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Biomarker responses of the freshwater clam *Corbicula fluminea* in acid mine drainage polluted systems

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Abstract

The environmental quality of an acid mine drainage polluted river (Odiel River) in the Iberian Pyrite Belt (SW Spain) was assessed by combining analyses of biomarkers (DNA strand breaks, LPO, EROD, GST, GR, GPx) in freshwater clams (*Corbicula fluminea*) exposed during 14 days and correlated with metal(loid) environmental concentrations.

Results pointed that enzymatic systems are activated to combat oxidative stress in just 24 hours. Along exposure, there were homeostatic regulations with the glutathione activity that influenced in lipid peroxidation oscillations, provoking significant DNA strand damage after 14 exposure days. EROD activity showed no changes throughout the exposure period.

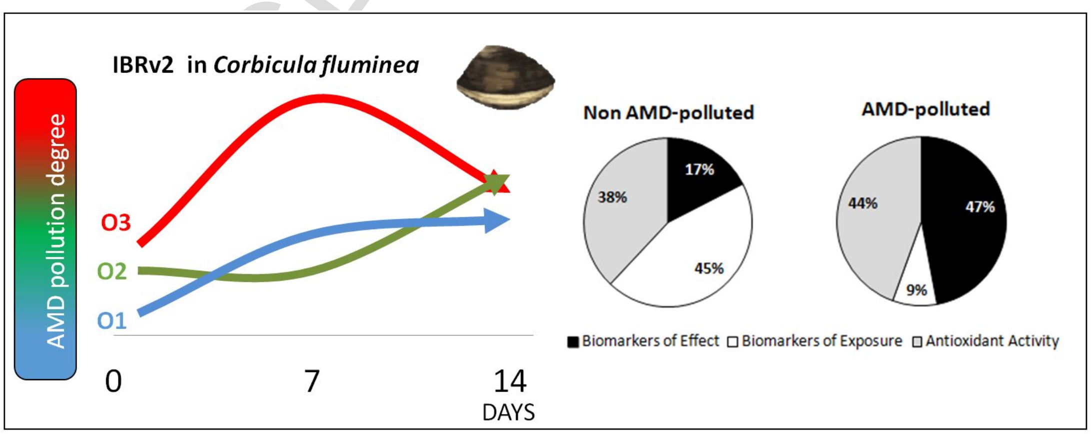
The Asian clam displayed balance biomarkers of exposure–antioxidant activity under non–stressfully environments; meanwhile, when was introduced into acid polymetallic environments, such as the acid mine drainage, its enzymatic activity was displaced towards biomarkers of effect and the corresponding antioxidant activity.

Keywords: Iberian Pyrite Belt; glutathione activity, DNA strand damage, EROD, lipid peroxidation; risk assessment; Integrated Biological Response (IBR_{v2}); metal contamination; Asian clam

Abbreviations

Usepa: United States Environmental Protection Agency; NOAA: National Oceanic and Atmospheric Administration; AMD: Acid Mine Drainage. IPB: Iberian Pyrite Belt. ROS: Reactive Oxygen Species. TP: Total Protein. S_{15}: Fraction separated at 15,000g. GSH: Reduced glutathione. GPx: Glutathione Peroxidase. GST: Glutathione S–Transferase. GR: Glutathione Reductase. EROD:Ethoxyresorufin–O–Deethylase. CAT: Catalase.LPO: Lipid Peroxidation. HF: Homogenate fraction. OD: Optical Density. TBARS:thiobarbituric acid reactive substances. TCA: trichloroacetic acid. *TU:* Toxic units. I_{tox}: Toxicity index. I_{bio}: Bioaccumulation Index. I_{con}: Contamination index. MPI_{12}: Metal Pollution Index. I_{geo}: Geoaccumulation index. C_d: Contamination degree. *PER:* Potential Ecological Risk. P_{triad}: Pollution index. IBR_{v2}: Integrated Biological Response version 2.

Graphical Abstract



1. Introduction

Contamination in aquatic environments is regulated by legislation through guidelines and thresholds by international and national environmental agencies and organizations, such as the NOAA or the USEPA. Nevertheless, these limitations do not always imply biological responses. Therefore, regulatory agencies, such as the European Union, emphasis on regulating biological effects inserted into the environmental monitoring. According to Cazenave et al. (2009), the data combining measurements of physiological damage, accumulation of chemicals in biological tissues and the presence of contaminants in sediments, provides a powerful tool to monitor bioavailability of pollutants and the ecological effects linked to sediments.

The metabolism of toxic compounds in organisms results in the formation of reactive oxygen species (ROS), which are neutralized by antioxidant defenses, antioxidant substances (glutathione, vitamin E and carotenoids) and enzymes (CAT, GR, and SOD). Oxidative stress occurs when the rate of generation of ROS exceeds the antioxidant defense system (Finkel and Holbrook, 2000), causing deleterious effects, such as protein and DNA oxidation as well as peroxidation of lipids in the cell membrane (Bigot et al., 2010; Cazenave et al., 2009,Zhou et al., 2008).

Biomarkers are known as “early warnings” of the potential adverse effects caused by xenobiotics to organisms (van der Oost et al., 2003). Biomarkers are able to measure either exposure or effects, but both types can provide early warning of potential meaningful ecological effects in combination with other lines of evidence (Martín–Díaz et al., 2004). There is an extensive list of literature using biomarkers for environmental quality assessment (Capolupo et al., 2017; Costa et al., 2012; Díaz–Garduño et al., 2018; Martín–Díaz et al., 2004, 2007). Among the sessile organisms, the bivalves are commonly used as sentinels species for studies of biological effects of environmental pollution since these filter–feeders are in contact with the contaminated compartments (sediment and water), and, therefore, they tend to accumulate large levels of metal(loid)s in their tissues, delivering an index of contamination with measurable cellular and physiological responses (Al–Subiai et al., 2011). Concretely, some studies employed the freshwater clam *Corbicula fluminea* as biomonitoring tool for metal contaminated environments (Baudrimont et al., 1999; Guo et al., 2018; Santos and Martinez, 2014), and rarely for acid mine drainage polluted environments (Bonnail et al., 2016a, 2016b, 2017; Sarmiento et al., 2016).

The purpose of the present study is to test the suitability of using a set of biomarkers with the freshwater clam *Corbicula fluminea* in order to assess the environmental quality of sediments affected by acid mine drainage contamination. This technique allow to determinate the effects of acid mine drainage polluted sediments (with several degrees of contamination) at different levels of biological organization, including the biochemical level, specific bioaccumulation in each tissue and changes at the organism level. To achieve this objective, the selected biomarkers were expressed throughout the Integrated Biomarker Response version 2 (IBR_{v2}) and linked to the contamination.

2. Materials and methods

2.1. Environmental approach

The Iberian Pyrite Belt (IPB) is a volcano–sedimentary massive–sulphide deposit in the Southwest Iberian Peninsula (Figure1). It contains reserves above 1700 Mt (Sáez et al., 1999) promoting its exploitation since remote times. Mining activity in the area has derived in the formation of acid mine drainage (AMD). This is an acid lixiviate containing high concentrations of trace elements and sulphates as result of high–sulphide wastes oxidation.

Part of the courses of the Tinto, Odiel and Guadiana Rivers run over the IPB. This last, in the western part of the region, just drains the IPB in the lower part of the basin; meanwhile the Tinto–Odiel basin contains mines in the head of the Odiel River, where mining discharges and sulphide residues deposits constitute the main pollution source.

Sediment samples were collected in agreement with a mining contamination scale (Figure 1). Out from the IPB and free from mining influences, the first sediment sample was collected in the Guadiana River basin (G), near Montijo. Three stations from the IPB, in the Odiel River basin, were selected as following: before any mining influence (O1), after the first mining discharge (O2), and the lowest part of the basin as the highest polluted transect of the river before reaching the salted plume influence (O3).

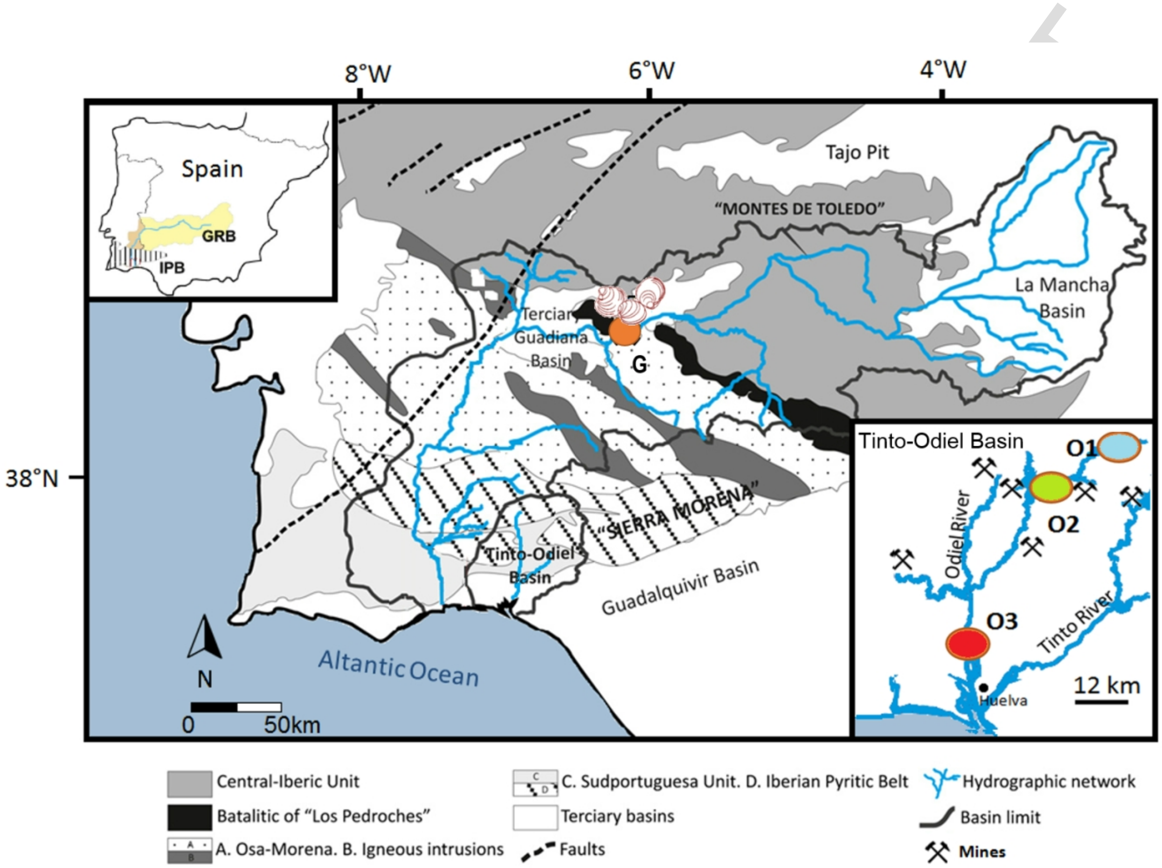


Figure 1. Map of the Southern area of the Iberian Peninsula showing the location of the Guadiana River and Tinto–Odiel River watersheds in the Iberian Pyrite Belt (IPB), including the sediment sampling points (circles: G, O1, O2 and O3) and clam collection site (clams, G).

2.2. Environmental characterization

Hydrochemistry (physico–chemical parameters: pH, Eh, T, DO, metal(loid) concentration–As, Cd, Co, Cr, Cu, Fe, Ni, Mn, Pb, Sb, Zn–, metal speciation) and geochemical (sediment characterization, OM, TOC, total metal(loid) concentrations, metal(loid) fractionation) matrix data, sampling procedures, sampling processing and analyses, are collected in Bonnail et al. (2016a).

2.3. Test species

Adults of the freshwater Asian clam *Corbicula fluminea* were collected in field from the non–contaminated Lácara River at the Guadiana River basin (G, Figure 1). Specimens were acclimatized for three days before the exposure under laboratory conditions in aired commercial water Natura® (Temperature= 20 ± 2ºC; pH = 8 ± 0.3; dissolved oxygen > 7.4 mg L^{-1}; 9 h light: 15 h dark; commercial *Artemia* sp. feeding). A representative group of organisms were kept under these conditions as a control test group.

2.4. Experimental sediment toxicity test

Sediment toxicity tests were conducted as outlined in Bonnail et al. (2016a,b). Briefly, the chambers were filled with a proportion 1:4 (v/v) of sediment–water and diet supply; physico–chemical parameters were monitored during the whole experiment. Clam samplings were developed in days 1, 7, and 14. The endpoints observed were reburial activity, lethality, metal bioaccumulation (Bonnail et al., 2016a), and biomarker responses.

2.5. Biochemical biomarker analysis

2.5.1. Sample processing

Clams were individually homogenized using an ULTRATURRAX® in a buffer solution of 1 mL of 140 mM NaCl, 25 mM HEPES–NaOH, 0.1 mM ethylene diamine tetra-acetic acid and 0.1 mM dithiothreitol (pH 7.5) following methodology described by Lafontaine et al. (2000).Homogenate samples (HF) were used to determine DNA strand damage and lipid peroxidation (LPO). An aliquot of HF was centrifuged at 15,000 g for 30 min at 4ºC to extract the S_{15} fraction destined to measure the enzymatic activity (Glutathione S–Transferase–GST; Glutathione Reductase– GR; Ethoxyresorufin–O–Deethylase– EROD). All samples were stored at –80ºC until analysed with a Tecan Infinite M200 PRO®

Multimode Microplate reader. Protein contents were analysed according to the methodology of Bradford (1976). All biomarkers responses measured were normalized by the total protein (TP) content of the pertinent fraction.

2.5.2. Total Protein Content

Total protein (TP) content was analysed according to an adaptation of the methodology of Bradford (1976) using the principle of protein–dye binding with bovine serum albumin for calibration. In transparent microplates (96 flat bottom wells), 20 µL of the supernatant fraction S_{15} or homogenate (10 µL sample+ 10 µL Milli-Q water) were incubated for 10 min with 200 µL of Bio–rad protein assay reagent (Bio–Rad® Laboratories GmbH Cat. No. 5000–0006) and absorbance was measured spectrophotometrically in microplate reader at 595 nm. Total protein concentration was expressed as mg mL^{-1}.

2.5.3. DNA strand breaks

DNA damage was assessed in accordance with the alkaline precipitation assay described by Olive (1988). It is based on the K–SDS precipitation of DNA–protein cross links followed by detection of DNA strands (Gagné et al., 1995). Salmon sperm DNA standard was used for calibration. The homogenate sample (25 µL) was added to 200 µL of SDS (prepared with a determinate concentration of SDS, EDTA, TRIS Base and NaOH. After 1 min of reaction at room temperature, 200 µL of KCl was added. The solution was heated at 60ºC for 10 min and then was incubated for 30 min in darkness at 4ºC. The sample was centrifuged 5 min at 8,000g. The supernatant (50 µL) was added to 150 µL of Hoechst solution and placed in dark microplates. DNA strand breaks were quantified by fluorescence (excitation λ=360 nm; emission λ=450 nm) in a microplate reader, using a blank containing the same reactors as the samples. Results were expressed as µg DNA mg^{-1} TP.

2.5.4. Lipid Peroxidation

Lipid peroxidation (LPO) was determined on homogenate samples using the thiobarbituric acid reactive substances (TBARS) method described by Wills (1987). Samples were prepared with 150 µL of homogenate sample mixed with 300 µL of trichloroacetic acid (TCA) (previously diluted at 10% in 1mM $FeSO_4$) and also with 150 µL of 0.67% thiobarbituric acid (TBA). After 10 min incubation at 70ºC, the TBARS were determined by absorbance reading in microplate (excitation λ=516 nm; emission λ=600 nm). Standard calibration curve was established using tetramethoxypropane (TMP). Results were expressed as µg TBARS mg^{-1} TP.

2.5.5. Antioxidant enzyme activity

The Glutathione S–Transferase (GST) activity was developed according to Boryslawskyj et al. (1998). The sample was centrifuged at 15,000 gand 25 µL of supernatant was added to 200 µL of GST buffer solution (1 mM L–glutathione reduced and 1 mM 1–chloro–2.4–dinitrobenzene in a buffer of 10 mM Hepes–NaOH, pH 6.5, containing 125mM NaCl) and absorbance was measured (340 nm every 5 min for 30 min). Homogenization buffer was used as a blank. Results were expressed as optical density (OD) min^{-1}mg TP^{-1}.

The Glutathione Reductase (GR)was determined according to the methodology described by McFarland et al. (1999) adapted by Martín–Díaz et al. (2007). GR kinetic was measured through the reduced glutathione (GSH) regeneration. 20 µL of S_{15} sample was added to the reaction buffer and spectrophotometrically measured (340 nm every 2 min for 10 min). Results were expressed as pmol min^{-1} mg^{-1} TP.

2.5.6. Ethoxyresorufin–O–Deethylase Activity

The Ethoxyresorufin–O–Deethylase (EROD) activity was measured using the adapted assay ofGagné and Blaise (1993). Briefly, 50 µL of supernatant (homogenate 15,000 g for 30 min), 10 µM 7–ethoxyresorufin, and 10 mM reduced NADPH in 100 mM KH_2PO_4 buffer (pH 7.4). The reaction was started by the addiction of NADPH, being allowed to proceed for 60 min at 30ºC, and stopped by the addition of 100 µL of 0.1M NaOH. The 7–hydroxyresorufin was determined fluorometrically (excitation λ=520 nm; emission λ=590 nm). The 7–hydroxyresorufin concentration in the samples was achieved through a standard calibration curve developed with concentrations of 7–hydroxyresorufin. Results were expressed as pmol mg^{-1} TP min^{-1}.

2.6. Statistical approach

Data sets of mortality (*N*=64 per chamber), sub-lethal responses (reburial activity (*N*=64 per chamber), and biomarker responses (*N*=6 per replicate)of *C.fluminea* were statistically treated.Mortality and reburial activity responses data sets were evaluated to determine normality of distribution and homogeneity of data, and subsequently evaluated using one–way Analyses of Variance (ANOVA) followed by Dunnett´s test. Biomarkers data were tested for normality of distribution using the Shapiro–Wilk test and homogeneity of variance with Levene´s tests. Significant variations between biomarker activities in the different sampling sites (Control, G, O1, O2, and O3) and days (D1, D7 and D14) were determined using a one–way ANOVA with Bonferroni multiple *post–hoc* comparisons performed using the Statgraphics Statistical Program. Log–transformations were applied for the non–normal distribution of LPO data.

Pair wise correlations were examined through Spearman's rank correlation analysis by splitting biomarker responses of samples in contact with AMD and non–polluted environments; a second division comprises data set related to per sediment sampling point. The significance level was set up at $\alpha>0.05$.

Biomarker Response Index proposed by Beliaeff and Burgeot (2002) and modified by Sánchez et al. (2013) as Integrated Biological Responses version 2 (IBR_{v2}) was calculated for the different stations and days against the control. Correlations between biomarker responses indexes (IBR_{v2}) calculated for *C.fluminea* in the different days and pollution indexes determined by theoretical calculations based on metal concentration in sediments (I_{con}: Contamination index; MPI_{12}: Metal Pollution Index; I_{geo}: Geoaccumulation index; C_d: Contamination degree; *PER:* Potential Ecological Risk; P_{triad}: Pollution index) and other biological responses of the clam in other organizational levels (*TU*: Toxic units; I_{tox}: Toxicity index based on reburial activity; I_{bio}: Bioaccumulation index–As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Zn) were undertaken using a two–tailed Pearson correlation analysis (α>0.05) using the statistical program GraphPad Prism 5.0.

A biological index was used to represent the load of the effects for each group of biomarkers in exposed organisms (Maranho et al., 2014; Díaz–Garduño et al., 2018). The biochemical responses were subdivided into three groups: exposure (phases I–EROD– and phase II–GST), oxidative stress(antioxidant enzymes –GR and GPx), and oxidative effects (LPO and DNA damage).Data were divided by the control value to determine the biological index; and then non AMD–polluted environment (G) and averaged of data after 14 days from the IPB (O) as AMD–polluted environment were compared by percentages.

3. Results

3.1. Sediment and water characterization

Characterization of sediments and water in the river and bioassays, together the theoretical calculations based on metal concentrations in sediments and other biological responses are detailed in Bonnail et al. (2016a, 2017).

3.2. Biological responses

3.2.1. Lethal responses

During 14 days of exposure to increasing AMD polluted environments (O1<O2<O3), the mortality observed was not significant (p<0.05) in duplicates of sediment, nor the control.

3.2.2. Sub–lethal responses

Results of the biochemical responses obtained from control organisms and clams exposed to Guadiana River (G)– as non AMD polluted environment– and Odiel River (O1, O2, and O3) –as AMD polluted environment– sediment samples exposed for 1, 7 and 14 days are represented in Figure 2.

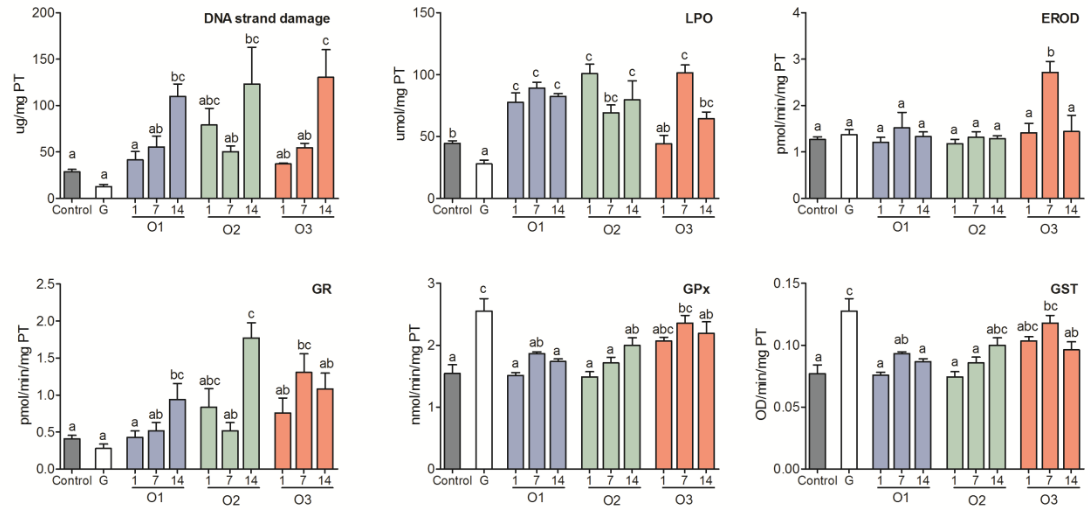


Figure 2. Mean and standard deviation values of DNA damage (strand breaks), lipid peroxidation (LPO), ethoxyresorufin–o–deethylase activity (EROD), glutathione reductase (GR),glutathione peroxidase (GPx) and glutathioneS–transferase (GST) analysed in *Corbicula fluminea* in days 1, 7, and 14 from laboratory exposure to sediment samples (G, O1, O2, and O3) and control. Different letters identify significant differences (Bonferroni test, $p>0.05$).

DNA strand breaks exhibited significant higher values ($p<0.05$) at the end of exposure in the three sediment samples from the Odiel. Clams exhibited damage in day 14 when exposed to sediments from O1 (3.81–fold increase), O2 (4.28–fold increase), and O3 (4.53–fold increase).

LPO determinations showed high levels of TBARS concentrations, but without any particular pattern. The most remarkable differences ($p<0.05$) in comparison with LPO control results were observed in clams from all days of O1 sediment sampling point (between 1.7 and 2–fold increase), in day 1(2.2–fold increase) and 14 (1.7–fold increase) in O2 sediment, and in day 7 ($p<0.001$) in O3 (1.4–fold increase).

Significant differences ($p<0.001$) were observed between control and clean sediment sample exposure (G) in glutathione enzyme activity (GPx, GST), except for GR. Regarding AMD–polluted environments, no significant differences in GST and GPx activities were shown in organisms exposed to sediment from O1 and control; although GR activity was significant higher in after 14 exposure days. In contrast, GR and GST was significantly different (*p*<0.05) in organisms exposed to O2 samples after 14 days; whereas these activities were significantly higher (*p*<0.001) after 7 days of O3 sediment exposure.

Organisms did not display any significant differences (*p*<0.05) of EROD activity from clams when exposed to any contamination grade or along the time, except for those individuals exposed to O3 after 7 days.

The correlation analyses (Table 1) were performed on biomarker responses to support the mechanisms of action, first distinguishing AMD affection in environment by dividing data into specimens exposed to AMD (Odiel River sediments: O1, O2, and O3) and non–exposed (control and Guadiana River, G); and secondly, into the different degrees of contamination from the IPB.

DNA damage positively correlated with LPO (*r*=0.683) and GR (*r*=0.806) at non–AMD polluted sites; however, this correlation was negative and not significant (*p*=0.207) with the rest of the analysed biomarkers. A positive significant correlation between GST and EROD in individuals exposed to these non-polluted environments. There is also a significant (*p*>0.05) positive correlation between GR and LPO in these environments (*r*=0.650). In contrast, there are some significant similarities (*p*>0.05) in the GST and GPx evolution activities (*r*=0.995, *p*=0.207) when analysing clams exposed to AMD–polluted systems. This GST-GR enzymatic behaviour was significant (*p*>0.05) when individually observed in the gradient for O1 (*r*=0.995), O2 (*r*=1.000) and O3 (*r*=0.998).

Table 1. Spearman rank correlations matrix obtained by using biomarker responses of AMD and non–AMD polluted environment, and individual stations from the IPB (O1, O2, and O3).

	DNA	LPO	GR	GST	GPX
Non–AMD polluted environment (N=12)					

LPO	**0.683**				
GR	**0.806**	**0.650**			
GST	–0.500	–0.334	–0.310		
GPx	–0.500	–0.333	–0.300	**1.000**	
EROD	–0.050	–0.200	–0.033	0.100	0.100
AMD–polluted environment (N=54)					
LPO	0.210				
GR	0.286	0.107			
GST	0.163	–0.071	0.325		
GPx	0.167	–0.080	0.323	**0.999**	
EROD	–0.209	0.123	0.389	**0.091**	0.117
Individual AMD–polluted environment					
O1 (N=18)					
LPO	0.182				
GR	0.530	–0.308			
GST	0.427	0.235	0.116		
GPx	0.398	0.224	0.105	**0.995**	
EROD	–0.191	–0.446	0.448	–0.131	–0 .147
O2 (N=18)					
LPO	0 .027				
GR	0 .576	0 .433			
GST	0 .235	–0 .280	0 .252		
GPx	0 .235	–0 .278	0 .273	**1 .000**	
EROD	–0 .329	0 .007	0 .291	0 .226	0 .264
O3 (N=18)					
LPO	0 .510				
GR	0 .164	**0 .580**			
GST	0 .118	0 .437	0 .151		
GPx	0 .145	0 .409	0 .150	**0 .998**	
EROD	0 .126	0 .612	0 .567	–0 .055	–0 .056

Significant correlations (*p*<0.05) are indicated in bold.

The IBR_{v2} values calculated for each site and time are show in Figure 3. According to the Sanchez et al. (2013), the $IBRv_2$ is obtained by sum the absolute values of A parameters calculated for each biomarker: $IBR_{v2}=\Sigma|A|$. In the radar type graphs, the area up to 0 reflects biomarker induction, and the area down to 0 indicates a biomarker inhibition. Thus, when calculating the IBR_{v2} for the non–polluted site Lácara River (G), the global activity supposes a value of 9.71, and the area below the setup of the control were included in the calculation of IBR_{v2} value. However, the area corresponding to the inhibition activity was deleted from the global index due to an overvalue promoted by the absolute value proposed by the index. As can be seen in graphs there is a high displacement of the areas. Therefore, by readjusting the value of the effective activity over the control, the value supposes 2.71 over the control. The synchronism among the AMD pollution degree and the IBR_{v2} is greater after 24 h of exposure ($1.33<3.59<5.21$). However, due to the homeostatic adjustment and the inhibitory responses of some biomarkers in presence of genotoxic substances do not allow showing correspondence between the IBR_{v2} values and the AMD degree in days 7 and 14.

As global view, the greatest biomarker activation is displayed by clams in contact with the highest AMD polluted environment (O3), this occurred after 7 days of exposure (13.14). But the activation of the antioxidant systems promotes the decrease of the IBR_{v2} almost to half of the value (7.94) one week later. In contrast to intermediate polluted system, this activation is observed after two weeks of exposure ($3.59<3.57<8.93$), being a more virulent response. Meanwhile, at the lowest AMD polluted environment, the biomarker responses assessed by theIBR_{v2}gradually grew on time ($1.33<5.63<6.47$).

Although the calculated IBR_{v2} for each station after exposure differed, the pattern or the figure drew in the sunray plots presented similar angles (Figure 3). Therefore, after 14 days in contact with different AMD polluted environments, the Asian clam displayed a similar biomarker behavior independently of the contamination degree.

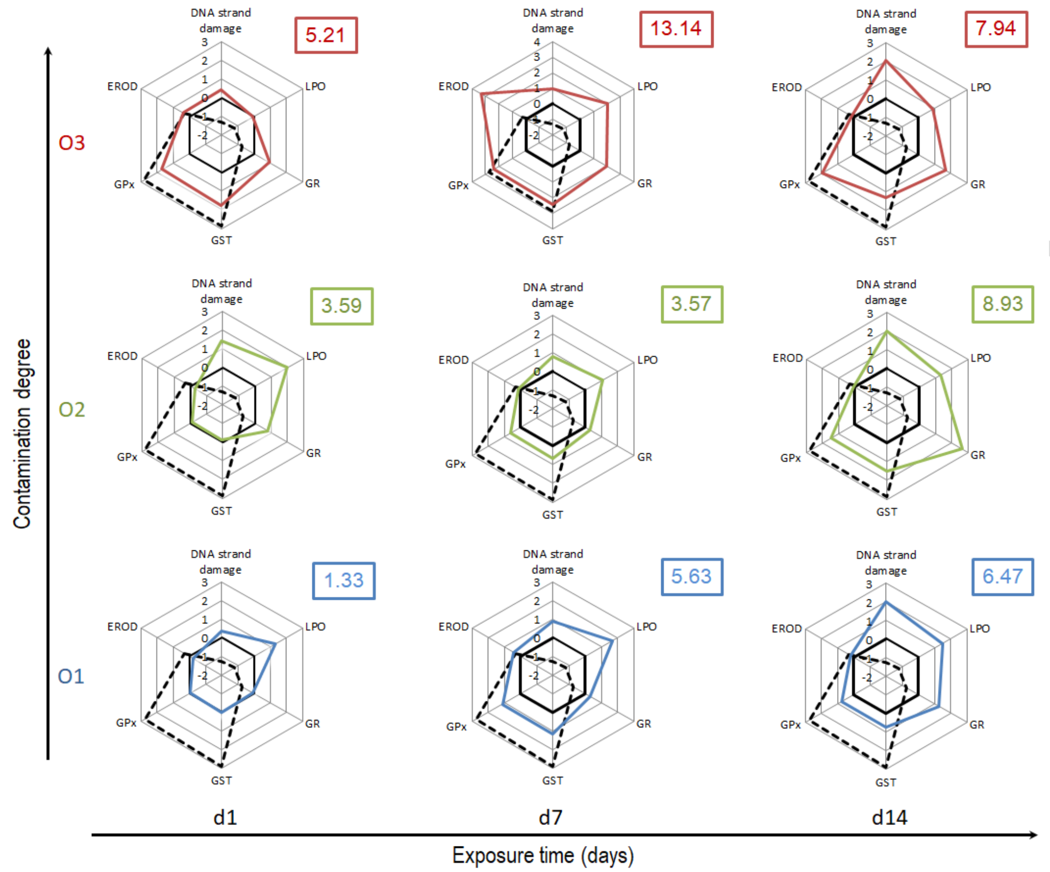


Figure 3. Sunray pots showing the integrated biomarker response index (*IBR*$_{v2}$– in boxes) based on the analysed biomarker (GST, GR, GPx, LPO, EROD, and DNA strand damage) in *C.fluminea* in a gradient of AMD sediment affection (O1<O2<O3) along exposure (days 1,7, and 14). Control in continuous black line (–), Lácara River exposure in discontinuous line (-–-).

Research about the response of *C.fluminea* to metal polluted environments has been focused in bioaccumulation and kinetics, leaving aside the assessment of the global behaviour of the clam in terms of biochemical, histological, and physiological level. This study aimed to cross the data obtained from previous studies that quantified the contamination through theoretical indexes and the characterization of the sampling sites based on the metal(loid) bioaccumulation and the reburial activity (Table 2). Therefore, by integrating the battery of biomarkers in the *IBR*$_{v2}$ analysed in the different days and sites, information was crossed with other toxic responses, bioaccumulation and metal(loid)s in environment (Table 3).

Table 2. Biological and pollution indexes calculated for *Corbicula fluminea* in the sediment sampling points.

	G	O1	O2	O3	Reference
Biological responses indexes					
$IBR_{v2}D1$	2.71	1.33	3.59	5.21	a
$IBR_{v2}\ D7$	2.71	5.63	3.57	13.14	a
$IBR_{v2}\ D14$	2.71	6.47	13.14	7.94	a
TU	0.3	0.78	1.45	18.3	b
I_{tox}	1	3.4	6	576	c
I_{bio}	1	1.33	1.24	2.7	c
Pollution indexes					
I_{con}	1	2.7	3.82	46	c
MPI_{12}	1.67	2.15	2.24	2.56	b
I_{geo}	n.c.	1.7	2.8	4.5	b
R	n.c.	4	9.9	77.7	b
C_d	n.c.	5	10.9	78.7	b
PER	n.c.	124	283	2907	b
P_{TRIAD}	1.30	6.31	13.9	12312	c

n.c.: not calculated. References: a) this study, b) Bonnail et al. (2016a); c) Bonnail et al. (2016b).

TU: Toxic units; I_{tox}: Toxicity index based on reburial activity; I_{bio}: Bioaccumulation index (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Zn); I_{con}: Contamination index; MPI_{12}: Metal Pollution Index (12 elements); I_{geo}: Geoaccumulation index; C_d: Contamination degree; *PER*: Potential Ecological Risk; P_{triad}: Pollution index.

Table 3. Pearson correlation coefficient matrix (*r*) and the signification level (*p–value*, 2–tailed) between the biological responses of the Asian clam in contact with sediments at the studied sites.

			Biological responses					
			$IBR_{v2}D1$	$IBR_{v2}D7$	$IBR_{v2}D14$	*TU*	*Itox*	*Ibio*
Biological responses	*TU*	*r*	0.8335	**0.9674**	0.1115			
		p–value	0.1665	0.0326	0.8885			
	I_{tox}	*r*	0.8218	**0.9666**	0.0649			
		p–value	0.1782	0.0334	0.9351			
	I_{bio}	*r*	0.7667	**0.9914**	0.1622			
		p–value	0.2333	0.0086	0.8378			
Pollution indexes	I_{con}	*r*	0.8274	**0.9702**	0.1089	**0.9999**	**0.9989**	**0.9896**
		p–value	0.1726	0.0298	0.8911	<0.0001	0.0011	0.0104
	MPI_{12}	*r*	0.6095	0.8172	0.6259	0.7651	0.7379	0.8342
		p–value	0.3905	0.1828	0.3741	0.2349	0.2621	0.1658
	I_{geo}	*r*	0.9763	0.8217	0.0881	0.9335	0.9224	0.8980
		p–value	0.1388	0.3861	0.9438	0.2335	0.2525	0.2900
	R	*r*	0.8544	0.9616	–0.2379	**0.9993**	**0.9977**	0.9919
		p–value	0.3479	0.1770	0.8471	0.0244	0.0434	0.0809
	Cd	*r*	0.8544	0.9616	–0.2379	**0.9993**	**0.9977**	0.9919
		p–value	0.3479	0.1770	0.8471	0.0244	0.0434	0.0809
	PER	*r*	0.8432	0.9672	–0.2584	**0.9999**	**0.9989**	0.9944
		p–value	0.3614	0.1635	0.8336	0.0109	0.0299	0.0674
	P_{TRIAD}	*r*	0.8204	**0.9661**	0.0587	**0.9986**	**1.000**	**0.9835**
		p–value	0.1796	0.0339	0.9413	0.0014	<0.0001	0.0165

In bolt $p<0.05$

4. Discussion

4.1. Biochemical response

In the current study, the freshwater clam *Corbicula fluminea* was introduced into a gradient of polluted sediments affected by AMD from the IPB. Attending the surrounding environment, the biochemical responses varied according to the obtained results in Figure 2. The biomarkers analysed in the Asian clam displayed exhibited significant induction regarding control. Antioxidant defences, represented by GR and GST activity induction, appeared to be moderately efficient, though. As increased LPO level indicate increased oxidative damage, which might correlate with increased DNA strand breaks. In accordance with the contamination degree in the environment, a (almost) linear correlation was observed in time with the deterioration of the DNA strand as final consequence of a fluctuant peroxidation of the bi–lipid cell membrane. Besides the glutathione activity was clearly activated when introducing the clam in the AMD environments, with several homeostatic fluctuations in time; contrary to EROD activity, which did not show significant variations for any site and time.

The presence of metal(loid)s and other stress parameters, such as high acidity or low dissolved oxygen promoted the induction of the oxidative stress. It is widely known the effect of xenobiotics in the activation of the enzymatic systems of animal cells in order to obtain a balance with the internal reactive oxygen species production (ROS: superoxide anion ($O^{\bullet}{}_2{}^{-}$), the hydroxyl radical ($HO^{\bullet}$), the perhydroxyl radical ($HO^{\bullet}{}_2$), the alkyl radical ($RO^{\bullet}$) and the radical peroxyl ($ROO^{\bullet}$)) and the antioxidant defence systems activation. The disequilibrium between the ROS and the antioxidant systems may have harmful effects on cells, such as the lipid peroxidation (loss of permeability and integrity of cell membrane–Catalá, 2009; Nigam and Schewe, 2000), damage in proteins (polypeptide chain modifications–Stadtman, 2006), and DNA strands damage. On the other hand, the presence of contaminants activated the GST systems in order to metabolize or conjugate compound to combat xenobiotic effects. These enzymes are considered as biomarkers of stress (detoxification) (Frova, 2006). Meanwhile GR and GPx act as antioxidant enzymes to deactivate ROS generated in normal metabolism (Livingstone, 2003)

According to previous studies, it is known that *C.fluminea* increases its enzymatic response as dose-response for individual metals spiked in the environment (Bigot et al., 2010; Cazenave et al., 2009).Vale et al. (2014) determined the increase of CAT, LPO, SOD, and

GST activities in presence of $nTiO_2$ and cadmium. Increasing doses of Cu in water promotes increase of MTs, GPx and DNA strand damage and inhibition of AChE and GST (Bonnail et al., 2016c). When exposed to higher concentrations of As, the Asian clam triggers effective regulatory mechanisms through MT induction and metal detoxification (Santos et al., 2007).Some other studies have demonstrated the increase of LPO in bivalves in the presence of higher metal concentrations (*Perna canilicutus*/ cadmium– Chandurvelan et al. 2013). Gills and digestive glands showed different responses or effects after exposure to several domestic landfill leachate concentrations while gills alterations occurred more rapidly, especially EROD inhibition and increased in GST activity (Oliveira et al., 2014). Particularly the EROD activity registered by *C.fluminea* in AMD polluted environment was not significant, independently from the pollution degree. Previous studies have also reported inhibitory effects of metals on EROD activity in bivalves (Zhang et al., 2010). Conversely, EROD activity is has been clearly involved in organic contaminates environments (Ramos-Gómez et al., 2011).

Once clams were exposed to contaminated sediments, the influence of metals bound to the sediments might have induce antioxidant defense systems leading to the inhibition after 14 days. This has been previously observed for *Ruditapes philippinarum* (Ramos-Gómez et al., 2011) for the same exposure time. Furthermore, Martín–Díaz et al. (2008) worked to address the relationship between sediment contamination, the bioavailability of contaminants and their associated sub–lethal effects, in two species of invertebrates, the clam *Ruditapes philippinarum* and the crab *Carcinus maenas*, following 28 days of exposure to sediments from four different Spanish ports; they found that it is possible to delineate patterns in the accumulation of contaminants and to relate this processes with associated effects. The same patterns were found by Peltier et al. (2009), with maximum levels during the first 28 days of experiment then a declined to constant level of Ni, Cu, Cd and Zn in *C. fluminea*. Care must be taken since Oliveira et al. (2014) observed in their study with clams exposed to contaminated dilutions that valves closure behavior due to hypoxic conditions, indicating may have contributed for different effects and responses observed. The same authors concluded that changes in the biomarkers may be also due to increased pH values of the exposure media. Results obtained by Bocchetti et al. (2008)

indicate that variations in seasons, as well as species–specific differences, should be considered into account on anthropogenic disturbance studies.

Nevertheless, the activation on the enzymatic system through multi-marker approach in *C.fluminea* under acid polymetallic environments is not widely studied; it has registered disparate responses in literature (Guo et al., 2018). Taylor et al. (2017) determined that, under metal contaminated Molonglo River, the total antioxidant capacity of *Corbicula australis* was mildly impaired, with corresponding increased LPO and lysosomal membrane destabilization at the higher tissue metal concentrations. Although metallothioneins (MTs) were not analysed in this study, it has been previously checked the antioxidant activation systems in tissue of the Asian clam in presence of Cd and Zn (Arini et al., 2014; Marie et al., 2006) and As (Costa et al., 2009; Santos et al., 2007).Also into organic polluted environments, such as ammonia (Costa and Guilhermino, 2015) or pharmaceuticals (Aguirre et al., 2015), *C.fluminea* showed sensibility and was used for multi–marker approach.

Previous research in bivalves found that the activation of enzymes would be stronger in determinate organelles attending to the impact degree. Gagné et al. (2006) studying the integration of biomarker response data into a biomarker index at the whole–individual level (morphometric characteristics) and for various organs (gill, digestive gland, and gonad) from *Mya arenaria* clams, revealed that, relative to the control site, morphological characteristics and gonadal activity were more affected at the most contaminated site, while the effects were more pronounced in the digestive gland and gill at moderately impacted sites.

4.2. Enzyme activity proportion

Biomarker responses mean values after 14–d exposure to AMD and non–AMD polluted environments were represented in pie charts in order to assess the enzyme activities (Figure 4). Biomarkers were subdivided into three different groups (Díaz–Garduño et al., 2018; Maranho et al., 2014): analyses were subdivided into groups according to biomarkers of metabolism called here as exposure (EROD and GST enzymatic activities), antioxidant responses (GR and GPx enzymatic activities), and effects (DNA damage and LPO).

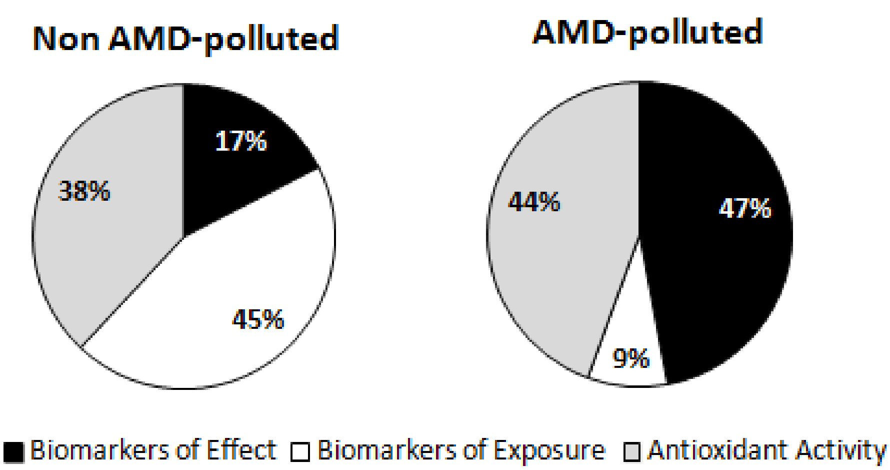


Figure 4. Pie charts of biomarkers responses index of *Corbicula fluminea* after 14 days of exposure. Results were distributed in three groups: biomarkers of exposure (EROD and GST), antioxidant activity (GPx and GR) and biomarkers of effect (DNA damage and LPO).

AMD exposure caused sub–lethal effects variation in *C. fluminea*. After 14 days of exposure to non AMD–polluted environment, almost half of the biochemical activation (45%) was focused in biomarkers of exposure in the freshwater clam. In contrast, the biomarker response in organisms exposed to AMD–polluted after 14 days was predominant in biomarkers of effect (47%) followed by the antioxidant activity (44%). The presence of metal(loid)s and acid conditions promoted the induction of the antioxidant and biomarkers of effect.

4.3. Contamination indexes Vs. Biological indexes

In the environment, contaminants are normally present as a complex mixture and there is no single biomarker that can yield a complete diagnosis of environmental degradation

(Cazenave et al., 2009). Our results permitted to calculate biological (Figure 2, 3, and Table 2) and geological indexes (Table 2) integrating all *C. fluminea* responses (at sub–cellular, tissue, and physiological levels).

Statistical results summarized in Table 3 pointed that the strongest correlation between the biological responses of *C.fluminea* in contact with the environment are shown by the enzymatic system activation after 7 days of exposure ($IBR_{V2}D7$) with the toxic units (*TU*, r=0.97, p<0.05); the physiological response of reburial ($I_{tox,}$ r=0.97, p<0.05), and the metal bioaccumulation (I_{bio}, r=0.97, p<0.05). Nevertheless, it is important to point out that, in spite of the *TU* is a theoretical index based on other organism's responses according to the metal concentration in sediment; this approach is valid for the day 7. Some other highlights, regarding the toxicity test length, the I_{tox} based on the reburial activity, with just few hours of observations might be providing an early warning response. The high sensitivity of the filter–feeder species, like *C. fluminea*, could be related with its trophic status, since individuals are exposed to both dissolved and particulate metallic pollution (Vranković, 2015). As evidenced on results, the interaction between the contamination in the environment (r=0.97, p<0.05) through the index of contamination in the different sites (I_{con}) and reflected by the metal accumulated in tissue (I_{bio}) and the biomarker response (IBR_{v2}) showed a strong correlation after one week of exposure, i.e., any of them might be providing a valuable ecotoxicological information. Besides, the P_{triad}, as index that integrate the index of contamination, toxicity and bioaccumulation, kept obviously correlation with the IBR_{v2}. However, surprisingly, this pollution index was calculated for the day 21 and the most significant correlation was found on day 7 of the IBR_{v2}. This fact might be pointing that, independently of the AMD pollution degree of a system, the analyses of the biomarker response in *Corbicula fluminea* exposed for 7 days to AMD polluted sediments provided information related to the contamination in the environment (R^2= 0.958).

It is important to point out, after 24 hours of exposure, that biomarker responses allow classing the AMD pollution degree based on the correspondence between P_{triad} and the multi-marker response. Whilst, after 7 exposure days the enzymatic system is fully activated, allowing recognizing the AMD pollution in the environment, but categorization is still a difficult task.

5. Conclusions

After 14–d exposure of the freshwater clam *Corbicula fluminea* to a gradient of AMD–polluted sediments from the IPB, in absence of mortality, biomarker responses (glutathione activity, EROD activity, lipid peroxidation and genetic damage) were analysed after 1, 7 and 14 days. Results threw information related to the homeostatic changes induced by the metal(loid) content and the acid environment. They conclude that the Asian clam generally experiments important positive deviations of glutathione activity related to the along the experimental time. Also LPO, in spite of being an irreversible issue, did not keep correlation with the contamination of the surrounding environment. The EROD activity registered in the clam did not show any particular change or correlation with the stressfully medium. However, since it has been found to be the responsible of the DNA damage, it is proposed as the best early warning biomarker for predicting AMD pollution presence in the environment after 7 days of exposure.

Results revealed that, into non stressfully environments, the normal enzymatic activity of the Asian clam displays balance between the biomarkers of exposure and the antioxidant activity; whereas, under acid polymetallic environments, this activity is displaced towards the biomarkers of effect and the corresponding antioxidant activity.

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Highlights

- Biomarker clearly discriminated between acid mine drainage polluted environments
- Biomarkers of effect are suitable early warning for acid mine drainage
- Acid mine drainage can damage DNA and cell membranes
- EROD activity analysed in the Asian clam is discarded for biomonitoring purposes
- IBR_{v2} showed correlation Vs. Geochemical indexes