible effects, alone or interacting with each other (min Pperm = 0.07 for the interaction "body size"×"location"). For each combination location-analyte, no inter-sex differences were observed (Mann-Whitney U tests, always $P > 0.05$). Further univariate tests showed that Bolsena crayfish were characterized by significantly lower Na and higher N, C, K, and Mg concentrations compared with Lake Trasimeno (Tab. 4).

Considerable inter-lake differences were apparent in the unstandardized $\delta^{13}C$ and $\delta^{15}N$ signature of crayfish (Fig. 4B). After normalization, however, the two populations showed similar trophic positions (Fig. 4A; Bolsena: TP = 2.37 ± 0.05; Trasimeno: TP = 2.31 ± 0.06) with negligible

inter-lake and inter-sex variations; similarly, no lake- or sex-related differences were observed in $\delta^{13}C_{st}$ signatures (Tab. 4).

3.4. Correlations profiles

In Tables A and B (supplementary online material) bivariate Spearman correlation coefficients among analytes are reported for each crayfish population. Correlations with individual wet weights and condition factors are included. Since previous analyses showed generally negligible differences between males and females, the factor "sex" was not considered further.

In general, Bolsena crayfish showed less than one half the significant correlations of Trasimeno (26 vs. 77), with positive relationships prevailing over negative ones (26 vs. 66, Bolsena and Trasimeno, respectively). Remarkable inter-populations differences in the nature and strength of analyte bivariate relationships were apparent. Specifically, *i*) within isotopic signatures, TP and $\delta^{13}C_{st}$ were unrelated in Bolsena, while they were negatively associated in Trasimeno; *ii*) within macroelements, in Bolsena C was positively related with N and negatively with Mg, the latter scaling positively with Ca; in Trasimeno C was associated only with N, and Mg was associated only with Ca; *iii*) within trace elements, positive associations were observed in Bolsena between: B and Cr/Ba/Cu/Mn/Sr/V/Zn; Cr and Fe/Mn/Ni/Zn; Fe and Zn/Ba/Cd; Li and Co/Sr/V; Mn and Ba/Ni. No significant associations were observed between Pb and any other analyte. Conversely, in Trasimeno Pb was positively related with Ba, Fe, and Li. The remaining trace elements were generally characterized by a high number of positive associations (Tab. B; 48 out of the possible 78), generally confirming other significant (e.g., Fe-Zn, Fe-Cr, Mn-Ni, Li-Sr) or nearly significant (e.g., Mn-Sr, Mn-Zn) associations observed for Bolsena crayfish.

Considerable differences were observed also in the relationships among trace-, macroelements, and isotopic signatures. In Bolsena, TP values scaled positively only with Zn, while $\delta^{13}C_{st}$ showed a negative association with Cd and Cu; Cr was related positively with Ca and negatively with K; positive associations were further observed between V and Mg/Na, the latter related

positively with Co and Li. In Trasimeno, Na was positively related with TP and with most of the trace elements with the exclusion of B, Li, Mn, Pb, and V. In addition, both N and C were negatively associated with Co/Cu/Ni; Ca was positively related with B/Cd/Cr/Sr; K scaled negatively with Cr/Li/Sr, while Mg was positively related with B/Co/Sr/V.

Notwithstanding the above-listed differences, a general consistency was observed in the nature and strength of the correlation coefficients characterizing the two populations (Fig. 5A). A Mantel test performed on the two correlation matrices indicated a significant correspondence between the two correlation matrices ($Z = 34.7$, $P = 0.001$); additionally, the procrustean analysis performed on 3d nMDS plots constructed using correlation coefficients as between-analytes distance measures eventually confirmed the similarity of the two crayfish populations in terms of inter-individual co-variation of analyte concentrations (Fig. 5B; PROTEST test: $m^2 = 0.77$, $P = 0.008$). From figure 5A it is apparent that the most substantial departures were observed for relationships involving the trace elements B, Li, and Pb, the macroelements C, N, K, and Ca, and the carbon isotopic signature $\delta^{13}C_{st}$. Procrustes analyses corroborated these qualitative observations and allowed to quantitatively rank the strength of the departure of each analyte, with the largest inter-lake residuals observed for C and N, followed by Li, N, K, Pb, $\delta^{13}C_{st}$, Ca, and B (Fig. 5C).

4. Discussion

4.1. Inter-lake variations in crayfish elemental fingerprints

Useful biomonitors are those species that accumulate a pollutant over the normal range in their tissues, integrate the pollution signal spatially or temporally in their tissues, and mirror ambient contaminant levels (Caro, 2010 after Beeby, 2001). In our study, location was the only factor affecting the elemental fingerprint of *Procambarus clarkii* muscle tissues. No effects were detected for sex and for other continuous covariates included in the analysis, confirming (sex: Elia et al., 2006; Kouba et al., 2010; body size: Bellante et al., 2015; Fikirdeşici Ergen et al.,

2015) or contradicting (trophic position: Larsson et al., 2007; Alcorlo and Baltanás, 2013) earlier investigations and reviews on crayfish. Furthermore, the negligible effect observed for the condition factor excluded significant influences of crayfish growth phase on analytes concentration and *vice versa* (see Cizdziel et al., 2002; Bervoets and Blust, 2003 for fish). *P. clarkii* was confirmed as an effective biomonitor species for both trace- and macroelements, capable of providing information predominantly reflecting environmental conditions while being relatively unaffected from other sources of inter-individual variations. Noticeably, the sediments of both lakes were characterized by heavy metal contents under standard threshold values (Tab. 2), further confirming *P. clarkii* as a model indicator species, capable to accumulate metals reflecting even low environmental concentrations (Alcorlo et al., 2006; Goretti et al., 2016). Inter-lake differences in trace elements in crayfish generally reflected those observed in plant detritus (Fig. 3). *P. clarkii* is omnivorous, including animal prey as well as plant material depending on their availability (Gherardi and Barbaresi, 2007; see also Mancinelli et al., 2013). Thus, the observed concordance may be attributed to a trophic relationship between a consumer and its resource. The only exceptions were Cu and Zn: they are essential to crayfish metabolism and their accumulation are generally independent from environmental concentrations (Alcorlo et al., 2006; Kouba et al., 2010).

Inter-lake differences in plant detritus elemental fingerprint were in turn related with local water or sediment geochemistry. For example, crayfish mirrored the considerable enrichment of Bolsena plant detritus in B and Li (Tab. 2 and 4). A high concentration of both elements characterizes Lake Bolsena waters, due to the volcanic origin of the basin (Mosello et al., 2004; Sappa et al., 2014); they may have been accumulated in living and dead plant material (Mitra, 2015), and ultimately transferred to crayfish via trophic consumption. Similarly, natural factors as well as anthropogenic contamination may have determined the high Mn content of Trasimeno sediments, while species-specific mechanisms of accumulation may have governed plant detritus concentrations (Schaller et al., 2011; Harguinteguy et al., 2016).

Local environmental conditions related with the nature and origin of trophic resources and with water chemistry are likely to have determined the macroelement profile of the two crayfish populations. Significant differences were observed in total carbon and nitrogen contents (Tab. 4). A similar trophic position characterized the two populations (Fig. 4), excluding that a differential contributions of animal and plant items in the diet affected tissue CN contents, as observed in other studies (Alcorlo and Baltanás, 2013). Alternatively, it can be hypothesized that site-specific CN concentrations of detrital resources may have influenced those of crayfish consumers. Changes in CN stoichiometry of trophic resources in lacustrine habitats have been indicated to generally reflect on benthivorous invertebrates and vertebrates (Cai et al., 2016; Tuckett et al., 2016); furthermore,variations in detritus CN stoichiometry have been shown to reflect on the body composition of *P. clarkii* (Adams et al., 2005). Indeed, the contribution to the detrital resource pool of aquatic macrophytes and riparian vegetation - widely differing in CN chemical quality (e.g., Enríquez et al., 1993) - varied considerably between the two lakes (Fig. A, supplementary online material). The hypothesis is further confirmed by the detritivorous amphipod *Echinogammarus* sp., used as isotopic baseline, that showed a similar variability in CN signatures between Bolsena and Trasimeno (N = 6.48 ± 0.28 vs. 5.53 ± 0.29; C = 39.07 ± 1.73 vs. 33.78 ± 1.41; t -tests for separate variances, P always < 0.05).

With the exception of Ca, the concentrations of K, Mg, and Na in crayfish tissue varied between lakes (Tab. 4). *P. clarkii* is generally known to be a good hyperosmoregulator in freshwater (Sarver et al., 1994). The significantly higher concentration of sodium observed in the Trasimeno population, however, indicates a direct response to water chemistry. The higher conductivity measured in November 2014 in Lake Trasimeno, compared with Lake Bolsena, actually reflects a generally higher concentration of the Na in water (Tab. 1), as in the last decades a net accumulation of salts has occurred in the lake (Ludovisi et al., 2013). Similarly, the significantly higher concentration of K observed in the Bolsena population reflects the higher water

concentration of the cation (Tab. 1), in turn determined by the potassic nature of rocks of the Bolsena volcanic district (Mosello et al., 2004).

Remarkably, the total equivalent concentration of Ca, K, Mg, and Na was virtually identical (741.2 vs. 741.8 meq kg^{-1}, Bolsena and Trasimeno, respectively); additionally, similar concentrations were cumulatively observed for the alkali K and Na (662.5 vs. 664.6 meq kg^{-1}) and the alkaline Ca and Mg (78.7 vs. 78.2 meq kg^{-1}) as well as the ratio alkali vs. alkaline (0.12 in both lakes).Thus, the different cationic composition of the waters seems to affect the relative abundance of the alkali metals in the muscle tissues of *P. clarkii*, determining statistically different fingerprints for them. However, the osmotic regulation by *P. clarkii* may be effective in maintaining similar, balanced concentrations in muscle tissues in spite of the different environmental conditions.

4.2. Correlation profiles

Analyte correlations in *Procambarus clarkii* tissue varied considerably between Lake Bolsena and Lake Trasimeno, with the former characterized by a lower number of statistically significant relationships. However, a qualitative scrutiny of inter-lake association patterns (Fig. 5A) as well as quantitative examinations performed using Mantel tests and procrustean analysis (Fig. 5B) indicated an overall consistency of analyte association patterns between the two lakes. Thus, our results indicate that the chemistry of the environment together with locally-determined physiological and metabolical variations occurring at the individual scale affect the strength and nature of relationships between specific analytes, but not general association patterns .

An in-depth scrutiny of each correlation is beyond the scope the present investigation; however, inter-lake correlations comparison (Fig. 5A) and, most importantly, procrustean residuals (Fig. 5C) indicated that carbon and nitrogen were characterized by the highest variability in the nature and strength of association with other analytes. Specifically, the relationships observed for Lake Trasimeno crayfish between C and N with trace elements may provide a key for a general

understanding of the results. the inverse scaling with Co, Cu, and Ni, suggested the occurrence of a somatic growth dilution effect (SGD: Karimi et al., 2007). Compared with the rest of the year, between October and November Trasimeno crayfish are characterized by high growth rates (Dörr et al., 2006; Dörr and Scalici, 2013). In the period preceding this study crayfish total biomass (and, in turn, total C and N) may thus have increased outpacing heavy metals gain from food and diluting their mass-specific concentrations in abdominal muscles (Karimi et al., 2010). No previous information are available on *P. clarkii* growth patterns in Lake Bolsena; however, in our study crayfish mean wet weight was significantly lower than that determined in Trasimeno; additionally, the lower condition factor indicated that, compared with Trasimeno, the population was in a reduced growth phase, and dilution effects may have been weaker. The generally positive, non-significant relationships of C and N with Co, Cu, and Ni observed in Bolsena and the nearly-significant negative relationships observed in Trasimeno between C and N and individual wet weight (Table A online information; $P = 0.08$ and 0.07, respectively) provide a further support to an SGD dilution effect.

Assuming an SGD framework, the interpretation of trace element correlation patterns are straightforward: in Bolsena the reduced growth phase of the crayfish population, coupled with the relatively low concentrations of most of the trace elements in the benthic matrices (Tab. 4), increased the "biological noise" due to physiological and metabolical variations at the individual scale and weakening the strength of bivariate relationships. Conversely, the relatively high trace element concentrations characterizing Lake Trasimeno reflected in the tissues of metabolically-active, still-growing crayfish individuals, ultimately reflecting in stronger couplings in metal-metal accumulation kinetics. Specifically, the general positive relationships observed between divalent metals (Pb, Cd, Cu, Zn), suggest that a shared divalent transporter such as DMT1 might be involved in their synergistic accumulation, as suggested by Fikirdeşici Ergen et al., (2015) for *Astacus leptodactylus*. In addition, correlations observed between Cr, Cu, Fe and Ni can be attributed to shared transport pathways between non-essential metals and their essential

counterparts, as these metals are all known to be transported by transferrins, a protein family that has been reported in crayfish and freshwater prawns (Liang et al., 1997; Toe et al., 2012). Transferrins play an important role in the transport of Fe in particular, but also facilitate the transport of Cr^{3+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} (Fikirdeşici Ergen et al., 2015 and literature cited).
The negative relationships observed between K and Cr, Li, Sr, and Zn eventually require a different explanation. In euryhaline crustaceans the osmoregulatory system is known to control metal uptake (Wildgust and Jones, 1998; Verslycke et al., 2003); thus, inter-individual variability in osmoregulation of K uptake may have determined the observed patterns. This line of reasoning can be extended to explain the number of positive associations between the cations Ca, Mg, and Na and trace elements (Tab. B online information); yet, it is necessary to emphasize that to date, little work has been done in this field, and variability at an inter-individual scale is actually unexplored. Two exceptions are represented by the associations Na-Cu and Ca-Cd. Copper uptake interacts with that of sodium (Paquin et al., 2002), while Cd uptake can increase along with Ca assimilation: Cd^{2+}, has an ionic radius of 0.97Å, very close to that of Ca^{2+} (0.99Å), and thus it is taken up through the gills by the apical Ca^{2+} channel and basolateral Ca^{2+} adenosine triphosphatase and $3Na^{+}/Ca^{2+}$ ion exchanger (Bondgaard and Bjerregaard, 2005; Wigginton and Birge, 2007).

4.3. Conclusions

Here an effort was made to analyze in *P. clarkii* from Lake Bolsena and Lake Trasimeno an extended set of analytes, including macronutrients and major cations, trace elements, as well as carbon and nitrogen stable isotopes. This allowed an advanced profiling of the elemental fingerprint of the crayfish and of its relationship with those characterizing non-living matrices of the benthic environment as well as with water chemistry. A further, previously unattempted confirmation of *P. clarkii* as a model bioindicator was provided by the scrutiny of analyte correlation profiles. The local chemistry of the benthic environment was shown to interact with

the growth phase characterizing the crayfish populations, ultimately varying the strength and, to some extent, the nature of the relationships. Nevertheless, the overall consistency of the analyte correlation patterns characterizing the two populations actually indicated that their ultimate nature is species-specific, and robust against biases induced by locally-determined inter-individual variations in metabolism and physiology.

As measurements were performed on crayfish muscle tissues, generally characterized by reduced trace element contents compared with e.g., hepatopancreas (Kouba et al., 2010), further investigations including different crayfish tissues are needed, in particular for Lake Bolsena. While several studies have focused in Lake Trasimeno on heavy metals in sediments, fish, and in *Procambarus clarkii* (e.g., Elia et al., 2010; Di Veroli et al., 2012; Goretti et al., 2016), for Lake Bolsena, besides the data presented here, available data are limited only to fish (Orban et al., 2006; Pujolar et al., 2012).

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Figure captions

Figure 1. Map of Italy indicating the position of Lake Bolsena and Lake Trasimeno. A digital elevation map of the area where the two lakes are located is shown in the inset; sampling sites are also reported.

Figure 2. 3d nMDS plots based on Euclidean distance similarity matrices of trace- and macroelement concentrations in the tail muscle tissue of *Procambarus clarkii* from Lake Bolsena (full circles) and Lake Trasimeno (empty circles). Letters in the plot indicate females (f) and males (m).

Figure 3. Relationships between trace element concentrations in *Procambarus clarkii* muscle tissues and in sediments (A) and in plant detritus (B). The Spearman correlation coefficient *r* of the relationships are reported; *r* values calculated excluding Cu and Zn are included in parentheses. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Please note the Log+1 scale of x and y axes in both graphs.

Figure 4. Isotopic biplot of trophic position and normalized $\delta^{13}C$ values of Lake Bolsena (full circles) and Lake Trasimeno (empty circles) *Procambarus clarkii* specimens (A). Associated box plots show means (symbol), standard errors (box), and standard deviations (whiskers) calculated for each population. In the inset (B) a box-plot showing the original $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures of specimens is reported. Squares indicate the mean isotopic signature (±1SE) of the gammaridean *Echinogammarus* sp., used as a baseline species to calculate *P. clarkii* trophic position and normalized $\delta^{13}C$ values.

Figure 5. (A) Correspondence between-analyte Spearman correlation coefficients in Lake Bolsena and Lake Trasimeno. Black squares: associations involving N; grey diamonds = C; grey squares = K; black circles = Ni; grey circles = $\delta^{13}C_{st}$; black triangles = Pb; grey triangles = Li. (B) Procrustean superimposition analysis of 3d distances among macro-, trace elements, and isotopic signatures of *P. clarkii* from Bolsena (arrows end) and Trasimeno (empty circles). Length of arrows represents the projection of residuals on the first two axes. Grey lines indicate the projection of the three rotation axes. (C) Ordination of unprojected procrustes residuals.

Table 1. Summary of the main characteristics of Lake Bolsena and Lake Trasimeno. Physical-chemical data were collected at off-shore sampling stations (Bolsena: 11°56'27.68" E, 42°35'29.21"N; Trasimeno: 12°06'46.57" E, 43°09'17.11"N) during monitoring activities of local Regional Agencies for the Protection of the Environment (ARPA) at the time of the present study (Lake Bolsena: 03/11/2014; Lake Trasimeno: 10/11/2014). Concentrations of major cations are from Mosello et al., 2004 (Lake Bolsena, winter 2003, epilimnion 0-15 m) and Ludovisi and Gaino, 2010 (Lake Trasimeno, spring 2005, surface layer). For Lake Bolsena, data for an off-shore site (42°34'59.05"N, 11°56'30.47"E) taken in March 2015 (Bruni, 2015) are also reported in brackets.

	Lake Bolsena	Lake Trasimeno
Coordinates	42°35'36.98"N, 11°56'24.62"E	43° 8'14.60"N, 12° 6'36.98"E
Origin	Volcanic	Tectonic
Area (km^2)	114	124
Volume (km^3)	9.2	0.59
Maximum depth (m)	151	6
Outlet discharge ($m^3\ s^{-1}$)	2.4	0.9
Residence time (years)	120 [a]	21
Trophic classification	Mesotrophic	Meso-Eutrophic
Alkalinity [mg L^{-1} $Ca(HCO_3)_2$]	220	297
Conductivity (µS cm^{-1}, 20°C)	487	1233
P-PO_4^{3-} (mg L^{-1})	0.28	0.03
Dissolved oxygen (mg L^{-1})	6.9	9
Temperature (°C)	17.1	15
Cations (meq L^{-1})		
Ca^{2+}	1.04 (0.98)	2.03
Mg^{2+}	1.28 (1.32)	2.93
Na^{+}	1.87 (1.86)	8.57
K^{+}	1.24 (1.25)	0.23

[a] Due to a recent decrease in the outflow of the Marta River effluent, residence time currently exceeds 400 years (Di Francesco et al., 2016)

Table 2. Summary of trace element concentrations (mg Kg^{-1}) in sediment and plant detritus samples (*n* =3 per site/type) collected in Lake Bolsena and Lake Trasimeno. Means, SE in brackets; Mann-Whitney U test results are included. For sediments, standard threshold values (TEC = threshold effect concentration; MEC = midpoint effect concentration; PEC = probable effect concentration) calculated according with Consensus Based Sediment Quality guidelines (MacDonald et al., 2000; Contaminated_Sediment_Standing_Team, 2003) are reported. In both tables, * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; NS = $P > 0.05$.

A) Sediment

	Lake Bolsena	Lake Trasimeno	U test	TEC	MEC	PEC
B	22.35 (2.69)	23.31 (2.13)	NS			
Cd	< 0.0001	< 0.0001	nd	0.99	3	4.98
Co	5.12 (0.16)	9.15 (0.17)	***			
Cr	22.47 (0.27)	73.75 (3.48)	**	43.4	76.5	111
Cu	7.38 (0.82)	15.69 (1.22)	**	31.6	91	149
Li	31.07 (0.07)	26.81 (0.87)	*			
Mn	244.59 (7.44)	329.76 (5.08)	**	460	780	1100
Ni	6.86 (0.20)	30.25 (1.05)	**	22.7	36	48.6
Pb	8.04 (0.04)	15.67 (7.10)	NS	35.8	83	128
V	44.01 (0.37)	52.87 (1.71)	*			
Zn	25.77 (0.32)	39.98 (0.75)	***	121	290	459

B) Plant detritus

	Lake Bolsena	Lake Trasimeno	U test
B	81.90 (5.13)	25.03 (0.10)	**
Cd	0.004 (0.001)	0.052 (0.003)	***
Co	1.43 (0.26)	2.39 (0.06)	*
Cr	3.23 (0.95)	14.07 (0.67)	**
Cu	10.40 (0.15)	13.74 (0.02)	***
Li	7.65 (0.82)	3.84 (0.26)	*
Mn	59.15 (7.69)	1001.52 (13.20)	***
Ni	0.04 (0.02)	6.00 (0.21)	***
Pb	3.40 (0.43)	4.56 (0.10)	NS
V	18.56 (1.84)	22.18 (0.46)	NS
Zn	50.70 (2.68)	72.91 (19.70)	NS

Table 3. Summary of biometric data of *Procambarus clarkii* specimens sampled in Lake Bolsena and Lake Trasimeno. Means, SE in brackets. Data refer to 25 specimens for Lake Bolsena Lake and 24 for Lake Trasimeno, as one was lost during preparation. For crayfish wet weight, carapace length and condition factor the results of 2-way ANOVA tests are included, with factor F1 = "location", and factor F2 = "sex"; the effects of the latter was always not significant ($P > 0.05$), alone or interacting with F1.

Parameter	**Lake Bolsena**	**Lake Trasimeno**	**ANOVA**
N. of analyzed specimens	25	24	
Wet weight range (min – max, g)	17.78 - 65.66	12.69 - 51.73	
Individual wet weight (g)	36.71 (2.74)	29.83 (0.04)	F1: $P = 0.02$
Carapace length range (min – max, mm)	45-67	40-64	
Individual carapace length (mm)	55.03 (1.29)	51.15 (1.33)	F1: $P = 0.03$
Condition factor	0.96 (0.02)	1.02 (0.02)	F1: $P = 0.03$

Table 4. Summary of element concentrations and isotope signatures of *Procambarus clarkii* specimens sampled in Lake Bolsena and Lake Trasimeno. Means, SE in brackets. Mann-Whitney U-test results are included. As in Table 3, data refer to 25 specimens for Lake Bolsena Lake and 24 for Lake Trasimeno.

Parameter/site	**Lake Bolsena**	**Lake Trasimeno**	**U-test [a]**
Macroelements (g Kg^{-1})			
N	119.12 (2.21)	104.44 (2.29)	***
C	398.83 (10.23)	345.73 (10.65)	**
Ca	3.29 (0.38)	3.58 (0.41)	NS
K	16.77 (0.38)	15.01 (0.39)	**
Mg	1.83 (0.04)	1.63 (0.05)	**
Na	5.37 (0.39)	6.43 (0.41)	*
Trace elements (mg Kg^{-1})			
B	1.4 (0.08)	0.84 (0.09)	***
Ba	1.63 (0.19)	2.63 (0.39)	**
Cd	0.03 (0.001)	0.05 (0.001)	*
Co	0.06 (0.02)	0.05 (0.02)	NS
Cr	0.20 (0.02)	0.29 (0.04)	*
Cu	29.11 (2.13)	39.14 (2.82)	*
Fe	22.04 (4.28)	39.35 (4.88)	***
Li	0.33 (0.05)	0.05 (0.01)	***
Mn	2.86 (0.35)	21.4 (7.53)	***
Ni	0.21 (0.02)	0.48 (0.05)	***
Pb	0.12 (0.02)	0.13 (0.01)	NS
Sr	26.73 (3.41)	11.61 (1.59)	***
V	2.45 (0.04)	2.33 (0.05)	NS
Zn	66.78 (2.59)	60.94 (2.37)	NS
Isotopic signatures [b]			
$\delta^{13}C_{st}$	-0.13 (0.11)	-0.07 (0.12)	NS[c]
TP	2.34 (0.06)	2.18 (0.05)	NS[c]

[a] * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; NS = $P > 0.05$;
[b] both variables are standardized
[c] refer to ANOVA results; no effect detected for the factor "sex" ($P > 0.05$)

Figure 1

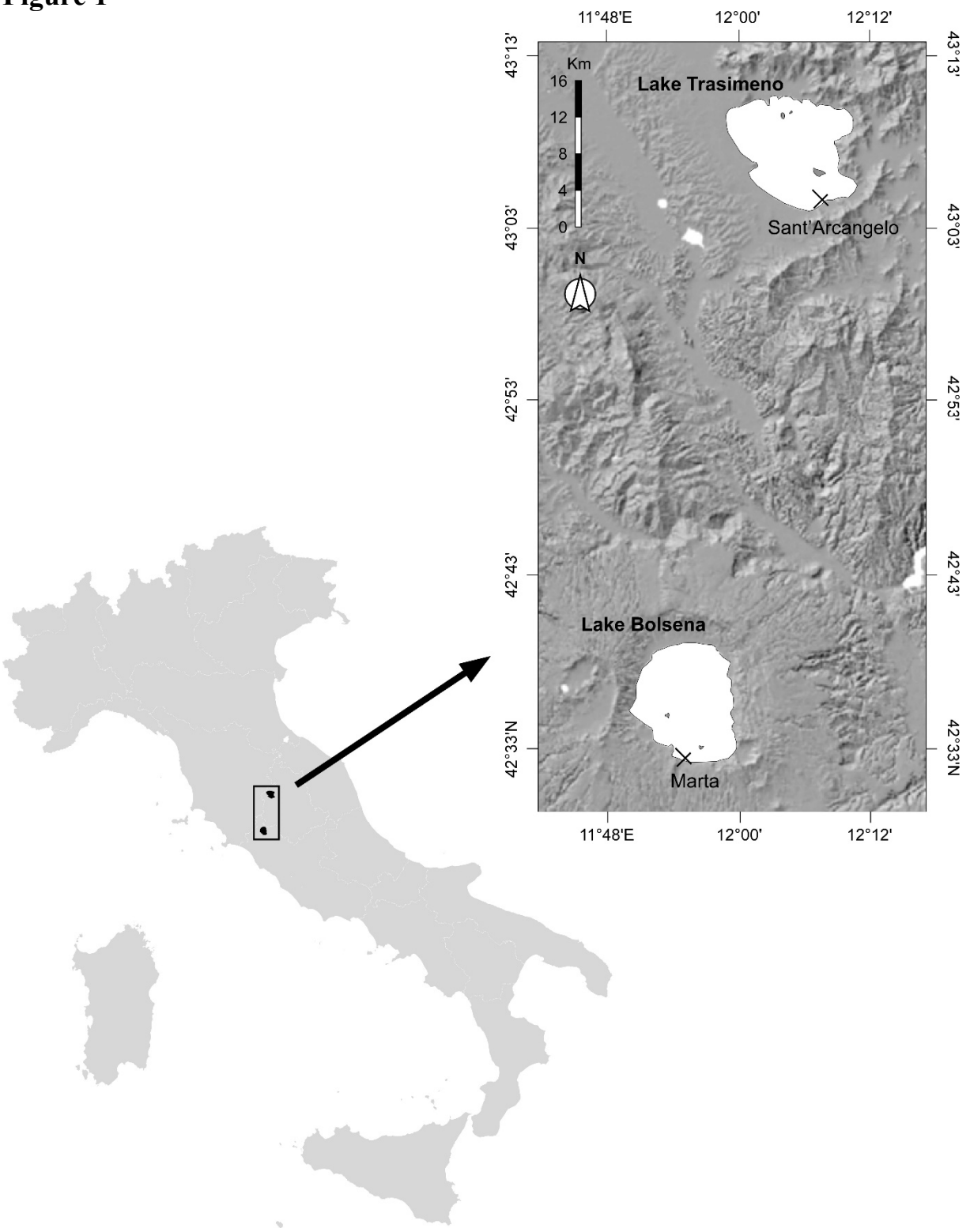

11°48'E
12°00'
12°12'
43°13'
43°03'
42°53'
42°43'
42°33'N
Km
16
12
8
4
0
N
Lake Trasimeno
Sant'Arcangelo
Lake Bolsena
Marta

Figure 2

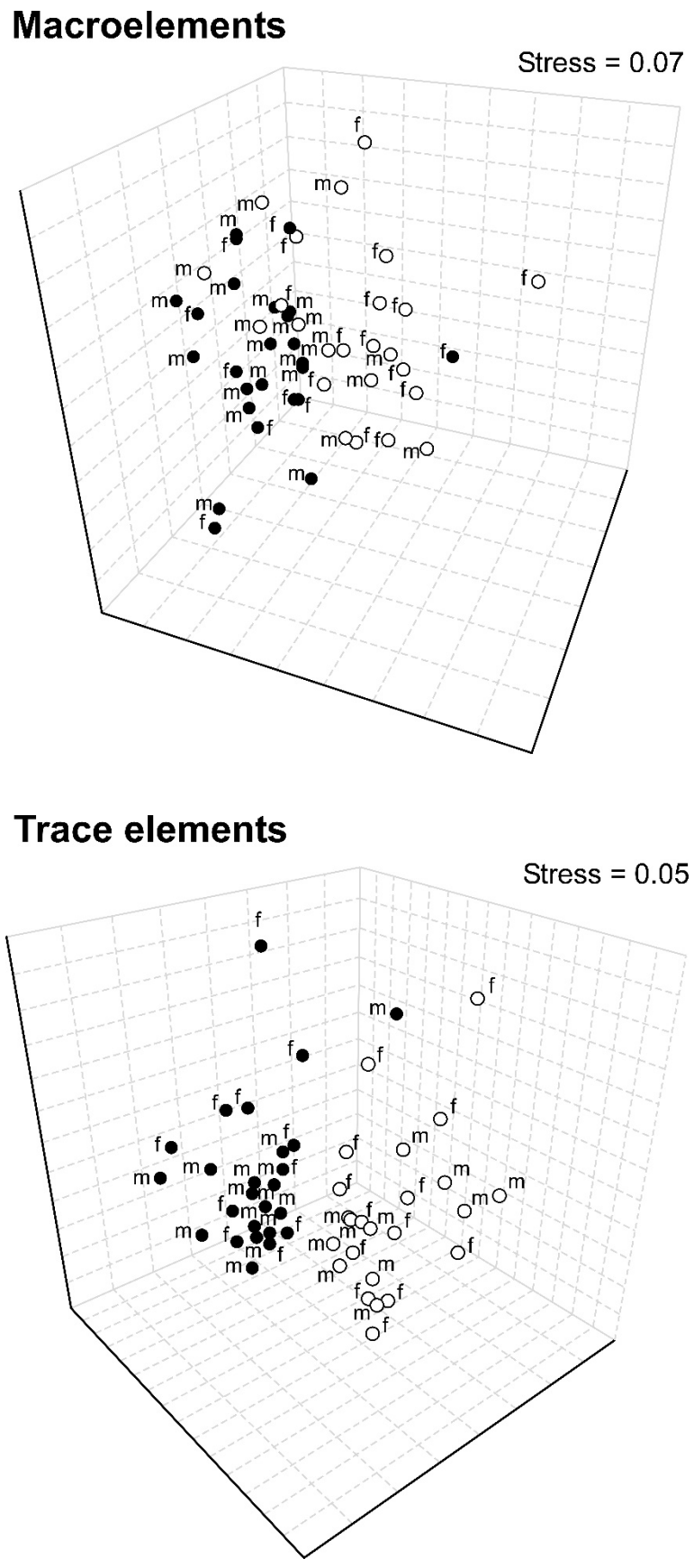


Figure 3

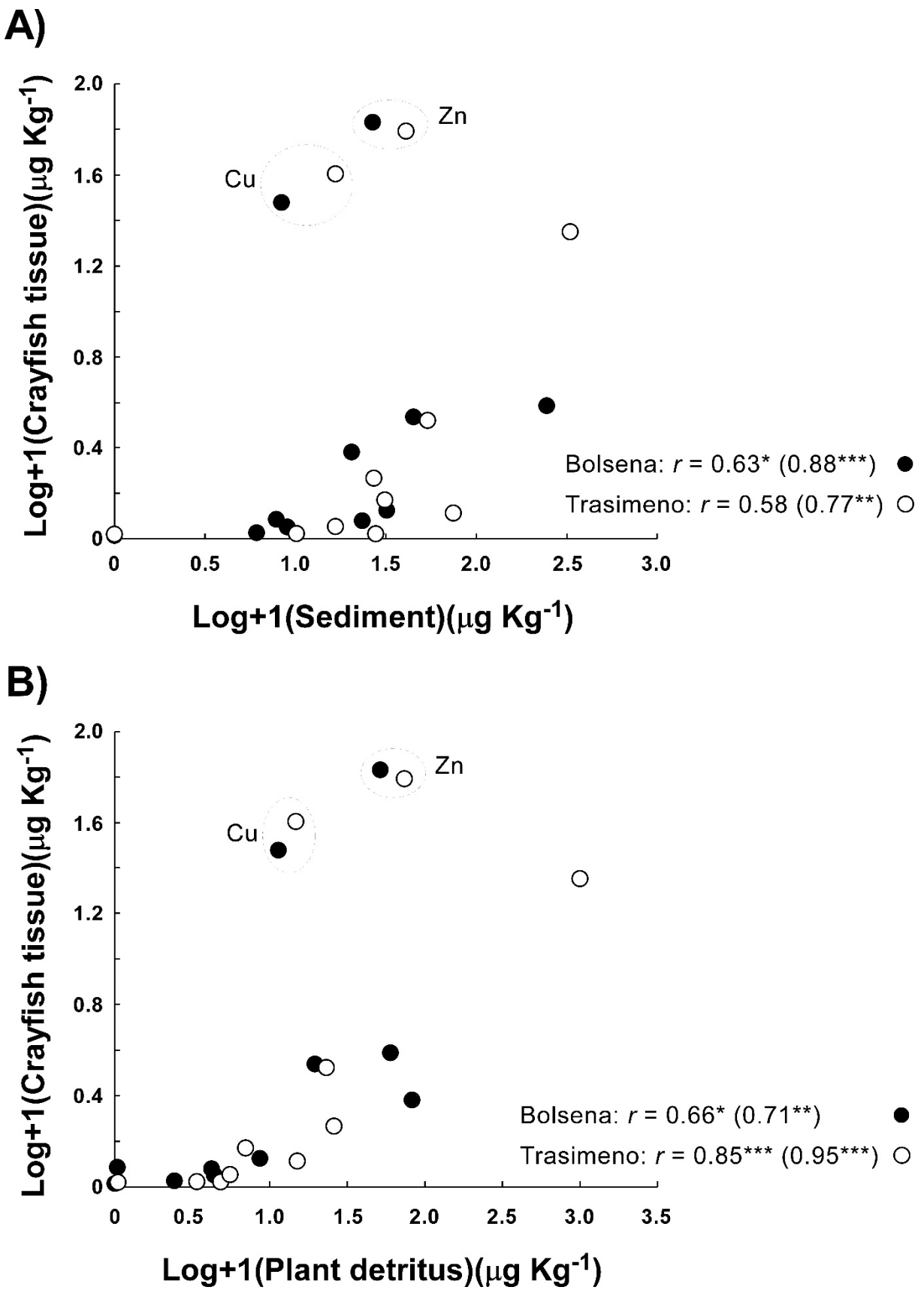

A)
Log+1(Crayfish tissue)(μg Kg-1)
2.0
1.6
1.2
0.8
0.4
0
0
0.5
1.0
1.5
2.0
2.5
3.0
Zn
Cu
Bolsena: r = 0.63* (0.88***)
Trasimeno: r = 0.58 (0.77**)
Log+1(Sediment)(μg Kg-1)
B)
Log+1(Crayfish tissue)(μg Kg-1)
2.0
1.6
1.2
0.8
0.4
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Zn
Cu
Bolsena: r = 0.66* (0.71**)
Trasimeno: r = 0.85*** (0.95***)
Log+1(Plant detritus)(μg Kg-1)

Figure 4

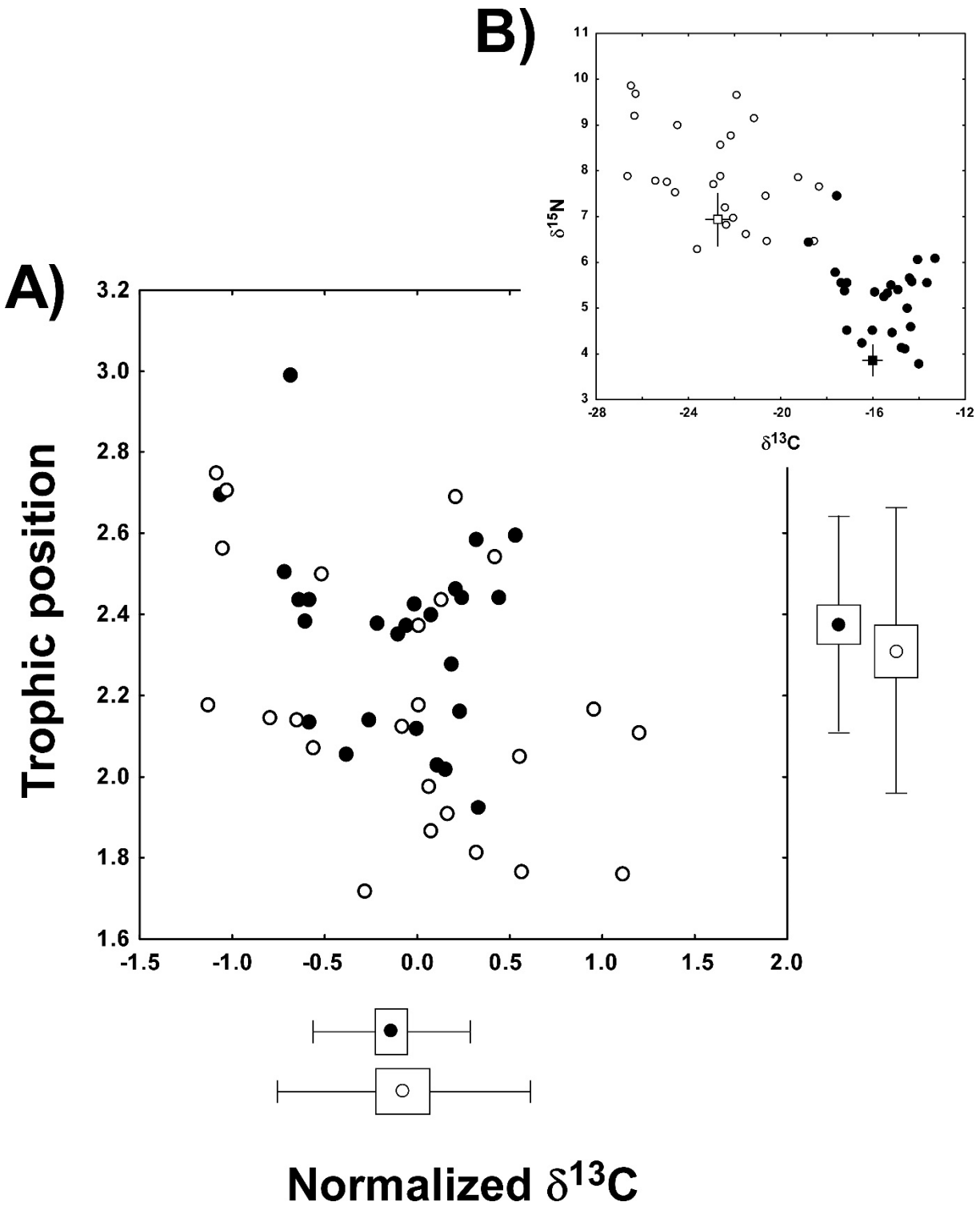
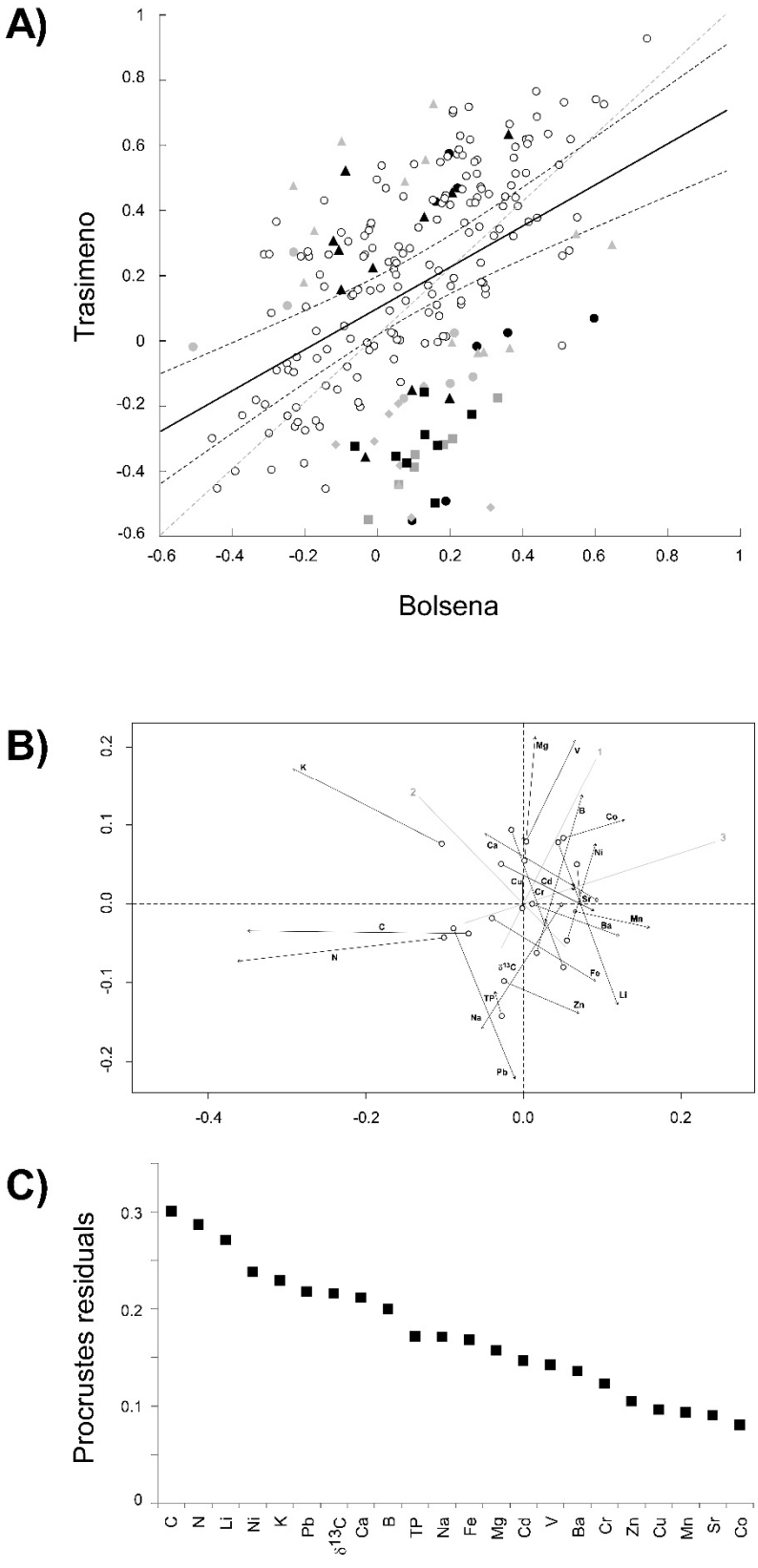

B)
A)
$\delta^{15}N$
$\delta^{13}C$
Trophic position
Normalized $\delta^{13}C$

Figure 5


A)
Trasimeno
Bolsena
B)
C)
Procrustes residuals
C
N
Li
Ni
K
Pb
δ13C
Ca
B
TP
Na
Fe
Mg
Cd
V
Ba
Cr
Zn
Cu
Mn
Sr
Co

Graphical abstract

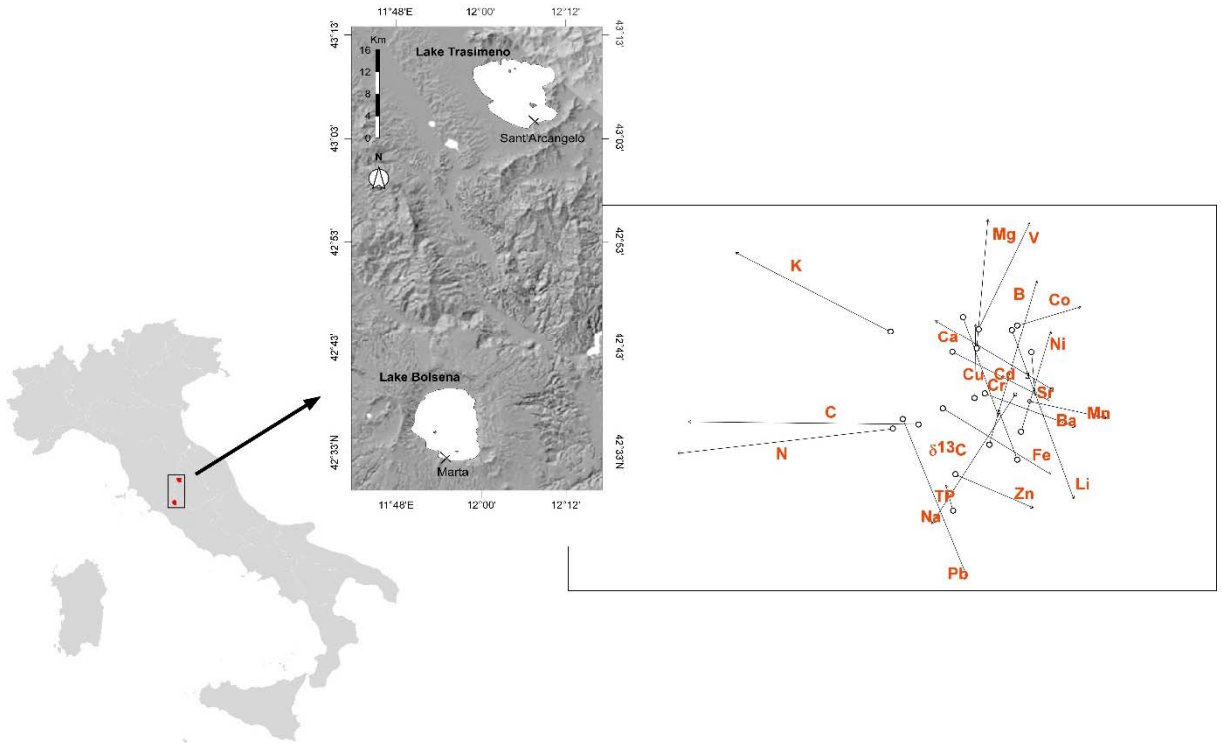


Figure A

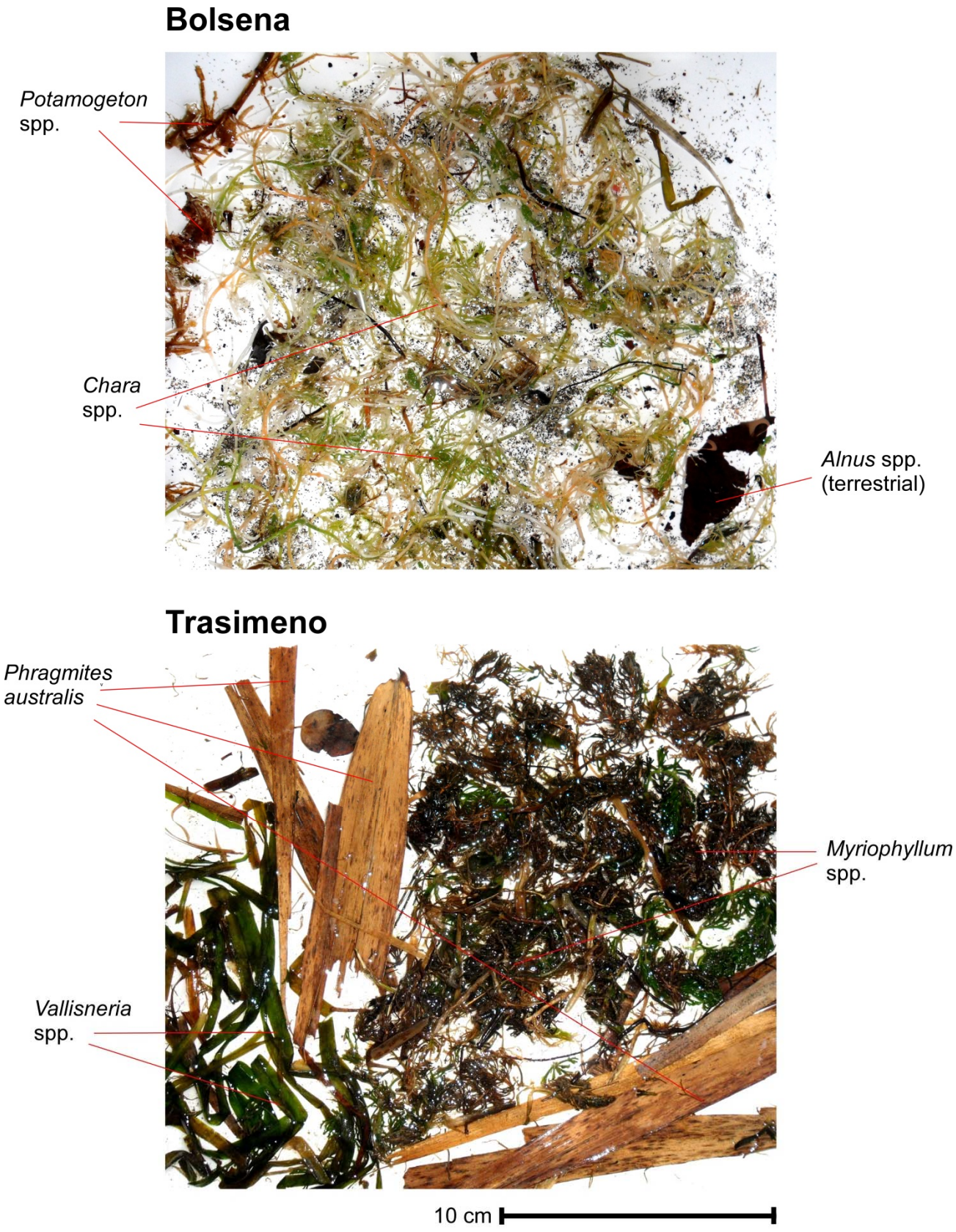

Bolsena
Potamogeton spp.
Chara spp.
Alnus spp. (terrestrial)
Trasimeno
Phragmites australis
Myriophyllum spp.
Vallisneria spp.
10 cm

Table A. Spearman correlation coefficients among trace-, macroelements, and carbon and nitrogen isotopic signatures (expressed as standardized trophic position TP and $\delta^{13}C_{st}$) for Lake Bolsena crayfish. Correlation coefficients between analytes and crayfish condition factor (CF), wet weight (WW) are also reported. Significant correlations (P <0.05) are in bold.

	CF	WW	TP	$\delta^{13}C_{st}$	N	C	Ca	K	Mg	Na	B	Ba	Cd	Co	Cr	Cu	Fe	Li	Mn	Ni	Pb	Sr	V
WW	0.29																						
TP	-0.05	**0.48**																					
$\delta^{13}C_{st}$	-0.15	0.23	-0.13																				
N	0.03	0.07	0.08	-0.19																			
C	-0.11	0.13	0.15	-0.10	**0.78**																		
Ca	0.01	-0.18	-0.18	0.23	0.06	0.08																	
K	-0.02	-0.30	-0.21	-0.07	0.31	0.19	-0.10																
Mg	-0.32	**-0.44**	-0.24	0.28	-0.28	**-0.45**	**0.40**	0.21															
Na	0.20	**-0.52**	-0.34	0.07	-0.21	-0.16	0.00	0.36	0.32														
B	0.02	0.07	0.06	0.22	0.18	-0.15	0.21	-0.24	0.27	-0.02													
Ba	0.10	-0.24	-0.09	-0.16	0.07	0.01	0.31	0.20	0.16	0.22	0.20												
Cd	0.29	-0.38	-0.18	**-0.44**	0.15	-0.03	0.17	0.12	-0.02	0.30	0.25	0.39											
Co	-0.03	-0.32	-0.20	0.01	-0.07	0.11	0.30	0.23	0.03	**0.40**	0.04	0.12	0.29										
Cr	0.22	0.10	0.05	0.16	0.15	-0.04	**0.44**	**-0.44**	-0.07	-0.17	**0.43**	0.31	0.23	0.01									
Cu	0.19	-0.20	0.07	**-0.51**	0.18	0.33	-0.08	-0.06	-0.32	0.24	-0.12	**0.54**	0.39	0.18	0.07								
Fe	0.29	-0.11	-0.01	-0.24	0.28	0.05	0.16	0.12	-0.01	0.29	0.28	**0.54**	**0.65**	-0.01	**0.44**	0.36							
Li	-0.12	-0.21	-0.19	0.30	-0.30	-0.13	0.23	0.08	0.39	**0.47**	0.32	0.15	0.09	**0.58**	-0.16	-0.04	-0.09						
Mn	0.06	-0.19	0.02	-0.16	-0.05	0.07	0.31	-0.19	-0.14	0.00	-0.02	**0.40**	0.28	0.33	**0.40**	0.56	0.25	0.17					
Ni	0.19	-0.06	0.05	0.38	0.11	0.21	0.30	-0.21	-0.06	0.24	0.30	0.32	0.09	0.18	**0.63**	0.22	0.22	0.25	**0.41**				
Pb	0.07	0.03	-0.09	0.06	0.05	-0.01	-0.33	0.23	-0.02	0.15	0.11	0.23	0.27	-0.22	0.11	0.00	0.38	-0.08	-0.11	0.15			
Sr	-0.03	-0.25	-0.15	0.09	-0.22	-0.13	**0.53**	-0.01	0.25	0.31	0.26	**0.63**	0.27	**0.56**	0.23	0.25	0.21	**0.50**	0.30	0.18	-0.09		
V	0.05	-0.28	-0.37	0.09	-0.39	-0.24	0.06	0.20	**0.46**	**0.54**	0.01	**0.44**	0.07	0.19	-0.19	0.17	0.07	**0.68**	0.13	0.18	0.22	0.37	
Zn	0.31	**0.48**	**0.58**	-0.22	0.10	0.08	-0.05	-0.29	-0.27	-0.31	0.23	**0.40**	0.24	-0.33	**0.43**	0.34	**0.46**	-0.22	0.20	0.19	0.38	0.09	-0.04

Table B. Spearman correlation coefficients among trace-, macroelements, and carbon and nitrogen isotopic signatures (expressed as standardized trophic position TP and $\delta^{13}C_{st}$) for Lake Trasimeno crayfish. Correlation coefficients between analytes and crayfish condition factor (CF), wet weight (WW) are also reported. Significant correlations (P <0.05) are in bold.

	CF	WW	TP	$\delta^{13}C$	N	C	Ca	K	Mg	Na	B	Ba	Cd	Co	Cr	Cu	Fe	Li	Mn	Ni	Pb	Sr	V
WW	0.33																						
TP	-0.07	0.05																					
$\delta^{13}C$	0.12	0.36	**-0.45**																				
N	-0.12	-0.36	-0.13	-0.27																			
C	0.03	-0.37	-0.14	-0.32	**0.93**																		
Ca	0.02	0.06	0.26	0.02	-0.06	0.00																	
K	0.18	0.27	-0.20	-0.08	0.18	0.08	-0.15																
Mg	-0.09	-0.01	-0.07	-0.11	-0.39	-0.30	**0.60**	0.01															
Na	0.22	0.32	**0.41**	0.00	-0.25	-0.24	0.29	-0.17	0.14														
B	0.17	0.17	0.02	-0.13	-0.32	-0.26	**0.45**	-0.09	**0.51**	0.27													
Ba	0.06	0.02	0.33	0.03	-0.35	-0.31	0.18	-0.32	0.23	**0.42**	**0.42**												
Cd	0.34	0.04	0.27	0.00	-0.29	-0.20	**0.43**	-0.35	0.32	**0.56**	**0.57**	**0.67**											
Co	0.14	0.13	0.26	-0.02	**-0.67**	**-0.54**	0.35	-0.30	**0.54**	**0.44**	**0.47**	**0.54**	**0.55**										
Cr	0.08	0.11	0.24	0.17	-0.16	-0.19	**0.60**	**-0.45**	0.31	**0.58**	**0.52**	**0.47**	**0.70**	0.36									
Cu	0.27	0.28	0.20	-0.02	**-0.50**	**-0.51**	0.05	0.14	0.23	**0.57**	0.27	0.26	**0.48**	0.37	0.24								
Fe	0.10	0.09	0.15	0.11	-0.23	-0.22	0.09	-0.39	-0.01	**0.42**	**0.42**	**0.73**	**0.73**	0.34	**0.62**	0.34							
Li	0.21	-0.22	0.18	-0.04	-0.19	-0.03	0.00	**-0.44**	-0.02	0.38	-0.03	**0.56**	**0.49**	0.33	0.34	0.16	**0.61**						
Mn	0.31	-0.07	0.16	-0.05	-0.32	-0.19	0.24	-0.37	0.17	0.35	0.26	**0.56**	**0.62**	**0.45**	0.32	0.28	**0.63**	**0.73**					
Ni	0.29	0.25	0.09	0.03	**-0.55**	**-0.49**	-0.02	-0.05	0.01	**0.47**	**0.44**	0.16	0.27	**0.43**	0.07	**0.57**	0.17	0.11	**0.41**				
Pb	-0.06	-0.04	0.16	0.25	0.03	-0.03	-0.18	-0.31	-0.36	0.38	-0.15	**0.45**	0.33	-0.10	0.28	0.22	**0.63**	**0.52**	0.31	-0.01			
Sr	0.02	-0.16	0.20	0.12	-0.26	-0.14	**0.54**	**-0.55**	**0.47**	**0.47**	0.36	**0.74**	**0.72**	**0.62**	**0.71**	0.12	**0.57**	**0.64**	**0.51**	0.00	0.28		
V	0.03	-0.17	-0.23	-0.18	-0.40	-0.23	0.22	0.01	**0.77**	-0.01	**0.50**	0.37	0.29	**0.55**	0.10	0.14	0.17	0.30	0.35	0.11	-0.18	**0.41**	
Zn	0.22	**0.47**	0.38	0.27	-0.37	-0.38	0.14	-0.28	-0.09	**0.64**	0.19	**0.51**	**0.59**	**0.41**	**0.62**	0.32	**0.69**	**0.48**	**0.44**	0.22	**0.44**	**0.44**	-0.11