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Does *Corbicula fluminea* Respond to Dissolved Metal(loid)s in Laboratory Exposure Bioassays?

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Abstract

Benthic organisms are widely used in metal contamination monitoring systems in aquatic environments. But the chemical concentration stored in their tissues depends on the environmental fate and the bioconcentration skills of the species. The freshwater clam *C. fluminea* is used to monitor metal pollution due to its great resistance to the contaminated environments. Nevertheless, it may act in contrarily when exposed to different polymetallic environments. This study aimed to understand the bioaccumulative behaviour of *Corbicula fluminea* when is exposed to different environments contaminated by individual elements (As, Cd, Cr, Co, Cu, Fe, Ni, Pb, Sb and Zn), environments containing a mixture of the elements in lower concentrations, and mining residues (acid mine drainage lixiviate). Acute toxicity bioassays were carried out to observe valve closure, clam mortality and metal bioconcentration in soft tissue. Results revealed that the Asian clam shows a greater metal(loid) bioconcentration response under polymetallic environments than under individual metal-contaminated environments.

Keywords: *Corbicula fluminea*; Dissolved metals; Spiked water; Metal bioconcentration; Valve closure; Laboratory tests

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Introduction

Environmental risk assessments (ERA) include the study of physical and chemical parameters with respect to the quality standards established by national and international regulatory bodies. Past research has indicated that biota demonstrate correlation with these parameters in the surrounding environment, meaning that the biota themselves can act as indicator of the "health" of the environment [1]. Therefore, the new ERA studies consist of integration of different compartments (water-sediment-biota), i.e., the new models include biological response analyses. As a result, integrative models incorporate three lines of evidence to evaluate the environmental risk: (a) chemistry, it provides information about the presence and levels of different chemicals of potential ecological concern, (b) toxic responses caused by the chemicals, (c) in-situ alterations deriving from this contamination. As such several aquatic organisms are able to provide integrated information about the environment; they constitute a new line of evidence to the environmental risk assessments. Information of chemical bioaccumulation introduces the concept of bioavailable substance and passage of harmful substances in other levels of the food chain [1].

The freshwater bivalve *Corbicula fluminea* is an invasive species from the Southern and Eastern of Asia, due to that it is as well-known as Asian/Asiatic clam. This species has wide capacity of adaptation to another extreme environment. This clam meets all the requirements to be a good bioindicator: Sedentary, widespread, long lifespan, and filter feeder that accumulates chemicals**,** with genetic variability and phenotypic plasticity, physiological tolerance to abiotic changes, opportunistic behaviour (r-strategist) and self-fertilizing hermaphrodites [2-6]. The clam can feed both from water and from sediments, and its tissue reflects ambient metal concentration over the time [7]. Comprehensive investigations have shown that the freshwater clam *C. fluminea* is capable of surviving exposure to polymetallic polluted environments and extreme polluted environments such as acid mine drainage effluents [7-14]. Acid mine lixiviates have an average pH of 2.64, electrical conductivity between 2.03-3.13

mS cm^{-1} and about 10% dissolved oxygen, its net acidity is about 1910 mg L^{-1} as $CaCO_3$ equivalent and contains average values of 596 mg L^{-1} of Fe, 112 mg L^{-1} of Al, 16 mg L^{-1} Cu, 12 mg L^{-1} Zn and 0.1-3 mg L^{-1} of As, Cr, Cd, Co, Ni [15].

The main aims of this study were: i) to investigate the toxic response (lethal and sublethal) of the Asian clam to different metal(loid) contaminated aquatic environments, ii) to determine the metal(loid) bioconcentration in the soft tissue of the clam and iii) determine the adequacy of the Asian clam as biomonitor of highly polymetallic environments. For this, screening acute toxicity bioassays were carried out for 72 h with individuals of *C. fluminea* exposed to several dissolved metalloids and metals (As, Cd, Cr, Co, Cu, Fe, Ni, Pb, Sb and Zn).

Materials and Methods

Clams collection and experimental design

Sized samples of specimens of *C. fluminea* were collected from an artificial reservoir in the South of Spain. Organisms were acclimatized in the laboratory for three days with the following conditions: Continuous aeration, 24 ± 1°C and photoperiod (9 h light: 15 h of dark) and commercial mineral water Natura™ (in mg L^{-1}): 197 HCO_3^-, 50.6 SO_4^{2-}, 13.9 Cl^-, 65.1 Ca^{2+}, 13.7 Mg^{2+}, 8.2 Na^+, 4.1 SiO_2. Physico-chemical properties in water were monitored with a multi-parametric probe.

Sterilized polyethylene glasses were filled with 100 mL of mineral water, and they were individually spiked with metal solutions (ICP Panreac) as reported in **Table 1**. Two types of experiments were carried out. Type I experiments were carried out with simulated single metal(loid) spiked in water. Type II experiments as result of combined mixtures of elements: Half of the concentrations of the metal(loid)s used in type I experiments (All Metals Together, AMT) and an Artificial Acid Mine Drainage (AAMD) was produced in the laboratory by dissolving $Fe_2(SO4)_3$ (800 mg Fe L^{-1} plus addition of the elements reported in **Table 1**).

Toxicity tests

Bioassays were conducted in triplicate by exposing three clams (*n*=9) to solutions plus a diet supply, temperature (24 ± 1°C) and photoperiod. The behaviour of the clams was observed (valve closure movement and mortality were monitored as endpoints) during experimentation. Temperature, electrical conductivity (EC), pH and dissolved oxygen saturation (DO) were daily checked. After 72 h, clams were sampled, rinsed with Milli-Q water and deep frozen (-80°C) **(Table 1)**.

Table 1 Experiment descriptions with the code name of each chamber and the principal metal spiked and its corresponding concentration.

Exp.	Code	Element	Spiked conc. (mg L^{-1})
Type I	D_{As}	As	1
	D_{Cd}	Cd	1
	D_{Co}	Co	1
	D_{Cr}	Cr	1
	D_{Cu}	Cu	1
	D_{Fe}	Fe	10
	D_{Ni}	Ni	1
	D_{Pb}	Pb	10
	D_{Sb}	Sb	1
	D_{Zn}	Zn	1
Type II	AMT	All Metals Together	Cu, Co, Zn, As, Cr, Ni, Cd (0.5), Fe, Pb (5)
	AAMD	Artificial Acid Mine Drainage	Fe (800), Pb (5), Cu, Co, Zn, As, Cr, Ni, Cd (0.5)

D-indicates spiked/doped element

The valve closure (VC) monitoring test is a toxicity bioassay advocated to be a cost-effective and useful method for use in screening the toxicity of pollutants in water. The valvometry system monitored to achieve a real-time observation of the valve closing/opening activities of the Asian clam [16-20]. In the current study, this experimental procedure test consisted of a cursory observation based on the aperture of the valve by assigning a numerical code of five aperture stages: Shells completely closed (0-state) means a protective strategy when stressor agents in the environment (metals in this case) overcome the protective mechanisms. Meanwhile, the 5-state leads to the highest contact of the clam with the environment; clam is admitting the metal concentration and does not recognize it as a hazard. Numerical data derived from VC monitoring were used to calculate the average of closure over the time and estimation based on the global behaviour of the population in the experimental time **(Figure 1a)**.

The lethality test consisted of recounting individual survival over the time. Results were statistically analyzed using the program Graph Pad Prism version 5.00 Software for Windows: Median lethal time (LT_{50} Median time required to reach 50% survival) was obtained from Kaplan-Meier graphs and compared survival curves using both the logrank (Mantel-Cox) test and the Gehan-Wilcoxon test.

Sample processing and analysis

Soft tissues samples were proceed as detailed in Sarmiento et al. [21]. Briefly, freeze-dried tissue was acid digested with HNO_3 and H_2O_2 by using a thermoregulatory plate. Trace element concentrations in digested samples (As, Ba, Bi, Cd, Co, Cr, Cu, Fe, Ga, Ni, Pb, Sb, Se, and Zn) were quantified using an Agilent Technologies 7700 inductively coupled plasma-mass spectrometer (ICP-MS Agilent 7700). The accuracy of metal analysis was checked by the certified reference material TORT-2 (National Research Council, Canada). The agreement of the analysis results and certified values was higher than 90%.

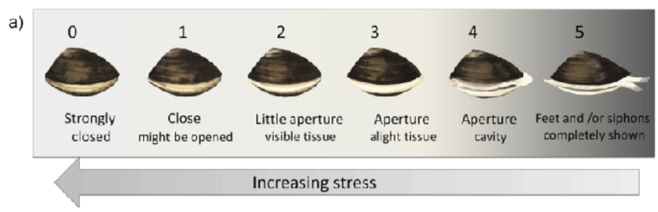

Figure 1a Valvometry conversion criteria: 0-Strongly closed. Non-filtering, 1- Closed, but might be opened, 2- Little aperture: Tissue visible, 3- Aperture with tissue alight, 4- Opened, 5- Feet and/or siphons completely shown filtering.

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Statistical data analysis

For bioconcentration experiments, data were tested for normality (Shapiro-Wilk´s test) and homoscedasticity (Bartlett´s test). Significant differences between individuals exposed to control and individuals exposed to spiked dissolved metal(loid) s were determined for each element (As, Cd, Co, Cr, Cu, Fe, Ni, Pb, Sb, Zn) using a one-way analysis of variance (ANOVA). Post-hoc examination of significant differences was carried out using multiple comparisons Dunnett's test by GraphPad Prism 5.0 Software. The relationship amongst variable was assessed by using a multivariate analysis approach by means of a factor analysis. Principal component analysis (PCA, Varimax normalized rotation) was used as an extraction procedure applied to the original set of variables: Element concentration in soft tissue of the clam (As, Ba, Bi, Cd, Co, Cr, Cu, Fe, Ga, Ni, Pb, Sb, Se, Zn), the biological parameters (shell length-SL and wet weight-WW), results of toxicity bioassays (VC and LT_{50}) and acid conditions (pH) using the statistical package XLSTAT-pro (v. 5.1).

Results and Discussion

The metal(loid) concentrations (As, Cd, Co, Cr, Cu, Fe, Ni, Sb, Pb and Zn) in soft tissue of *C. fluminea* obtained after 72 h of exposure to spiked water as described in table are summarized in **Figure 2**. The metal concentration in soft tissue after experimentation was statistically compared to basal levels (concentrations in control clams) **(Table 1)**.

Regarding type I experiments, Cr, Cd, Cu, Ni, Pb, Zn ($p<0.001$) and Sb ($p<0.01$) were significantly bioconcentrated by *Corbicula fluminea*. Previous studies found association between exposure and bioaccumulation in soft tissue of *C. fluminea*, e.g. Cd, Zn [10,22]. However, As, Co and Fe did not display significant ($p<0.05$) bioconcentration in the tissue of the clams from the type I experiments (D_{As}, D_{Co} and D_{Fe}) respect to the controls. In contrast, clams from AMT experiments showed a significant ($p<0.05$) bioconcentration for all the studied elements except Fe. Thus, despite the concentration of the elements was lower, the combination of elements in the environment promoted the influx of them (except Fe). Nevertheless, in an environment highly contaminated by Fe (AAMD: 800 mg Fe L^{-1}), uptake of iron by clams was mine-water concentration dependent as previous research observed [12]. *C. fluminea* displayed a direct dose-response registering 8.5 mg Fe g^{-1} dw without lethal effects, some elements (Co, Cd, Cr, Ni, Pb) were significantly bioconcentrated by the clam. Meanwhile, some others (As, Cu, Sb, and Zn) were exposed to an antagonistic release. Soucek et al. also carried some in situ studies with the Asian clam to detect AMD and nutrient inputs in low-order streams, their results were positive for AMD inputs and positive for nitrate concentrations [13]. In addition, clam survival was significantly determined by pH, conductivity, Al and Fe.

A decay in the as concentration in the clam tissue when exposed to as contaminated environments (DAs and AAMD) is in agreement with previous findings [23]. Arsenic is rapidly taken up and eliminated from the soft tissue of the Asian clam in the first exposure period [20]. The clam is able to regulate this metalloid by accumulating and eliminating the excess of as in tissues, thanks to metallothionein induction as part of a primary defense mechanism [24]. Nevertheless, the clam bioconcentrated as ($p<0.001$) in presence of some other elements with the same concentration (AMT).

Samples from D_{Co} registered Co concentrations below the background concentrations. Fraysse et al. explained that Zn presence shows an inhibitory effect on the uptake of Co [25]. Nevertheless, this phenomenon was not observed in the metal-mixture experiments (Type II). What is more, the Co bioconcentration displayed a synergistic effect in presence of other metal(loid)s: Co was significant ($p<0.001$) bioconcentrated in samples from the AMT and the AAMD **(Figure 2)**.

Biological effects (Valvometry and mortality)

The results obtained from the averaged valve movement recording along the experimental time are shown in **Figure 1b**. Ortmann and Grieshaber´s research employed a complex monitoring instrument called Mosselmonitor™ and made chemical analysis to measure metabolism [26]. They admitted that shell closure reduced the metabolism rate in the *C. fluminea*. However, the metabolism remained aerobic for many hours, but when time overpasses 5-10 h the metabolism became anaerobic and a succinate accumulation can be observed in the tissues. This accumulation promoted clam aperture and finally death. Hartmann et al. established three states (open, closed and avoidance behavior), where the closed shell corresponds to both resting behaviour and active evasive behavior [27]. In contrast, this study determined five phases: Linking the state 0 with the complete rejection of a toxic environment and 5 with the greatest acceptance to the environment as previously explained. In this study, the VC was understood as an immediate protection response. The non-filtration activity was observed in clams from the AAMD and AMT experiments, this might be the last state before death [26]. Clams prevailed closed for many days in the case of D_{Cu} and 6 h for D_{As}. This corroborates the inductance-based valvometry measurement technique for dynamic biomonitoring system for potential in-situ detection of waterborne as concentrations by a valve daily activity in *C. fluminea* [17,20]. Liao et al. reported that *Corbicula fluminea* requires 5 h to detect and show valve closure response under environments polluted with 300-400 µg L^{-1} for As, 2.3-8.8 µg L^{-1} for Cu, 16 µg L^{-1} for Cd [20]. Tran et al. also demonstrated that valve closure was induced by Cd concentrations between 16 and 50 µg L^{-1} after 5 h of exposure [28]. In contrast, in this study clams from D_{Cd} did not experimented important closure movements,

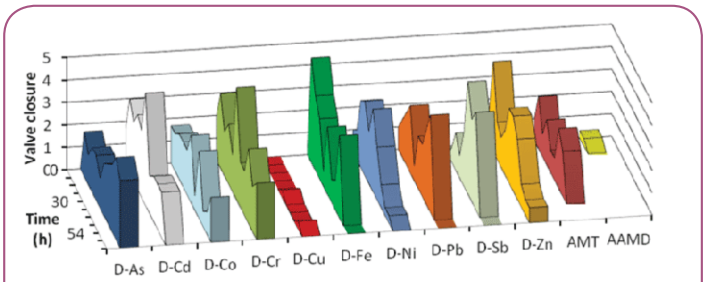

Figure 1b Averaged valve aperture evolution of the Asian clam in time (h) for the different experiments (Type I and II) as described in **Table 1**.

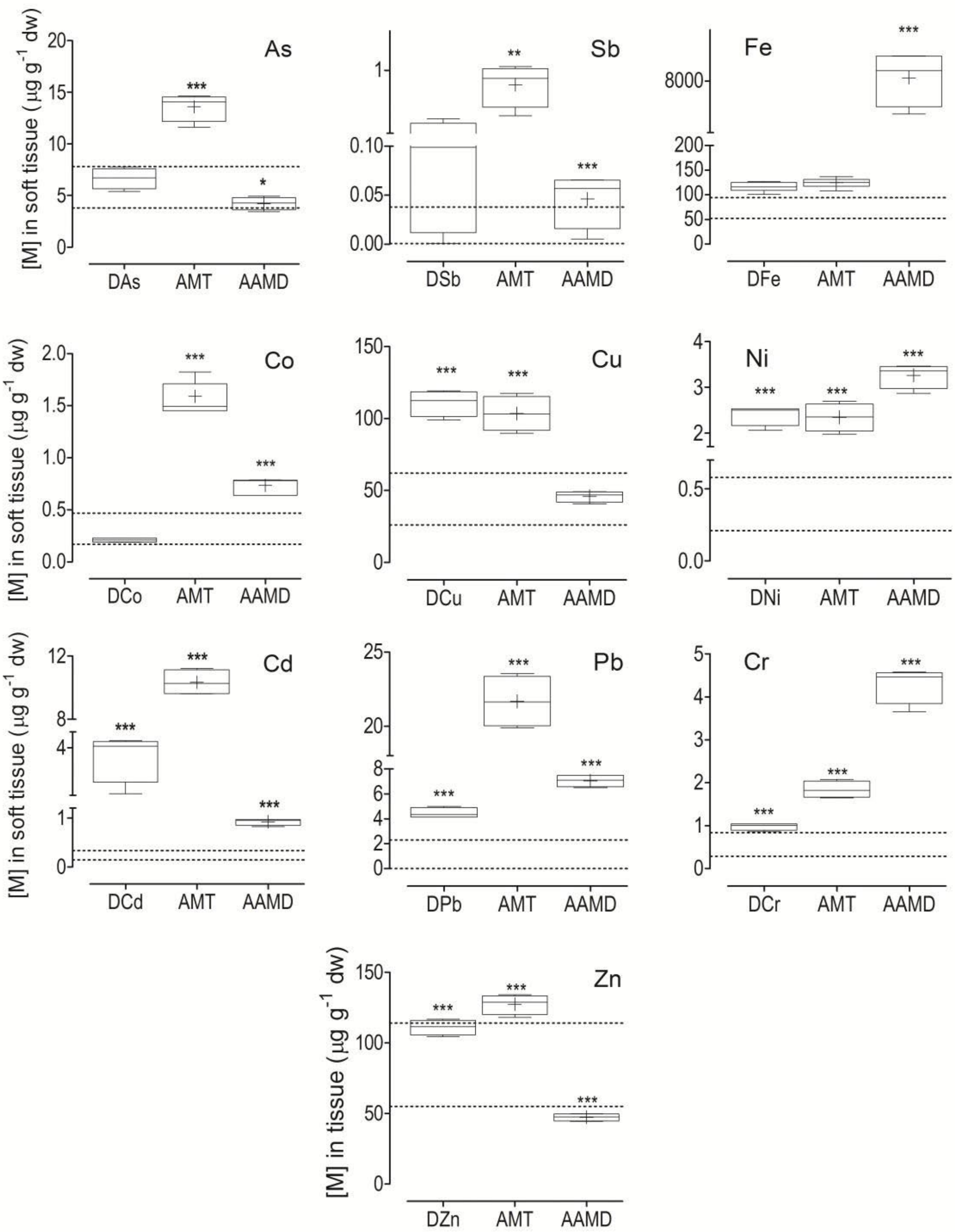

Figure 2 Box plots showing the metal concentration in soft tissue (µg g^{-1} dw) of *C. fluminea* after the experimentation for the spiked experiments (Type I D_{As}, D_{Cd}, D_{Co}, D_{Cr}, D_{Cu}, D_{Fe}, D_{Ni}, D_{Pb}, D_{Sb}, D_{Zn}) and Type II (the AMT and the AAMD) experiments. Maximum and minimum threshold concentrations found in control clams are marked by dots.

Statistical analyses showed some significant differences in the Dunnett´s test (*p>0.05, **p>0.01, ***p>0.001) using control clams for comparison

probably due to the fact the clam did not recognize the metal as toxic **(Figure 1b)**.

Based on lethality, the AAMD experiment displayed the lowest LT_{50} value (720 min), apart from the high metal concentrations simulating the most polluted scenario (AMD), water conditions registered a pH of 2.18 and an electrical conductivity of 1345 µS cm^{-1}. Also, the averaged VCAAMD response (0.22) informed about a rejection of the environment by the clam. Nevertheless, the second most polluted environment (the AMT experiment) showed an average valve closure slightly high (VC_{AMD}=1.63) as sign of a greater acceptance, although the median lethal time did not overcome 50 h. The pH reached was 6.32 and the electrical conductivity was 525 µS cm^{-1} in the AMT experiment. In contraposition, the median lethal time in Type I-experiments was greater (LT_{50}>3240 min). Attending to valve opening/closing rhythm, one of the strongest of avoidance responses was observed in the D_{Cu} (VC=0.06), despite that, clams registered a significant (p<0.001) Cu bioconcentration in soft tissue. Cu uptake in *C. fluminea* was detectable after 24 h with a low rate constant of loss [29] **(Figure 2)**.

Statistical approach

It is understood that elements found in soft tissue (e.g. Ba, Bi, Ga) were residual concentrations from the collection site. The factor analysis reorganized the data of the original data set into three principal factors, which together explained 77% of variance in the original data set. The loadings of the variables following varimax rotation for these three factors are represented in **Table 2**.

The predominant factor (F1) accounted for 41.6% of the variance and related the two toxic responses LT_{50}, VC, the acidity and some element concentrations (Ba, Bi, Co, Cr, Cu, Fe, Ga, Ni and Pb). The second factor (F2) represented 23.8% of the total variance and correlated concentrations of a second group of metal(loid)s (As, Cd, Co, Cu, Sb and Zn). Lastly, the third factor (F3) represented 11.7% of the total variance and grouped the biological characteristics (WW and SL) with Zn and Se concentration in tissues.

The PCA indicates cause-effect correlations amongst the variables that are associated to the same axis (factor) [30]. By representing the PCA in a two-dimension graph it is possible to observe relationships. All the elements (except Zn and Se) present an inverse relationship with pH and LT_{50}. Indeed, acidification in the environment causes mortality and these two rise with the concentration of metal(loid) in the environment. These both variables also maintain an inverse relationship with a first group of elements, such as Ba, Bi, Cr, Fe, Ga and Ni **(Figure 3a)**.

These elements were classified as non-toxic due to the inversely relationship (F1<0) with the toxic responses (VC and LT_{50}). A second group of elements (As, Cd, Co, Cu, Pb, Sb and Zn) were also inversely (F2>0) allied with pH and mortality. These elements tend to for a new group that they may show different bioconcentration behaviour from that already mentioned for the first group. It is well known that elements such as As, Cd, Co, Cu, Pb and Zn are associated to AMD processes, the origin of these elements is attributed to the oxidation of sulphurs [31] **(Table 2)**.

In a biplot graph representation of the observations, three groups were randomly divided according to the new variables. Results revealed an enhancement of the elements common from AMD polluted environments (As, Cd, Co, Cu, Pb and Zn) with low pH and high mortality. In contrast, the vector of AAMD is displaced towards the Fe concentration, as pointed by the ANOVA. On the other hand, the F3 distinguishes the valve closure and the weight of the clams as inversely linked to Se and Zn concentrations. This

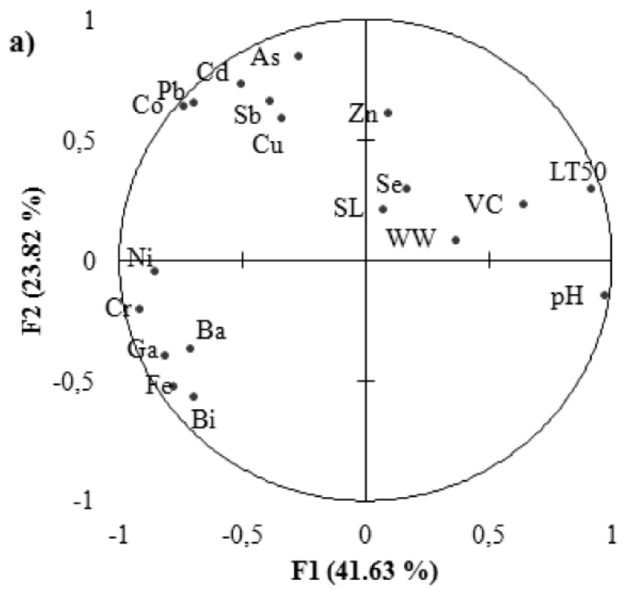

Figure 3a Principal analysis score plot of the samples subjected to the different experiments. The variables are represented in a bi-plot by a vector (the direction from the origin and length of vector indicate how each variable contributes to the principal components in the plot).

Table 2 Sorted rotated factor loadings (pattern) for the three principal factors resulting from the multivariate analysis of results obtained from *Corbicula fluminea*. Factors are numbered consecutively from left to right in order of decreasing variance. Only loading above 0.5 are shown in table.

	F1	F2	F3
% Variance	41.62	23.81	11.73
As	—	0.85	—
Ba	-0.72	—	—
Bi	-0.70	-0.56	—
Cd	-0.51	0.73	—
Co	-0.74	0.64	—
Cr	-0.92	—	—
Cu	—	0.59	—
Fe	-0.78	-0.51	—
Ga	-0.82	—	—
Ni	-0.86	—	—
Pb	-0.70	0.65	—
Sb	—	0.66	—
Se	—	—	-0.53
Zn	—	0.61	-0.58
pH	0.96	—	—
LT_{50}	0.90	—	—
SL	—	—	0.81
WW	—	—	0.82
VC	0.63	—	—

LT_{50}: Median Lethal Time; SL: Shell Length; WW: Wet Weight; VC: Valve Closure fact is reflected in the biplot with the Type I experiments due to the similar metal concentration of Cu and Zn (~110 mg L^{-1} dw) bioconcentrated by the clam in environments spiked with 1 mg L^{-1} (DCu and DZn). The Asian clam strongly accumulates Cu due to a low rate constant of loss consistently the clam size [29,32]. Previous studies have determined the rapid depuration of Zn and the diet dependence of its accumulation in tissues, but the elevated presence of Fe in AMD polluted environments might be blocking the Zn uptake [11,33]. The Asian clam revealed a high sensitivity of metallothionein response along polymetallic gradient pollution, that it might be blocking the uptake of metals as defense barrier [10,34]. Metallothioneins play an important role in the homeostasis regulation of the essential metals such as Cu, and Zn and non-essential metals, such as Cd [35] **(Figures 2 and 3b)**.

The Cd and Zn bioaccumulation and the metallothionein response in the Asian clam along a polymetallic gradient have been previously studied [10]. Bivalves accumulated lower concentrations of Cd on mixture of Zn and Cd than exposure to Cd. Recent investigations have also determined the importance of hydrodynamic conditions in the extracellular accumulation of Cd of *C. fluminea* [36].

It is important to highlight that these experiments were carried in laboratory and there are discrepancies in results that might be found between controlled laboratory experiments and field (in-situ) studies. The prediction of metal(loid) bioaccumulation by the bivalves in field and laboratory was improved by considering the metal partitioning within the surface sediments as pointed by Belzunce-Segarra et al. [37]. Therefore, this study might be understood as a first appraisal approach of the Asian clam in dissolved metal contaminated environments.

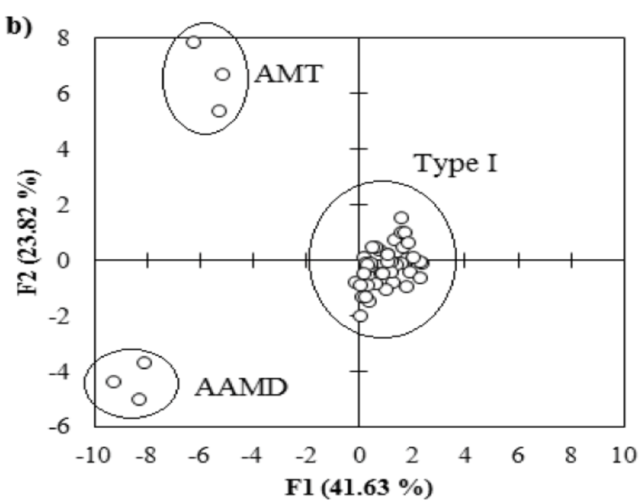

Figure 3b Observations scheme that illustrates samples groups.

Conclusion

In the present study, clams were introduced for 72 h to different environments contaminated with individual metal(loid)s and two different degrees of metal contamination [38]. The results of laboratory studies suggest that individuals of the species *C. fluminea* registered different toxicological responses based on valve closure, mortality and assimilation of elements within soft tissue.

The freshwater clam *Corbicula fluminea* has demonstrated to be able to survive in environments polluted with 1 mg L^{-1} As, Cd, Cr, Co, Cu, Ni, Sb and Zn and environments polluted with 10 mg L^{-1} of Fe and Pb up to 72 h. In contrast, under polymetallic environments, lethality works as crucial endpoint for monitoring [38-40]. The valvometry toxicity test is been demonstrated to be a complementary quick observable response.

The Asian clam successfully assimilates Cd, Co, Cr, Cu, Ni, Pb, Zn from environments individually contaminated with 1 mg L^{-1} after 72 h of exposure. Nevertheless, under polymetallic polluted environments it suffers a synergistic uptake effect of some metal(loid)s (As, Cd, Co, Cr, Cu, Sb, Ni, Sb, Pb and Zn) that promotes other toxicological responses (valve closure and mortality) [41].

In summary, *C. fluminea* is a recommendable organism to biomonitor the water quality in high acidic and metal contaminated environments due to its resistance to adverse environmental conditions and its bioaccumulative response to elements in solution. The toxicity of metals and metalloids seems to cause synergistic effects even with low concentrations.

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