Ecotoxicology and Environmental Safety 103 (2014) 17–23

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

# Assessment of domestic landfill leachate toxicity to the Asian clam *Corbicula fluminea* via biomarkers

Luciana F Oliveira [a], Sandra M C P Silva [b], Claudia B R Martinez [a,*]

[a] Laboratório de Ecofisiologia Animal, Departamento de Ciências Fisiológicas, Universidade Estadual de Londrina, C.P. 10011, 86057-970, Londrina, Parana, Brazil
[b] Laboratório de Hidráulica e Saneamento, Departamento de Construção Civil, Universidade Estadual de Londrina, C.P. 10011, 86057-970 Londrina, Parana, Brazil

ARTICLE INFO

*Article history:*
Received 7 October 2013
Received in revised form
26 January 2014
Accepted 27 January 2014
Available online 19 February 2014

*Keywords:*
Biotransformation
Bivalve
Lipid peroxidation
Metallothionein
MXR mechanism

ABSTRACT

In order to evaluate the effects of domestic landfill leachate to bivalves *Corbicula fluminea*, clams were exposed to different leachate concentrations (v/v): 2, 3, 6 and 10 percent, corresponding to dilutions observed along a stream that receives this effluent, or only to clean water for comparisons. After 5 and 15 days of exposure the activity of the biotransformation enzymes 7-ethoxyresorufin-*O*-deethylase (EROD) and glutathione S-transferase (GST), the multixenobiotic resistance mechanism (MXR) and lipid peroxidation (LPO) in gills and digestive gland and metallothionein (MT) content in gills were evaluated. Differences in biomarkers responses were observed between gills and digestive gland, except for MXR that decreased in both tissues of clams exposed to 6 percent for 5 days. EROD activity in gills was reduced in all leachate concentrations after 5 days and only in 2 percent after 15 days exposure, while an EROD increase was observed in digestive gland after 15 days exposure to 6 percent. GST activity increased only in the gills of clams exposed to 10 percent for 5 days. LPO varied between tissues and different conditions. A significant increase in LPO was observed in the gills, after 5 days exposure to 2 and 6 percent, and in digestive gland after 5 and 15 days exposure to 2 and 3 percent. MT content in the gills increased after 15 days exposure to 2 percent. In conclusion, different leachate concentrations tested here caused biochemical changes in *C. fluminea*, but due to the observed variability in biomarkers responses among leachate concentrations, it was difficult to determine patterns or thresholds concentrations.


## 1. Introduction

Leachate is a complex mixture produced by the decomposition of wastes and can contain dissolved organic matter, inorganic macrocomponents, metals and xenobiotic organic compounds (Christensen et al., 2001). Risk assessment of this mixture is often difficult due to its complex and highly variable composition and should be based not only on chemical analysis but the interactions among its chemicals and/or toxic degradation products with the biota as well (Tsarpali et al., 2012).

Bioassays have already been successfully performed for the evaluation of landfill leachate toxicity (Thomas et al., 2009; Ribé et al., 2012), but biomarkers application is not frequent. Biomarkers are assessment and monitoring tools able to detect effects of chemical exposure in aquatic organisms before they become significant in conservation or ecological terms (Long et al., 2004), however there are only few studies regarding biomarkers application to assess landfill leachate effects on bivalves (Tsarpali and Dailianis, 2012; Toufexi et al., 2013). These animals have been considered as good bioindicators (O'Connor, 2002) as they exhibit alterations in response to contaminant exposure present in the leachate, like metals (Marie et al., 2006; Zhang et al., 2010) and hydrocarbons (Large et al., 2002). In particular, the Asian clam *Corbicula fluminea* has been the focus of several toxicological studies, including biomarkers approach (Santos and Martinez, 2014).

Several biomarkers can be used to evaluate leachate toxicity in aquatic organisms, such as the mechanisms related to the biotransformation of xenobiotics, which usually consist of three phases including numerous different enzyme systems and several types of substrates. Phase I reactions involve oxidation, reduction and hydrolysis, catalyzed mainly by cytochrome P450, that facilitate the excretion of compounds, transforming them into more water-soluble compounds and also serving as a substrate for phase II reactions that increase the excretion rate (Stegeman et al., 1992). As well stablished for fishes, several studies showed that the

* Corresponding author. Fax: +55 43 3371 4467.
*E-mail addresses:* cbueno@uel.br, claudiabrmartinez@gmail.com (C.B. Martinez).

http://dx.doi.org/10.1016/j.ecoenv.2014.01.034
0147-6513 

activity of 7-ethoxyresorufin-*O*-deethylase (EROD) should be used in bivalves as an indirect measurement of the catalytic activity of one P450 family, CYP1A (Binelli et al., 2006; Faria et al., 2009). Glutathione S-transferase (GST) is an enzyme of phase II responsible for conjugation reactions which is frequently used as biomarker (Hodgson et al., 2008). Related to phase III, the multixenobiotic resistance mechanism (MXR) is responsible for the elimination of xenobiotics or its metabolites through ATP-dependant proteins and could be alterated after exposure to contaminants (Bard, 2000). Osaki et al. (2006) showed that in the medaka fish, *Oryzias latipes*, the exposure to landfill leachate induced EROD activity, but the inhibition of the same biomarker was already been observed in the fish cell line RTG-2 by Pablos et al. (2011). This evidenced that variable composition of landfill leachates can promote different alterations in biomarkers.

In addition, contaminants could stimulate reactive oxygen species (ROS) production and the resultant oxidative stress has been indicated as a mechanism of toxicity in aquatic organisms exposed to a broad range of contaminants (Livingstone, 2003). Damage caused by ROS can be considered proportional to the levels of tissue lipid peroxidation (Almeida et al., 2005), which makes this an important tool to assess generic toxicity of mixtures such as landfill leachates. Tsarpali and Dailianis (2012) and Toufexi et al. (2013) recently showed that hemocytes of *Mytilus galloprovincialis* treated with relevant concentrations of leachate showed a significant enhancement of lipid peroxidation products (malondialdehyde (MDA)).

Metals within landfill leachate are also of concern due to their high toxicity (Thomas et al., 2009) and metallothionein (MT) is a commonly used biomarker usually related to the presence of metals (Amiard et al., 2006). Other chemical compounds, particularly oxidizing agents, also induce MT, since it also works as an antioxidant (Viarengo et al., 1999). It was previously observed that landfill leachate increases MT content in gills and digestive glands of *M. galloprovincialis* (Tsarpali and Dailianis, 2012).

Thus, the purpose of this work was to evaluate the effects promoted by leachate from domestic waste landfill on the freshwater clam *C. fluminea* measuring biochemical biomarkers such as biotransformation enzymes, MXR mechanism, lipid peroxidation and metallothionein content in gills and digestive glands. These organs were chosen for biomarkers analyses considering that digestive gland is a primary organ for bioaccumulation and is involved in pollutant detoxification and homeostasis maintenance (Cappello et al., 2013), while the gills are the main entrance of contaminants present in the environment (Rocha and Souza, 2012).

## 2. Material and methods

### 2.1. Experimental design

Adult bivalves of the species *C. fluminea* ($n=120$), measuring $3.9 \pm 0.06$ cm in length and $2.74 \pm 0.06$ cm in height (mean $\pm$ SEM), were collected from an urban lake in the municipality of Londrina (PR, Brazil) and acclimated in the laboratory for 7 days before the start of toxicity tests. During acclimation the specimens were maintained under 12 h:12 h photoperiod in 70 L glass aquaria containing 30 L of clean aerated and dechlorinated water with the following characteristics (mean $\pm$ SE, $n=4$): temperature: $16.65 \pm 0.37$ °C, conductivity: $72.25 \pm 1.98$ μS $cm^{-1}$, dissolved oxygen: $6.83 \pm 0.15$ mg $L^{-1}$ $O_2$, pH: $7.08 \pm 0.21$. After this period, animals were exposed to different concentrations of leachate for a period of 5 and 15 days in 5 L glass containers, with 12 animals in each, filled with 1 L of clean water (control group or CTR) or with 1 L of different leachate concentrations (2, 3, 6 and 10 percent v/v). The longer tests (15 days) were performed in semi-static conditions with water renewal after 7 days. For each period of exposure (5 and 15 days) toxicity tests were run independently, not simultaneously.

The raw leachate used in the present study was obtained from the controlled landfill of the municipality of Londrina before the aerobic treatment and was maintained in plastic container protected from the light at room temperature.

**Table 1**
Physical and chemical characteristics of raw leachate from the controlled landfill of the municipality of Londrina collected before the aerobic treatment, analyzed in accordance to APHA, AWWA and WEF (2005).

| Characteristics | Values (units) |
|---|---|
| Electrical conductivity | 8865 μS $cm^{-1}$ |
| Biochemical oxygen demand (BOD) | 593 mg $O_2$ $L^{-1}$ |
| Dissolved organic carbon (DOC) | 3025 mg $O_2$ $L^{-1}$ |
| Ammonia | 957 mg $L^{-1}$ |
| Total Kjeldahl nitrogen (NKT) | 1052 mg $L^{-1}$ |
| Chlorides | 1751 mg $L^{-1}$ |
| Total phosphorus | 2 mg $L^{-1}$ |
| Total aluminum | 0.435 mg $L^{-1}$ |
| Total cooper | 0.081 mg $L^{-1}$ |
| Total chromium | 0.06 mg $L^{-1}$ |
| Total iron | 8.74 mg $L^{-1}$ |
| Total manganese | 1.68 mg $L^{-1}$ |
| Total nickel | 0.017 mg $L^{-1}$ |
| Total silver | 0.401 mg $L^{-1}$ |

Some physical and chemical analyses of the leachate were made in accordance to APHA, AWWA and WEF (2005) and the results are presented in Table 1.

For the experiments the landfill leachate was diluted in different proportions with declorinated water to obtain the following concentrations (v/v): 2, 3, 6 and 10 percent. The conductivity of these concentrations corresponds to the conductivity observed along the stream that receives the leachate from the controlled landfill of the municipality of Londrina, determined in a previous study performed in our laboratory (data not published).

### 2.2. Sampling

After the experimental periods (5 and 15 days) the anterior adductor muscles of the clams were cut allowing the valves to open and the gills and digestive glands were removed. The tissues were frozen at −72 °C for subsequent analysis of the biomarkers.

### 2.3. Biomarkers

The gills and digestive gland removed from the clams ($n=6$–8) were individually homogenized in potassium phosphate buffer (0.1 M, pH 7) and then centrifuged (14,000*g*; 20 min, 4 °C) for the determination of 7-ethoxyresorufin-*O*-deethylase (EROD), glutathione S-transferase (GST) and lipid peroxidation (LPO) in the supernatant.

#### 2.3.1. Ethoxyresorufin-O-deethylase (EROD)

The CYP1A activity was determined by analyzing the EROD activity, which was estimated by the rate of conversion of 7-ethoxyresorufin to resorufin, according to the protocol of Eggens and Galgani (1992), with modifications. The reaction was initiated by adding the sample to the reactive mixture (0.1 M potassium phosphate buffer, pH 7.6; 2 mM NADPH and 0.1 mM 7-ethoxyresorufin). The progressive increase in fluorescence resulting from the formation of resorufin was measured at 1-min intervals for 30 min (ex/em: 530/590 nm). The EROD activity was expressed in pmol resorufin $min^{-1}$ mg of $protein^{-1}$.

#### 2.3.2. Glutathione S-transferase (GST)

The GST activity was determined using the method described by Keen et al. (1976). This method is based on the GST catalyzed conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). The increase in CDNB conjugate was monitored for 1 min in a spectrophotometer at 340 nm and the enzyme activity was expressed in nmol CDNB conjugate $min^{-1}$ mg of $protein^{-1}$.

#### 2.3.3. Multixenobiotic resistance mechanism (MXR)

The test for rhodamine B fluorescence accumulation (substrate of P-gp) was performed according to Kurelec et al. (2000) to evaluate the multixenobiotic resistance mechanism (MXR). In this assay, an increase in accumulated fluorescence represents a reduction in MXR. The gills and digestive glands were kept *ex vivo* in a solution of 1 μM of rhodamine B fluorescent dye for a period of 2 h. After this period, the tissues were washed in saline (26 mM NaCl; 4.3 mM sucrose; pH 7.4), homogenized in distilled water (1:7–w/v) and centrifuged (1700*g*; 7 min; 4 °C). The fluorescence corresponding to accumulated rhodamine was measured (ex/em: 544/590 nm) and the concentrations were determined by a rhodamine standard curve, with the data expressed in μM rhodamine mg of wet $tissue^{-1}$.

#### 2.3.4. Lipid peroxidation (LPO)

The thiobarbituric acid reactive substances (TBARS) assay was performed according to Camejo et al. (1998) as a measure of lipid peroxidation. Butylated hydroxytoluene (BHT 1 M), phosphate buffered saline (2 mM KCl; 1.4 mM $NaH_2PO_4$; 357 mM NaCl; 10 mM $Na_2HPO_4$; pH 7.4), trichloroacetic acid (TCA 50 percent) and thiobarbituric acid (TBA 1.3 percent) dissolved in 0.3 percent NaOH were added to the supernatant and the mixture was kept in an incubator at 60 °C for 1 h. A fluorescence reading was then made (ex/em: 535/590 nm) and the TBARS concentration was determined from a malondialdehyde (MDA) standard curve. The TBARS concentration was expressed in nmol TBARS mg of protein$^{-1}$.

#### 2.3.5. Metallothionein-like proteins (MT-like)

The MT-like content was determined only in the gills, following the methodology described by Viarengo et al. (1997) with modifications. MT content in digestive gland was below the detection limit of the assay. The gills were homogenized (1:5-w/v) in buffer (0.5 M sucrose, 26 mM Tris, 0.5 mM phenylmethylsulfonyl fluoride, 1.3 mM β-mercaptoethanol) and centrifuged for 45 min (18,650g; 4 °C), and the supernatant was subjected to ethanol/acid chloroform fractionation to obtain a partially purified metalloprotein fraction. In this fraction sulfhydryl groups (–SH) were quantified in a spectrophotometer at 412 nm, using Ellman's reagent. Reduced glutathione (GSH) was used as standard and the metallothionein content was expressed in nmol GSH mg of protein$^{-1}$.

#### 2.3.6. Total proteins

The protein concentration was determined by the Bradford (1976) method, based on the reaction of proteins with Coomassie Brilliant Blue G-250 dye. The calibration curve was prepared with bovine serum albumin (BSA) and absorbance was read in a spectrophotometer at 595 nm. For biomarker analysis, protein extract concentration was approximately 5 mg mL$^{-1}$ in gills and 10 mg mL$^{-1}$ in digestive glands.

### 2.4. Statistical analysis

Data were first tested for normality and homogeneity of variance to check statistical demands. The parametric analysis of variance (ANOVA) was used, followed by a multiple comparison test (Newman–Keuls test), when indicated, to evaluate significant differences ($P<0.05$) among the different experimental conditions (CTR × 10 percent × 6 percent × 3 percent × 2 percent), for each experimental period (5 and 15 days).

## 3. Results

The characteristic of the water from 5 and 15 days experiments, for each experimental treatment, were pooled together, as they did not differ from each other (*t*-test: $P>0.2$ for all parameters, $n=4$). Water temperature (17.4 ± 0.2 °C) and dissolved oxygen (7.0 ± 0.1 mg mL$^{-1}$) were kept constant in the different experiments conditions, but significant variations occurred in water conductivity and pH (Table 2). Water conductivity was significantly higher in all leachate concentrations than in control aquaria while water pH from 6 and 10 percent was higher than control and the others leachate concentrations (2 and 3 percent). After exposure, it was observed a mortality rate less than 20 percent among the clams exposed to all leachate concentrations, except for the concentration of 10 percent in 15 days, in which there was a 100 percent mortality rate. It was observed that already in the first day of exposure, the animals exposed to 6 and 10 percent showed a behavior of closing their valves leaving a portion of the foot exposed and stiff.

**Table 2**
Conductivity and pH values measured in the water from control (CTR) and experimental treatments groups: dilutions of domestic landfill leachate (2, 3, 6 and 10 percent). Data from 5 and 15 days experiments were pooled together, as they did not differ from each other (*t*-test: $P>0.2$ for all parameters, $n=4$).

| Treatments | Condutivity (μS cm$^{-1}$) | pH |
|---|---|---|
| CTR | 72.8 ± 3.8$^{a}$ | 7.1 ± 0.2$^{a}$ |
| 2% | 236.8 ± 9.2$^{b}$ | 7.8 ± 0.2$^{a}$ |
| 3% | 279.4 ± 9.9$^{b}$ | 7.8 ± 0.2$^{a}$ |
| 6% | 383.2 ± 25.6$^{c}$ | 7.9 ± 0.2$^{b}$ |
| 10% | 665.0 ± 29.7$^{d}$ | 8.0 ± 0.1$^{b}$ |

Values are mean ± SE, $n=8$. Different letters indicates significative difference between treatments ($P<0.05$).

Significant reduction in EROD activity was detected in the gills of clams exposed to all leachate concentrations for 5 days and those exposed to 2 percent for 15 days (Fig. 1a). On the other hand, the digestive gland showed increased EROD activity after 15 days of exposure to the highest concentration (10 percent) (Fig. 1b). GST activity increased only in the gills of animals exposed for 5 days to 10 percent and no significant change in GST activity was found in the digestive gland (Fig. 1c and d). The MXR mechanism was evidenced by the accumulation of the fluorescent dye rhodamine and the significant higher accumulation of rhodamine in the gills and digestive gland of clams exposed for 5 days to 6 percent indicates a reduction of this mechanism (Fig. 1e and f).

Lipid peroxidation was found to increase in the gills of bivalves exposed to 2 and 10 percent for 5 days (Fig. 2a) and in the digestive gland after 5 and 15 days exposure to 2 and 3 percent (Fig. 2b). A significant increase in MT-like content was only found in the gills of *C. fluminea* exposed for 15 days to the lowest leachate concentration (2 percent) (Fig. 3).

## 4. Discussion

Biomarkers are important tools that allow the early diagnosis of the quality of environments to which animals are exposed and can give information about the toxicity of liquid effluents produced by human action (Long et al., 2004). The study of biomarkers for the investigation of complex mixtures is interesting because they may act, for example, as parameters to monitoring or analyze the result of an effluent treatment (Tsarpali and Dailianis, 2012). The biomarkers evaluated in *C. fluminea* exposed to dilutions of leachate from a domestic landfill indicated the presence of contaminants that produced effects on these animals.

In the present work, only the higher leachate concentration (10 percent) was lethal to *C. fluminea* after 15 days exposure and at the others experimental conditions the mortality was never higher than 20 percent. Tsarpali and Dailianis (2012) determined that 96 h $LC_{50}$ for *M. galloprovincialis* was 0.526 percent v/v of leachate, a much lower concentration comparing to those used in the present work and that did not kill *C. fluminea* neither after 5 or 15 days exposure. This might happened because the physical and chemical parameters of the leachate used in the present work presented, in general, lower values, such as electrical conductivity which was 8865 μS cm$^{-1}$ while in the other leachate was 10,570 μS cm$^{-1}$ or the ammonia concentration which were 957 mg L$^{-1}$ and 1526 mg L$^{-1}$, respectively. In addition, it could be an indicative that the marine bivalve presents lower resistence to the leachate contaminants than the freshwater clam.

The results of the present work indicated that the leachate was able to induce in *C. fluminea* lipid peroxidation and inhibition of phase I and III biotransformation processes, EROD and MXR. Activation of phase I and II biotransformation enzymes was only observed at the higher concentrations (6 and 10 percent).

Biotransformation is an important response of organisms being exposed to lipophilic compounds, as it generally leads to the formation of more hydrophilic compounds which are more easily excreted than the parent compounds (Van der Oost et al., 2003). Compounds such as PAHs (Gowland et al., 2002) and PCBs (Binelli et al., 2006; Faria et al., 2009) can increase EROD or GST activities in bivalves. The leachate increased GST activity in gills after 5 days of exposure and increased EROD in the digestive gland after 15 days, but this occurred only at the higher concentrations (10 and 6 percent, respectively). These results indicate the presence of compounds that are biotransformed in phases I and II. However,

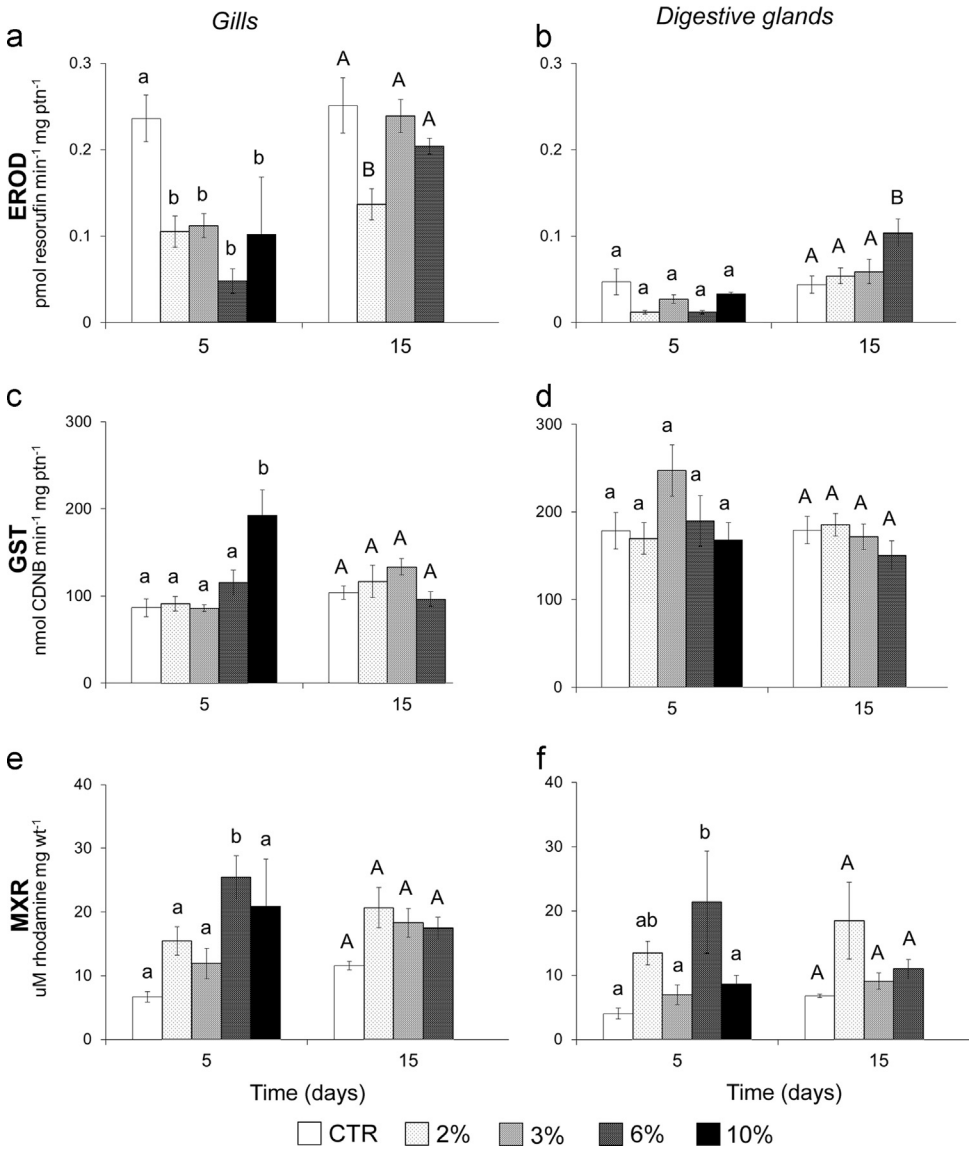


**Fig. 1.** Activities of 7-ethoxyresorufin-*O*-deetilase (EROD) (a) & (b) and glutathione S-transferase (GST) (c) & (d) and multixenobiotic resistance mechanism (MXR) (e) & (f) in gills and digestive gland of *C. fluminea* exposed for 5 and 15 days only to water (CTR) or to different dilutions of domestic landfill leachate (2, 3, 6, 10 percent). Values are presented as mean ± SEM (*n*: 6–8). Different letters indicate significant difference between treatments for 5 days (lowercase) and 15 days (uppercase) ($P < 0.05$).

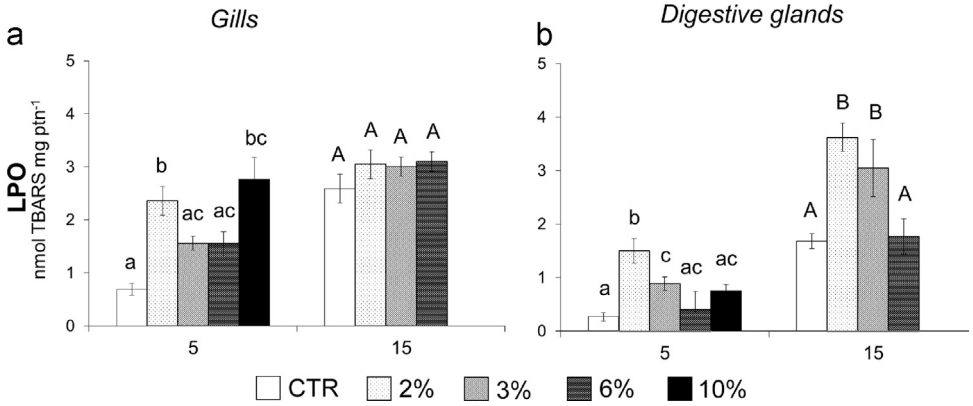


**Fig. 2.** Lipid peroxidation (LPO) in gills (a) and digestive gland (b) of *C. fluminea* exposed for 5 and 15 days only to water (CTR) or to different dilutions of domestic landfill leachate (2, 3, 6, 10 percent). Values are presented as mean ± SEM (*n*: 6–8). Different letters indicate significant difference between treatments for 5 days (lowercase) and 15 days (uppercase) ($P < 0.05$).

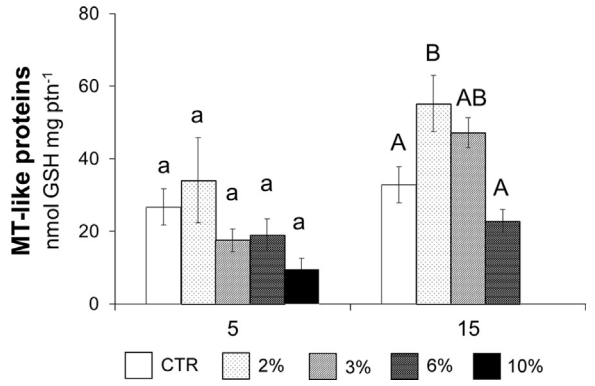


**Fig. 3.** Metallothionein-like proteins (MT-like) in gills of *C. fluminea* exposed for 5 and 15 days only to water (CTR) or to different dilutions of domestic landfill leachate (2, 3, 6, 10 percent). Values are presented as mean ± SEM ($n$: 4–6). Different letters indicate significant difference between treatments for 5 days (lowercase) and 15 days (uppercase) ($P < 0.05$).

a concentration equal to or lower than 3 percent did not promote activation of these processes, or would have required more than 15 days for this activation to occur. In another works, it was demonstrated the potencial of landfill leachate to increase EROD and GST activities in fish (Osaki et al., 2006), gastropod (Li et al., 2008) and daphnids (Jemec et al., 2012).

Unlike the response presented by the digestive gland, the gills of *C. fluminea* showed diminished EROD activity at all the leachate concentrations. Various compounds, such as metals and pharmaceutical products can inhibit the induction of EROD in aquatic organisms (Navas and Segner, 2000; Oliveira et al., 2004; Kirby et al., 2007; Beijer et al., 2010), as well as conditions such as oxidative stress (Barouki and Morel, 2001) or an excess of enzyme substrates may also be responsible for this inhibition. Similar to these results, Mdegela et al. (2010) observed the induction of EROD enzyme only in the liver, and not in gills of the fish *Clarias gariepinus* exposed to a sewage lagoon. As suggested by these authors, this may be explained by the presence of chemical compounds in the water that somehow inhibit EROD activity in gills affecting the phase I of biotransformation reactions in this tissue, and hence, larger amounts of this enzyme inducers reach the liver.

The MXR mechanism is considered an important defense of aquatic organisms against xenobiotics, since it contributes to reduce their accumulation within the cells. This mechanism depends on the activity of P-glycoproteins (P-gp), which are ATP-dependent, and transport a variety of substrates with different structures and functions out of the cell. The compounds that interact with this mechanism are usually moderately hydrophobic, planar, or natural products (Bard, 2000). In the present study, MXR was found to be reduced in bivalves exposed to 6 percent for 5 days. This may have been due to the presence in the leachate of compounds called chemosensitizers, which are capable of inhibiting the action of MXR (Smital and Kurelec, 1998). These chemosensitizers include pesticides, personal care products, products of microbial degradation (Smital et al., 2004), Pb (Rocha and Souza, 2012) and some fragrances (Luckenbach et al., 2004; Smital et al., 2004). The inhibition of this mechanism can also be related to the high concentration of dissolved organic carbon (DOC) in leachate, as demonstrated by Kurelec et al. (1998) in extracts of solid wastes in a municipal landfill, mainly of household wastes. The MXR mechanism was not found to be reduced at 10 percent, which leads to the hypothesis that, at this concentration, new transporters may have been synthesized to compensate the inhibition caused by the compounds present in the leachate. The inhibitions of both EROD activity and MXR in gill of clams exposed to 6 percent represent an impairment of biotransformation process and an increase in bivalves' susceptibility to contaminants (Beijer et al., 2010).

The increase in MT-like content in gills of *C. fluminea* occurred only after 15 days of exposure to 2 percent, but not to 3 and 6 percent. These low molecular weight proteins are rich in cysteines and are therefore considered metal ligands (Amiard et al., 2006). The function of these proteins is the capture and release of metals, exchange metals, transfer metals from proteins to other biomolecules, and they also have redox activity (Capdevila et al., 2012). The metals Cu, Ag and Fe, which were detected in the tested leachate, have affinity for MTs thiol groups (Capdevila et al., 2012). Tsarpali and Dailianis (2012) also observed an increase in MT content in *M. galloprovincialis* tissues exposed to a landfill leachate, but this increase occurred in minor concentrations (0.01 and 0.1 percent v/v) and after 96 h exposure. These authors observed the same increase in TBARS. However, in the present work, TBARS did not increase concomitantly with the increase in MT-like content, which leads to two hypotheses. The first is that the increase in MT-like content was stimulated directly by the presence of metals and this effect was observed only after 15 days exposure to the lower leachate concentration, while the second hypothesis is that the increase in MT-like protein possibly contributed to protect against oxidative stress, and avoiding the occurrence of LPO in the gills after 15 days.

The increase in phase I biotransformation of xenobiotics normally promotes augmented generation of ROS, which can cause oxidative damage such as lipid peroxidation. In this study, an increase in EROD activity was observed only in digestive gland after 15 days of exposure to 6 percent, but TBARS did not increase in this tissue under this condition. The MT-like content, which may also be associated with oxidative stress, was also augmented in the gills in conditions in which no increase in EROD occurred. These factors indicate that contaminants that are biotransformed in phase I were not responsible for the oxidative stress established in digestive gland after exposure to 3 and 6 percent. This must have occurred by other pathways, such as impairment of the electron transport chain by lipophilic contaminants or metal ions, or by impairment of the antioxidant defenses (Livingstone, 2003).

The presence of metals such as Cu (Liao et al., 2007) and Al (Kádár et al., 2001) in water can cause the valves of freshwater bivalves to close. Liao et al. (2007) showed that 60 percent of the *C. fluminea* individuals exposed to 0.03 $\mu g\ L^{-1}$ of Cu closed their valves after 9 h of exposure. In the present study, the animals exposed to leachate also displayed a valve closure behavior, particularly at the higher concentrations (6 and 10 percent). This fact leads to the hypothesis that the more concentrated leachate causes this behavior, which bivalves may use as a means of protection at 6 and 10 percent. This was not permanent, which means that despite of valve closure, clams were able to filter the medium. This behavior may be one of the factors that explain the unchanged values of MT-like proteins and TBARS in digestive glands and the reduction of MXR after 15 days exposure to 6 percent. In this context, at some moments clams exposed to these dilutions could be facing a hypoxic condition that could be responsible for biomarkers changes, as demonstrated by Vidal et al. (2002) in *C. fluminea*. These authors showed that in a hypoxic condition catalase and GST activities increase and peroxidized lipid can decrease in Asian clam.

The observed variation in the conductivity and pH of the exposure media could cause changes in the availability of contaminants, acting for example, on metal speciation and on the percentage of un-ionized ammonia, which is more toxic (Alabaster and Lloyd, 1982). Ammonia is an important compound in the leachate, however the pH alteration from 7.8 to 8.0, as observed among the different leachate concentrations tested, lead to calculated concentrations of

un-ionized ammonia that follow the same concentration gradient of the leachate (2 percent: 0377 mg $NH_3\,L^{-1}$; 3 percent: 0.565 mg $NH_3\,L^{-1}$; 6 percent: 1.410 mg $NH_3\,L^{-1}$; 10 percent: 2.950 mg $NH_3\,L^{-1}$). These calculated concentrations are all below the maximum concentrations of un-ionized ammonia for Unionid mussels, set at 6 mg $L^{-1}$ for the same conditions of pH and temperature (EPA, 2013). On the other hand, a larger difference in pH was observed between control (pH 7.1) and the groups exposed to 6 and 10 percent of leachate (pH 7.9–8.0). Vidal et al. (2002) showed that pH is a factor that alters lipid peroxidation, besides the activity of antioxidant enzymes in *C. fluminea*. Thus, the observed changes in the biomarkers in this study may also be due to increased pH values of the exposure media due to the presence of the leachate.

During the experiments, the bivalves were submitted to a starvation period and that could cause some side effects at 15 days test. In this context, it was observed in control animals the increase in TBARS after 15 days exposure in comparison to the shorter exposure period. Some works showed an increase in lipid peroxidation or alterations in oxidative stress parameters in aquatic animals, such as fish (Morales et al., 2004; Bayir et al., 2011) and bivalve (Ansaldo et al., 2007) after starvation. However, that fact did not exclude the differences observed between control and experimental groups.

## 5. Conclusions

Even the lowest concentration of the leachate (2 percent) tested in this study caused alterations in *C. fluminea* biomarkers. However, in comparison to anothers works, the leachate studied here probably had minor concentrations of organic pollutants, since the EROD and GST activities increased only at the highest concentrations and clams mortality occurred only at higher concentrations than those observed in other studies. In general, gills and digestive glands did not show similar responses or effects after exposure to different leachate concentrations and gills alterations occurred more rapidly, especially EROD inhibition and increased GST activity. In gills, these two biomarkers could be considered as good parameters for monitoring contamination by leachate because they are fast and show consistent results. Despite of the observed alterations, it was dificult to indicate patterns of effects and responses or threshold concentrations for the studied leachate, especially because of the variability of biomarkers through time and between concentrations. This variability may be consequence of the pH and conductivity increase, following the increase of leachate concentrations, starvation period, following the increase of exposure time and a possible hypoxia caused by valve closure. Valves closure behavior may have contributed for different effects and responses observed in clams exposed to higher concentrations (6 and 10 percent) and should be considered as an important issue.

## Acknowledgments

This work is part of L.F. Oliveira's master's dissertation. The authors acknowledge the Brazilian research agency CAPES for a scholarship granted to L.F. Oliveira. C.B.R. Martinez is research fellow from CNPq and member of the National Institute of Science and Technology in Aquatic Toxicology (INCT-TA, CNPq 573949/2008-5).

## References

Alabaster, J.S., Lloyd, R., 1982. Water Quality Criteria for Freshwater Fish, 2nd ed FAO, Cambridge, UK

Almeida, E.A., Bainy, A.C.D., Dafre, A.L., Gomes, O.F., Medeiros, M.H.G., Di Mascio, P., 2005. Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed. J. Exp. Mar. Biol. Ecol. 318, 21–30.

Amiard, J.C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S., 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76160–202

Ansaldo, M., Sacristán, H., Wider, E., 2007. Does atarvation influence the antioxidant status of the digestive gland of *Nacella concinna* in experimental conditions? Comp. Biochem. Physiol. C 146, 118–123.

APHA (American Public Health Association), AWWA (American Water Works Association) and WEF (Water Environment Federation), 2005. Standards Methods for the Examination of Water and Wastewater, 21st ed. Washington, DC.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357–389.

Barouki, R., Morel, Y., 2001. Repression of cytochrome P450 1A1 gene expression by oxidative stress: mechanisms and biological implications. Biochem. Pharmacol. 61, 511–516.

Bayir, A., Sirkecioglu, A.N., Byir, M., Haliloglu, H.I., Kocaman, E.M., Aras, N.M., 2011. Metabolic responses to prolonged starvation, food restriction, and refeeding in the brown trout, *Salmo trutta*: oxidative stress and antioxidant defenses. Comp. Biochem. Physiol. B 159, 191–196.

Beijer, K., Abrahamson, A., Brunström, B., Brandt, I., 2010. CYP1A inhibition in fish gill filaments: a novel assay applied on pharmaceuticals and other chemicals. Aquat. Toxicol. 96, 145–150.

Binelli, A., Ricciardi, F., Riva, C., Provini, A., 2006. New evidences for old biomarkers: effects of several xenobiotics on EROD and AChE activities in Zebra mussel (*Dreissena polymorpha*). Chemosphere 62, 510–519.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Camejo, G., Wallin, B., Enojärvi, M., 1998. Analyses of oxidation and antioxidants using microtiter plates. In: Amstrong, D. (Ed.), Free radical and Antioxidants Protocols. Humana Press, New Jersey, pp. 377–387.

Capdevila, M., Bofill, R., Palacios, Ò., Atrian, S., 2012. State-of-the-art of metallothioneins at the beginning of the 21st century. Coord. Chem. Rev. 256, 46–62.

Cappello, T., Maisano, M., D'Agata, A., Natalotto, A., Mauceri, A., Fasulo, S., 2013. Effects of environmental pollution in caged mussels (*Mytilus galloprovincialis*). Mar. Environ. Res. 91, 52–60.

Christensen, T.H., Kjeldsen, P., Bjerg, P.L., Jensen, D.L., Christensen, J.B., Baun, A., Albrechtsen, H., Heron, G., 2001. Biochemistry of landfill leachate plumes. Appl. Geochem. 16, 659–718.

Eggens, M.L., Galgani, F., 1992. Ethoxyresorufin-O-deethylase (EROD) activity in flatfish: fast determination within a fluorescence plate-reader. Mar. Environ. Res. 33, 213–221.

EPA – Environmental Protection Agency, 2013. Aquatic life ambient water quality criteria for ammonia – Freshwater.

Faria, M., Carrasco, L., Diez, S., Riva, M.C., Bayona, J.M., Barata, C., 2009. Multibiomarker responses in the freshwater mussel *Dreissena polymorpha* exposed to polychlorobiphenyls and metals. Comp. Biochem. Physiol. C 149, 281–288.

Gowland, B.T.G., McIntosh, A.D., Davies, I.M., Moffat, C.F., Webster, L., 2002. Implications from field study regarding the relationship between polycyclic aromatic hydrocarbons and glutathione S-transferase activity in mussels. Mar. Environ. Res. 54, 231–235.

Hodgson, E., Das, P.C., Cho, T.M., Rose, R.L., 2008. Phase I metabolism of toxicants and metabolic interactions. In: Smart, R.C., Hodgson, E. (Eds.), Molecular and Biochemical Toxicology, 4th ed. John Wiley & Sons, New Jersey, pp. 173–204

Jemec, A., Tisler, T., Zgajnar-Gotvajn, A., 2012. Assessment of landfill leachate toxicity reduction after biological treatment. Arch. Environ. Contam. Toxicol. 62, 210–221.

Kádár, E., Salánki, J., Jugdaohsingh, R., Powell, J.J., McCrohan, C.R., White, K.N., 2001. Avoidance responses to aluminium in the freshwater bivalve *Anodonta cygnea*. Aquat. Toxicol. 55, 137–148.

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the glutathione-S-transferase. J. Biol. Chem. 20, 6183–6188.

Kirby, M.F., Smith, A.J., Rooke, J., Neall, P., Scott, A.P., Katsiadaki, I., 2007. Ethoxyresorufin-O-deethylase (EROD) and vitellogenin (VTG) in flounder (*Platichthys flesus*): system interaction, crosstalk and implications for monitoring. Aquat. Toxicol. 81, 233–244.

Kurelec, B., Britvić, S., Pivčević, B., Smital, T., 1998. Fragility of multixenobiotic resistance in aquatic organisms enhances the complexity of risk assessment. Mar. Environ. Res. 46, 415–419.

Kurelec, B., Smital, T., Piveeviæ, B., Eufemia, N., Epel, D., 2000. Multixenobiotic resistence, p-glycoprotein and chemosensitizers. Ecotoxicology 9, 307–327.

Large, A.T., Shaw, J.P., Peters, L.D., McIntosh, A.D., Webster, L., Mally, A., Chipman, J.K., 2002. Different levels of mussel (*Mytilus edulis*) DNA strand breaks following chronic field and acute laboratory exposure to polycyclic aromatic hydrocarbons. Mar. Environ. Res. 54, 493–497.

Li, X., Lin, L., Luan, T., Yang, L., Lan, C., 2008. Effects of landfill leachate effluent and bisphenol A in glutathione and glutathione-related enzymes in the gills and digestive glands of the fresgwater snails *Bellamya purificata*. Chemosphere 70, 1903–1909.

Liao, C., Lin, C., Jou, L., Chiang, K., 2007. Linking valve closure behavior and sodium transport mechanism in freshwater clam *Corbicula fluminea* in response to copper. Environ. Pollut. 147, 656–667.

Livingstone, D.R., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Rev. Méd. Vét. 154, 427–430.

Long, S., Svendsen, C., Shore, R., Osborn D., Pottinger, T., Allen., Y., Thomas, K., Chipman, K., 2004. Evaluation of Potential Use of Biomarkers as Long-term Monitoring Tools in Assessing Ecological Quality in Terrestrial and Aquatic Environments of the UK, vol. 593. English Nature Report, 26 pp.

Luckenbach, T., Corsi, I., Epel, D., 2004. Fatal attraction: synthetic musk fragrances compromise multixenobiotic defense systems in mussels. Mar. Environ. Res. 58, 215–219.

Marie, V., Baudrimont, M., Boudou, A., 2006. Cadmium and zinc bioaccumulation and metallothionein response in two freshwater bivalves (*Corbicula fluminea* and *Dreissena polymorpha*) transplanted along a polymetallic gradiente. Chemosphere 65, 609–617.

Mdegela, R.H., Braathen, M., Mosha, R.D., Skaare, J.U., Sandvik, M., 2010. Assess of pollution in sewage ponds using biomarker responses in wild African sharptooth catfish (*Clarias gariepinus*) in Tanzania. Ecotoxicology 19, 722–734.

Morales, A.E., Pérez-Jiménez, A., Hidalgo, M.C., Abellán, E., Cardenete, G., 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol. C 139, 153–161.

Navas, J.M., Segner, H., 2000. Modulation of trout 7-ethoxyresorufin-O-deethylase (EROD) activity by estradiol and octylphenol. Mar. Environ. Res. 50, 157–162.

O'Connor, T.P., 2002. National distribution of chemical concentrations in mussels and oysters in the USA. Mar. Environ. Res. 53, 117–143.

Oliveira, M., Santos, M.A., Pacheco, M., 2004. Glutathione protects heavy metal-induced inhibition of hepatic microsomal ethoxyresorufin O-deethylase activity in *Dicentrarchus labrax* L. Ecotoxicol. Environ. Saf. 58, 379–385.

Osaki, K., Kashiwada, S., Tatarazako, N., Ono, Y., 2006. Toxicity testing of leachate from waste landfills using medaka (*Oryzias latipes*) for monitoring environmental safety. Environ. Monit. Assess. 117, 73–84.

Pablos, M.V., Martini, F., Fernández, C., Babín, M.M., Herraez, I., Miranda, J., Martínez, J., Carbonell, G., San-Segundo, L., García-Hortigüela, P., Tarazona, J.V., 2011. Correlation between physicochemical and ecotoxicological approaches to estimate landfill leachates toxicity. Waste Manage. 31, 1841–1847.

Ribé, V., Nehrenheim, E., Odlare, M., Gustavsson, L., Berglind, R., Forsberg, A., 2012. Ecotoxicological assessment and evaluation of a pine bark biosorbent treatment of five landfill leachates. Waste Manage. 32, 1886–1894.

Rocha, C.T., Souza, M.M., 2012. The influence of lead on different proteins in gill cells from the freshwater bivalve, *Corbicula fluminea*, from defense to repair biomarkers. Arch. Environ. Contam. Toxicol. 62, 56–67.

Santos, K.C., Martinez, C.B.R., 2014. Genotoxic and biochemical effects of atrazine and Roundup®, alone and in combination, on the Asian clam *Corbicula fluminea*. Ecotoxicol. Environ. Saf. 100, 7–14.

Smital, T., Kurelec, B., 1998. The chemosensitizers of multixenobiotic resistance mechanism inaquatic invertebrates: a new class of pollutants. Mutat. Res. 399, 43–53.

Smital, T., Luckenbach, T., Sauerborn, R., Hamdoun, A.M., Veja, R.L., Epel, D., 2004. Emerging contaminants pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms. Mutat. Res. 552, 101–117.

Stegeman, J.J., Brower, M., Di Giulio, R.T., Fórlin, L., Fowler, B.A., Sanders, B.M., Van Veld, P.A., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett, R.J., Kimerle, R.A., Mehrle , P.P., Bergman, H.L. (Eds.), Biomarkers. Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis Publishers, Chelsea, pp. 235–335

Thomas, D.J.L., Tyrrel, S.F., Smith, R., Farrow, S., 2009. Bioassays for the evaluation of landfill leachate toxicity. J. Toxicol. Environ. Health B. Crit. Rev. 12, 83–105.

Toufexi, E., Tsarpali, V., Efthimiou, I., Vidali, M., Vlastos, D., Dailianis, S., 2013. Environmental and human risk assessment of landfill leachate: an integrated approach with the use of cytotoxic and genotoxic stress indices in mussel and human cells. J. Hazard. Mater. 260, 593–601.

Tsarpali, V., Dailianis, S., 2012. Investigation of landfill leachate toxic potency: an integrated approach with the use of stress indices in tissues of mussels. Aquat. Toxicol. 124–125, 58–65.

Tsarpali, V., Kamilari, M., Dailianis, S., 2012. Seasonal alterations of landfill leachate composition and toxic potency in semi-arid regions. J. Haz. Mater. 233–234, 163–171.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, F., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Meditarranean and Antartic mollusks. Mar. Environ. Res. 44, 69–84.

Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein agaist oxidative stress in the mussel *Mytilus galloprovincialis*. Am. J. Physiol. 277, 1612–1619.

Vidal, M., Bassères, A., Narbonne, J., 2002. Influence of temperature, pH, oxygenation, water-type and substrate on biomarker responses in the freshwater clam *Corbicula fluminea* (Muller). Comp. Biochem. Physiol. C 132, 93–104.

Zhang, Y., Song, J., Yuan, H., Xu, Y., He, Z., Duan, L., 2010. Biomarker responses in the bivalve (*Chlamys farreri*) to exposure of the environmentaly relevant concentration of lead, mercury, cooper. Environ. Toxicol. Pharmacol. 30, 19–25.