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Bioaccumulation and biochemical response in South American native species exposed to zinc: Boosted regression trees as novel tool for biomarkers selection

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ABSTRACT

The aim of this study was to evaluate the response of a wide battery of biomarkers in two native species, the freshwater shrimp *Palaemonetes argentinus* and the macrophyte *Potamogeton pusillus*, experimentally exposed to zinc in order to establish the potential use of selected species as bioindicators of aquatic pollution. For this purpose, we propose the use of integrated biomarker index (IBR) with a previous selection of biomarkers using boosted regression trees (BRTs) as a new tool in ecotoxicology. Organisms were collected from a reference site, acclimated and exposed at relevant environmental zinc levels (control, 5, 50 and 500 μg Zn L^{-1}) for 96 h. Biomarkers were measured in cephalothorax and abdomen of shrimp as well as in leaf, stem and root of plants.

Significant zinc accumulation was observed in cephalothorax of *P. argentinus* from 50 μg Zn L^{-1} and from 5 μg Zn L^{-1} in stem and root of *P. pusillus*, when compared with control condition. Those effect biomarkers with significant differences among treatments were pre-selected to run out the BRTs model for each species. In *P. argentinus*, microsomal acetylcholinesterase activity, metallothioneins and superoxide dismutase activity measured in cephalothorax, as well as glutathione reductase activity in abdomen, showed the higher capacity to explain or predict the zinc exposure concentration. On the contrary, in *P. pusillus*, only chlorophyll *a* measured in leaf and H_2O_2 measured in root were the more representative of exposure concentrations, at least, within the biomarkers tested in the present study. Thereafter, IBR was calculated with the selected biomarkers in *P. argentinus* and showed in a sole value the organism stress, which also correlates with zinc exposure and accumulation.

Natives species tested displayed a sensitive response to metal exposure, which represents an important characteristic for biomonitoring programs. Our findings suggest that the BRTs and IBR are useful and robust run tools to select the better biomarkers in toxicological studies and indicate the organism stress.

1. Introduction

Physical and especially chemical methodologies quantify pollutants in detail, but lack the ability to judge their toxicity impact on biota. Therefore, biomarkers, more precisely biochemical, physiological and histological responses of organisms, were developed to assess the impact of environmental pollutants in terms of exposure and/or damage to organisms (Lam and Gray, 2003; Van der Oost et al., 2003).

Many authors have pointed out the importance of the use of a wide battery of biomarkers when assessing the biological effects in impacted environments, since a single biomarker may not reflect the health status of a sentinel species (Cazenave et al., 2009 and

Abbreviations: Accu, zinc accumulation; α-Toco, alpha-tocopherol; BRTs, boosted regression trees; cAChE and mAChE, cytosolic and microsomal acetylcholinesterase; CAT, catalase; cBChE, cytosolic butyrilcholinesterase; cGST and mGST, cytosolic and microsomal glutathione-S-transferase; ChE, cholinesterase; Chl, chlorophyll; CP, carbonyl content in proteins; CV, cross validation; dw, dry weight; GPx, glutathione peroxidase; GR, glutathione reductase; H_2O_2, hydrogen peroxides; IBR, Integrated Biomarkers Response; ICP-MS, inductively coupled plasma-mass spectrometry; MTs, metallothioneins; P, insoluble fraction; Pheo, pheophytins; POD, guaiacol peroxidase; ROS, reactive oxygen species; S, soluble fraction; SOD, superoxide dismutase; TBARs, thiobarbituric acid-reactive substances; ww, wet weight; [Z1], 5 μg Zn L^{-1}; [Z2], 50 μg Zn L^{-1}; [Z3], 500 μg Zn L^{-1}.

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other authors therein referenced). However, the interpretation of data provided by such a multibiomarker approach is difficult without an integrated overview that globally assesses the potential influence of contaminants and environmental conditions in an organism. In this context, stress indexes, as the Integrated Biomarkers Response (IBR), constitute practical and robust tools to assess the susceptibility to pollutants using multiple biomarker responses (Beliaeff and Burgeot, 2002; Serafim et al., 2012). Nevertheless, the calculation of IBR needs a previous selection of biological responses to be considered. Beliaeff and Burgeot (2002) suggested that only careful selection of an appropriate combination of biomarkers can provide information about global adverse environmental effects. Previous studies about the use of IBR do not explain how to select biomarkers to be included in this mathematical tool index. Usually, those biomarkers are selected according to capability to be measured. Although, which are the best biomarkers? How should be carried out an independent selection of them? In this way, the use of boosted regression trees (BRTs) could be a valid tool to select those biomarkers to be used in IBR, with higher capacity to predict contaminant exposure. Boosted methods such as BRTs differ substantially from regression-based methods such as Generalized Linear Models and General Additive Models that have been used widely over the last decade (Yuan, 2004; Iwasaki and Brinkman, 2015). While these latter methods seek to identify a single "best" model describing relationships between the response and the predictor variables, boosting progressively builds a sequence of models of increasing complexity, each one fitting the training data slightly better than its predecessor (Leathwick et al., 2008).

In South America, the use of biomarkers to evaluate aquatic contamination is increasing, but not always in native species (Carriquiriborde and Bainy, 2012). The study of biomarkers in native bioindicators results relevant since the sensibility to a toxic compound is specie dependent (Van der Oost et al., 2003) and only few previous studies indicated negative effects of water contamination on native biota, particularly for invertebrates and plants (Hued and Bistoni, 2005; Brodeur et al., 2011). The decapod *Paleomonetes argentinus* showed sensitivity to pollution in laboratory test (Galanti et al., 2013; Griboff et al., 2014; Bertrand et al., 2015), and consequently some authors have proposed that this species might be used as a bioindicator crustacean to provide information on environmental quality (Montagna and Collins, 2007). This species is known to be widely distributed in South America from estuaries to large river systems in Brazil, Uruguay and Argentina, among others countries (http://www.iucnredlist.org; Spivak, 1997). In a similar way, *Potamogeton pusillus* is proposed as sentinel organism as it is an aquatic macrophyte of ecological importance within the aquatic ecosystem, providing shelter and habitat for young fishes and other aquatic animals. Additionally, *Potamogeton* genus has a cosmopolitan distribution (Novara, 2003, http://www.iucnredlist.org).

Environmental exposure to metal pollutants can provoke several toxic effects on individuals and may have community wide consequences (Wallace et al., 2000). Several investigations around the world showed the occurrence of various levels of heavy metal in aquatic areas with the predominance of Zn, Pb, Cd, Cu and Cr (Cheung et al., 2003; Smolders et al., 2003; Zhou et al., 2008).

Therefore, the aims of this work were: (1) evaluate the response of biomarkers of exposure and effect in two native species, the freshwater shrimp *P. argentinus* and the macrophyte *P. pusillus*, experimentally exposed to zinc; (2) apply an integrated biomarker index (IBR) with a previous selection of biomarkers using boosted regression trees as a new tool in ecotoxicology, in order to determine the potential use of selected species as bioindicators of aquatic pollution.

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, blanks and artificial freshwater employed for metal exposition. All glassware and aquaria were appropriately washed to avoid metal contamination.

AccuStandard® atomic absorption spectrometry standard solution (1000 mg L^{-1} in 1% nitric acid) was used as stock solution for calibration of metal quantification equipment.

2.2. Specimens

Adult freshwater shrimps, *P. argentinus*, and macrophyte, *P. pusillus*, were collected from a reference site located in Suquía River basin (Córdoba, Argentina; Monferrán et al., 2009; Galanti et al., 2013). Shrimps were acclimated during two weeks in 40 L glass aquaria filled with artificial freshwater and maintained at constant laboratory temperature (25 ± 1 °C), under a light/dark regime of 12 h:12 h and fed daily *ad libitum* with commercial food for fish (Vita Fish) complemented until 54% proteins through the addition of lyophilized shrimp (Griboff et al., 2014). Zinc content of the formulated diet was 0.066 μg Zn mg^{-1} food. Plants were also placed into a 40 L tank but containing 10% Hoagland's solution and sediment (1/4) from the same sampling area. Plants were grown for two weeks under a light/dark photoperiod of 14 h:10 h before starting the exposures (Monferrán et al., 2012).

2.3. Experimental exposure

Two days before the beginning of metal exposition, organisms were relocated into the exposure aquaria. Adults (body length >2.2 cm) of *P. argentinus* (0.195 ± 0.011 g wet weight (ww); 2.815 ± 0.050 cm) were transferred to aquaria filled with artificial freshwater (ultra-pure water containing 0.100 g L^{-1} sea salt, 0.200 g L^{-1} $CaCl_2$ and 0.103 g L^{-1} $NaHCO_3$), maintaining a relation of two individuals per liter. Meanwhile, plants were kept in 1 L beakers (three plants per beaker, 5–8 g ww per liter) containing 10% Hoagland's solution prepared without Zn.

The experimental design involved four experimental conditions: Control (not metal exposed) and organisms exposed to 5 μg Zn L^{-1} (7.65×10^{-2} μM, [Z1]), 50 μg Zn L^{-1} (7.65×10^{-1} μM, [Z2]) and 500 μg Zn L^{-1} (7.65 μM, [Z3]). Zinc exposition was carried out for 96 h at similar temperature and photoperiod than during acclimation. Heavy metal concentrations tested were selected according to relevant environmental levels (1.9–12,000 μg L^{-1} in contaminated environments; Cheung et al., 2003; Smolders et al., 2003) and the concentration of Zn established for the protection of the aquatic biota in freshwaters by the Argentinean Environmental Water Quality Guidelines (9.7–45 μg L^{-1} depending on the water hardness; AEWQG, 2003).

For each treatment, 7–11 organisms were used to measure Zn accumulation, while 4–7 individuals were used to assess the other biomarkers responses. Organisms were taken randomly from nine aquaria or 21 beakers prepared for each exposure condition. A total of 166 shrimps and 128 plants were used for the experimental exposure. All measurements were performed in triplicate.

A stock metal solution was prepared using $ZnSO_4 \cdot 7H_2O$ (99.5%, Merck) and specific aliquots were taken to provide nominal metal concentrations. Exposure concentrations were tested at time 0 and 96 h for each experimental condition. Zinc concentrations in exposure media were measured on an inductively coupled plasma–mass spectrometry (ICP–MS) (Agilent Technology

7500cx, USA) equipped with an auto-sampler ASX-100 (CETAC Technologies, USA; limit of detection = 0.8 $\mu g\,L^{-1}$; limit of quantification = 1.2 $\mu g\,L^{-1}$; Bertrand et al., 2015).

The mean water quality parameters in the exposure aquaria of *P. argentinus* were pH 7.66 ± 0.06, conductivity 395 ± 3 $\mu S\,cm^{-1}$, water temperature 22 ± 1 °C, dissolved oxygen 7.06 ± 0.09 mg $O_2\,L^{-1}$ and total hardness 79.9 ± 0.9 mg $CaCO_3\,L^{-1}$ showing stable conditions along the whole test. The mean water quality parameters in beakers where plants were exposed were pH 7.13 ± 0.16, conductivity 307 ± 5 $\mu S\,cm^{-1}$, water temperature 23 ± 1 °C, and total hardness 43.9 ± 3.5 mg $CaCO_3\,L^{-1}$, also showing stable conditions along the exposure period.

Quantified Zn concentrations ($\mu g\,L^{-1}$) at 0 h were: Control = 13.9 ± 4.1; [Z1] = 16.9 ± 3.5; [Z2] = 55.8 ± 12.3; and [Z3] = 527.4 ± 63.5. After 96 h, a significant decline in metal concentration was observed for [Z2] and [Z3], varying this decay between 50 and 70%. This decay could be due in part to speciation of Zn^{2+} in the aquarium freshwater, which could form complexes with OH^-, HCO_3^-, CO_3^{2-} and Cl^- modifying the metal solubility (Stumm and Morgan, 1995), and to the interaction with the exposed organisms. Therefore, the similar Zn levels observed at control and [Z1] could be due to equilibrium of Zn between the organisms (with a basis content of Zn, since it is an essential metal) and the exposure medium (with low Zn concentrations; Luoma and Rainbow, 2008).

During metal exposure shrimps were fed with 3.5 mg of formulated diet per organism per day. Mortality of *P. argentinus* varied from 1% in the control group to 8% in [Z3].

At the end of metal exposure shrimps were cryoanesthetized, and both shrimps and plants were washed three times with ultrapure water, weighed and dissected (cephalothorax and abdomen for shrimps; leaf, stem and root for plants; Bertrand et al., 2015). Finally, samples were frozen with liquid nitrogen and kept at −80 °C until analysis.

2.4. Biomarkers analysis

Exposure and effect biomarkers were measured in shrimp and plant samples. Unless particularly explained, the methodology described below was applied in both studied species.

2.4.1. Exposure biomarkers

2.4.1.1. Metal accumulation. Metal accumulation (Accu) has been determined in body sectors of shrimps (cephalothorax and abdomen) as well as in all sections of plants (leaf, stem and root). For shrimps, samples digestions were carried out according to Chappaz et al. (2012) with some modifications, using subboiled HNO_3 and H_2O_2 (30%, Merck). For plants, samples digestions were carried out according to Monferrán et al. (2012) with *aqua regia* (1HNO_3:3HCl). All tissue samples were previously dried at 38 °C until constant weight. Concentrations of Zn were determined by triplicate using atomic absorption spectroscopy (Perkin Elmer 3110). Quality assurance and quality control were done using spiked control samples for shrimps and using a certified reference material (peach leaves, NIST1547) for plants. The percentages of recovery of Zn from tissues were 104.0 ± 0.6% and 92.5 ± 10.6% for shrimps and plants, respectively. Results were reported in $\mu g\,Zn\,g^{-1}$ dry weight (dw).

2.4.1.2. Metal concentration in soluble and insoluble fractions of P. argentinus. The subcellular compartmentalisation of Zn includes the metal bound to proteins (metallothionein-like proteins and heat-sensitive proteins, named as soluble (S) fraction) and the metal bound to organelles, cellular debris, and metal-rich granules, named as insoluble (P) fraction (Wallace and Luoma, 2003). Therefore, in cephalothorax and abdomen samples, Zn concentration was additionally determined in S and P fractions. These fractions were obtained after homogenization, centrifugation (25,000 × *g* for 55 min at 4 °C), and digestion of the samples in accordance to Mouneyrac et al. (1998) and Bertrand et al. (2015). Quality assurance and quality control were done using spiked control samples. The average of the assay recoveries were 94 ± 8%. The metal levels in soluble and insoluble fractions were expressed in $\mu g\,Zn\,g^{-1}$ wet weight (ww).

2.4.2. Effects biomarkers

2.4.2.1. Peroxides. Hydrogen peroxides (H_2O_2) concentration was determined according to Jana and Choudhuri (1981) in extracts of shrimps and plants prepared in sodium phosphate solution (50 mM, pH 6.5). The pertitanic acid (H_2TiO_4) generation, after the reaction of H_2O_2 with titanium sulfate dissolved in a H_2SO_4, was measured using a spectrophotometer at 415 nm. Results were expressed in mg $H_2O_2\,g^{-1}$ ww.

2.4.2.2. Lipid peroxidation. Lipid peroxidation was estimated by determining the thiobarbituric acid-reactive substances (TBARs) content in aqueous extracts of *P. argentinus* and *P. pusillus* according to Heath and Parker (1968). The amount of present TBARs was calculated using an extinction coefficient of 155 $mM\,cm^{-1}$. Results were expressed in $mmol\,g^{-1}$ ww.

2.4.2.3. Protein carbonyl groups in P. argentinus. Carbonyl content in proteins (CP) was quantified in extracts by the reaction of this functional group with 2,4-dinitrophenylhydrazine (Levine et al., 1990). 2,4-Dinitrophenylhydrazone generation was measured at 366 nm, using a microplate reader (Bio-Tek, Synergy HT), considering a molar absorption coefficient of 22,000 $M^{-1}\,cm^{-1}$. Results were expressed in μmol carbonyl group μg^{-1} proteins.

2.4.2.4. Enzyme extraction and measurement. Enzyme extracts of *P. argentinus* and *P. pusillus* were prepared according to Wiegand et al. (2000) and Monferrán et al. (2009), respectively. After removal of cell debris (10 min at 13,000 g, 4 °C), the membrane fraction of the extracts was separated by centrifugation at 105,000 × *g* for 60 min. The remaining supernatant, defined as the soluble (cytosolic) and the re-suspended pellet (microsomal) fractions were used for enzyme measurement. Enzymatic activities were determined spectrophotometrically using a microplate reader (Bio-Tek, Synergy HT).

The activity of cytosolic and membrane bound glutathione-S-transferase (cGST and mGST; EC 2.5.1.18) was measured according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The glutathione reductase activity (GR; EC 1.8.1.7) was assayed according to Tanaka et al. (1994). 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. Catalase activity (CAT; EC 1.11.1.6) was measured in shrimp using H_2O_2 as substrate, immediately after enzymes extraction (Beutler, 1982). In plants, the guaiacol peroxidase (POD) activity was measured using guaiacol and H_2O_2 (Bergmeyer, 1983).

Superoxide dismutase (SOD) activity was measured in shrimps using the Ransod kit (Randox Laboratories, UK; Suttle, 1986). SOD activities were expressed as units per milligram of protein; where 1 unit of SOD activity being defined as the amount of protein causing 50% inhibition of the rate of 2-4-iodophenyl-3-(4-nitrophenol)-5-phenyltetrazolium chloride reduction.

The activities of cholinesterase using acetylcholine and butyrilcholine as substrates were determined as reported by Ellman et al. (1961). Cholinesterase isoforms with capacity to hydrolyze acetylcholine and butyrilcholine will be referenced as acetylcholinesterase (AChE) and butyrilcholinesterase (BChE), respectively. ChE activities were measured in cytosolic and microsomal fractions (cAChE, cBChE, mAChE and mBChE).

The activity of all enzymes assessed (except for SOD) 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. The protein quantification was performed using bovine serum albumin as standard.

2.4.2.5. Pigments in P. pusillus. 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 by visible spectrophotometry using a microplate reader (Chl = 649 and 665 nm; Pheo = 654 and 666 nm, after hydrochloric acid addition). Concentrations were calculated and reported in μg pigment g^{-1} ww.

2.4.2.6. Metallothioneins in P. argentinus. The level of metallothioneins (MTs) was determined by differential pulse polarography (DPP), a technique based on SH-compound determination according to the Brdicka reaction (Brdicka, 1933) as described by Olafson and Olsson (1991). A MDE 150 Stand Polarographic (Radiometer, Copenhagen) Tracelab 50, controlled by the computer software Tracemaster 5 through a Polarographic analyzer POL 150 was used. MTs results were reported in μg MTs g^{-1} ww.

2.4.2.7. Alpha-tocopherol in P. argentinus. Tocopherol extraction was performed in shrimp samples as described by Griboff et al. (2014). The tocopherol content was determined by high performance liquid chromatography with fluorescence detection (excitation wavelength 276 nm and emission wavelength 316 nm) as described by Griboff et al. (2014). α-tocopherol concentrations were reported in mg gr^{-1} ww.

2.5. Data analysis

2.5.1. Statistical treatment

Data were expressed as the average ± standard deviation (SD). The tests of Shapiro Wilks and Levene were used to check the normality and the homogeneity of the data, respectively. Analysis of variance was afterwards used to assess a significant effect on all means treatment at $p < 0.05$. A posteriori test was used to prove for significant differences between the means of controls and other treatments ($p < 0.05$). Pearson correlation test was used to establish the association between different variables. Infostat (Version 2013p, Di Rienzo et al., 2013) was used for all statistical analysis.

2.5.2. Boosted regression trees

Our aim was to determine those effect biomarkers with higher capacity to explain or predict the exposure concentration of organism in order to be further used in biomonitoring studies. Thus, before run out the boosted regression trees, a pre-selection of biomarkers was made: effect biomarkers without significant variation respect to control condition were ruled out. Boosted regression trees model were constructed with the GBM packages (Ridgeway, 2013) in R (version 0.98.953), using the code described by Elith et al. (2008). This method builds a sequence of models of increasing complexity, in order to describe relationships between the response (exposure zinc concentration) and the predictor variables (biomarkers). The model building process in BRT is referred to a "forward stagewise", which reflects the fact that at each step a term is added to the model to slightly decrease the deviance. The terms added are in the form of small regression trees, which are fit to the gradient of the deviance (generalized residuals). Results from this ensemble of regression trees are the averaged to form a final prediction (Leathwick et al., 2008). Over-fitting of the model was avoided by Cross Validation (CV), therefore the model was trained on 9 of 10 subsets of the data and tested on the remaining subset (independent; Elith et al., 2008; Hale et al., 2014). Model parameters were also adjusted to maximize performance. The "learning rate" controls the degree at which model complexity is increased. Smaller values result in the fitting of a larger number of trees, each of lower individual influence and generally giving superior predictive performance in the growing model. The proportion of data randomly selected at each iteration of the CV procedure represented by the "bag fraction", and the "tree complexity" indicating the number of interactions (nodes) in each tree (Elith et al., 2008). Considering our sample size, we set tree complexity at 2. The performance of the model was estimated using the CV correlation (the correlation between predicted and raw data withheld from the model) and explained deviance (an indication of goodness of fit, calculated by predicting to data not used in model training, and comparing between observed and predicted values; Leathwick et al., 2008).

Finally, PRESS function was applied to confirm the capacity of each biomarker selected to predict the metal exposure concentration. Those biomarkers with PRESS function values higher than 0.5 were candidate to be used in the IBR calculation. BTR were applied to the datasets of each species.

2.5.3. Integrated biomarker response

One general stress index, termed "Integrated Biomarker Response", was afterwards calculated with the selected biomarkers in *P. argentinus*. This IBR was performed in accordance to Beliaeff and Burgeot (2002) with modifications by Devin et al. (2014). 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. The integrated biomarker response for each condition was calculated *via* the following formula:

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

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 (Devin et al., 2014). Pearson correlation (Zn exposure levels *vs.* IBR median) and Kruskal–Wallis test to identify IBR difference between treatments were carried out. In the present study, several biomarkers showed significant variation between treatments. Therefore, the four more important biomarkers selected with BRT and with a good capacity to predict (PRESS >0.5), were used in the IBR calculation.

3. Results and discussion

3.1. Biomarkers responses

3.1.1. P. argentinus

Zinc is an essential metal required in small quantities by all living organisms for metabolic functions; however it can generate deleterious effects when internal concentrations exceed the requirements of the organism and its detoxification capability (Viarengo et al., 1999).

Metal bioaccumulation and subcellular distribution as well as biological responses (MTs, oxidative stress biomarkers and cholinesterases activities) measured in cephalothorax and

Table 1
Biomarkers measured in cephalothorax and abdomen of *P. argentinus* exposed to different concentrations of Zn for 96 h: Zinc Accumulation (Accu), Soluble Zinc (S), Insoluble Zinc (P), Metallothioneins (MTs), Hydrogen peroxides (H_2O_2), Thiobarbituric Acid Reactive Species (TBARS), Carbonyl Proteins (CP), alpha-Tocopherol (α-Toco). The data represent mean ± standard deviation (SD). The same letter indicates no significant differences ($p > 0.05$) among treatments.

		Cephalothorax			Abdomen					Cephalothorax			Abdomen		
		Means	SD		Means	SD				Means	SD		Means	SD	
Accu μg Zn g^{-1} dw	Control	147	21	a	94	14	a*	mg H_2O_2 g^{-1} ww	Control	3.5	0.5	a	3.6	0.7	a
	[Z1]	145	23	a	83	13	a*		[Z1]	5.2	1.1	b	4.9	1.0	a
	[Z2]	233	69	b	115	32	a*		[Z2]	5.5	1.5	b	4.5	1.1	a
	[Z3]	369	50	c	184	38	b*		[Z3]	5.4	0.7	b	4.2	0.8	a
S μg Zn g^{-1} ww	Control	11	1	a	8	3	a	TBARs nmol g^{-1} ww	Control	0.029	0.005	a	0.010	0.003	a*
	[Z1]	9	2	a	5	1	a		[Z1]	0.039	0.006	ab	0.013	0.003	a*
	[Z2]	15	4	a	6	3	a		[Z2]	0.034	0.018	a	0.007	0.004	a*
	[Z3]	38	6	b	12	2	b		[Z3]	0.042	0.013	b	0.014	0.004	a*
P μg Zn g^{-1} ww	Control	11	3	a	10	3	a	μmol CP g^{-1} total prot	Control	7.1	2.1	a	4.5	3.1	a
	[Z1]	15	5	ab	8	1	a		[Z1]	10.4	1.8	a	4.6	2.4	a
	[Z2]	25	8	bc	17	6	b		[Z2]	10.1	2.2	a	19.8	3.7	c*
	[Z3]	33	6	c	33	3	c		[Z3]	9.4	1.0	a	13.4	4.2	b
μg MTs g^{-1} ww	Control	0.518	0.045	a	0.093	0.023	a*	μg α Toco g^{-1} ww	Control	40	25	a	29	15	a
	[Z1]	0.516	0.085	a	0.104	0.018	a*		[Z1]	52	3	a	55	12	a
	[Z2]	0.550	0.082	ab	0.084	0.016	a*		[Z2]	54	12	a	34	12	a
	[Z3]	0.651	0.025	b	0.086	0.015	a*		[Z3]	48	29	a	41	17	a

* Statistically differences between cephalothorax and abdomen ($p < 0.05$).

Table 2
Enzymes activities in cephalothorax and abdomen of *P. argentinus* exposed to different concentrations of Zn for 96 h: Catalase (CAT), Superoxide dismutase (SOD), cytosolic and microsomal Glutathione S-Transferase (cGST and mGST), Glutathione Peroxidase (GPx), Glutathione Reductase (GR), cytosolic and microsomal Acetylcholinesterase (cAChE and mAChE) and cytosolic Butyrylcholinesterase (cBChE). Enzymes activities are expressed in nanokatal per milligram of proteins except SOD that is expressed in units per milligram of protein. The data represent mean ± standard.

		Cephalothorax			Abdomen					Cephalothorax			Abdomen		
		Means	SD		Means	SD				Means	SD		Means	SD	
CAT nkat mg^{-1} prot	Control	12.2	2.0	ab	10.6	0.6	a	GPx nkat mg^{-1} prot	Control	0.597	0.141	a	0.585	0.182	b
	[Z1]	9.6	1.6	a	10.8	1.7	a		[Z1]	0.469	0.092	a	0.293	0.044	a*
	[Z2]	14.2	1.7	b	12.2	1.3	a		[Z2]	0.495	0.109	a	0.373	0.067	a
	[Z3]	18.2	1.9	c*	12.9	3.6	a		[Z3]	0.468	0.090	a	0.353	0.047	a
SOD U mg^{-1} prot	Control	1.03	0.30	a	2.19	0.58	b	GR nkat mg^{-1} prot	Control	0.036	0.010	a	0.024	0.006	a
	[Z1]	0.55	0.27	a	0.42	0.11	a		[Z1]	0.042	0.012	a	0.021	0.006	a*
	[Z2]	1.25	1.01	a	0.59	0.11	a		[Z2]	0.039	0.014	a	0.027	0.004	a
	[Z3]	2.87	0.96	b*	0.65	0.19	a		[Z3]	0.037	0.012	a	0.051	0.006	b
cGST nkat mg^{-1} prot	Control	0.21	0.01	b	0.26	0.03	c*	cAChE nkat mg^{-1} prot	Control	0.011	0.002	b	0.012	0.002	b
	[Z1]	0.15	0.02	a	0.16	0.02	a		[Z1]	0.007	0.001	a	0.007	0.001	a
	[Z2]	0.18	0.03	a	0.18	0.02	ab		[Z2]	0.009	0.001	ab	0.008	0.001	a
	[Z3]	0.25	0.04	c	0.21	0.04	b*		[Z3]	0.006	0.001	a	0.007	0.001	a
mGST nkat mg^{-1} prot	Control	0.37	0.07	a	0.29	0.07	c*	mAChE nkat mg^{-1} prot	Control	0.010	0.002	b	0.003	0.001	a*
	[Z1]	0.35	0.02	a	0.18	0.04	a*		[Z1]	0.011	0.002	b	0.003	0.001	b*
	[Z2]	0.37	0.06	a	0.26	0.04	bc*		[Z2]	0.010	0.002	b	0.002	0.001	ab*
	[Z3]	0.39	0.05	a	0.21	0.03	ab*		[Z3]	0.005	0.002	a	0.003	0.001	b*
								cBChE nkat mg^{-1} prot	Control	0.014	0.004	b	0.013	0.002	b
									[Z1]	0.008	0.002	a	0.008	0.001	a
									[Z2]	0.008	0.002	a	0.010	0.001	a
									[Z3]	0.007	0.001	a	0.009	0.002	a*

* Statistically differences between cephalothorax and abdomen ($p < 0.05$).

abdomen of *P. argentinus* exposed to Zn, are shown in Tables 1 and 2. Our results indicate a differential response pattern between body sectors of *P. argentinus*.

In cephalothorax, the accumulation of total Zn (Accu) and Zn in the P fraction increase significantly from exposure levels of [Z2] (50 μg Zn L^{-1}). The increase of Zn in P fraction could be indicating a cellular metal detoxification strategy. Ahearn et al. (2004) reported in invertebrates, including crustaceans, the presence of granules containing sulfur or phosphorus complexed with a metal like Zn. The cytosolic Zn (S fraction) rise occurred only at [Z3] and it was closely correlated ($r^2 = 0.52$, $p < 0.01$) with MTs induction. MTs serve a key role in heavy metal detoxification when intracellular metal concentrations exceed those necessary for metabolic functions (Ahearn et al., 2004). At [Z3] the Zn in the S fraction triplicated its concentration observing simultaneously a significant increase in MTs concentration. This result, would confirm the well reported strong capacity of Zn to induce MTs transcription (Pourang et al., 2004). The increase in P fraction and the MT induction could be indicating the co-existence of different subcellular strategies to regulate Zn concentration in this organism.

In abdomen, a significant rise in total Zn concentration occurred only at [Z3], while the increase in Zn concentration in P fraction was observed from [Z2]. In the same way, the Zn in the S fraction increased significantly at the higher exposure concentration without a significant MT induction. S fraction in abdomen was three times lower than concentration measured in cephalothorax which could explain the absence of MT induction. If a body sector comparison it is made, our results indicate higher concentration of Zn

has been found in cephalothorax than in abdomen. That could be explained by the presences of hepatopancreas in cephalothorax sector; an organ with metal detoxification functions (Ahearn et al., 2004).

Some authors have proposed Zn as an antioxidant, while others suggest that an excess of Zn is related to reactive oxygen species (ROS) generation or the decline of antioxidant enzymes in several organisms including invertebrates (Quintaneiro et al., 2015 and authors therein referenced).

In cephalothorax, evidence of lipid damage (TBARs) has been found at [Z3] even though the significant increase of H_2O_2 levels was detected from [Z1] (Table 1). Conversely, no significant damage on proteins was detected in this body sector. Instead, in abdomen, no significant rises of H_2O_2 and TBARs levels have been observed when compared to Control, but a significant damage on proteins was quantified at [Z2] and [Z3], where the CP formation occurred at lower exposure concentration than the bioaccumulation of total Zn. Muscle predominance in abdomen, and therefore protein content, could explain the higher sensibility of this body sector to suffer protein damage under oxidative stress conditions.

Among nonenzymatic antioxidant mechanisms to protect organisms from oxidative damage are the antioxidant molecules, such as α-tocopherol (vitamin E – vit-E; Lushchak, 2011). The concentrations of this molecule in both body sectors of *P. argentinus* did not show a significant variation at the different experimental conditions indicating the possibility of lower oxidative stress levels than necessary to produce decay in concentration of antioxidant molecule.

Another defensive mechanism comprises antioxidant enzymes including glutathione-dependent peroxidases (GPx), GST, SOD, CAT, DT-diaphorase and associated ones providing needed cofactors as GR and glucose-6-phosphate dehydrogenase (Lushchak, 2011).

The TBARs increase observed at [Z3] in cephalothorax probably induces the enhanced response of cytosolic GST, an enzyme with capacity to reduce lipid hydroperoxides (Regoli et al., 2011). SOD is the enzyme responsible for the removal of O_2^- to H_2O_2, which is then reduced to H_2O by CAT. Both enzymes increased significantly their activity at [Z3] when H_2O_2 and TBARs presented higher levels, probably as a mechanism to reduce ROS concentrations and oxidative stress. The enzymes GPx, GR and mGST did not change their activity when shrimps were exposed to Zn (Table 2).

An inverse pattern was observed for antioxidant enzymes in abdomen, where GPx, SOD, microsomal and cytosolic GST were inhibited from the lowest exposure concentration (5 μg L^{-1}). Inhibitory effect of Zn on GPx and GST has been previously reported (Atli and Canli, 2010). Conversely, GR raised its activity only at [Z3] probably to maintain levels of reduced glutathione, another nonenzymatic antioxidant molecule. Finally, no changes in CAT activity were observed in this body sector.

Cholinesterase (ChE) family members are responsible for controlling the nerve impulse, detoxification and various developmental functions. They are a major target of pesticides and others contaminants, including metals (Andreescu and Marty, 2006; Johnson and Moore, 2012). In cephalothorax of *P. argentinus* a significant inhibition of mAChE was reported at the highest concentration of Zn tested [Z3], when compared to control condition. This response could be indicating a negative effect of this essential metal on the nervous system and probably over the cholinergic neurotransmission. The potential of some metallic ions, such as Hg^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}, to depress the activity of AChE of fish and invertebrate, included crustaceans, *in vitro* and *in vivo* conditions, has been demonstrated in several studies (Frasco et al., 2005; Lionetto et al., 2011). According to previous report, the inactivation caused by Zn on cholinesterase has reversible characteristics (Tomlinson et al., 1981).

An opposite pattern response was observed in abdomen of *P. argentinus* where a low, but significant increase in mAChE occurred. Tomlinson et al. (1981) reported the inducting capacity of some ion metals like Mg^{2+}, Na^+, Mn^{2+} and Ca^{2+}, however, the capacity of Zn to induce AChE activity has not been reported before.

No cholinesterase activity has been reported in the microsomal fraction obtained from both body sectors with the substrate butyrilcholine.

Inhibition of cAChE and cBChE activities was observed in cephalothorax and abdomen of *P. argentinus* at all Zn exposure levels studied (Table 2). Inhibition of cytosolic ChE has been reported at similar Zn concentration (550 μg Zn L^{-1}) in the crustacean *Daphnia magna* (Diamantino et al., 2003). By the contrary, a study carried out in a chironomid species did not show a significant inhibition of AChE (total fraction) after *in vivo* and *in vitro* exposure to Zn (Ibrahim et al., 1998).

Cytosolic ChEs inhibition in organisms exposed to a toxic compound has been associated with the sequestration of toxic, as a mechanism of detoxification (Johnson and Moore, 2012). In both body sectors, cytosolic ChEs showed a rapid response with inhibition from [Z1] which can explain their protective and detoxifying roles over microsomal ChEs directly linked with nervous impulse.

Our results indicate a differential response of cytosolic and microsomal ChE, indicating higher sensibility of the first isoforms respect to the other one. The significant effect of an essential metal like Zn over ChE, indicate the potential toxicity and biological damage on *P. argentinus*.

3.1.2. P. pusillus

Metal bioaccumulation and biological responses (oxidative stress biomarkers and pigments) measured in leaf, stem and root of *P. pusillus* exposed to Zn, are shown in Table 3. Our results indicate a differential response pattern among leaf, stem and root in *P. pusillus*. This macrophyte presented a fast response in Zn accumulation, with a significant increase in metal tissue concentration from [Z2] in leaf and from [Z1] in stem and root. Although metal accumulation in leaf occurred at higher exposure concentrations than in the others plant sections, the values of accumulation at [Z3] were significantly larger than measured in stem and root. Zn concentration in leaf exposed at [Z3] was five times higher than control condition, while others plant sections increased only three times their metal concentration respect to control. Similar concentrations of Zn were reported in *Lemna minor* after controlled exposure (Lahive et al., 2014) and in *Phalaris arundinacea* naturally exposed (Polechońska and Klink, 2014).

However, Zn accumulation in plant sections was different in *P. pusillus* when compared to *P. arundinacea*. According to Polechońska and Klink (2014) trace metal concentrations found in *P. arundinacea* samples generally decreased in the following order: root < leaf < stem.

The bioaccumulation of metals in higher plants is often accompanied by the induction of several cellular changes, some of which directly contribute to metal tolerance capacity of plants (Monferrán et al., 2009). Even though a significant rise in H_2O_2 has been observed in leaf and root from [Z1], no significant variations in TBARs concentration were detected. The higher levels of H_2O_2 were measured in root at [Z3], being this concentration three times greater than in the control condition.

Previous studies reported the diminution in pigments concentrations, like chlorophylls and pheophytins, as indicator of oxidative stress in *P. pussillus* when exposed to Cu (Monferrán et al., 2009). In line with these results, when this macrophyte was exposed to Zn, levels of Chl-a decrease significantly in leaf at the higher exposure concentration. However, no significant

Table 3
Biomarkers measured in leaf, stem and root of *P. pusillus* exposed to different concentrations of Zn for 96 h: Zinc Accumulation (Accu), Hydrogen peroxides (H_2O_2), Thiobarbituric Acid Reactive species (TBARs), Guaiacol Peroxidase (POD), Glutathione Reductase (GR), Glutathione Peroxidase (GPx). nd, no detected activity. Table present mean ± standard deviation (SD). The same letter indicates no significant differences ($p > 0.05$) among treatments. nm, not measured. nd, not detected.

		Leaf			Stem			Root		
		Means	SD		Means	SD		Means	SD	
Accu μg Zn g^{-1} dw	Control	198	34	a*	177	56	a	146	83	a
	[Z1]	233	43	ab	265	119	b	206	78	b
	[Z2]	311	51	b	303	93	b	188	41	b*
	[Z3]	1063	208	c*	676	165	c	554	120	c
mg H_2O_2 g^{-1} ww	Control	0.28	0.09	a	0.13	0.06	a	0.33	0.06	a
	[Z1]	0.51	0.17	a	0.16	0.08	a	0.55	0.14	ab
	[Z2]	0.48	0.09	a	0.13	0.08	a	0.53	0.07	ab
	[Z3]	0.50	0.12	a	0.15	0.09	a	0.92	0.19	b
TBARs nmol g^{-1} ww	Control	0.0116	0.0009	a	0.0092	0.0052	a	0.0054	0.0013	a*
	[Z1]	0.0096	0.0013	a	0.0034	0.0017	a	0.0027	0.0019	a*
	[Z2]	0.0091	0.0013	a	0.0072	0.0019	a	0.0045	0.0009	a*
	[Z3]	0.0089	0.0010	a	0.0085	0.0016	a	0.0052	0.0009	a*
POD nkat mg^{-1} prot	Control	502	107	a*	114	43	a	148	42	a
	[Z1]	401	64	a*	155	16	a	113	33	a
	[Z2]	309	40	a*	139	29	a	114	16	a
	[Z3]	317	53	a*	119	26	a	121	30	a
GR nkat mg^{-1} prot	Control	0.56	0.16	cb	nd	nd		0.52	0.13	b
	[Z1]	0.71	0.17	c	nd	nd		0.53	0.12	b*
	[Z2]	0.47	0.23	ab	nd	nd		0.30	0.14	a
	[Z3]	0.30	0.08	a	nd	nd		0.22	0.09	a
GPx nkat mg^{-1} prot	Control	0.71	0.13	c*	0.33	0.14	a	0.67	0.04	b*
	[Z1]	0.59	0.12	b	0.64	0.17	b*	0.41	0.11	a*
	[Z2]	0.54	0.14	cb*	0.64	0.11	b	0.50	0.15	ab
	[Z3]	0.43	0.11	a*	0.34	0.19	a	0.49	0.10	a
Chl-a μg g^{-1} ww	Control	399	35	b	51	10	ab*	nm	nm	
	[Z1]	501	80	b	36	11	a*	nm	nm	
	[Z2]	465	37	b	51	2	ab*	nm	nm	
	[Z3]	191	55	a	57	6	b*	nm	nm	
Chl-b μg g^{-1} ww	Control	152	32	ab	22	8	a*	nm	nm	
	[Z1]	168	44	ab	21	1	a*	nm	nm	
	[Z2]	191	42	b	23	3	a*	nm	nm	
	[Z3]	131	19	a	28	12	a*	nm	nm	
Pheo-a μg g^{-1} ww	Control	312	44	a	59	17	a*	nm	nm	
	[Z1]	310	131	a	51	13	a*	nm	nm	
	[Z2]	387	49	a	43	8	a*	nm	nm	
	[Z3]	396	69	a	54	11	a*	nm	nm	
Pheo-b μg g^{-1} ww	Control	79	7	a	17	7	a*	nm	nm	
	[Z1]	88	31	a	25	10	a*	nm	nm	
	[Z2]	101	14	a	20	7	a*	nm	nm	
	[Z3]	102	17	a	32	15	a*	nm	nm	

* Statistically differences between leaf, stem and root ($p < 0.05$).

variations among control and others treatments were observed in Chl-b, Pheo-a or Pheo-b levels measured either in leaf or stem. Chlorophyll a is well known to be more sensitive to oxidative stress than others pigments (Kong et al., 1999). Probably, the decrease in Chl-a concentration in leaf is related with the increased H_2O_2 levels before mentioned. In stem concentrations of Chl-a remained constant. The Zn concentration at which a significant effect over pigments could be detected is species dependent. In *Potamogeton pectinatus*, a significant decay in chlorophylls levels were observed at 6.5 mg Zn L^{-1} after 24 h exposure (Tripathi et al., 2003). By the contrary, *Ceratophyllum demersum* required concentrations higher than 13 mg Zn L^{-1} to affect the photosynthetic system, included pigments (Aravind and Prasad, 2004). Our results indicate the occurrence of damages over Chl-a in *P. pusillus* exposed to [Z3].

Antioxidant enzymes activities were also measured in different plant sections (Table 3). GPx activity showed a significant inhibition from the lowest Zn concentration tested in root and leaf, while in stem the enzyme activity increased at the lowest and intermediate exposure concentrations. The activity of GR in leaf diminished at [Z3], while in root the same effect was observed at [Z2] and [Z3]. The capacity of Zn to inhibit the activity of GR has been reported in plants by Schaedle and Bassham (1977). This response could be indicating a negative effect over enzymatic antioxidant mechanism. According to Van Assche and Clijsters (1990) toxic concentrations of Zn induce the activity of POD in plants, particularly in leaves, due to increase in ROS levels. In *P. pusillus*, the absence of variation in POD activity (Table 3) could be indicating than exposure concentrations, and ROS generated, were not sufficient to activate this enzyme of the antioxidant system.

All results considered, the leaf showed a higher resistance in inhibition of enzymes than root. Despite the increase in H_2O_2 levels the enzymes analyzed did not show activation as indicating of antioxidant and protective system. This could be due to non-enzymatic antioxidant mechanisms able to neutralize ROS compounds and not measured in the present study. No enzymatic activity was detected for GST in microsomal or cytosolic fractions, as well as for GR in the stems.

Table 4
Boosted regression trees (BRT) and PRESS values for *P. argentinus* and *P. pusillus*: Percentages (%) of *Y* variability (exposure concentration) explained by each predictor variable (*Xs*). Press values were calculated for each *Xs* as an indicator of variable capacity to predict. PRESS function with value from 0 to 1, where 0 is no capacity to predict and 1 is the higher capacity to do it. Bold values indicate selected biomarkers in each species.

	P. argentinus				*P. pusillus*		
	Body Sector	BRT (%)	PRESS		Body Sector	BRT (%)	PRESS
mAChE	Cephalothorax	**33.5**	**0.64**	Chl-a	Leaf	**42.2**	**0.74**
GR	Abdomen	**30.3**	**0.83**	GR	Root	**17.7**	**0.26**
MTs	Cephalothorax	**18.7**	**0.49**	GR	Leaf	**16.6**	**0.31**
SOD	Cephalothorax	**11.9**	**0.64**	H_2O_2	Root	**10.8**	**0.60**
cGST	Cephalothorax	1.7	0.49	GPx	Leaf	5.0	0.30
cAChE	Cephalothorax	1.6	0.27	Chl-b	Leaf	4.3	0.14
H2O2	Cephalothorax	0.6	0.01	GPx	Stem	2.8	0.04
CP	Abdomen	0.4	0.02	GPx	Root	0.7	0.06
mAChE	Abdomen	0.4	0.05				
cBChE	Abdomen	0.2	0.05				
cGST	Abdomen	0.2	0.07				
TBARs	Cephalothorax	0.2	0.06				
cBChE	Cephalothorax	0.1	0.11				
mGST	Abdomen	0.1	0.01				
cAChE	Abdomen	0.1	0.14				
GPx	Abdomen	0.0	0.01				
SOD	Abdomen	0.0	0.03				

3.2. Boosted regression trees

In *P. argentinus and P. pusillus*, BRTs were carried out by species considering the biomarkers of effect with responses significantly different to the control condition, measured in all body sectors. Table 4 shows the percentages of *Y* variability (exposure concentration) explained by each predicting variable (biomarker). According to our results, in *P. argentinus*, mAChE, MTs and SOD measured in cephalothorax, as well as GR activity in abdomen, explained an important percentage of *Y* variation (94.4%) and showed a good PRESS value (≥0.5, Table 4). The remainder biomarkers measured in both body sectors presented no satisfying BRT (<10%) and PRESS (<0.5) values, indicating a low capacity to explain *Y* variability and to predict the metal exposure concentration suffered by *P. argentinus*. In *P. pusillus*, the analysis indicates that Chl-a and GR measured in leaf as well as H_2O_2 and GR activity in root, were the best biomarkers to explain the variation in Zn exposure concentrations (Table 4). Considering those four biomarkers, 87.3% of exposure concentration variability could be explained in the macrophyte. Nevertheless, two of the four responses before mentioned did not show a good capacity to predict exposure concentration (PRESS <0.5; Table 4). BRTs plots for both species are presented as Supplementary material.

If we compare the two native species, the biomarkers pointed out in shrimp represent a set of biological responses (enzyme activity and metallothioneins induction) with good capacity to predict exposure concentrations. Conversely, in the macrophyte, biological responses explain a good percentage of variability of *Y* but only two had the ability to predict Zn exposure concentrations.

Table 5
Integrated Biomarker Response (IBR) at different exposure concentrations in *P. argentinus* exposed to Zn. The same letter indicates no significant differences ($p > 0.05$) among treatments.

	IBR					
	Median		Media	SD	Min	Max
Control	0.90	a	0.80	0.15	0.60	0.90
[Z1]	0.70	a	0.47	0.36	0.00	0.70
[Z2]	1.30	b	1.27	0.05	1.20	1.30
[Z3]	3.90	b	4.17	0.66	3.60	5.00

3.3. Integrated biomarker response

Biomarkers previously selected by BRTs method have been used to calculate IBR in *P. argentinus*. The obtained results are shown in Table 5 and Fig. 1A, where the area in gray integrates the IBR for each treatment and is represented as star plot. Significant increased IBR values were observed at Z2 and Z3 when compared to control. Similar IBR values where obtained by Kim et al. (2010) when the common carp *Cyprinus carpio* was exposed to perfluorinated organic compounds.

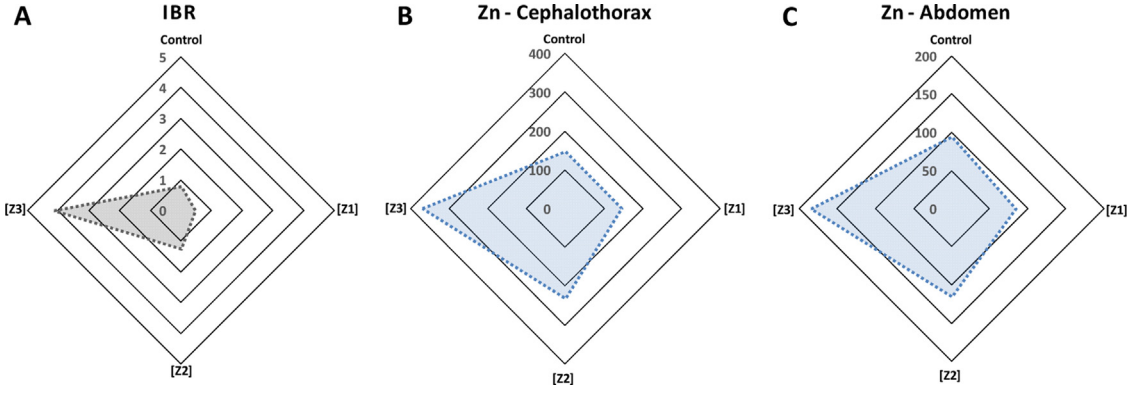

Fig. 1. (A) Integrated biomarker response; (B) zinc accumulation in cephalothorax of *P. argentinus* (μg g^{-1} dw); (C) zinc accumulation in abdomen of *P. argentinus* at different exposure concentrations: Control (not metal exposed), [Z1] (5 μg Zn L^{-1}), [Z2] (50 μg Zn L^{-1}) and [Z3] (500 μg Zn L^{-1}).

Moreover, a significant correlation was obtained between IBR and the concentration of Zn in the exposure media ($R^2 = 0.97$, $p < 0.0001$). This association evidenced that the IBR, as well as the biomarkers selected by BRTs, are pertinent to evaluate tested treatments.

In order to compare IBR with Zn accumulation in *P. argentinus*, star plots of metal concentration in cephalothorax and abdomen are also shown in Fig. 1B and C. Higher values of IBR occurred in those conditions with maximal levels of metal accumulation in both body sectors showing the agreement among the measured concentration and IBR.

For *P. pusillus* the biomarkers selected by BRTs did not allow us to calculate IBR values as only two presented good percentage of *Y* variability explained and suitable PRESS value.

Overall, the results here presented suggest that IBR might be a useful tool for quantification of multiple biomarker responses induced by Zn in the native species *P. argentinus*. In *P. pusillus*, the use of additional biomarkers could allow the selection of at least four biomarkers with good capacity to predict exposure concentration and further calculation of IBR values.

4. Conclusions

Natives species tested displayed a sensitive response to metal exposure, which represents an important characteristic for biomonitoring programs. The differential reaction between body sections of *P. argentinus* justifies the performing dissection and improves the biomarker response evaluated. Our results allow us to recommend the measurement of exposure and effect biomarkers in cephalothorax section of *P. argentinus* for biomonitoring. On the contrary, in *P. pusillus*, accumulation levels of metal in any plant section have been the more representative of exposure concentrations, at least, within the biomarkers tested in the present study. While the strong performance of boosted methods has been known for a numbers of years, only in few instances they have been applied to the analysis of ecotoxicological data. Our findings suggest that the BRTs are a useful tool to select the better biomarkers in toxicological studies. Moreover, the selected biomarkers measured in *P. argentinus* were used to calculate an IBR which integrates those results in a sole value indicating the organism stress, and which also correlates with zinc exposure. The measurement of additional effect biomarkers with good capacity to predict metal exposure in *P. pusillus* would allow applying this index.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2016.03.048.

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