Usefulness of a freshwater macrophyte (*Potamogeton pusillus*) for an environmental risk assessment in a multi-source contaminated basin.

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HIGHLIGHTS

- *Potamogeton pusillus* proved to be a good tool for active biomonitoring.
- Macrophyte stress was associated with Cr, Cu, Cd, Pb and Al concentrations.
- Macrophyte stress was associated with total pharmaceuticals concentration in water.
- Hg in sediments, Pb, Al, As, B, Hg in water, surpassed environmental guidelines.
- First report of integrated biomarker response for a macrophytes active biomonitoring.

GRAPHICAL ABSTRACT
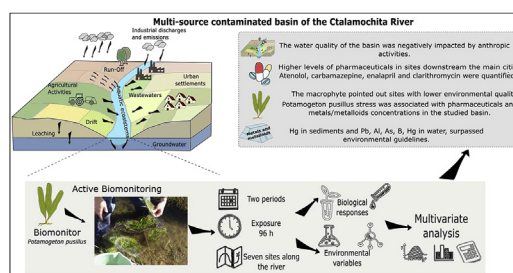
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ABSTRACT

Chemical analysis of pollutants in environmental matrices cannot always offer powerful evidence on their impact and toxicity on the aquatic biota. This paper reports the ability of *Potamogeton pusillus* to highlight in an aquatic ecosystem those sites with higher environmental degradation through an active biomonitoring. Organisms were exposed for 96 h in seven sites along a river where variation in pollutants mixtures and levels could be expected due to different pollution sources. Different anthropogenic activities favoured the presence of pharmaceutical compounds along the basin (atenolol and carbamazepine showed the highest levels in water) as well as metals in water (Pb, Al, As, B, Hg) and sediments (Hg) surpassing local and international environmental guidelines. Through several biomarkers responses, *P. pusillus* showed sensitivity when exposed to different environmental scenarios and succeeded indicating the most contaminated sites. An integrative biomarker response index (IBR) confirmed to be a useful tool to obtain a holistic interpretation of response expressed by organism despite being little used for macrophyte studies. The Principal Components Analysis (PCA) analysis showed consistently a positive association between the IBR and pollutants concentrations, being this linkage strongest when a higher compounds levels occurred in the basin (cold period). Moreover, multivariate analysis indicated that IBR values were boosted when the concentration of metal in water and in the bioavailable fraction of

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sediments, increased. Finally, the obtained results indicate that the use of *P. pusillus* in combination with chemical analysis could be considered a promising tool for environmental management of aquatic ecosystems contaminated by multi-source.

1. Introduction

Chemical analysis of the environmental matrices, such as water and sediments, are the most direct approach to reveal the contamination status in the environment. However, it cannot always offer powerful evidence on the integrated impact and the possible toxicity of such toxic compounds on the organisms and ecosystem. In developed countries, current environmental risk assessment practices comprise the integration of chemical and biological measurements, usually through indices, to assess the status of inland and coastal waters as recommended by the Water Framework Directive, established by the European Union (**EU; 2000/60/EC**). In consequence, several authors propose organisms from numerous taxa as bioindicators of environmental quality (Mertens et al., 2005).

Aquatic plants, including macrophytes, are worldwide distributed organism in numerous aquatic environments, including freshwater ecosystems. They play a key role in wetland biogeochemistry representing the principal living accumulators of contaminants. Besides, they are responsible for the primary production, fulfilling a fundamental trophic role in aquatic ecosystems (O'Brien et al., 2014). Several studies evaluated the biological responses of aquatic plants exposed under controlled conditions to different compounds such as metals (Costa et al., 2018; Xu et al., 2010), pesticides (Garanzini and Menone, 2015) and pharmaceutical compounds (Brain et al., 2004; Kummerová et al., 2016) with the aim to evaluate mainly their usefulness to remove water pollutants or, their sensitivity and ability as bioindicator tools to be used in monitoring programs.

Surveys could be carried out by using either passive or active monitoring. Passive monitoring refers to organisms sampled in local populations whereas active implies the exposure of organisms in sites to be monitored for a period of time. Nevertheless, most of the biomonitoring surveys developed until now have been focused on the passive monitoring methodology, with the use of vertebrates and invertebrates (Ghisi et al., 2017; Ronci et al., 2016; Ruchter and Sures, 2015), as well as some aquatic plants species (Bonanno et al., 2017; Demirezen and Aksoy, 2004; Gonzalez et al., 2013; Harguinteguy et al., 2016; Pérez et al., 2017) as bioindicators. Passive biomonitoring has been widely used for its sampling simplicity, long-term measurements and existing guidelines. Simultaneously, disadvantages have been previously pointed out, including the effective availability of the selected organism at the sampling sites, the variability in the exposure time, age and size of sampled individuals, among others. The active biomonitoring not only avoids mentioned drawbacks but also allows the choice of organisms' origin, the repeatability of exposure, and to predict the cost and duration of experimentation (Besse et al., 2012). Nevertheless, active biomonitoring has been less developed and, in most of the cases, performed with animal species (Ballesteros et al., 2017; Bertrand et al., 2018; Ciliberti et al., 2017; Tan et al., 2018).

The presence of complex pollutants mixtures in aquatic ecosystems requires an expensive chemical analysis if hundreds of pollutants want to be analysed, but also, a significant investment of time. Instead, the development of biomonitoring programs, able to evaluate environmental quality, searches for the lowest cost and quick procedure trying to avoid, as far as possible, the use of animals (Sneddon et al., 2017). To the best of our knowledge, only a few studies tested and reported the usefulness and ability of macrophyte to point out environmental pollution through an active monitoring procedure in a real scenario where a complex mixture of pollutants occurs (Nimptsch et al., 2005).

The *Potamogeton* genus includes perennial monocotyledonous hydrophytes, with a cosmopolitan distribution (Novara, 2003). Vegetative propagation of *Potamogeton* occurs via growth and fragmentation of rhizomes and shoots as well as by dormant apex; sexual reproduction has been described for this genus as well (Nichols and Shaw, 1986). The *Potamogeton* genus is also a good candidate as test species in non-target plant studies for the development of protection guidelines (http://www.iucnredlist.org). Previous studies reported *Potamogeton* capacity to evidence pollutants exposure under laboratory conditions (Bertrand et al., 2016, 2017; Costa et al., 2018; Monferrán et al., 2009). In a similar way, others authors described the usefulness of *Potamogeton* in passive monitoring programs, showing to be useful as a biological indicator while evaluating environmental deterioration (Demirezen and Aksoy, 2004; Harguinteguy et al., 2016; Munteanu and Munteanu, 2007). Nevertheless, the ability of *Potamogeton,* or another macrophyte genus, to reflect environmental degradation associated with multi-sources pollution through short term exposures in active monitoring studies are scarce or unavailable to the extent of our knowledge.

Given this background, the present study proposes to evaluate the ability and usefulness of *Potamogeton pusillus* to reflect environmental quality through an active biomonitoring procedure, and additionally, to establish the aquatic ecosystems status through risk indexes. To achieve our goal, organisms from a pristine site were exposed during two different seasons in seven river sites with different land uses, and where spatio-temporal variations in pollutants mixture are expected. During each monitoring campaign, water and sediments samples were collected for physicochemical and pollutants determination. In parallel, exposure, defence and damage biomarkers were measured in tissues from exposed organisms. The results of this study were used to obtain a holistic and integrative overview of the studied scenario, and ultimately, to evaluate the usefulness of *P. pusillus* for environmental risk assessment in aquatic systems.

The hypothesis is that *Potamogeton pusillus* biochemical responses are able to point out sites with higher environmental degradation due to multi-sources pollutants mixtures in river system.

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. Study site

The Ctalamochita River basin (area ~ 3000 km², Córdoba, Argentina) was proposed as a case of study of human activities impact on an aquatic ecosystem. Ctalamochita River basin is one of the most important water resources of the central Argentinian region. The water quality of the basin decreases from the upper basin located in a mountainous area to a plain area. The river receives waste wasters discharges from industrial and agricultural activities developed along the basin, but also associated with important urban settlements (DiPAS, UNC, 2007; O'Mill, 2012). For the present study, seven monitoring sites were selected according to an expected variation in pollutants mixtures and levels due to different pollution sources (Fig. 1 and Supplementary Material, SM, Table SM1 and Figure SM1). The site located upstream Santa Rosa de Calamuchita town (S1, upper basin) was considered as the reference site due to lower anthropogenic activities.

2.3. Monitoring

Individuals of South American native macrophyte, *P. pusillus*, were collected from a reference site located in a tributary (Yuspe River) of the Suquía River upper basin based on an earlier study (Bertrand et al., 2017). Organisms were acclimated during two weeks in glass aquaria filled with 10% Hoagland's solution, sediments (1/4) from the same sampling area, and they were maintained at $25 \pm 1\,°C$ under a natural light: dark regime.

Acclimated organisms were transported in tanks to monitoring area where groups of 24 macrophytes were deposited at each site in perforated plastic envelopes with an adequate water circulation (SM, Figure SM1). Envelopes were maintained at a depth of 0.5—0.7 m, simulating environments usually colonized by the selected macrophyte species. Organisms were exposed for four days in two sampling campaigns. The exposure period was selected in accordance to previous laboratory testing (Bertrand et al., 2016, 2017). Sampling dates were chosen to take into account the rain seasonality and thermal variation for the proposed basin: cold (July, CP) and warm periods (December, WP), according to previous studies (SM, Table SM2; Bertrand et al., 2018; O'Mill, 2012). After exposure, macrophytes were collected, counted, washed three times with ultrapure water, frozen with liquid nitrogen, and kept at $-80\,°C$ until analysis.

Samples of superficial (0—15 cm depth interval) water and sediment were taken at each sample site during both campaigns in appropriate receptacles depending on the analysis to be carried out. Sample collection, containers, stabilization, and transportation to the laboratory, as well as sample storage for physicochemical and metal determinations, were done in accordance with Merlo et al. (2011), while the procedure for pharmaceutical residues determination in water was conducted as described by Valdés et al. (2014). All samples were collected by duplicate.

2.4. Physicochemical analysis

2.4.1. Water

Portable equipment (WTW, Multiline F/Set 3) was used for *in situ* water parameters measurement (water dissolved oxygen, pH, temperature, and conductivity) while, nitrate, nitrite, sulphate, total phosphorus, chlorides, ammonium, and total coliform bacteria were measured in water samples according to Pesce and Wunderlin (2000) and APHA (1998). Results were expressed in $\mu g\,L^{-1}$ except Cl^- expressed in $mg\,L^{-1}$ and coliform bacteria as the most probable number (MPN) for 100 mL of water.

2.4.2. Sediments

The content of organic matter (COM; APHA, 1998) and pH (Klute, 1986) were determined in sediment samples. For granulometry, samples were dried at 38 °C up to constant weight. Afterwards, 100 g of material were weighed and sieved using metallic meshes (IRAM 1501) obtaining following fractions: gravel (>2 mm), sands (>0.075 mm and < 2 mm) and limes and clays (<0.075 mm). Proportion of each fraction was calculated.

2.4.3. Pharmaceutical residues determination in water samples

Thirteen compounds were selected to be studied, considering different therapeutic classes of pharmaceuticals and steroid hormones, as follows: β-blockers: atenolol (ATE), propranolol (PROP); antibiotics: ciprofloxacin (CIPR), clarithromycin (CLAR); diuretics— antihypertensive: enalapril (ENAL); antiepileptic: carbamazepine (CBZ), oxcarbazepine (OXCZ); androgens: androstenedione (AND), testosterone (T), dihydrotestosterone (DHT), methyltestosterone (MT) and estrogens: 17β-estradiol (E_1), estrone (E_2). Water samples were extracted using a Strata-X® SPE cartridges (500 mg/6 mL) and analysed by high performance liquid chromatography coupled to mass spectrometry using a quadrupole time-of-flight analyzer, with an electrospray ionization source operated in either positive or negative modes (HPLC—ESI—qTOF, Agilent—Bruker Daltonics, Valdés et al., 2014). Quantification was done by external five-point calibration curves run in each sampling batch. Recoveries for each compound, evaluated in river water, ranged from 40 to 83%. The method limit of detection (LOD) ranged from 0.1 to 16 ng L^{-1}.

2.4.4. Metals and metalloids in water and sediment samples

Twenty elements, including seventeen metals and three metalloids were selected and quantified in collected samples according to procedure previously described by Bertrand et al. (2018). An elements sequential extraction was carried out with sediment samples with the aim to obtain bioavailable (mobile A1 + mobilisable A2) and pseudo-total (Ps) fractions of metals previous drying (38 °C) and sieving (63 μm acrylic meshes) of them (Supplementary material, SM, Figure SM2). The analysis of water samples and sediments extracts were 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 stock solution for calibration of metal quantification equipment. Results were expressed in $\mu g\,L^{-1}$ for water samples and $\mu g\,g^{-1}$ dried materials for sediments.

The concentrations of studied elements were determined in triplicate. Quality assurance (QA) and quality control (QC) 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 higher than 95% in all the case.

2.4.5. Water quality index

With physicochemical and bacteriological determinations in water samples, a water quality index (WQI) was calculated according to Pesce and Wunderlin (2000); which included the parameters normalization and the application of a weighting factor according to the importance of each measured parameter on the water quality. Obtained WQI gives then a non-dimensional number being 100 and 0 the best and worst water quality, respectively.

2.4.6. Assessment of potential ecological risk associated with sediments

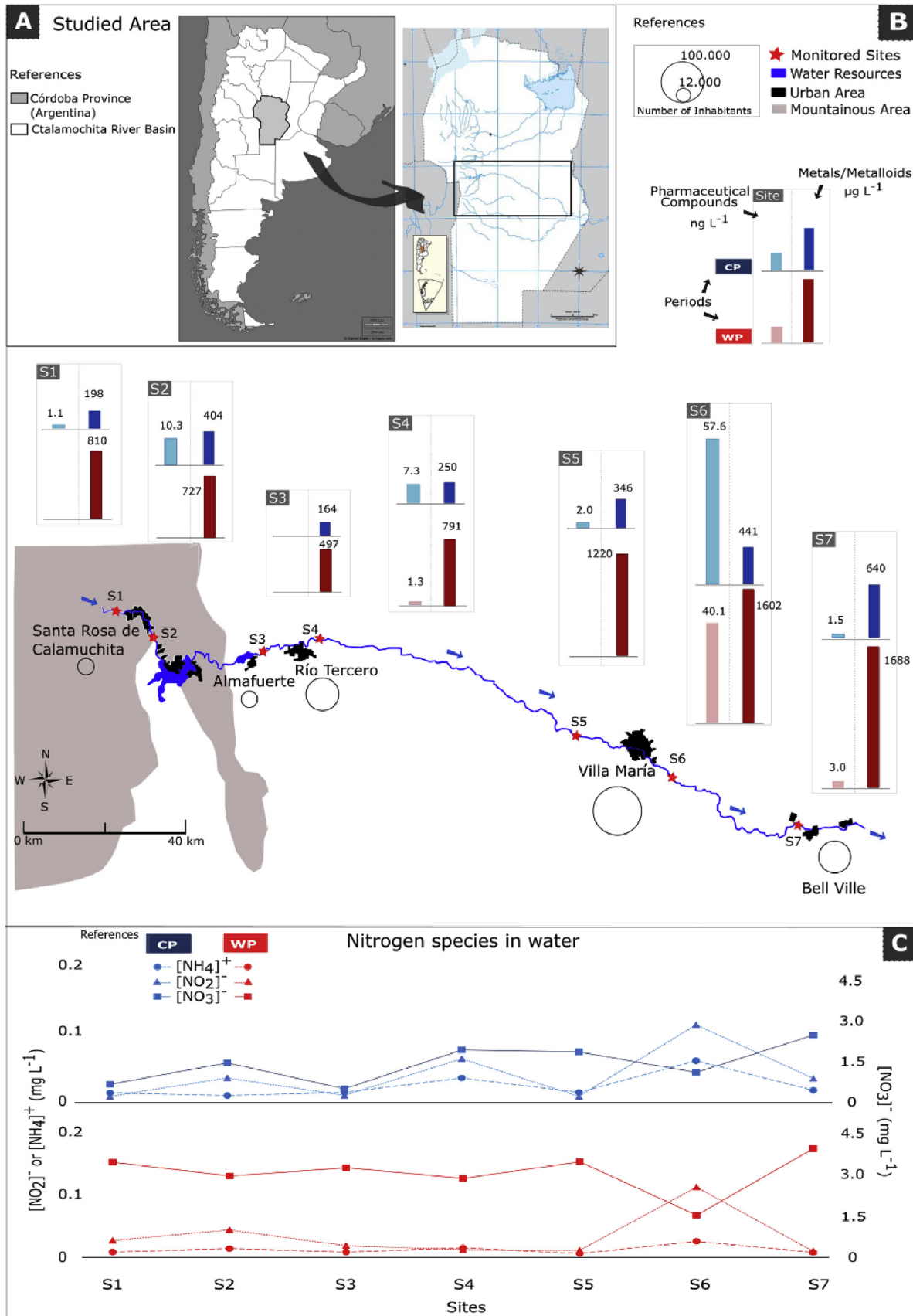Sediments are well known to be reservoirs for metals. Therefore,

Fig. 1. Studied area and concentrations of measured pollutants: **A-**The Ctalamochita River basin (Córdoba Province, Argentina). **B-**Detail of the Ctalamochita River Basin: location of the monitored sites, inhabitants (circle area) of principal urban settlement and towns of the basin. Total concentration in water of metals/metalloids ([TMetW], μg L^{-1}) and of pharmaceutical compounds ([TPharW], ng L^{-1}) are shown for cold (CP, blue) and warm (WP, red) monitored periods. **C-** Nitrogen species concentrations including ammonia nitrogen ([NH$_4$]$^+$, mg L^{-1}), nitrites nitrogen ([NO$_2$]$^-$, mg L^{-1}) and nitrates nitrogen ([NO$_3$]$^-$, mg L^{-1}), measured in water samples, are shown for cold (CP, blue) and warm (WP, red) monitored periods. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the potential ecological risk index (RI) and the mean probable effect concentration quotient (mPEC-Q) were calculated to assess the pollution degree and biological effect of multiple heavy metal presence in Ctalamochita River sediments. The RI was calculated according to Hakanson (1980), which is based on eight parameters (polychlorinated biphenyls (PCB), Hg, Cd, As, Pb, Cu, Cr and Zn). In the present study, PCB concentrations were excluded. Therefore, RI is calculated as the sum of all risk factors for total metals measured in sediment (A1 + A2 + Ps) during this study as indicated in SM, Formula SM1, SM2, and SM3. According to the obtained values, the potential ecological risk could be classified between different levels as explained below (3.1.4.2).

2.4.7. Enrichment factor and geo-accumulation index

The enrichment factor (EF) is useful to indicate the dynamics of an inorganic pollutant or chemical element of the earth's crust that can be transported by natural or anthropogenic process. It was calculated following Ghrefat et al. (2011) with the aim to assess the level of contamination and the possible anthropogenic impact in the sediments. In our study, Fe has been used as a conservative tracer to differentiate natural from anthropogenic components (used formula shown in SM, Formula SM4).

Besides, possible sediment enrichment of metals in aquatic sediments was evaluated in terms of the geoaccumulation index (Igeo) described by Müller (1979) (used formula is shown in SM, Formula SM5).

2.5. Biomarkers

Exposed organisms were dissected in leaf, stem and root for the later biomarker determination in each plant section.

2.5.1. Metals and metalloids accumulation

For metals and metalloids, accumulation samples were dried up to constant weight at 38 °C. Digestions were carried out according to Bertrand et al. (2016). Briefly, tissues (aprox. 0.02 g dw) were ground and homogenized with mortar, digested with 8 mL of *aqua regia* (HNO_3: HCl; 1:3) and heated at 160 °C overnight in Teflon tubes. Obtained samples were filtered with 0.45 μm nitrocellulose membrane filter and studied elements were measured in plant extracts as described in 2.4.4. Quality assurance and quality control were performed using a certified reference material (peach leaves, NIST1547). The percentages of recovery from tissues were $102 \pm 16\%$. Results were reported in μg g^{-1} dry weight (dw).

2.5.2. Pigment content

Chlorophyll (Chl) and pheophytins (Pheo) concentrations were determined in leaf and stem of *P. pusillus* according to Wintermans and de Mots (1965). Concentrations of pigments in plant extracts were measured in triplicate by visible spectrophotometry using a microplate reader (Bio-Tek, Synergy HT; Chl = 649 and 665 nm; Pheo = 654 and 666 nm, after hydrochloric acid addition). Concentrations were calculated and reported in μg g^{-1} wet weight (ww).

2.5.3. Enzyme activity

Enzyme extracts of *P. pusillus* were prepared according to Bertrand et al. (2017). After removal of cell debris (10 min at 13,000 g, 4 °C), the membrane fraction of the extracts were separated by centrifugation at 105,000 g for 60 min. The remaining supernatant, defined as the soluble (cytosolic) fraction was used for enzyme measurement. Enzymatic activities were determined in triplicated by spectrophotometry, using a microplate reader (Bio-Tek, Synergy HT). The activity of glutathione peroxidase (GPx; EC 1.11.1.9) was determined as reported by Drotar et al. (1985) using

hydrogen peroxide (H_2O_2) as substrate. The guaiacol peroxidase (POD) activity was measured using guaiacol and H_2O_2 as substrates (Bergmeyer, 1983). Superoxide dismutase activity was determined by using the photochemical *p*-nitroblue tetrazolium chloride (NBT) reduction method as described by Aiassa et al. (2010) (SOD; EC 1.15.1.1). The activity of all enzymes assessed was calculated in terms of the protein content of the sample extract (Bradford, 1976) and is reported in nanokatals per milligram of protein (nkat mg prot^{-1}), where 1 nkat is the conversion of 1 nmol of substrate per second. Likewise, one unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT. The protein quantification was performed using bovine serum albumin as standard.

2.5.4. Integrated biomarker response

One general stress index, the integrated biomarker response (IBR), was calculated as it was described in Bertrand et al. (2018) following the methods proposed by Beliaeff and Burgeot (2002). A higher value of IBR means a greater response expressed by the exposed organisms.

2.6. Data analysis

2.6.1. Statistical treatment

Generalized Linear Mixed Models were fitted for biomarkers responses for each studied period, including all macrophyte sectors when indicated, using Infostat Software (Di Rienzo et al., 2017). Then, normality and variance homogeneity were tested, being a variance function applied if necessary. A posteriori test LSD Fisher, with Bonferroni errors' correction when necessary, was used to determine significant differences between the means ($p < 0.05$). Correlations and Kruskal Wallis analysis were carried out with mentioned software. Discriminant analysis (DA) was performed with Statistic Software 8.0 with the aim to select those biomarkers with higher contribution to the discrimination between groups (monitored sites). Finally, principal component analysis (PCA) was carried out using "factoextra" R package of R Studio Software (Version 1.1.153) to investigate the associations between the macrophyte stress levels and the environmental variables in the studied sites.

With the aim to evaluate which pollutants explained best the distribution of the dependent variable (stress levels expressed as IBR), multiple linear regression models (MLRM) were fitted with R Studio software using "car" and "Rcpp" packages. The collinearity was tested with the "vif" function and variables with high values (>10) were ruled out in each built model. The best model was selected using a "both" function where the independent variables (Xs) selection combine forward and backward methods leaving those Xs that explain best the distribution of the dependent variable (Y). Those variables with values below the limit of detection in all sites were not considered. Pharmaceutical compounds could not be included in MLRM due to the low individual frequency of quantification in monitored sites. Therefore, four models were fitted for metals and metalloids concentrations in water and in the bioavailable fraction of sediments during CP and WP. For this statistical analysis, a value of LOD/2 was considered for all values below the LOD.

3. Results and discussion

3.1. Environmental scenario of the Ctalamochita River basin

3.1.1. Physicochemical characteristics

The quality of freshwaters has been identified as a conditioning factor of resources uses as well as for biota protection and survival.

A spatio-temporal variation in water quality index (WQI, Table 1) could be observed along the basin, with lower values measured during the WP when compared to the CP. In general, a decrease in water quality could be observed mostly in those sites downstream cities (S2, S4, S6), being S6 the site with the lowest WQI in both monitored periods. As expected, high concentrations of nitrogen species including ammonia ($[NH_4]^+$), nitrites ($[NO_2]^-$) and nitrates ($[NO_3]^-$) were found in the same sites (S2, S4, and S6, Fig. 1, Table SM2). Ammonia concentrations are an indication of organic contamination, coming from domestic sewage, industrial waste and fertiliser run-off, among other sources. High nitrate concentrations are associated with diffuse rather than point sources (Pasquini et al., 2011). The higher levels of $[NH_4]^+$ and $[NO_2]^-$ in S6 could be explained by the proximity of this site to Villa María city sewage discharge (<3.5 km) indicating that nitrogen species were not yet oxidized. The WQI value in S6 during WP (49%) indicates an inadequate condition for healthy aquatic life, mainly due to low dissolved oxygen concentration ($0.3\,mg\,L^{-1}$), increased concentration of NH_4^+ ($0.027\,mg\,L^{-1}$) and TCB values (3150000 MPN per 100 mL) (SM, Table SM2). Our results evidence the occurrence of water pollution due to inadequately, or not, sewage treatment and, probably, industrial wastewaters discharges.

As for the sediments, no significant differences, in pH and organic matter (OM) content, between studied period were observed along the basin (CP: 6.5 ± 0.4 pH, $9.6 \pm 2.8\%$ OM; WP: 6.8 ± 0.4, $8.9 \pm 2.7\%$ OM). Finally, granulometric fractions in samples were: sands > limes and clays > gravel during CP and WP.

3.1.2. Pharmaceutical compounds in water

Few studies describe the contamination of water with pharmaceuticals in Argentina (Elorriaga et al., 2013; Ondarza et al., 2019; Valdés et al., 2014). Moreover, the bioaccumulation of pharmaceuticals and their effects on native biota, have been barely reported for Argentina and South America (Maranho and Pereira, 2017; Ondarza et al., 2019; Valdés et al., 2016). Therefore, the ability of native species including macrophyte to bioindicate this kind of contamination has been poorly assessed.

In the present study four out of thirteen studied compounds were quantified: ATE, CBZ, and ENAL in both periods and CLAR in CP (Table 1). Fig. 1 shows temporal differences in [TPharW], with higher concentrations in CP respect to WP. Lower levels of pollutants due to the greater rainfall occurred during the WP (120 ± 30 mm monthly accumulated) when compared to CP (3.6 ± 3.8 mm monthly accumulated). Unsurprisingly, the highest concentrations of [TPharW] were measured downstream the main cities (S2, S4 and S6, Fig. 1 and Table 1) while no pharmaceuticals were detected at S3, as well as S1, S5 and S7 showed the lowest values. The occurrence of pharmaceutical compounds was in accordance with previously described nitrogen species changes, supporting the anthropic origin of pollutants, mainly through discharges of urban wastewater with low or null treatment.

Within the quantified compounds, ATE and CBZ were those with highest concentrations ($35 \pm 10\,ng\,L^{-1}$ and $20 \pm 2\,ng\,L^{-1}$, respectively). Both compounds are characterized by a low removal rate in sewage treatment processes and have been proposed as indicators of urban pollution in previous studies (Clara et al., 2004; Cleuvers, 2005; Miège et al., 2009). Quantified concentrations of ATE, ENAL ($2.1 \pm 0.3\,ng\,L^{-1}$) and CBZ were within the reported range for others regions of the world. In freshwater rivers, Gavrilescu et al. (2015) mentioned concentrations of CBZ ranging from 9 to $157\,ng\,L^{-1}$ in Europe, from 25 to $34.7\,ng\,L^{-1}$ in countries from Asia and Australian region and from 2.7 to $113.7\,ng\,L^{-1}$ in North America. The same authors informed concentrations of ATE ($314\,ng\,L^{-1}$) for European region whereas others authors reported concentrations from 5.8 to $1437\,ng\,L^{-1}$ in South American countries including Brazil and

Argentina (de Sousa et al., 2014; Quadra et al., 2017; Valdés et al., 2014). However, the only former report of pharmaceutical compounds in Ctalamochita River basin accounts for the presence of CBZ and ATE (Elorriaga et al., 2013). Therefore, the present study is the first report of ENAL and CLAR for this basin.

Pharmaceutical guidelines for aquatic biota protection have not been established at the time of the present study. Nonetheless, negative effects on animal and macrophyte, due to the presence of this kind of pollutants in aquatic environment have been described by Valdés et al. (2016) and Brain et al. (2004), among others. Therefore, the negative impact of domestic sewage on the water quality will probably condition the water uses and aquatic biota health status.

3.1.3. Metals and metalloids in water and sediments

3.1.3.1. Water.
Studied basin evidenced spatiotemporal variability in the total concentrations of metals and metalloids in water ([TMetW], Fig. 1). Lower values were observed in the site located immediately downstream a chain of reservoirs (S3), during both periods, which probably favoured the sedimentation of particles and metals as well as the occurrence of thermal stratification especially during the cold period (Wetzel, 2001; Yi et al., 2011). The intense rainfalls occurred during WP, could explain the higher [TMetW] quantified during this period, compared to CP. Rainfalls would have favoured the surface runoff from riversides and surrounding lands increasing the suspended materials and soluble metals in water.

Some elements (Pb, Al, As, B, Hg; Table 1) were quantified in concentrations higher than that suggested mainly for the protection of aquatic biota in freshwaters, but also for water resources used as drinking water source as well as crop irrigation (AEWQG, 2003; USEPA, 1988).

3.1.3.2. Sediments.
Sediments in aquatic ecosystems were described as metals deposit from where the elements could be remobilised to the water column depending on hydrodynamics, biogeochemical processes and environmental conditions (Luoma and Rainbow, 2008).

Concentrations of more environmentally relevant elements were compared with sediments quality guidelines (MacDonald et al., 2000; SM Table SM3 and Figure SM3). Results point out the total Hg concentration (A1+ A2 +Ps) as the unique metal surpassing, at five sites including S1, the concentration above which adverse effects are expected to be frequent ($1.06\,mg\,kg^{-1}dw$) during the CP. For the WP, concentrations of Hg in sediments diminished and probable effect concentration (PEC) value was not surpassed. The worrisome Hg concentrations in the Ctalamochita Basin have already been described in a previous study, at least for one season (Bertrand et al., 2018). Mercury sources include industrial activities and pesticides use but also a significant natural contribution such as volcanoes emissions and the presence of volcanic sediments. Therefore, significant concentrations of Hg in sites with low anthropic activities, like S1, could be explained by atmospheric transport and its later deposition in addition to the natural background.

Elements concentrations in sediments will depend on rocks and parent materials levels, pedological processes but also anthropogenic activities. Pearson correlations among metals in sediments evidenced the existence of close and significant positive correlations among Ag, Cd, Cr, Cu, Hg, Pb and Zn during both periods (SM, Table SM4) which are usually characterized by reaching rivers from common sources including coal burning, industrial activities and an intense use of pesticides (Li et al., 2009; Ruchter and Sures, 2015). On the other hand, Fe could be considered as a reference metal and, in most cases, anthropogenic sources are smaller compared to

Table 1
Metals, metalloids and pharmaceutical compounds concentrations in water samples (n = 3) collected in seven sites of Ctalamochita River basin during cold (CP) and warm (WP) periods. Concentrations (media ± standard deviation) are expressed in μg L^{-1} for measured elements and in ng L^{-1} for pharmaceutical residues. Values of water quality index (WQI) for each monitored site, and studied periods, are shown. Concentrations of metals and metalloids surpassing environmental guideline are indicated in bold.

		Period	S1	S2	S3	S4	S5	S6	S7
Metals and Metalloids	Ag	CP	<LOD	<LOD	<LOD	<LOD	**0.33 ± 0.02** [a]	<LOD	<LOD
		WP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Al	CP	56.8 ± 1.2	44.9 ± 0.5	<LOD	27.0 ± 0.5	84.5 ± 0.6	**112.2 ± 0.2** [b]	**105.3 ± 0.4** [b]
		WP	**131.7 ± 4.8** [b]	**169.9 ± 3.6** [b]	<LOQ	**98.8 ± 1.4** [b]	**415.2 ± 8.5** [b]	**578.5 ± 2.5** [b]	**465.8 ± 12.7** [b]
	As	CP	0.19 ± 0.01	0.71 ± 0.01	1.95 ± 0.06	1.95 ± 0.05	2.06 ± 0.08	3.45 ± 0.08	13.3 ± 0.22
		WP	0.64 ± 0.05	1.19 ± 0.06	2.39 ± 0.10	2.27 ± 0.05	2.76 ± 0.02	5.46 ± 0.13	**27.18 ± 0.40** [a]
	B	CP	<LOD	84.2 ± 0.1	47.3 ± 0.6	51.2 ± 0.3	48.6 ± 0.7	60.6 ± 0.2	**186.3 ± 4.0** [d]
		WP	**135.2 ± 9.4** [d]	**147.1 ± 4.2** [d]	**204.5 ± 14.7** [d]	**206.7 ± 11.9** [d]	**202.6 ± 4.7** [d]	**193.8 ± 13.2** [d]	**353.2 ± 2.6** [d]
	Ba	CP	11.7 ± 0.3	27.9 ± 0.5	10.4 ± 0.1	13.9 ± 0.1	17.9 ± 0.3	18.5 ± 0.3	24.4 ± 0.2
		WP	7.1 ± 0.2	13.7 ± 0.4	12.7 ± 0.3	16.5 ± 0.2	26.0 ± 0.2	32.3 ± 0.6	32.8 ± 0.8
	Cd	CP	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ
		WP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Co	CP	0.10 ± 0.01	0.09 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.15 ± 0.01
		WP	0.19 ± 0.03	0.17 ± 0.02	0.06 ± 0.01	0.11 ± 0.01	0.29 ± 0.01	0.42 ± 0.02	0.33 ± 0.02
	Cr	CP	<LOD	<LQ	<LOD	1.45 ± 0.03	0.53 ± 0.01	0.64 ± 0.01	0.34 ± 0.01
		WP	1.14 ± 0.03	0.31 ± 0.01	<LOQ	1.05 ± 0.02	1.30 ± 0.03	1.49 ± 0.01	1.00 ± 0.07
	Cu	CP	1.97 ± 0.13	<LOD	<LOD	1.22 ± 0.11	1.39 ± 0.07	<LOD	0.82 ± 0.11
		WP	<LOD	<LOD	<LOD	3.44 ± 0.06	<LOD	<LOD	<LOD
	Fe	CP	23.1 ± 0.2	56.2 ± 0.8	14.9 ± 2.2	30.2 ± 0.2	70.6 ± 1.2	106.1 ± 0.5	100.1 ± 0.5
		WP	231.9 ± 7.7	261.3 ± 5.4	114.7 ± 1.3	235.7 ± 4.8	388.1 ± 6.1	518.3 ± 6.1	474.9 ± 13.8
	Hg	CP	<LOQ	<LOD	<LOD	<LOD	**5.55 ± 0.21** [a,c]	**1.03 ± 0.09** [a]	<LOQ
		WP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Mn	CP	2.64 ± 0.04	14.29 ± 0.28	4.14 ± 0.24	11.09 ± 0.28	13.09 ± 0.09	24.19 ± 0.15	28.35 ± 0.27
		WP	250.76 ± 7.70	55.44 ± 1.14	51.42 ± 0.75	77.48 ± 0.49	34.87 ± 0.80	101.52 ± 1.44	43.21 ± 1.18
	Mo	CP	0.33 ± 0.04	0.90 ± 0.06	0.91 ± 0.04	2.27 ± 0.03	1.91 ± 0.08	1.42 ± 0.02	4.22 ± 0.11
		WP	0.72 ± 0.09	0.70 ± 0.08	1.69 ± 0.12	2.42 ± 0.07	2.61 ± 0.12	1.91 ± 0.10	6.91 ± 0.13
	Ni	CP	0.23 ± 0.04	<LOD	<LOQ	0.39 ± 0.01	0.40 ± 0.01	0.42 ± 0.01	0.34 ± 0.02
		WP	0.43 ± 0.09	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Pb	CP	**6.91 ± 0.19** [a]	1.01 ± 0.04	0.45 ± 0.01	0.47 ± 0.01	0.57 ± 0.01	0.54 ± 0.01	0.82 ± 0.02
		WP	0.09 ± 0.01	0.08 ± 0.03	0.79 ± 0.03	**3.90 ± 0.08** [a]	1.47 ± 0.05	1.44 ± 0.05	1.01 ± 0.05
	Rb	CP	0.68 ± 0.02	0.69 ± 0.03	1.03 ± 0.03	1.21 ± 0.02	1.11 ± 0.03	1.53 ± 0.05	1.53 ± 0.09
		WP	0.95 ± 0.01	1.10 ± 0.02	1.20 ± 0.02	1.37 ± 0.09	2.18 ± 0.06	3.29 ± 0.03	3.73 ± 0.09
	Se	CP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
		WP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Sr	CP	63.51 ± 0.34	160.70 ± 0.20	74.73 ± 0.35	86.19 ± 1.76	82.87 ± 0.49	96.41 ± 0.45	146.22 ± 2.75
		WP	34.98 ± 0.51	66.73 ± 0.54	100.96 ± 1.04	110.67 ± 2.08	124.35 ± 1.48	132.52 ± 1.08	222.83 ± 1.59
	V	CP	1.22 ± 0.03	3.36 ± 0.02	2.36 ± 0.04	2.34 ± 0.03	3.28 ± 0.04	6.34 ± 0.01	19.4 ± 0.11
		WP	1.77 ± 0.07	3.39 ± 0.09	2.87 ± 0.02	3.69 ± 0.04	6.21 ± 0.14	11.92 ± 0.16	44.18 ± 1.23
	Zn	CP	29.83 ± 0.96	8.33 ± 0.29	5.34 ± 0.19	18.93 ± 0.29	10.72 ± 0.59	7.09 ± 0.19	8.07 ± 0.35
		WP	12.01 ± 0.66	5.45 ± 0.75	4.04 ± 0.19	26.83 ± 0.76	11.93 ± 0.13	19.41 ± 0.07	10.55 ± 0.89
PharmaceuticalCompounds	ATE	CP	<LOD	2.3 ± 0.6	<LOD	7.3 ± 0.2	<LOD	36 ± 2	<LOQ
		WP	<LOD	<LOD	<LOD	1.29 ± 0.01	<LOD	35 ± 10	<LOD
	CBZ	CP	1.1 ± 0.1	<LOD	<LOD	<LOD	2.0 ± 0.4	20 ± 2	1.54 ± 0.01
		WP	<LOD	<LOD	<LOD	<LOD	<LOQ	3 ± 1	3 ± 1
	CLAR	CP	<LOD	8.0 ± 1.0	<LOD	<LOD	<LOD	<LOD	<LOD
		WP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	ENAL	CP	<LOD	<LOD	<LOD	<LOD	<LOD	1.6 ± 0.3	<LOD
		WP	<LOD	<LOD	<LOD	<LOD	<LOD	2.1 ± 0.3	<LOD
WQI		CP	87	81	80	82	82	72	76
		WP	85	74	79	71	76	**49**	73

Metals and Metalloids: **LOD**: below limit of detection, for each element being (μg L^{-1}): Ag = 0.13; Al = 8.2; B = 4.7; Cd = 0.44; Cr = 0.15; Cu = 0.81; Hg = 0.32; Ni = 0.19; Se = 1.6.**LOQ**: below limit of quantification, for each element being (μg L^{-1}): Al = 19.40; Cd = 0.48; Cr = 0.38; Hg = 0.85; Ni = 0.22.

Pharmaceutical Compounds: ATE: atenolol, CBZ: carbamazepine, CLAR: clarithromycin and ENAL: enalapril. **LOD**: below limit of detection, for each compound being (ng L^{-1}): ATE = 0.2, CBZ = 0.2, CLAR = 0.3, ENAL = 0.1. CLAR and ENAL concentrations are estimated values due to percentages of recovery lower than 50%. Others nine compounds were below the limit of detection throughout studied water samples: propranolol (LOD = 0.5), ciprofloxacin (LOD = 16), oxcarbazepine (LOD = 3), androstenedione (LOD = 0.8), testosterone (LOD = 0.6), dihydrotestosterone (LOD = 2), methyltestosterone (LOD = 0.1), 17β-estradiol (LOD = 6), and estrone (LOD = 2).

Water Quality Index (WQI): calculated for seven monitored sites in the Ctalamochita River basin during cold and warm periods. Sites with values < 50 (in bold) indicate a poor water quality and inadequate condition for aquatic life.

[a] Concentrations higher than recommended for the protection of aquatic biota in freshwaters by the Argentinean Environmental Water Quality Guidelines (AEWQG, 2003): Pb < 1.59 μg L^{-1}; As ≤ 15 μg L^{-1}; Hg ≤ 0.029 μg L^{-1}, Ag ≤ 0.028 μg L^{-1}.

[b] Concentrations higher than recommended for the protection of aquatic biota in freshwaters by the United States Environmental Protection Agency (USEPA, 1988): Al < 89 μg L^{-1} for chronic exposure.

[c] Concentrations higher than recommended for water resources used to purification and human consumption by the Argentinean Environmental Water Quality Guidelines (AEWQG, 2003): Hg ≤ 4 μg L^{-1}.

[d] Concentrations higher than recommended for water resources used to crop irrigation by the Argentinean Environmental Water Quality Guidelines (AEWQG, 2003): B ≤ 100 μg L^{-1}.

naturally present levels. In the present study, Al, Rb, Co, Ni showed a positive and significant correlation with Fe in CP and WP (SM, Table SM4) probably indicating a natural source of these metals.

A geoaccumulation index (Igeo) and an enrichment factor (EF) were calculated to assess the possible anthropogenic impact in sediments of the studied river (Ghrefat et al., 2011). According to the Igeo results, surface sediments were not contaminated with Cr, Cu, Cd, Pb, Zn, and As (Igeo< 0) in both monitored periods. On the contrary, a moderate contamination for Ag (1 < Igeo<2) and a strong contamination for Hg (3 < Igeo< 4) occurred in the CP, whereas Igeo decayed in the WP for both metals, reaching moderate or uncontaminated levels.

The EF results showed a different pattern and decreased according to the following order Hg > Ag > Cd > Pb > Zn > Cu > Cr for the CP and Ag > Hg > Pb > Zn > Cu > Cr > Cd for the WP. Mercury showed from moderately severe (EF = 5–10, S2 and S4) to extremely severe (EF > 50, all others monitored sites) enrichment in the CP, and a severe (EF = 10–25, S5 and S6) or very severe (EF = 25–50, S4) enrichment in the WP (data not shown). Silver presented similar EF in both periods, varying between minor enrichment (EF = 1–3) to very severe enrichment (EF = 25–50), especially in sites located in the lower basin (from S4 to S6 in both periods, as well as S1 and S7 in CP). Cadmium showed EF between minor enrichment (2.9) and severe enrichment (10.3) in CP, whereas no enrichment (EF < 1) was detected in WP for all the studied sites. Lead showed EF values included between moderate (EF = 3–5) and moderately severe (EF = 5–10) enrichment in both periods. Finally, Zinc varied between a minor and moderately severe enrichment being S4 that with maximal value (9.9) in the basin. Therefore, according to EF, the metal contamination of basin sediments due to anthropogenic activities (EF > 1) is mainly associated with Hg, Ag, Pb, Cd, and Zn levels. Consequently, these results show the usefulness of different approaches in enrichment assessment since the interpretation could vary.

3.1.4. Risk assessment of metals and metalloids in sediments

3.1.4.1. Metals and metalloids in bioavailable fractions. The potential mobility and bioavailability of metals and metalloids in sediments determine their toxicity for organisms. Therefore, considering only the total level of elements probably, can not accurately reflect its risk in the environment. For the Ctalamochita River, the obtained results showed a significant variation in A1 + A2 fraction (bioavailable) among elements. During the CP, the bioavailable fraction of Cr, Hg and As represented lower than 10% of the total

quantified concentration (SM, Table SM3). In a different way, for Cd, Cu, Zn and Pb, the bioavailable fraction reached up to 36% of total metal. Conversely, in the WP the observed pattern was similar only for As (bioavailable fraction <10%). For the other metals, A1 + A2 increased, varying from 20% to 70%, according to the following order: Pb < Cu < Cd < Zn < Cr < Hg. In S1 and S2, Hg showed exceptional high proportion bioavailable compared with other sites. Those results evidence that despite significant higher total concentrations of Hg in sediments during the CP (4.16 ± 3.89 mg kg^{-1}dw) when compared to WP (0.37 ± 0.32 mg kg^{-1}dw), the bioavailability reaches the highest percentages in WP, probably due to the variation in water temperature and oxygen levels in sediments (Yang et al., 2014).

3.1.4.2. Risk index of metals. In order to evaluate the combined pollution risk in sediments associated with the presence of As, Cd, Cr, Cu, Hg, Pb, Zn, the potential ecological risk index (RI) and the mean probable effect concentration quotient (mPEC-Q) were calculated for Ctalamochita River basin. Fig. 2 shows that in the CP, a "very high" risk index was observed in S1, S5, S6 and S7, while in S3 the risk index was "considerable". The others sites presented a "low" risk index. Those metals that contributed most to the RI values (monomial potential ecological risk factor, E^i_r) were Hg and, to a lesser extent, Cd (data not shown). In particular, for Hg, moderate values of E^i_r were calculated in S2 and S4 ($40 \leq E^i_r \leq 80$), while obtained E^i_r were very high in all the others sites ($E^i_r \geq 320$). On the other hand, in the WP, the RI decreased in most of the sites compared with CP. However, S4 showed a "considerable" RI due to a moderate E^i_r associated with Hg. In a similar way, S5 and S6 presented also a moderate E^i_r related to Hg concentrations, although the RI values were "low". The mPEC-Q in sediment varied from 0.08 to 1.27 with an average value of 0.63 in the CP, while for the WP, the mPEC-Q in sediment varied from 0.04 to 0.20 with an average of 0.11. The S2, for the both studied periods, was classified as the least toxic site, in relation to metals in sediments. Sites from S3 to S7 in CP and S4, S5, and S6 in WP presented mPEC-Q values between 0.1 and 1 (Fig. 2) indicating an incidence probability of toxicity with a range of 15–29% (Long et al., 2006). In the agreement, described sites presented a "considerable" (S3) and "high" RI during CP, whereas in WP, the RI was "moderate" in S4 and "low" for the others sites. Similar to RI, the highest value of mPEC-Q in CP was found at site S1 (1.27) indicating that adverse biological effects can potentially occur.
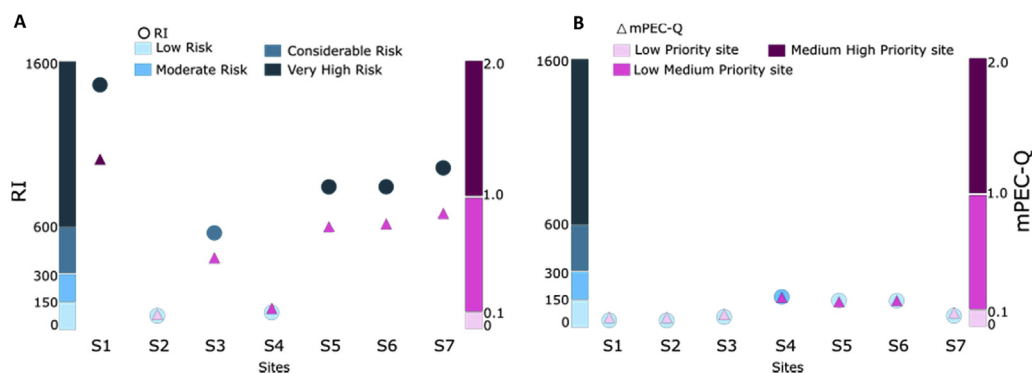

Fig. 2. The potential ecological risk index (RI) and the mean probable effect concentration quotient (mPEC-Q) of As, Cd, Cr, Cu, Pb, Hg, Ni and Zn in sediments from seven sites of the Ctalamochita River basin in CP (A) and WP (B). The mPEC-Q is classified into four levels: low priority site (<0.1), low-medium priority site (0.1–1.0), medium-high priority site (1.0–5.0), and high priority site (45.0), indicating <14%, 15–29%, 33–58% and 75–81% of probability to being toxic, respectively (Long et al., 2006). Four classes of RI were defined: low risk (RI < 150), moderate risk (150 > RI < 300), considerable risk (300 > RI < 600), and very high risk (RI > 600) (Hakanson, 1980).

3.2. Biomonitor responses

3.2.1. Metals and metalloids accumulation in macrophyte

Exposed macrophyte showed different patterns of metal accumulation depending on to the plant sector considered (SM, Table SM5). In general, the levels of metals and metalloids in macrophyte sections showed the following order: stem < root < leaf in the CP and the inverse pattern during WP: leaf < root < stem. During the CP, the maximum accumulation concentrations in leaf and root occurred in S4, thus exceeding 7 and 10 times the accumulation values of the reference site (S1), respectively. On the other hand, maximum accumulation levels in stem were observed in S5, doubling S1. In WP, the maximum concentrations of total accumulation in leaf and root occurred in S3, doubling the values at S1. In contrast, stem showed the highest concentrations of total metals accumulated in S4 and the lowest in S6 (SM, Table SM5). An acute laboratory exposure of *P. pusillus* to Zn showed higher metal accumulation in leaf when compared to stem and root, a similar pattern to the observed in the CP (Bertrand et al., 2016). Previously, Duman et al. (2007) have reported a seasonal variation in the accumulation of metals in aquatic macrophytes due to environmental factors but also physiological changes in photosynthetic organisms. Moreover, Duman et al. (2006) reported variations in metal accumulation in *Potamogeton lucens* depending not only on seasons but also plant sections and elements analysed. In agreement with the pattern described for the WP (summer time) in the present study, Duman et al. (2006) reported for most of the studied metals higher concentrations in root or stem sections than in leaf tissues during de summer season. These results could be indicating an attempt of the plant to protect leaves tissues over the stem or roots. Translocation from leaf to stem, or lower uptake rates in the foliar region compared with stem or roots, could be possible strategies to avoid plant damages. Unfortunately, there is no data available for winter season to be a compared with *P. pusillus* results from CP.

Associations between accumulated elements in the biomonitor and environmental levels were analysed through Pearson correlations (Table 2). In general, the accumulation in leaf and stem showed a significant correlation with metals water concentrations, for both monitored periods. The wider surface of leaves and stems in contact with water could explain the observed correlation for dissolved Al, As, Ba, Cr, Pb, Sr, and V (Table 2). By the contrary, the accumulation of metals in roots was correlated with the metals measured in the bioavailable and Ps fractions of the sediments. Considering *P. pusillus* is a rooted plant, the high capacity of roots to

accumulate metals and metalloids from sediments could be also expected. The results showed significant correlation for Cd, Co, Pb, V, and Zn. In particular, the correlation observed for Al in WP (leaf vs. water) correspond with the quantification of concentrations of this element in levels higher than recommended for the protection of aquatic biota (USEPA, 1988, Table 1). Previously described results would be reflecting the *P. pusillus* ability to point out, at least in part, metals pollution in aquatic ecosystem and its potential to be used as an active biomonitoring tool. Nevertheless, others elements surpassing environmental guidelines (Ag, As, Hg, Pb) seem to not be reflected, at least when metal accumulation is considered. The results obtained in the present study show the complexity of responses in organisms exposed to pollutants mixture being environmental factors (changes in metal concentrations, interactions between metals or with other elements, pH, conductivity, temperature, among others) and plant physiological mechanisms (ex. dilution of metal contents due to growth) the main responsible of variability in the metal uptake (Bonanno et al., 2017; Duman et al., 2006).

3.2.2. Responses of defence and damage biomarker responses

3.2.2.1. Pigments. Different patterns of biomarker responses were observed in monitored sites for both periods. In CP, pigments concentrations (Chl-a, Chl-b, Pheo-a, and Pheo-b) decreased significantly or showed a tendency to diminish, in S2, S4, S5, and S6, when compared to the reference site (S1; SM- Figure SM4). Pheo-b also showed a significant decay at S7 in the same period. During the WP the response was less clear, in particular for Chl-a, Chl-b, and Pheo-b. A significant decline was observed at S2 in Chl-a, Chl-b, and Pheo-a. Pheo-a also declined significantly at S4, S5, and S7. On the contrary, the concentrations of Chl-a, Chl-b, Pheo-a, and Pheo-b increased at S6 when compared to the reference site. Also, Pheo-b increased at S3. Diminution of pigments content, mainly chlorophylls, has been described as an indicator of biomolecule damages under stress conditions in macrophytes tissues, including *P. pusillus,* when it was exposed to pollutants (Bertrand et al., 2017; Dogan et al., 2018). On the contrary, the increase in pigment concentrations could be associated with an attempt of the macrophyte to maintain photosynthetic rates in water resources with high turbidity.

3.2.2.2. Antioxidant enzymes. Regarding antioxidant enzymes, once more, different patterns of response were observed at each monitoring site depending on the period of sampling, tissue or biomarker measured. When comparing the antioxidant enzymes

Table 2
Significant correlation coefficients between metals accumulated in *P. pusillus* (leaf, stem and root) and metal concentrations in water and sediments (bioavailable and pseudo total fractions). Level of significance = *p < 0.05 and ** for p < 0.01.

			CP			WP		
		Elements	**Leaf**	**Stem**	**Root**	**Leaf**	**Stem**	**Root**
Water		**Al**				0.78*		
		As	0.77*				0.92**	
		Ba					0.86*	
		Cr	0.94**	0.43**	0.87**			
		Pb				0.84*		
		Sr	0.82*				0.91**	
		V		0.76*			0.99**	0.77*
Sediments	Bioavailable fraction (A1+A2)	**Cd**						0.85*
		Pb						0.76*
		V				0.98**		
		Zn	0.89**		0.87**			
	Pseudototalfraction	**Co**			0.83*			
		V			0.88**			
		Zn	0.82*		0.78*			

responses at S2-S7 with S1, a greater number of significant responses were observed for WP than for CP, and they were measured in leaf and root (SM, Figure SM5).

GPx activity was not detected in leaf during the CP (SM, Figure SM5). However, in stem and root during CP a significant inhibition was detected at S7. Instead, in WP, this enzyme was inhibited in leaf at S4 and activated in root at S5, in both cases when compared to S1. Guaiacol peroxidases (POD) were also inhibited in root at S2 during CP when compared with S1. On the contrary during the WP, the POD activity was significantly induced only in leaf at S6. Finally, the activity of SOD increased significantly in leaf at S6 in CP and from S2 to S6 in WP, compared with S1. Conversely, in the stem, a significant decay of SOD activity was observed from S4 to S6, during the CP. Described results would be indicating the occurrence of significant oxidative stress in macrophyte exposed in Ctalamochita River, particularly in those sites with urban settlements (S4 and S6) as well as in those sites with an intense industrial and agricultural activities (S5 and S7).

3.2.3. Integrated biomarkers response (IBR)

The individual interpretation of biomarkers in field studies is complex due to the different patterns observed for each of them. Therefore, IBR was used as a tool to integrate and interpret responses obtained along the basin to achieve a comprehensive understanding of the biomonitor response (Fig. 3). The index was calculated with those biomarkers selected through discriminant analysis (SM- Table SM6). A Kruskal Wallis test revealed strong differences among monitored stations ($p < 0.0001$). During both monitored periods, S5 and S6 showed the highest levels of macrophyte response or suffered stress. As it was expected, the reference site (S1) was the one with the lowest IBR value, despite being the site with higher RI and mPEC-Q during the CP mainly associated with Hg concentrations. Nevertheless, the bioavailable fraction represented less than 10% of the total concentration of Hg, in the CP. This result would be pointing out limitations of RI to reflect bioavailable pollutants in the ecosystem. Moreover, the stressor response levels in *P. pusillus* increased along the basin from S1 to S5 or S6 with a slight or strong decay at S7 during CP and WP, respectively. The higher values of IBR in S5 and S6 could be associated with an increased complexity of pollutants mixture as a result of multi-sources discharges expected in both sites. Pollutants surpassing freshwater guidelines (Ag, Al, and Hg) and highest

values of [TPharW] in S6, could be responsible to induce an attempt by macrophyte to diminish toxicological effects through an increase of biological responses. In addition, in S5, S6, and S7 a significant metal enrichment in sediments (mainly Cd, Pb, Hg, Zn and Ag) due to human activities was observed during the CP (3.1.3.2). The higher conductivity and salinity described in the lower basin could be responsible forthe IBR decrease in S7, since those physicochemical parameters were described to promote variations in the speciation and bioavailability of pollutants (Luoma and Rainbow, 2008). By last, during both periods, S2 showed IBR values significantly increased compared with the reference site which indicates a significant impact of Santa Rosa de Calamuchita town on the aquatic biota also in the upper basin.

To the best of our knowledge, only few field studies performed with phytoplankton (Pereira et al., 2009; Liu et al., 2014) considered IBR as a tool to obtain a holistic overview of how pollutants mixtures affect photosynthetic organisms, and ultimately, to assess their usefulness for environmental biomonitoring in aquatic systems. According to previous studies, IBR is able to evidence stress levels in exposed organisms but also it could be interpreted as the capacity of them to cope with pollutants mixtures (Potet et al., 2018). Previous passive monitoring using *Mytilus* described strong stress levels in organisms with IBR values higher than nine (Bocquené et al., 2004). Even when inter-species comparisons of IBR values are not suitable due to variability in biological and physiological responses, *P. pusillus* surpassed mentioned value up to eleven. Pereira et al. (2009) described IBR values close to twenty when *Ulva* sp. suffered the highest stress level after being exposed in sites with strong metals pollution. A comparison between IBR values obtained from studies using the same genus, or others macrophytes would be more appropriate but, these IBR values are not available at the time of the present study. *P. pusillus* results are the first approach about its potentiality to be used as a useful tool for monitoring programs of aquatic ecosystems. Nevertheless, the limited studies available are pointing out the need of future field studies using the described methodology in photosynthetic organisms for a better understanding of obtained responses and their usefulness for biomonitoring. The final consequences of pollutants on aquatic plant communities are mostly unknown and should be explored (Coutris et al., 2011).
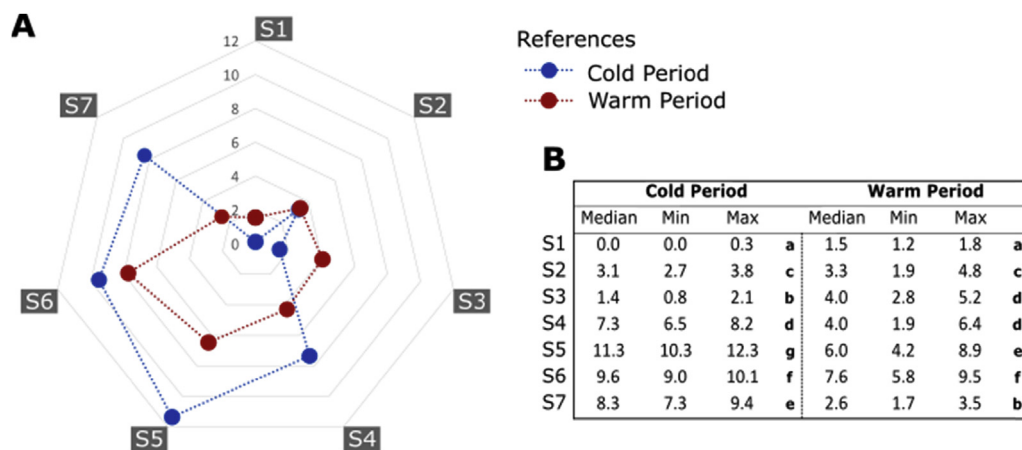

Fig. 3. A- Radar graph for the calculated Integrated Biomarker Response (IBR) index for *Potamogeton pusillus* exposed in seven sites at the Ctalamochita River basin during cold (blue) and warm (red) periods. **B-** Media, minimal (min) and maximal (max) for each site during studied periods. Different letters indicate significant differences among sites, analysed for each period by non parametric pairwise comparisons following a Kruskal-Wallis Test ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Multivariate analysis

3.3.1. Principal component analysis

With the aim to investigate the associations between the macrophyte stress, or response, levels (IBR) and the environmental variables in the studied sites, a principal component analysis (PCA) was performed. The first four dimensions of PCA analysis explained the 94% and 95% of variability for CP and WP, respectively. Fig. 4 shows the biplot of PCA performed for the CP (A), where a 66.0% of the total inertia was explained by the first two axes. The monitoring sites are segregated into four groups or quadrants: I. including S6, which was associated with [TPharW], [TMetW] and IBR; showing the IBR a negative correlation with the WQI. II. including S5 and S7, which were associated with mPEC-Q, and RI. III. including the reference site (S1), showing a good association with WQI and low [TPharW] and [TMetW]. IV. including S2, S3 and S4 associated with the total metals in the bioavailable fraction [TBioS] and total metals in the pseudo-total fraction [TPsS] of the sediments.

In a similar way, in the WP the two first axes explained 74.3% of the total inertia (Fig. 4 B). The monitored sites were segregated in the following groups: I. S1 and S3, strongly associated with WQI, as well as with [TBioS] and [TPsS] toa lesser extent. II. Including S4 and S5, related with RI and mPEC-Q, as well as with IBR. III: including S6 associated with [TPharW] and [TMetW]. IV. Including S2 and S7, negatively associated with RI, mPEC-Q and IBR. To sum up, in both periods the reference site (S1) shows the best environmental quality and lowest stress levels for *P. pusillus*. On the contrary, S5, S6 and S7 in CP as well as S4, S5 and S6 in WP, show the highest IBR values for the macrophyte.

The PCA analysis consistently reflected a positive and significant association between the degree of biomonitor response and the concentrations of pharmaceuticals and metals in the environment,

being this linkage strongest in the CP than WP, probably due to a greater pollutants levels. Harguinteguy et al. (2016) also reported an association between metal accumulation in *P. pusillus* through passive monitoring and sediment levels downstream Río Tercero city. However, the literature is limited for ecotoxicity data, especially on plants exposed to pharmaceuticals. The first results are promising considering that *Lemna* genus showed antioxidant response to chlorpromazine, paracetamol, and diclofenac (Alkimin et al., 2019). A higher understanding of the macrophyte response capacity to pharmaceutical compounds exposure will probably improve and promote the use of *P. pusillus* in monitoring programs of aquatic environments. Moreover, the quantification of other pollutants as pesticides and polycyclic aromatic hydrocarbons not available in the present study, would also help to characterize the biomonitor response.

3.3.2. Multivariate regression analysis

Finally, with the aim to evaluate those concentrations of pollutants with higher capacity to explain the stress suffered by exposed macrophytes, multivariate regression analysis (MRA) were fitted for both periods.

Firstly, we quantified the contribution of water metals and metalloids concentrations on the stress levels suffered by the biomonitor. Models showed a significant effect on the IBR value due to $Cr > Cu > Hg > Al > Pb$ in CP, and to $Pb > Al > As > Cr > Cu$ during WP. Chromium and Pb were the parameters with higher influence on the IBR values during CP and WP, respectively (Table 3). The analysis of coefficients shows that the biomonitor response was boosted when exposure concentrations of Cr and Pb increased. As well as, the negative coefficients of Pb in CP and As, Cr, and Cu in WP, could probably indicate a decrease in the ability of the monitor to cope with pollutant mixture. According to the models, the Cr, Cu, Hg, and Al during the CP as well as Pb and Al for the WP increased
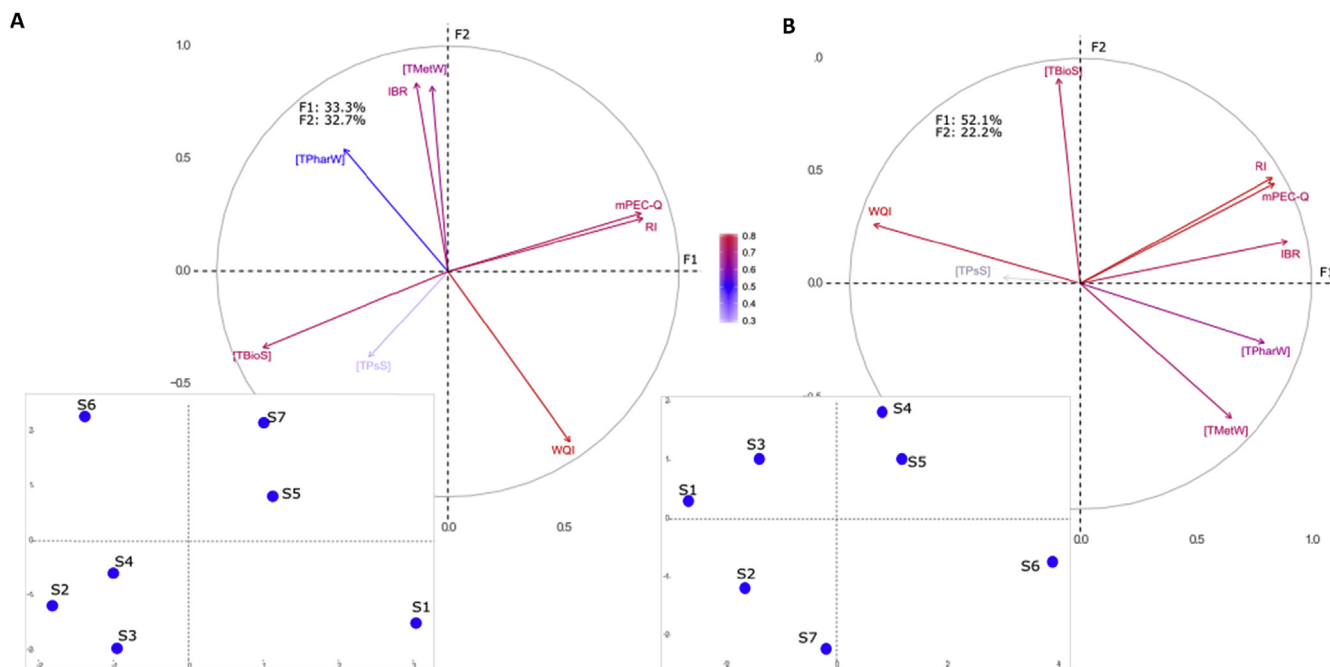

Fig. 4. Biplot of the two first principal components generated by the principal component analysis (PCA) performed considering environmental variables and calculated IBR for *P. pusillus* exposed in seven sites of the Ctalamochita River basin. In the biplot are the indications of: a- Correlation circles for A. cold (CP), and B. warm periods (WP); b- Distribution of monitored sites in the first two axis (F1 and F2). A scale of colours, red (r = 1), blue (r = 0.5) and white (r = 0), indicates the correlation of variables with axis. Environmental variables considered include: water quality index (WQI), total concentration of pharmaceutical compounds [TPharW] and metals [TMetW] in water, total concentracion of bioavailable [TBioS] and pseudototal [TPsS] metals in sediments, Risk index (RI) and mean probable effect concentration quotient (mPEC-Q) in sediments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Multiple regression models run to evaluate effects of metals and metalloids in water and sediments on integrated biomarker response values calculated for *P. pusillus* exposed in the Ctalamochita River basin during cold (CP) and warm periods (WP). Values in bold indicate the statistical significance ($p < 0.05$) of the coefficient (β) in the obtained model. Values of the constant (β) in the obtained model, adjusted R^2 (Adj R^2) and significance of the model (p-Value) are shown.

Compartment	Period	Model	Constant	β for Al	β for As	β for Ba	β for Cd	β for Cr	β for Cu	β for Hg	β for Ni	β for Pb	β for Sr	Adj R²	p-Value
Water	CP	IBR ~ Al + Cr + Cu + Hg + Pb	0.84	**0.06**	–	–	–	**2.47**	**1.39**	**0.51**	–	**−1.02**	–	0.99	<0.001
Water	WP	IBR ~ Al + As + Cr + Cu + Pb	3.48	**0.01**	**−0.17**	–	–	**−2.09**	**−2.17**	–	–	**2.56**	–	0.98	<0.001
Sediments-bioavailable	CP	IBR ~ Al + Ba + Cd + Sr	−23.92	**0.31**	–	**1.67**	**256.37**	–	–	–	–	–	**0.75**	0.87	<0.001
Sediments-bioavailable	WP	IBR ~ Al + As + Ba + Cd + Ni + Pb	5.53	**−2.77**	**−1.99**	**−0.39**	**53.76**	–	–	–	**2.05**	**−1.06**	–	0.95	<0.001

the IBR values observed along the basin; which would agree with surpassed environmental guidelines for aquatic biota protection, previously informed (Table 1; Al, Pb and Hg during CP as well as, Al and Pb during WP) and described positive correlation between metals accumulation (Cr, Al, Pb) and environmental levels (Table 2). Therefore, in this complex environmental scenario, *P. pusillus* was able to respond to some of these troublesome metals.

In a second instance, we evaluated the contribution of bioavailable elements concentrations in sediments on IBR value. Significant influence of elements decreased according to Cd > Ba > Sr > Al during CP, and Cd > Ni > Ba > Pb > Al for WP. For both periods, the cadmium was the metal with higher significant effect on IBR values. Most of the coefficients were negative in WP (Al, Ba, and Pb) which could be associated with lower concentrations of metals and metalloids in sediments during this period. Accordingly, if EF, mPEC and RI are considered, concentrations of elements and the associated risk, decreased during WP in the studied basin compared with CP.

It is important to point out that elements like Ag and B were ruled out the model due high collinearity with others variables, probably indicating a limitation of the analysis, at least from a statistical point of view.

4. Conclusions

The present study is one of the few studies, and the first one using *Potamogeton* genus, evaluating through an active monitoring procedure the ability of freshwater macrophytes as biomonitors. Through the presented results, it was possible to evidence the potentiality and usefulness of *P. Pusillus* to be used as a biomonitor highlighting the pollution hotspots of aquatic ecosystems.

The environmental scenario of Ctalamochita River basin confirmed a significant decay in environmental quality along the basin with the worst conditions in those sites located downstream the main cities. The results of the present study evidenced a significant anthropogenic impact on the aquatic ecosystem and possible water uses. This impact was demonstrated by surpassed environmental quality guidelines, and also by the obtained EF and RI values calculated for sediments. The severe Hg contamination, as well as Ag, Cd, Pb, Zn, Cu and Cr concentrations in the Ctalamochita River basin confirms the need for an environmental management of the water resource.

Potamogeton pusillus, showed sensitivity to pollutants mixture pointing out those sites with lower environmental quality during both studied periods. The IBR confirmed, once more, to be a useful tool to obtain a holistic interpretation of response, or stress, levels suffered by organism. A positive association was observed between IBR values and analysed pollutants in environmental matrices, mainly in CP where higher concentrations of contaminants were measured. Concentrations of metals, like Cr, Cu, Hg, Al and Cd, displayed significant effect on biomonitor response which was in concordance with the described decay in environmental quality along the basin. The present study confirms the complexity to evaluate the impact of pollutants mixture on aquatic biota. As it was showed, chemicals analysis are not enough to evaluate environmental quality and risk index or enrichments factors are not always able to reflect bioavailability and therefore, toxicological effects on organisms. Finally, future field studies promoting the use of macrophytes as biomonitor will be suitable for the development of environmental management tools, like active biomonitoring programs, for multi-source contaminated scenarios.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.02.018.

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Abbreviations

$[NH_4]^+$: ammonia nitrogen
$[NO_2]^-$: nitrites nitrogen
$[NO_3]^-$: nitrates nitrogen
[TBioS]: Total concentration in the bioavailable fraction in sediments of metals/metalloids
[TMetW]: Total concentration in water of metals/metalloids
[TPharW]: Total concentration in water of pharmaceutical compounds
[TPsS]: Total concentration in the pseudototal fraction in sediments of metals/metalloids
A1: mobile fraction
A2: mobilisable fraction
AEWQG: Argentinean Environmental Water Quality Guidelines
AND: androstenedione
ATE: atenolol
CBZ: carbamazepine
Chl: Chlorophyll
CIPR: ciprofloxacin
CLAR: clarithromycin
CP: Cold Period
CRMs: certified reference materials
CW: Warm Period
DA: Discriminant analysis
DHT: dihydrotestosterone
dw: dry weight
E_1: estrogens: 17β-estradiol
E_2: estrone
EF: enrichment factor
ENAL:: enalapril
GPx: glutathione peroxidase
HPLC−ESI−qTOF: Liquid chromatography coupled to mass spectrometry using a quadrupole time-of-flight analyzer, with an electrospray ionization source
IBR: Integrated Biomarker Response
ICP-MS: Mass Spectrometer Inductively Coupled Plasma
Igeo: geoaccumulation index
LOD: limit of the detection
LOQ: limit of quantification
MDL:: method detection limit
MLRM: multiple linear regression models
mPEC-Q:: mean probable effect concentration quotient
MPN: most probable number
MT: methyltestosterone
NBT: p-nitroblue tetrazolium chloride
OXCZ: oxcarbazepine
PCA: principal component analysis
PEC: probable effect concentrations
Pheo: Pheophytins
POD: guaiacol peroxidase
PROP: propranolol
Ps: pseudo-total fraction
QA: Quality assurance
QC: quality control
RI: ecological risk index
SM: Supplementary Material
SOD: Superoxide dismutase
SQGs: sediment quality guidelines
T: testosterone
TEC: threshold effect concentrations
USEPA: United States Environmental Protection Agency
WQI: water quality index
ww: wet weight