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# Native crustacean species as a bioindicator of freshwater ecosystem pollution: A multivariate and integrative study of multi-biomarker response in active river monitoring

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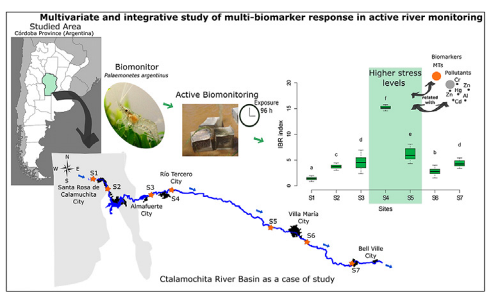
## HIGHLIGHTS

- The studied basin showed a significant metal pollution (Al, Ag, Hg, Pb, B).
- Total metals measured in *P. argentinus* showed correlation with metals in water.
- MTs responded to Hg, Cr, Cd, Hg, V levels in soluble fractions of shrimp tissues.
- MTs evidenced being a good indicator of stress due to pollutant mixture exposure.
- The multivariate approach pointed out a pollution gradient in the studied area.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The aim of this study was to evaluate the ability of *Palaemonetes argentinus* to evidence the environmental degradation due to pollutants mixture in a freshwater aquatic ecosystem. For this purpose, an active monitoring (96 h exposure) was carried out in seven sites along the Ctalamochita River basin (Córdoba, Argentina), as a case of study.

Our results evidenced sewage discharges impact in the water quality index, as well as metal pollution in water (Ag, Al, B, Pb, Hg) and sediments (Hg) with a potential effect on aquatic biota. The accumulation of total metals measured in exposed P. argentinus showed significant correlation with metals in water. Also, metallothioneins in cephalothorax showed significant changes along the basin, correlating with soluble concentrations of Cr, Zn, Cd, Hg, and V measured in shrimp tissues, which would be reflecting their bioavailability in the environment. In addition, the increase in antioxidant and detoxifying enzymes suggests the occurrence of oxidative stress in exposed shrimps. The integrative biomarker response index (IBR) pointed out the effect of metals on P. argentinus but also the occurrence of others pollutants.

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Finally, a high consensus was observed for water, sediments, and shrimps through the multivariate analysis (90%), indicating that P. argentinus can reflect changes in the abiotic matrixes. Moreover, studied sites were grouped according to their environmental quality.

The use of active biomonitoring and the integration of biological responses through an IBR confirm that native biota could be a useful monitoring tool for bioavailable pollutants in aquatic ecosystems constituting a highly valuable approach.

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## 1. Introduction

Biota in aquatic ecosystems is usually exposed to diverse stress conditions, like natural environmental variations and anthropological disturbs including the discharges of pollutants in water resources. Therefore, it is likely that a complex mixture of organic and inorganic pollutants reaches rivers and lakes from diffuse and point sources. Chemical analyses on water samples give a snapshot of the occurrence of pollutants in the environment but do not provide information about their bioavailability or their potentially toxic effects on aquatic biota. At the same time, chemical analysis is usually expensive and some chemicals and metabolites, particularly of emerging contaminants (ex. nanometals) are not yet accessible to analyze. Alternatively, the determination of pollutants bioaccumulation and biological responses in organisms confers an integrative measurement of their exposure and effect to ecologically available fractions of environmental pollutants over a time period (Rainbow, 2007). In concordance, previous studies described the importance of the use of bioindicators as tools capable to determine the overall effects of the pollutants mixtures occurring in aquatic systems where complex interactions arise.

Several biomonitoring programs, mention the importance to use sentinel and native species. In this context, fishes, submerged and floating plants as well as invertebrates like bivalves and crustaceans were described as suitable bioindicators of water pollution (Ghisi et al., 2017; Kumar et al., 2017). In the last years, active monitoring approaches based on transplanted organisms were developed with the aim to solve some limitations of passive methodologies including the effective presence of the native organisms at the sampling sites, variability in the exposure time, age and size of sampled organisms, among others (Besse et al., 2012). Nevertheless, most of the active biomonitoring studies published use fish and bivalves species as bioindicators organisms while, others taxa, including insects and crustaceans, are usually considered in a lesser extent for freshwater monitoring programs (Ballesteros et al., 2017; Vieira et al., 2017). According to previous studies, crustaceans have proved to be sensitive organisms for assessing the pollution of aquatic ecosystem (Lebrun et al., 2015; Ronci et al., 2016). For example, species from Amphidod family are widely used due to their small size, abundance in the freshwater ecosystem, and their ecologically relevant function being an important source of food for macroinvertebrates, fish, birds, and amphibians (Ciliberti et al., 2017). Furthermore, the multi-biomarker approach, including the Integrative Biomarker Index (IBR), was described to be useful in environmental quality assessment to evaluate the impact of environmental conditions on the health of organisms and their physiological status (Potet et al., 2016). In particular, IBR can offer a more holistic interpretation of biological responses from organisms exposed to stress gradients mainly due to a complex mixture of pollutants with different concentrations of exposure in aquatic ecosystems (Pain-Devin et al., 2014). The IBR showed usefulness to distinguish among sites located along a stress gradient, while individual biomarkers were not able to do so.

The decapod *Palaemonetes argentinus,* a small freshwater shrimp with South American distribution, demonstrated in previous studies to be a sensitive species (Bertrand et al., 2016a, 2016b). Studies conducted with *P. argentinus* in laboratory conditions, such as exposure to environmental concentrations of organic and inorganic pollutants, evidenced the sensitivity of the shrimp after a short time of exposure (96 h). Those pollutants were able to induce significant responses in several biomarkers including metallothioneins (MTs), cholinesterase (ChEs) and antioxidant enzymatic activities as well as in other biomarkers of damage (Bertrand et al., 2016a, 2016b; Galanti et al., 2013). Consequently, this species has been proposed as a bioindicator to provide information on environmental quality (Montagna and Collins, 2007). Nevertheless, no active biomonitoring experiments in rivers were previously carried out to test the usefulness of this decapod as a bioindicator of freshwater pollution.

In our field study, a large set of environmental data was obtained in the seven sites monitored including the concentration of 20 metals and metalloids in water and sediments samples as well as physicochemical parameters of both compartments in Ctalamochita River (Córdoba, Argentina). In shrimps exposed for 96 h in the sampling sites, a set of biomarker responses representative of metal exposure (accumulation, MTs), antioxidant system response (catalase activity), detoxification mechanisms (cytosolic cholinesterase and glutathione S-transferase activities, MTs) and damage occurrence (microsomal cholinesterase) were measured. In this context, first, we propose a univariate analysis to understand the biological responses occurred in exposed organisms. Then, a multivariate analysis is proposed to integrate the obtained information to reach a more holistic view of the aquatic system and to evaluate the *P. argentinus* usefulness to reflect the environmental degradation. The hypothesis underlying this work is that *P. argentinus* will be able to evidence environmental impact on the Ctalamochita River basin, selected as a case of study. This approach will potentially provide data for a better management of freshwater resources, supporting future regulatory guidelines, especially in South America countries, but also to implement biomonitoring programs including this or similar crustacean species.

## 2. Materials and methods

### 2.1. Reagents and materials

All reagents were of analytical grade supplied by Sigma–Aldrich, Merck, and Sintorgan (Argentina). Ultra-pure water (Arium 611 UV system, Sartorius) was used to prepare standard solutions, dilutions, and blanks. All materials were appropriately washed to avoid metal and organic contamination.

### 2.2. Site description

The Ctalamochita River basin (Córdoba, Argentina), with a drainage area of around 3300 $km^2$, represents a multi-stressor scenario for aquatic organisms. The river flows for approximately 300 km from a relatively mountainous pristine area with low anthropological activities (in the west), to sites with dense industrial and agricultural activities as well as urban settlements (in the east) with an average annual flow of 27 $m^3 s^{-1}$.

Seven sites were selected along the river according to developed activities in the riversides and their locations in the basin (Fig. 1 A). Therefore, different levels of metals were expected. The site located in an area with a low density of anthropic activities and situated near the mountainous area in the upper basin (upstream Santa Rosa Calamuchita town, S1) was considered as the reference site.

### 2.3. Biomonitoring procedure

Adult freshwater shrimps, *Palaemonetes argentinus*, were collected from a quasi-pristine site located in Suquía River basin (Córdoba, Argentina; Galanti et al., 2013). Organisms were acclimated during two weeks in 200 L glass aquaria filled with dechlorinated tap water, sediment and plants from the same sampling area and maintained at 25 ± 1 °C, under a natural light/dark regime. Organisms were fed daily *ad libitum* with commercial food for fish (Vita Fish) complemented until 54% proteins through the addition of lyophilized shrimp (Bertrand et al., 2015).

Acclimated organisms were transported to the monitored area with supplementary aeration and four groups of 12 shrimps (n = 48) were deposited in each site in plastic cages with an adequate water circulation. The cages were laid at the depth of 0.7–1.0 m, simulating environments usually inhabited by the selected species (Collins et al., 2007). Organisms were exposed during four days (96 h) according to previous laboratory testing (Bertrand et al., 2016a, 2016b) in August 2013. At the end of the exposure time, shrimps were collected, counted, washed three times with ultrapure water, dissected in cephalothorax and abdomen sections (Bertrand et al., 2016a), frozen with liquid nitrogen, and kept at −80 °C until analysis.

### 2.4. Physicochemical properties of water and sediments

Water dissolved oxygen, pH, temperature, and conductivity were measured *in situ* using a portable equipment (WTW, Multiline F/Set 3). Also, nitrate ($[NO_3]^-$), nitrite ($[NO_2]^-$), sulphate, total phosphorus, chlorides, ammonium ($[NH_4]^+$), and total coliform bacteria were measured in water samples according to Pesce and Wunderlina (2000) and APHA (2005). All results were expressed in μg $L^{-1}$ with the exception of chlorides expressed in mg $L^{-1}$ and total coliform bacteria as the most probable number per 100 mL of water. With these physicochemical variables measured in water samples, a water quality index (WQI) was calculated according to Pesce and Wunderlina (2000). The construction of WQI requires normalization for each parameter on a 0–100 scale to avoid interferences arising from different magnitudes of measured parameters, being 100 the optimal and 0 the worst water quality. Further to normalization, WQI requires the application of a weighting factor to each measured parameter, reflecting its importance on the water quality. WQI gives then a non-dimensional number that can be associated with a quality percentage.

In sediment samples, the content of organic matter (COM; APHA, 2005) and pH (Klute, 1986) were measured.

### 2.5. Metals and metalloids in water and sediments

Seventeen metals (Aluminium (Al), Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (Fe), Cobalt (Co), Nickel (Ni), Copper (Cu), Zinc (Zn), Rubidium (Rb), Strontium (Sr), Molybdenum (Mo), Silver (Ag), Cadmium (Cd), Barium (Ba), Mercury (Hg) and Lead (Pb) and three metalloids (Boron (B), Arsenic (As) and Selenium (Se)) were quantified in collected samples. These metals were selected according to land use, basin characteristics, and previous studies (O'Mill, 2012).

Before measurements, water samples were filtered using 0.45 μm nitrocellulose filters (Sartorius, Göttingen, Germany; Monferrán et al., 2016). Sediments were dried at 38 °C and sieved to 63 μm using acrylic meshes. A sequential extraction was carried out according to Maiz et al. (2000) using 1 g of the <63 μm dried material. First, the mobile fraction of metals (A1, metals immediately available to be absorbed by biota) was extracted using a $CaCl_2$ 0.01 M solution. Secondly, the metal mobilizable fraction (A2, metals susceptible to be absorbed by some species) was obtained adding DTPA (diethylene triamine penta acetic acid) 0.005 M, $CaCl_2$ 0.01 M, TEA 0.1 M with pH = 7.3 solution. The addition of A1 and A2 was called bioavailable fraction. Last, *Aqua regia* 1 $HNO_3$: 3 HCl was used to extract the pseudo-total (Ps, metals not immediately available for biota) fractions of elements from the collected samples.

The analysis of the extracts was performed with a Mass Spectrometer Inductively Coupled Plasma (ICP-MS), X Series, Thermo-Elemental X7 series (Thermo Fisher Scientific, Bremen, Germany), equipped with an ASX-100 autosampler (CETAC Technologies, Omaha, NE). AccuStandard® atomic absorption spectrometry standard solution (1000 mg $L^{-1}$ in 2% nitric acid) was used as a stock solution for calibration of metal quantification equipment. Results were expressed in μg $L^{-1}$ for water samples and mg $kg^{-1}$ of dried material for sediments.

The concentrations of studied elements were measured in triplicate. Quality assurance and quality control were performed using certified reference materials (CRMs) for water: NIST 1640, and NIST 1643e. Replicate analysis of these reference materials showed good accuracy. Recoveries from CRMs were 101 ± 5% and 101 ± 2%, respectively.

### 2.6. Biomarkers in Palaemonetes argentinus

The biomarkers were measured in both body sectors of *P. argentinus* (cephalothorax and abdomen, Bertrand et al., 2016a).

#### 2.6.1. Metals and metalloids accumulation

The concentration of the same seventeen metals measured in water (Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Mo, Ag, Cd, Ba, Hg and Pb) and three metalloids (B, As and Se) were quantified in cephalothorax (0.015 ± 0.004 g dry weight (dw)) and abdomen (0.018 ± 0.005 g dw), separately. The digestion of sample was carried out according to Bertrand et al. (2016a) with some modifications. Briefly, the exoskeleton was removed in both body sectors, tissue samples were dried at 38 °C until constant weight, ground with a mortar and digested with sub-boiling $HNO_3$ and $H_2O_2$ (30%, Merck, Germany) into Teflon tubes at 160 °C. The quantification of the elements was performed as explained in 2.5. Quality assurance and quality control were done using spiked control samples of shrimps with the standard solution mentioned in Section 2.5. The percentages of recovery from tissues were 96.3 ± 14.1%. The concentrations were reported in μg $g^{-1}$ dry weight (dw).

#### 2.6.2. Metallothioneins and metal compartmentalization

For MTs and metal compartmentalization analysis, individual samples (cephalothorax and abdomen of each individual) were homogenized at 4 °C in 20 mM Tris, 10.5 mM β-mercaptoethanol, 150 mM NaCl, solution adjusted to pH 8.6 (4 mL per g of soft tissue), and inhibitor protease cocktail (Sigma–Aldrich, Germany). The

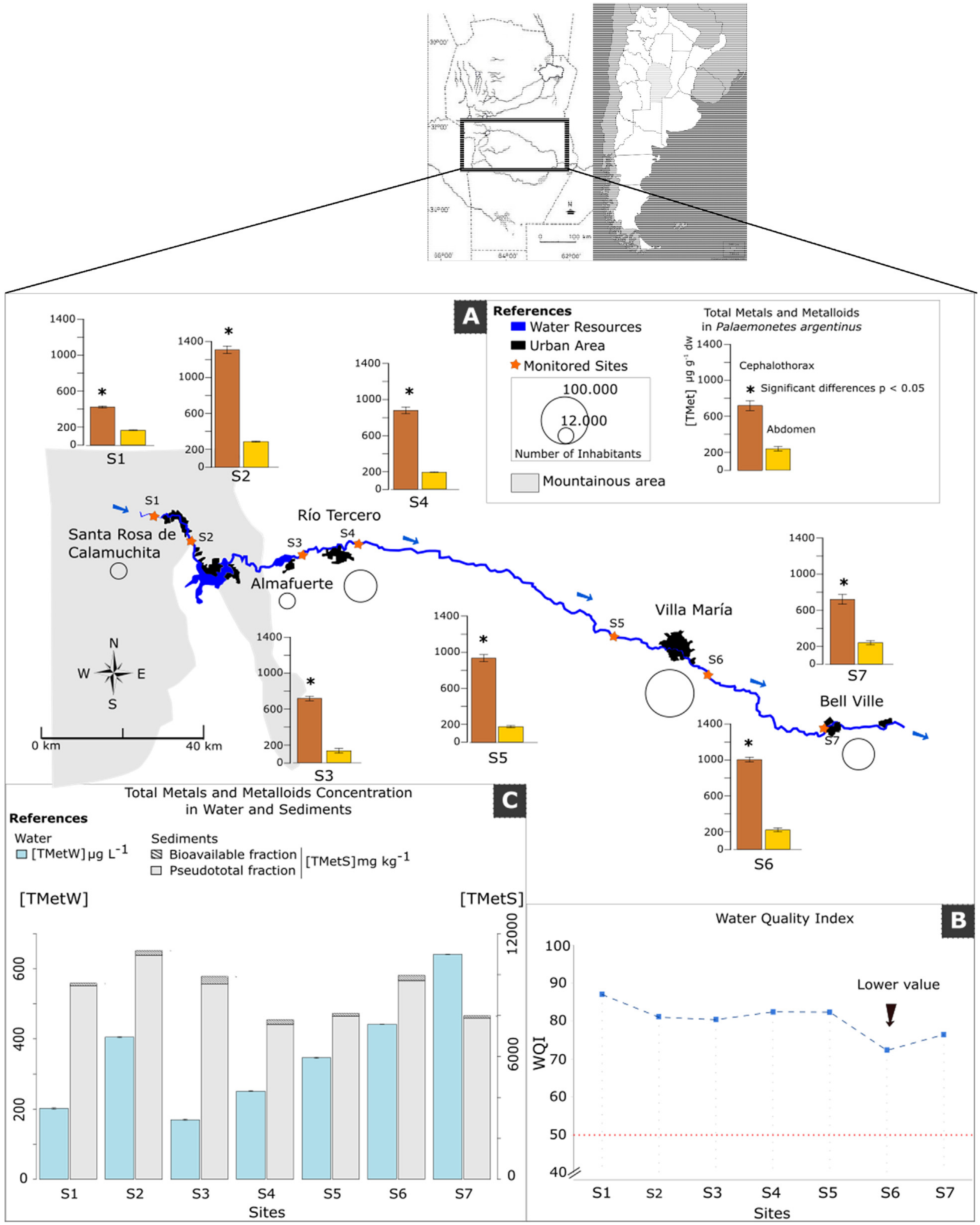


**Fig. 1.** Studied area and location of the seven sampling sites in the Ctalamochita River basin (Córdoba, Argentina). The S1 was considered as the reference site due to the low anthropological activities developed upstream. **A**: Number of inhabitants in principal cities and towns of the studied area, the total concentration of metals/metalloids ([TMet]) in body sectors of exposed shrimps, expressed in μg g$^{-1}$ dry weight (dw). **B**: Water quality index (WQI), being 100 the optimal water quality and values lower than 50 (red line) waters with an inadequate quality for aquatic biota. **C:** Total concentration of metals and metalloids in water samples ([TMetW], expressed in μg L$^{-1}$) and sediments ([TMetS], expressed as bioavailable (A1+A2) and pseudo-total (Ps) fractions in mg kg$^{-1}$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

soluble (S1) and insoluble (P1) fractions were separated by centrifugation (25,000 g for 55 min at 4 °C). The cytosolic heat-stable compounds including MT (S2) were isolated by centrifugation of an aliquot of the soluble fraction (S1) (15,000 g for 10 min at 4 °C) after heat-treatment (75 °C for 15 min) (Mouneyrac et al., 1998). The MTs were measured in S2 by differential pulse polarography (DPP) according to Bertrand et al. (2015). This method is based on SH-compound determination according to the Brdicka reaction as described by Olafson and Olsson (1991). MTs results are reported in mg MTs $g^{-1}$ wet weight (ww).

Metals can be distributed subcellularly bounded to proteins (metallothionein-like proteins and heat-sensitive proteins, named as soluble (S) fraction) and bounded to organelles, cellular debris, and metal-rich granules, named as insoluble (P) fraction (Wallace and Luoma, 2003). The concentrations of nine metals (Zn, Cd, Cr, Al, Cu, Co, V, Hg, Pb) were determined in the soluble (S1) and insoluble (P1) fractions. These elements were selected according to the total accumulated concentrations (2.5) and their capacity to induce MTs in organisms (Luoma and Rainbow, 2008). Concentrations of metals in S1 and P1 were measured by ICP–MS. Quality assurance (QA) and quality controls (QC) were done using spiked S1 and P1 control samples. Samples were processed as in 2.6.1 without removing the exoskeleton. The percentage of recovery from S1 and P1 fractions was $103.5 \pm 6.4$.

The soluble fraction S1 includes both the cytosolic metals and part of elements which are initially bound (adsorbed) onto the exoskeleton (White and Rainbow, 1984). In order to evaluate the contribution of these external metals to the soluble fraction S1, desorption tests were carried out in organisms exposed in all the studied sites (Supplementary material, SM, Appendix A). Concentrations of desorbed metals were taken into account for S1 concentrations calculations for each metal and body sector. Metals concentrations in S1 and P1 are expressed in mg $g^{-1}$ ww.

#### 2.6.3. Enzyme activity

Enzyme extracts of cephalothorax and abdomen of *P. argentinus* were prepared according to Wiegand et al. (2000). Tissue samples were homogenized in sodium phosphate buffer (NaP, 0.1 M, pH 6.5) containing glycerol (20%), 1,4-dithioerythritol (DTE, 1.4 mM) and ethylene diamine tetra acetic acid (EDTA, 1 mM). After removal of cell debris (10 min at 13,000 g, 4 °C), the membrane fraction of the extracts was separated by centrifugation at 105.000g for 60 min. The remaining supernatant, defined as the soluble (cytosolic) and the pellet (microsomal) fractions, previously re-suspended in NaP (20 mM, pH 7) containing glycerol (20%) and DTE (1.4 mM), were used for enzyme measurement. Enzymatic activities were determined by spectrophotometry, using a microplate reader (Bio-Tek, Synergy HT). The activity of catalase (CAT) was measured immediately after extraction in the cytosolic fraction, while the activities of glutathione-S-transferase (GST) as well as cholinesterases (ChEs) using acetylcholine and butyrylcholine as substrates, were determined in cytosolic and microsomal fractions (SM, Appendix B). The activity of all enzymes assessed was calculated in terms of the protein content of the sample extract (Bradford, 1976) and are reported in nanokatals per milligram of protein (nkat mg $prot^{-1}$), where 1 nkat is the conversion of 1 nmol of substrate per second. The protein quantification was performed using bovine serum albumin as standard.

### 2.7. Integrated biomarkers response

A general stress index, termed “Integrated Biomarker Response”, was calculated considering the responses of the antioxidant system, detoxification, and damage biomarkers measured in *P. argentinus*. Variables with strong correlation ($r \geq 0.8$, $p < 0.05$) were excluded from the analysis to keep non-redundant biological responses. Then, to select an acceptable number of biomarkers (no more than eight, Devin et al., 2014) a discriminant analysis was applied to select those biomarkers with higher capacity to separate the monitored sites. With selected variable, the IBR was calculated in accordance to Bertrand et al. (2016b) using R Studio. Briefly, the mean value ($X_i$), the general mean ($m_i$) and standard deviation ($SD_i$) for each biomarker at each exposure condition were calculated. The value $X_i$ was then standardized to obtain $Y_i$, where $Y_i = (X_i - m_i)/SD_i$. Subsequently, $Z_i = -Y_i$ or $Z_i = Y_i$ were computed in the case of a biological effect corresponding, respectively to inhibition or activation. The minimum value ($min_i$) of $Z_i$ for each biomarker was obtained for each exposure condition. Finally, the score S was calculated as $S_i = Z_i + |min_i|$, where $|min_i|$ is the absolute value. These S values thus represent the gradient of values for each biomarker in the different exposure conditions, with highest values corresponding to the highest biological effects. The integrated biomarker response for each condition was calculated via the following formula:

$$IBR = S1*\frac{S2}{2} + S2*\frac{S3}{2} + \cdots + Sn - 1*\frac{Sn}{2} + Sn*\frac{S1}{2}$$

in which the obtained score for each biomarker ($S_i$) is multiplied with the score of the next biomarker ($S_{i+1}$), arranged as a set, dividing each calculation by 2 and summing-up of all values.

Several IBRs were calculated from the same data changing the order of the biomarkers and using the median of all the index values as the final index value as recommended by Devin et al. (2014).

### 2.8. Statistical treatment

Generalized Linear Mixed Model was used for the statistical analysis. Models were fitted, using Infostat Software (Di Rienzo et al., 2017), and then normality and variance homogeneity were tested and variance function was applied if necessary. A posteriori test LSD Fisher, with Bonferroni errors' correction when necessary, was used to determine significant differences between the means ($p < 0.05$).

## 3. Results and discussion

### 3.1. Physicochemical properties of the water and sediments

The conductivity of water samples varied between 163 and 520 μS $cm^{-1}$ with highest values observed in sites located downstream. Chloride ions concentrations also showed a gradual increase along the basin, being the highest values measured in S7. The increase in conductivity and chlorides could be associated with urban run-off, natural drainage and the expansion of the agricultural activity in some areas that would result in a greater contribution of sediment from erosion processes intensified by deforestation (Wunderlin et al., 2001; Bonansea et al., 2013). Water temperature, ranging between 6.6 and 13.4 °C, was significantly lower ($p = 0.03$) in S1 when compared with the other sites, except when it was compared with the temperature in S2. Differences in water temperatures would be due to the mountainous characteristics of sites S1 and S2, located in the upper basin. The pH values fluctuated between 7.9 and 9.0, and no statistically significant differences were observed among monitored sites ($p > 0.05$). Dissolved oxygen ranged between 8.2 and 12.4 mg $L^{-1}$ with highest levels in S3, and significantly lowest ones in those sites located downstream urban settlements (S4, S6, and S2, Fig. 1 A, $p = 0.009$). The total coliform bacteria values also showed dissimilarities along the basin varying between 2200 and 800000 CFU100 $mL^{-1}$ being the S6 the site with the highest value, probably due to the inadequate functioning of a sewage treatment plant located approximately 4 km upstream the site. In concordance with total coliform bacteria levels, the highest values of less oxidized nitrogen species $[NH_4]^+$ and $[NO_2]^-$ occurred in S6, followed by S4 and S2, indicating a significant recent input of discharges with elevated nutrients loading (Figure S1). The concentration of sulfates presented maximal levels in S7 and S2 probably due to domestic and industrial wastewaters input in addition to natural sources. Moreover, total phosphorus levels increased markedly along the basin also indicating a relevant input of nutrients in the aquatic ecosystem (Figure S1). Finally, the WQI showed a decay along the basin, with the highest value in S1, the reference site, and the lowest one in S6 (Fig. 1 B).

Considering sediments samples, pH values were similar along the river and varied from 6.0 (S5) to 7.1 (in S1). The percentages of organic matter ranged from 7 to 14%, with the lowest values in S4, S5, and S7, and the highest one in S2 (SM, Table S1).

### 3.2. Metal and metalloids levels in water and sediments

To evaluate the environmental pollution due to metals and metalloids, the concentration of twenty elements was measured in water and sediment samples (Tables S2 and S3). Although the entire basin suffered an important contribution of volcanic loess from the Cordillera de los Andes with a significant increase of salinity and As levels in groundwater in the lower basin (Nicolli et al., 1989), there are probably local variations in the composition of the soils that contribute with metals to the river. In addition, it is expected that the development of anthropic activities, starting from the city of Río Tercero (located between S3 and S4) and the rest of the lower basin, will favor punctual discharges (sewage liquids and industrial effluents) and diffuse discharges (runoff from agricultural areas) of a wide variety of compounds, including metals. Accordingly, a spatial variation in the total concentration of metals and metalloids in water ([TMetW]) was observed along the river (Fig. 1 C). Particularly, increasing levels of Al, As, B, Ba, Co, Fe, Mn, Mo, Rb, Sr and V in water were observed from S3 to S7. The site S3 showed the lowest values of the basin for most of the studied metals while S1 exhibited similar concentrations to S3, although higher for Al, Ba, Co, Cu, Fe, Hg, Ni, Pb, and Zn. On the other hand, S2 showed concentrations much higher than S1 and S3. Low levels observed in S3 could probably be due to its location, downstream a chain of reservoirs, which could promote the metal sedimentation, especially in winter were thermal stratification could be occurring (Yi et al., 2011). Major levels of pollution were associated with concentrations of Ag in S5, Al in S6 and S7, B in S7, Hg in S5 and S6, and Pb in S1 in water, surpassing environmental guidelines (AEWQG, 2003; USEPA, 1988).

Total metals and metalloids concentrations in sediments ([TMetS]) were calculated as the addition of the bioavailable fraction (A1 + A2) and the pseudo-total fraction (Ps) of all the elements measured. The highest levels of [TMetS] were observed in S2 ($11177 \pm 129$ mg $kg^{-1}$ dw), S6 ($9960 \pm 5$ mg $kg^{-1}$ dw) and S3 ($9914 \pm 1097$ mg $kg^{-1}$ dw), while the lowest values were measured in S4 ($7795 \pm 539$ mg $kg^{-1}$ dw) and S7 ($7991 \pm 379$ mg $kg^{-1}$ dw) (Fig. 1 C). The bioavailable fraction represented between 3.7 and 1.1% of the [TMetS] measured in sediments. If only the bioavailable fraction is considered, the highest concentrations occurred in S3 ($368 \pm 59$ mg $kg^{-1}$ dw) followed by S6 ($261 \pm 5$ mg $kg^{-1}$ dw); while the lowest levels were found in S1 ($106 \pm 3$ mg $kg^{-1}$ dw) and S7 ($128 \pm 4$ mg $kg^{-1}$ dw).

The consensus-based sediment quality guidelines (SQGs) provided a unifying synthesis of the existing SQGs to assess the potential ecological toxicity of individual heavy metals in freshwaters ecosystem (CCME, 2001; MacDonald et al., 2000). The total concentrations of As, Cd, Cr, Cu, Pb, Hg, Ni, Zn in sediments were compared with two consensus-based SQGs: the threshold effect concentrations (TEC, concentration below which adverse biological effects are expected to be rare), and the probable effect concentrations (PEC, concentration above which adverse effects are expected to be frequent) for the metals in freshwaters ecosystems. For sediments with metal concentrations between TEC and PEC adverse effects occasionally occur on biota (CCME, 2001; MacDonald et al., 2000). In the present study, Hg (A1+A2+Ps) was the only metal surpassing the reference levels (TEC: 0.18 mg $kg^{-1}$ dw and PEC: 1.06 mg $kg^{-1}$ dw). Five sites, including the reference site (S1), exceed the PEC value and the others two (S2 and S4) presented concentrations between TEC and PEC. Described results evidence a worrisome Hg pollution of sediments in Ctalamochita River basin, with associated potential effect on the biota of this aquatic ecosystem. The presence of chlorine-alkali and metal industry, petroleum products combustion, a significant waste incineration and, probably, the intense use of pesticides, could explain, at least in part, the described situation of pollution in the studied area. Likewise, a natural contribution of Hg, including volcanoes emissions, cannot be ruled out. In this sense, Nriagu and Becker (2003) reported South and Central America as the regions of the world with the highest annual flux of Hg from volcanoes (34 t $year^{-1}$). Even though the Ctalamochita River basin does not present active volcanoes, the presence of materials with volcanic origin (loess and igneous rocks) has been reported (Pérez-Carrera and Fernández Cirelli, 2013). This natural source of Hg could be contributing to the pollution in sites where Hg presence was not expected (S1).

### 3.3. Metals and metalloids bioaccumulation in *Palaemonetes argentinus*

Previous studies carried out in laboratory studies with *P. argentinus* showed the potentiality of the species as a suitable bioindicator of metals exposure. When it was exposed to Zn, *P. argentinus* accumulated significantly higher concentrations of metal in cephalothorax compared with the abdomen (Bertrand et al., 2015, 2016b). Different patterns of accumulation observed between the body sectors could be associated with the presence of hepatopancreas in the cephalothorax (hepatopancreas showed high capacity to accumulate and detoxify metals; Amiard et al., 2006), and the predominance of muscle in abdomen (muscle showed low capacity to accumulate metals; Bertrand et al., 2015, and authors therein referenced).

In agreement with previous studies, shrimps exposed in the Ctalamochita River basin showed in cephalothorax a total concentration of metals and metalloids accumulated ([TMet]) significantly higher than in the abdomen ($p < 0.05$) (Fig. 1 A). From S3 to S6, accumulated concentrations in cephalothorax increased along the basin, with a diminution in S7 probably associated with the higher conductivity and salinity of water when compared with the other of studied sites (3.1). Other authors described the effect of salinity on metals accumulation in aquatic invertebrates due to changes in metal speciation and the increase in osmoregulation in the organism when the level of ions rises in the water (Capparelli et al., 2017; Luoma and Rainbow, 2005). Shrimps exposed in S2 showed the highest values of total metal accumulation in both body sectors while those exposed in S1, considered as the reference site, displayed the lowest ones. The concentration of each metal referenced to the total concentration of metals [TMet] measured in *P. argentinus* it allows to indentify three categories of accumulated elements: I- Major elements for those which represent more than the 10% of the total metal accumulated: Al, Cu, Fe and Zn; II- Intermediate elements when they represent between 10 and 1% of the total metal accumulated: As, Ba, Mn, Ni, Rb and Sr; and finally, III-Minority elements for those which represent <1% of the total metal accumulated: Ag, Cd, Co, Cr, Hg, Pb, Se and V (Table S4 and Figure S2).

Boro and Mo in both body sectors, as well as Cd in the abdomen, showed concentrations below the limit of detection ($LOD_B = 4.5\ \mu g\ g^{-1}$ dw; $LOD_{Mo} = 0.3\ \mu g\ g^{-1}$ dw; $LOD_{Cd} = 0.02\ \mu g\ g^{-1}$ dw). Cephalothorax, despite a multi-metals exposure in the river, keeps the capacity to accumulate a higher proportion of metals than the abdomen (Fig. 2). Moreover, all the individual elements maintained the described pattern of accumulation in both body sectors. Among these results the concentrations of Cr accumulated, especially in cephalothorax, became noticeable. Its concentration in cephalothorax was $5.8 \pm 1.5$ and $2.6 \pm 0.7\ \mu g\ g^{-1}$ dw in S4 and S6, respectively, being up to six times higher than in sites with lower levels (Fig. 2). This result could be associated with the presence of tannery activities in Rio Tercero City, upstream S4. Cadmium in cephalothorax showed the lowest concentrations in S1 and S6 and the highest in S5. Aluminium showed concentrations above $400\ \mu g\ g^{-1}$ dw in tissues of shrimps in S2 and varied between 250 and $360\ \mu g\ g^{-1}$ dw in S4, S5, and S6. Mercury showed concentrations significantly higher in cephalothorax in S5 and S7, as well as in abdomen in S6, compared with Hg accumulated levels in S1. Finally, the levels of Pb in exposed *P. argentinus* were lowest in S4 and highest in S6.

The accumulation pattern of total metals measured in *P. argentinus* exposed in the studied sites is very similar to the one described for the [TMetW]. Therefore, when lineal regressions were made between the [TMetW] (regressor variable) and [TMetCef] and [TMetAbd], both were statistically significant, with a higher $R^2$ coefficient for the cephalothorax ($R^2_{Cef} = 0.638$, $p < 0.00001$; $R^2_{Abd} = 0.439$, $p < 0.0004$), respectively). In cephalothorax, [TMetW] explained 63.8% of the variability in the [TMetCef] of exposed organisms, while in the abdomen it explained only 43.9% of the variability in [TMetAbd]. These models were adjusted using data from sites S1 to S6, since the relationship between the variables showed a different response in S7, probably due to higher conductivity levels as previously explained. On the contrary, the linear regressions made between [TMetS] and [TMetCef] and [TMetAbd] were not significant ($p > 0.05$). Similar results were obtained when linear regressions were performed between the concentration of metals in shrimps and the metal bioavailable fraction of sediments. Therefore, the content of metals in sediments was not able to explain the variability of metal accumulation measured in exposed shrimps. One possible explanation may be that cages were not in contact with the sediments since they were placed in the water column. Another possibility could be that the exposure time has not been sufficient to achieve equilibrium between the exposure concentration and the concentration in *P. argentinus* tissues.

### 3.4. Metallothioneins and metal compartmentalization in *Palaemonetes argentinus*

Previous studies recognized MTs as useful biomarkers of metal exposure and proposed their use in biomonitoring studies (Luoma and Rainbow, 2008). Moreover, MTs show antioxidant functions in aquatic vertebrates and invertebrates associated with the susceptibility of sulfhydryl groups of their cysteine residues, to sequester ROS through oxidation (Buico et al., 2008; Klein et al., 2017). The exposure of *P. argentinus* to Zn demonstrated the usefulness of MTs as a biomarker of metal occurrence (Bertrand et al., 2015). The antioxidant function in *P. argentinus* was also evidenced when shrimps were exposed to low concentrations of an organophosphorus pesticide like chlorpyrifos (Bertrand et al., 2016b). In both cases, the MTs in cephalothorax showed a higher capacity to respond than the MTs in the abdomen, probably associated with the presence of hepatopancreas in this body sector.

The response of MTs in shrimps exposed in the Ctalamochita River basin is shown in Fig. 3. As observed in laboratory studies, MTs showed significantly higher levels in cephalothorax than in abdomen. Moreover, the abdomen maintained similar MTs levels along all the monitored sites. On the contrary, significant changes were observed in cephalothorax. The highest MTs levels were observed in S4, followed by S7, S5, and S3, being S6 the site with the lowest MTs levels. In order to identify possible links between metal exposure and the induction of MTs in shrimps, several linear regression analyses were performed. No significant associations were observed between MTs in cephalothorax and [TMetW], [TMetS], total metals in the bioavailable fraction of sediments, or individual metals recognized as strong inductors of MTs (V, Cu, Cd, Zn, Co, Cr, Hg, Al, and Pb) in water or bioavailable fraction.

According to Mason and Jenkins (1995), the accumulation of metals in soft tissues occurs in two phases: dissolved in the cytosol ($S_1$), mainly as complexes with metal-binding proteins like MTs, or incorporated into metal-rich granules and other insoluble forms ($P_1$). In concordance with that, the intracellular partitioning of these nine metals recognized as strong inductors of MTs was measured. Fig. 4 shows the distribution of metals between soluble and insoluble fractions in both body sectors of exposed shrimps. Results show two patterns of distribution according to the metal considered: I. Pattern S1 ~ P1: where metals remained approximately equally distributed between both fractions; this pattern was observed for Co, Cu, Hg, Cr, Zn and Cd in cephalothorax (Fig. 4 A) as well as the Co, Cu, and Hg in the abdomen (Fig. 4 B). II. Pattern P1 ≫ S1: where metals remained more in the insoluble fraction than in the soluble one; this second pattern was observed for V, Al and Pb in cephalothorax (Fig. 4 C), and for V, Al, Pb, Cr, Zn, and Cd in the abdomen (Fig. 4 D). In cephalothorax, the concentration of metals in the soluble fraction was significantly higher ($p < 0.05$) than in the abdomen, $0.027 \pm 0.014\ mg\ g^{-1}$ ww and $0.010 \pm 0.001\ mg\ g^{-1}$ ww, respectively, which could be explaining the induction of MTs only in cephalothorax. These results are in agreement with the response observed in shrimps exposed in laboratory experiments to Zn (Bertrand et al., 2015). In cephalothorax of exposed organisms in Ctalamochita River basin, significant Spearman correlations were observed between MTs levels and the concentration of Cr ($r^2 = 0.5$, $p = 0.008$), Zn ($r^2 = 0.42$, $p = 0.007$), Cd ($r^2 = 0.45$. $p = 0.003$), Hg ($r^2 = 0.38$, $p = 0.020$) as well as V ($r^2 = 0.41$, $p = 0.008$) in the dissolved fraction ($S_1$). What is more, Zn showed a similar subcellular pattern distribution in shrimps exposed in Ctalamochita River basin, where multi-metals exposition occurred, and in those organisms exposed under laboratory conditions to Zn. These results could be suggesting the need to maintain adequate soluble Zn concentrations in tissues probably due to essential functions of this metal.

### 3.5. Enzymatic biomarkers response in *Palaemonetes argentinus*

Previous studies in *P. argentinus* showed significant responses of different enzymatic systems in organisms exposed to environmentally relevant concentrations of pollutants including organic and inorganic ones (Griboff et al., 2014; Bertrand et al., 2016a, 2016b). Based on this evidence, some enzymes that participate in the antioxidant system response, in detoxification mechanisms and

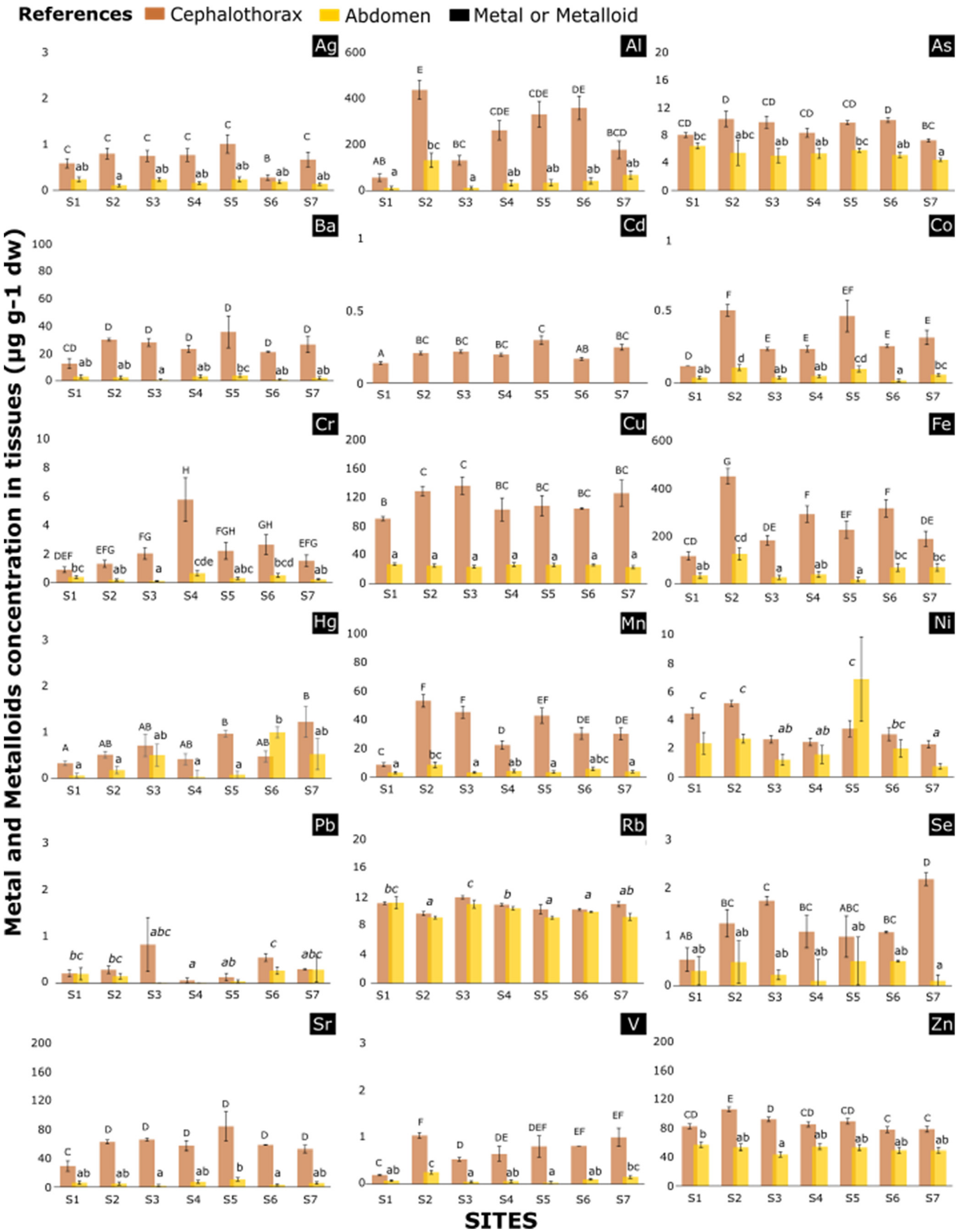


**Fig. 2.** Metals and metalloids accumulation, expressed in μg $g^{-1}$ dry weight (dw), in cephalothorax and abdomen of *Palaemonetes argentinus* exposed for 96 h in the Ctalamochita River basin. Statistical differences among sites and between body sectors are expressed in capital letter for cephalothorax, and minuscule for the abdomen. For Ni, Pb and Rb only differences among sites are shown in italics since there were no significant differences in the interaction sites*body sectors. The concentrations of Ni, Pb, and Rb were significantly higher in cephalothorax than in the abdomen along the basin ($p < 0.05$).

damage occurrence were selected for this study.

A wide literature recognizes the inhibition of cholinesterase activity as an indicator of organophosphorus and carbamate pesticides exposure in organisms including crustacean species (Krieger, 2010). Previous studies have also associated an increase in activities of cytosolic cholinesterase forms with a strong function in pollutants detoxification, including metals, with the aim to prevent or avoid the inhibition of microsomal forms linked to nervous impulse transmission (Bertrand et al., 2016b). In the present study, the activity of cytosolic forms of cholinesterase, cAChE, and cBChE (Fig. 5), showed similar activity levels and response patterns along the basin, and in both body sectors. In S3 and S6 the activity of cAChE increased significantly compared to the activity observed in S1, S2, and S5. The cBChE activity also showed the highest activity in S3 and the lowest in S5. Both enzymes showed in S7 an activity similar to the activity measured in shrimps exposed in the upper basin (S1 and S2). In contrast, mAChE (Fig. 5) displayed higher activity in cephalothorax than in abdomen. Nevertheless, no significant differences were observed among sites in none of the body sectors. The increase in cytosolic cholinesterases can be due to the de novo synthesis of these enzymes by the compensation metabolism, which results from the presence of anticholinesterasic compounds to compensate for functional defects in the cholinergic system (Bonansea et al., 2016; Yang et al., 2013). The activity of cytosolic enzymes could be associated with a protection response, and accordingly, the lack of effects observed on mAChE. Furthermore, differences observed between laboratory exposures to individual pollutants and field studies, where multi pollutants exposure occurred, could be pointing out a limitation in the sensitivity of mAChE as biomarker.

The enzymatic antioxidant system participates in the control of the imbalance between endogenous and exogenous reactive oxygen species (ROS) with the aim to avoid oxidative damage on essential biomolecules including proteins, lipids, and nucleic acids. Catalases are tetrameric heme-containing enzymes with the capability to dismutate $H_2O_2$ into $H_2O$ and $O_2$, and are indispensable for ROS detoxification during stressed conditions (Di Giulio, 1991). In *P. argentinus*, CAT was identified as the main enzyme responsible for avoiding the accumulation of ROS in the organism exposed to the organophosphates fenitrothion and to chlorpyrifos (Lavarías and García, 2015; Bertrand et al., 2016b). In the present study, the activity of CAT in cephalothorax (CAT, Fig. 5) increased significantly in all the sites from S3 to S7 compared to S1 and S2, being the higher activity registered in S4. In the abdomen, the enzyme showed a similar response pattern along the basin but, in most of the monitored sites, the activity was lower when compared to cephalothorax.

On the other hand, the detoxifying activity of mGST and cGST (Fig. 5) showed a similar response pattern along the basin. In both cases and in all monitored sites, the activity was higher in cephalothorax respect to the abdomen. In cephalothorax, the mGST activity showed only a tendency to increase from S2 to S4 if compared with S1; while in the abdomen, a significant increase occurred in all monitored sites respect to S1, with the exception of S6. The activity of cGST rose significantly in S2 and S4 in the cephalothorax when compared with the other sites, while the activity in abdomen maintained similar activity all along the basin (Fig. 5).

The increase in activities of antioxidant and detoxifying enzymes like CAT and cGST in shrimps exposed in the Ctalamochita River basin suggests that organisms suffered oxidative stress, especially in S4 where maximal activities occurred. Probably, the induction of CAT in exposed organisms is indicating an attempt to reduce $H_2O_2$ levels, while increased cGST could be indicating an attempt to make more water-soluble xenobiotics in order to better eliminate them.

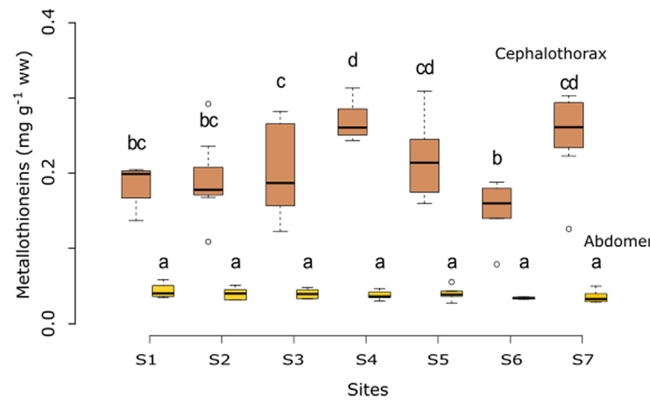


**Fig. 3.** Metallothioneins expressed in mg $g^{-1}$ ww, in cephalothorax and abdomen of *Palaemonetes argentinus* exposed for 96 h in seven sites of Ctalamochita River basin. Different letters indicate significant differences ($p < 0.05$) among monitored sites and body sectors.

### 3.6. Integrated analysis of biomarker responses in Palaemonetes argentinus

With the aim to obtain a more holistic interpretation of biological effects suffered by the exposed organisms, we calculated an Integrated Biomarkers Responses index (IBR). In our study, six biomarkers were selected through a discriminant analysis (SM-Table S5): MTs, cAChE, cBChE, mGST in cephalothorax, as well as CAT and cBChE in the abdomen. Shrimps exposed from S2 to S7 showed IBR values significantly higher than the ones exposed in S1 (Fig. 6). The highest values were observed in the middle basin, being the maximal stress observed in organisms exposed in S4. A significant correlation was obtained between IBR values and MTs levels in cephalothorax ($r^2 = 0.77$, $p < 0.05$). This result would be suggesting that MTs are good indicators, not only of metals exposure but also of stress levels suffered by shrimps. This is also in agreement with previous results obtained in *P. argentinus* exposed to chlorpyrifos where oxidative stress produced MTs induction (Bertrand et al., 2016a). On the other hand, the lowest level of WQI in S6 was not reflected by the IBR. Moreover, no significant correlations were obtained between IBR and [TMetW], [TMetS] or total metals in the bioavailable fraction of sediments. Nevertheless, significant correlations were obtained between IBR values and Cr in water ($r^2 = 0.77$, $p < 0.01$) and Zn in the bioavailable fraction of sediments ($r^2 = 0.62$, $p < 0.05$). Lowest levels of WQI are usually associated with sewage discharges, while probably IBR better indicates the presence of toxic pollutants from other sources.

### 3.7. Multivariate analysis

Generalized procrustes analysis (GPA) points out the relationship among four groups of analyzed variables in biotic and abiotic samples: water (including WQI and metals); bioavailable fraction of metals in sediments; pseudo-total metals in sediments; and shrimps (including IBR and bioaccumulated metals). Through this analysis, the obtained multivariate space was able to segregate studied sites in at least four groups, obtaining a 90.1% of consensus and explaining 49.7% of total variability through the first two components (PC1 and PC2, Fig. 7). The reference site (S1) was clearly isolated in the GPA analysis indicating differences in abiotic and biotic variables measured with regard of the rest of the sites in the basin, showing the lowest levels of stress (IBR) and higher values of water quality (WQI). Sites S2 and S3 were grouped by GPA even though the site located downstream Almafuerte Town (S3)

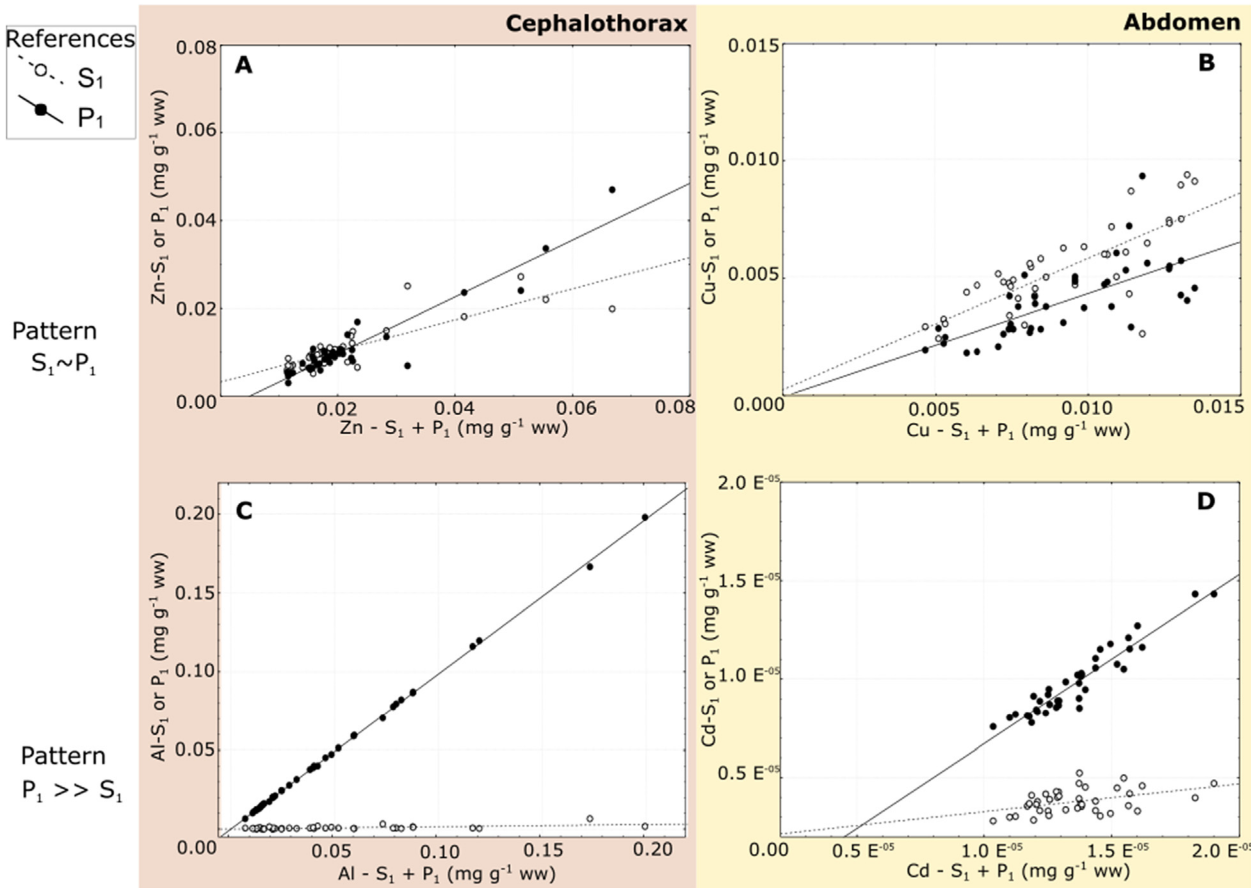


**Fig. 4.** Metal distribution in cephalothorax (A, C) and abdomen (B, D) of *Palaemonetes argentinus* after exposure for 96 h in seven sites of Ctalamochita River basin. Total metal: S1 + P1; cytosolic $S_1$ (○, dotted line) or insoluble $P_1$ (●, continuous line) metal. Measured metals showed different patterns of distribution according to the body sector considered: **Pattern $S_1$~$P_1$** observed for Co, Cu, Hg, Cr, Zn and Cd in cephalothorax as well as for the Co, Cu, and Hg in abdomen; **Pattern $P_1$ >> $S_1$** observed for the V, Al, and Pb in cephalothorax and for the V, Al, Pb, Cr, Zn and Cd in abdomen. Metals concentrations are expressed in mg $g^{-1}$ wet weigh (ww).

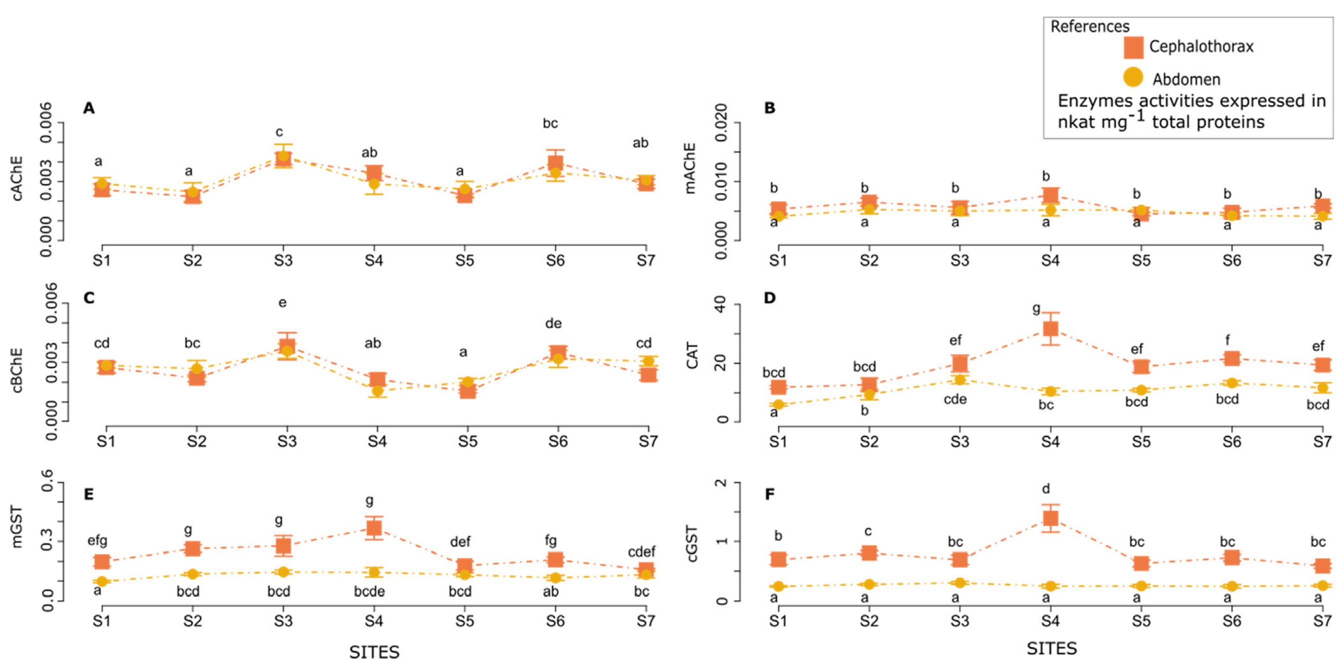


**Fig. 5.** Enzymes activities in cephalothorax and abdomen of *P. argentinus* exposed for 96 h in seven sites of Ctalamochita River basin. Cytosolic and microsomal Acetylcholinesterase (cAChE (**A**) and mAChE (**B**)), cytosolic Butyrylcholinesterase (cBChE, **C**), catalase (CAT, **D**), microsomal and cytosolic Glutathione S-Transferase (mGST (**E**) and cGST (**F**)) activities are expressed in nkat $mg^{-1}$ total proteins. Different letters indicate significant differences among monitored sites and body sectors ($p < 0.05$).

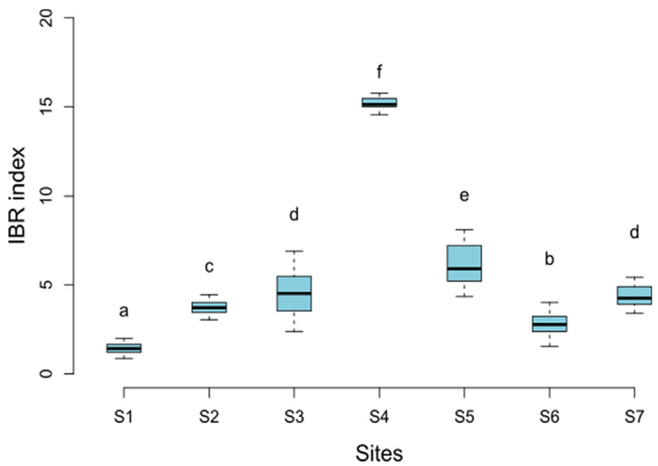


**Fig. 6.** Box plot for the calculated IBR index according to selected biomarkers measured in *Palaemonetes argentinus* exposed in seven sites of the Ctalamochita River basin. Different letters indicate statistically significant differences among monitored sites ($p < 0.05$).

receives higher agricultural impact than the site located downstream Santa Rosa Calamuchita Town (S2), characterized to be affected mostly by the urban settlement. This difference was probably indicated by a higher IBR in S3 than in S2, which could be associated with pesticide presence. The sites located in the lower basin (S6 and S7) were represented in the same plot sector. Both sites showed similar WQI and share particular physicochemical properties mainly due to natural sources. Shrimps were not sensible to the water quality decay due to sewage discharges observed in S6. Finally, the sites located downstream Río Tercero City (S4) and upstream Villa María City (S5) showed high similarity in the GPA space, being both sites situated in an area with intense agricultural and industrial activities. Both sites showed the highest values of IBR indicating important stress levels in *P.argentinus*. In concordance with the IBR values, levels of MTs also augmented significantly in S4 and S5 probably as a good biomarker of organic and inorganic pollutants exposure.

## 4. Conclusions

This study is the first report of the use of *P. argentinus* in an active freshwater biomonitoring and was able to demonstrate the ability of the proposed species to point out sites with lower environmental quality. The proposed case of study showed environmental problems including the decay in the water quality mainly due to sewage and wastewater discharges, but also to levels of metals (Ag, Al, B, Hg, and Pb) surpassing environmental guidelines in abiotic matrices. After 96 h of exposure, significant biological responses were observed along the basin in the proposed biomonitor. As it was described in previous studies, MTs responded in cephalothorax and correlated significantly with soluble metals concentrations (Cr, Zn, Cd, Hg, and V) in tissues, reflecting their bioavailability in the environment where a complex mixture of metals and metalloids occurs. Besides, a significant induction of antioxidant and detoxifying enzymes like CAT and cGST was observed, suggesting the occurrence of oxidative stress in exposed organisms. Finally, the usefulness of integrative tools like IBR and GPA was evidenced. The IBR was able to point out sites with significant agricultural and industrial activities (S4 and S5), where complex contaminants mixtures, including metals, metalloids but also others compound not measured in the present study, are expected.

The results of the present study demonstrated the usefulness of native biota in the biomonitoring but also that physicochemical analysis and environmental pollutants levels are not enough to

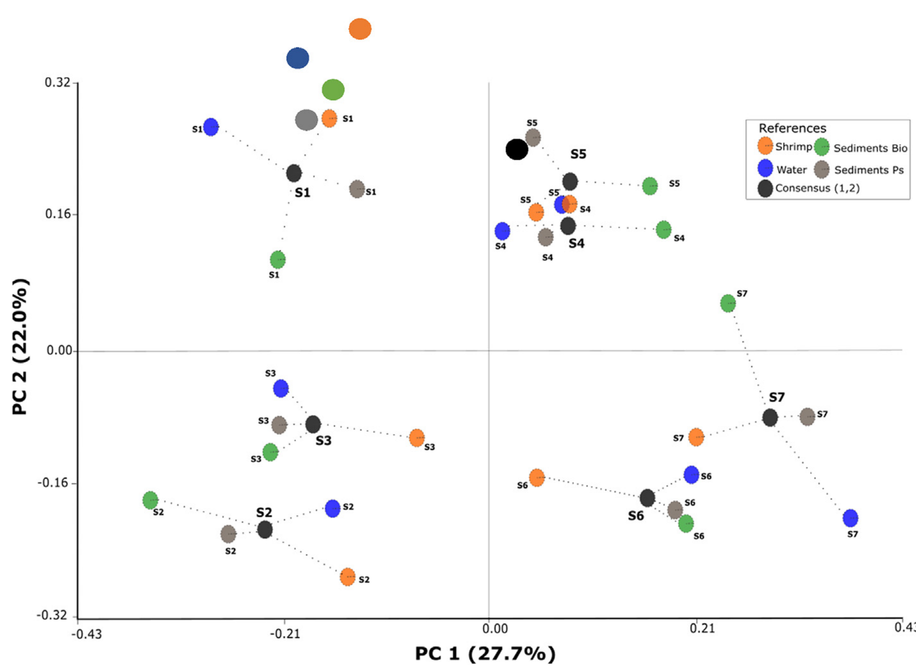


**Fig. 7.** Generalized Procrustes Analysis of four groups of variables measured in seven sites of Ctalamochita River basin: Shrimps (orange circle, including IBR and bioaccumulated metals and metalloids), Water (blue circle, including WQI and metals and metalloids concentrations), Sediments Bio (green circle, bioavailable fraction of metals and metalloids in sediments), Sediments Ps (grey circle, pseudo total fraction of metals and metalloids in sediments). The consensus among groups is shown (black circle) for all monitored sites. S1: Upstream Santa Rosa Calamuchita Town, S2: Downstream Santa Rosa Calamuchita Town, S3: Downstream Almafuerte Town, S4: Downstream Río Tercero City, S5: Upstream Villa María City, S6: Downstream Villa María City, S7: Upstream Bell Ville City. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interpret potential risk and effect on organisms. Future biomonitoring studies evaluating native species responses and their ability to evidence environmental degradation, are necessary challenges to set this methodology in South America and others developing regions.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.05.002.

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