Arch Environ Contam Toxicol (2016) 71:278–285
DOI 10.1007/s00244-016-0281-9

Multiple Biomarker Responses in *Corbicula fluminea* Exposed to Copper in Laboratory Toxicity Tests

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Received: 13 December 2015 / Accepted: 4 April 2016 / Published online: 18 April 2016

Abstract This study evaluated the alteration of the enzymatic system of the freshwater Asian clam exposed to different copper concentrations. Individuals of *Corbicula fluminea* were exposed to different concentrations of dissolved Cu (0.5, 1, and 2 mg L^{-1}) for 7 days, then, biomarkers of oxidative stress (GST, GPx, GR), exposure (MTs), effect (AChE), and damage (LPO, DNA strand breaks) were quantified. Results showed positive correlations between dissolved metal concentrations and GPx, MTs, and DNA damage, and negative correlation with GST and AChE. In contrast, no clear trend was found for GR and LPO. In general, the established mechanisms of protection might have a beneficial effect on the decreasing ROS attack on membrane and the activation of the metallothioneins. Integrated biomarker analysis revealed that the measured alterations are well correlated with the levels of increasing dissolved copper concentrations in water, demonstrating the effectiveness of this organism for biomonitoring approach purposes.

A biomarker is stated as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) that may be related to exposure or toxic effects of environmental chemicals redefined the terms, biomarker, bioindicator, and ecological indicator (van der Oost et al. 2003). Biomarkers provide valuable information in field or semi-field testing and could be used to measure a wide range of physiological responses to chemicals at the biochemical, cellular, or tissue level. They have been extensively used in environmental sciences since the late 1980s (Bayne et al. 1985; Huggett et al. 1992; Paekeall and Shugart 1993; Stagg 1998).

Aquatic organisms inhabiting contaminated environments can regulate the excess of metals by means of different mechanisms. Exposure to contaminants may induce or inhibit the enzyme synthesis or activity, or both (van der Oost et al. 2003). Metals can damage the organisms by increasing the internal levels of reactive oxygen species (ROS: peroxides-H_2O_2, superoxide-O_2^-, hydroxyl radical-OH^-) and, consequently, cause oxidative stress, lipid peroxidation (LPO), DNA damage, and, finally, cell death. Thus, the mechanisms to avoid the ROS involve antioxidant enzymes that metabolize (biotransform) contaminants through two phases. The phase I consists of an oxidative step, involving the lipotransformation of the functional molecules throughout the cytochrome P450. The phase II entails the conjugation reaction of the formed metabolite with an endogenous molecule (such as glutathione) to be excreted. The glutathione is a tripeptide (L-Y-glutamil-L-cisteinil-glicina) that participates in the cellular homeostasis regulation, including the metal detoxification and the oxi-radical release (Ringwood and Conners 2000). This is known as a defense mechanism against the oxidative stress. The glutathione conjugation with the metals avoids their harmful effects to other cellular components (Mason and Jenkins 1995).

The biotransformation mechanism involves the glutathione-S-transferase (GST) activity, an enzyme that

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catalyzes the conjugation of reduced glutathione (GSH) with a wide group of compounds carrying electrophilic centers. Additionally, the glutathione peroxidase (GPx) plays an important role in detoxification of cells from H_2O_2 through the reaction:

$$2GSH + H_2O_2 \rightarrow (GPx) \rightarrow GSSG + 2H_2O$$

The glutathione reductase (GR) also participates in the process by catalyzing the reduction of oxidized glutathione (GSSG) to GSH, using NADPH cofactor as an electron supplier (Doyotte et al. 1997).

$$GSSG + NADPH + H^+ \rightarrow (GR) \rightarrow NADP^+ + 2GSH$$

The measurement of GSH concentrations can signal that oxidative stress is occurring in response to metal or organic contaminant exposure (Stohs and Bagchi 1995). This is an endogenous tripeptide composed by amino acid a glutamate, cystine, and glycine rolled out of conjugation. It might have a double function: (1) transporting compounds to the place of phase I reaction; (2) or forming covalent bounds with activated epoxides preventing the activation of DNA or other macromolecules.

In contrast, increasing levels of xenobiotics may promote the inhibition of cholinesterases' activity. The acetylcholinesterase (AChE) in *Corbicula fluminea* (Mora et al. 1999) is mainly involved in neurotransmitter hydrolysis (Viarengo et al. 2007).

The metallothioneins (MTs) are low-molecular-weight proteins present in the cytosol, which participate in the regulation of the essential metals Cu and Zn (Roesijadi and Robinson 1994). The MTs also may play an antioxidant role, because they can bind the intracellular ionic Cu to prevent cytotoxicity (Luza and Speisky 1996).

Copper is a common metal in environments affected by mining activities. It is an essential element necessary for life, because it works as cofactor of several enzymes, such as the cytochrome c-oxidase, pyruvate hydrolase, bismutase, etc. Copper might be found in nature in the oxidized or reduced state, i.e., cupric (Cu^{2+}) or cuprous (Cu^+). The free Cu ions are involved in the formation of ROS (Gaetke and Chow 2003). However, the divalent form has greater electron affinity, and therefore, it more effectively binds to organic molecules. This form has a high affinity for the sulphydryl groups of enzymes and structural proteins (Viarengo and Nott 1993). Cu^{2+} can catalyze the hydroxyl radicals (OH^-) transformation into hydrogen peroxide (H_2O_2); this last product causes deleterious effects in cells and membranes as previously explained.

The tolerance of organisms to copper depends on their own ability to regulate the metal cation concentration inside the cell and to accumulate exceeding metals in nontoxic forms (Frausto da Silva and Williams 2001). Four mechanisms of homeostasis are identified in aquatic invertebrates to deal with excess of metals: (1) binding to specific soluble ligand (such as metallothioneins); (2) compartmentalization; (3) formation of insoluble precipitates; (4) enzyme active removal (ATP-ases and carboxilases).

The European Union establishes values below 1.3 mg Cu L^{-1} through the Water Framework Directive (WFD, Directive 2000/60/EC) for drinkable water standards, whereas the Brazilian water quality standards are limited to 2 mg Cu L^{-1}. Although the environmental protection are limited to 0.009 mg Cu L^{-1} (waters classified as 1 and 2) and 0.013 mg L^{-1} (Class 3) by the Brazilian Conselho Nacional do Meio Ambiente (CONAMA 357/2005).

The main objective of this study is to evaluate the enzymatic responses in the freshwater clam (*C. fluminea*) acutely exposed to a gradient of copper concentration to obtain initial data of the biochemical behavior of the clam. These data will be used to determine if oxidative stress may be induced by the metal contamination of concern at realistic exposure concentrations in highly metal polluted environments. Furthermore, this investigation aims to address the effectiveness of biomarkers to be used in monitoring studies related to environmental risk assessment.

Materials and Methods

Similarly sized clams (17 ± 2-mm shell length) were collected in the Ribeira de Iguape River (Sete Barras, 24°23′16″S, 47°55′33″W). Clams were transported to the laboratory in site water, and they remained 24 h at room temperature before being transposed into aired commercial water, where they remained for 3 days before toxicity tests. The clam sampling site has been previously characterised below the water National Standards for Cu (<0.009 mg L^{-1}) (Morais et al. 2013) and below the TEL values of the USEPA for Cu in freshwater sediment (Abessa et al. 2014). Copper concentrations in soft tissue of the clam in the sampling site registered values of 55.1 ± 8.7 mg kg^{-1} dw.

Laboratory toxicity tests are generally used to predict the possible further effects, as opposed to simply monitoring the effects after they have occurred. The toxicity tests were conducted with concentrations according to a gradient of 0.5, 1, and 2 mg L^{-1} Cu spiked water for 7 days of exposure (termed as 0.5 Cu, 1 Cu, and 2 Cu, respectively). Therefore, a prepared Cu stock solution was

made by diluting $CuCl_2$ with commercial water. Fifteen individuals were placed in the test tanks for 7 days and kept under a photoperiod of 16 h light/8 h dark, at 20 °C room temperature, with aeration and no food supply. Mortalities were daily checked using the commercial water as control. Clams were sampled at two different time points during the experiment: initial time (24 h after sampling collection: C24h) and after exposure (0.5 Cu, 1 Cu, 2 Cu from the test tanks) and control (at the end of the tests: C10d).

After 7 days of exposure and to evaluate the enzymatic activity response, the whole soft bodies of the clams were removed and individually homogenized in a buffer solution of 50 mM TRIS, 1 mM dithiothreitol (DDT), 1 mM EDTA, and 50 mM sacarose and 150 mM KCl (pH 7.6). A part of the homogenate fraction was reserved for lipid peroxidation (LPO) and DNA strand damage measurements, whereas the other part was centrifuged (15,000 rpm for 30 min at 4 °C) providing a supernatant fraction (S_{15}) destined to glutathione related enzymes (GR, GPx, and GST), and acetylcholinesterase (AChE) analyses. Another fraction was separated for the determination of metallothionein-like proteins, which was homogenized with 20 mM Tris–HCl buffer supplemented with 0.5 M sucrose, 0.01 % β-mercaptoethanol, and centrifuged at $15{,}000\times g$ for 30 min at 4 °C. Total protein contents in homogenized and S_{15} fraction were analyzed according the dye-binding principle (Bradford 1976).

Lipid Peroxidation

Lipid peroxidation (LPO) was measured according to the protocol described by Wills (1987), which evaluates the homogenates for the amounts of thiobarbituric acid reactive substances (TBARS). The TBARS were determined by fluorescence at 516 nm for excitation and 600 nm for emission using a fluorescence microplate reader after 10 min incubation at 70 °C. Results were obtained through a calibration curve and expressed as µg of TBARS per mg of total protein.

Glutathione Metabolism

The procedure used to determine glutathione activity was described by McFarland et al. (1999). The glutathione peroxidase (GPx) activity was measured spectrophotometrically (340 nm, every 10 s for 3 min at 30 °C) with addition of 1 mM of cumene hydroperoxide and 1.25 mM of hydroperoxide as fresh substrate. The GPx assay buffer had 50 mM potassium phosphate (pH 7), 0.1 mM EDTA, and 0.15 mM sodium azide. The procedure utilised for the determination of the glutathione S-transferase (GST) activity was described by McFarland et al. (1999) and adapted by Martín-Díaz et al. (2009). The glutathione reductase (GR) kinetic was measured in the same wave length every 2 min for 10 min at 30 °C. The buffer was prepared with a potassium phosphate (pH 7.6); GSSG (30 mM) and NADPH (0.8 mM) worked as substrate. Results were expressed as µmol NADPH min^{-1} mg^{-1} total protein.

Acetylcholinesterase

Acetylcholinesterase (AChE) activity was analyzed using a colorimetric method (Ellman et al. 1961). Absorbance readings (at 415 nm) were made during every 5 min during 20 min. A phosphate buffer (0.1 M, pH 7.6), acetylcholine iodide (0.075 M), and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, 10 mM) were used for these analyses. Results were expressed as µmol DTNB min^{-1} mg^{-1} total protein.

DNA Strands Breaks

DNA damage was assessed by an alkaline precipitation assay (Olive 1988) based on the alkaline K-SDS precipitation of DNA protein cross-link. DNA quantification was achieved using 100 nM Hoescht dye (pH 8.5) in 0.1 M of Tris–HCl, 40 mM of NaCl, and 4 mM of sodium cholate. Salmon sperm genomic DNA was used as standard and fluorescence readings were taken at 360 nm excitation, 450 nm emission, and 10 flashes. The results were expressed as µg of DNA mg^{-1} total protein.

Metallothionein Protein Content

Metallothionein concentration was evaluated utilizing a partially purified metalloprotein fraction obtained by the acidic ethanol/chloroform fractionation of the homogenized samples according to the procedures described in Viarengo et al. (1997). The concentration of metallothionein protein content (MTs) was quantified at 412 nm using Ellman's reagent containing reduced glutathione (GSH) as a reference standard (Viarengo et al. 1997).

The isolated responses of biomarkers were statistically analyzed by one-way ANOVA compared the control 24 h with a Dunnetts post hoc test ($p < 0.05$). The relationship between the Cu concentration and the biomarker responses were extrapolated by a first-order linear regression using average enzymatic responses for each treatment.

Finally, the integration of biomarker data into multiple-biomarker indices for the evaluation of contaminant-induced stress was performed through the Biomarker Response Index (BRI) proposed by Beliaeff and Burgeot (2002) and modified by Sánchez et al. (2013) as Integrated Biological Responses version 2 ($IBRv_2$). For a single site, parameters were reported in a star-plot to represent

reference deviation of each investigated biomarker. The area up to 0 reflects biomarker induction, and the area down to 0 indicates a biomarker inhibition.

Results and Discussion

As previously mentioned, biomarkers are used in risk assessment studies as tools of early diagnosis to analyze the environment health. The biomarkers assessed in the Asian clam exposed for 7 days to concentrations of dissolved Cu concentrations produced effects on the exposed animals.

In the present work, no mortality was registered in the control tank and in 0.5 Cu and 1 Cu spiked tanks. Nevertheless, two deaths were recorded on the 5th and 6th days in the 2 mg L^{-1} Cu tank.

Figure 1 shows the results obtained from the biomarker dose–responses in clams exposed to the 0.5, 1, and 2 mg of L^{-1} of dissolved copper and the controls. Among the antioxidants measurements, the enzymes of glutathione (GST, GR, GPx) were significantly altered in clams exposed to the different Cu treatments (Fig. 1). Glutathione concentrations were significantly elevated ($p < 0.05$) in clams from the high-exposure treatment after 7 days. No significant effects ($p < 0.05$) on glutathione were observed

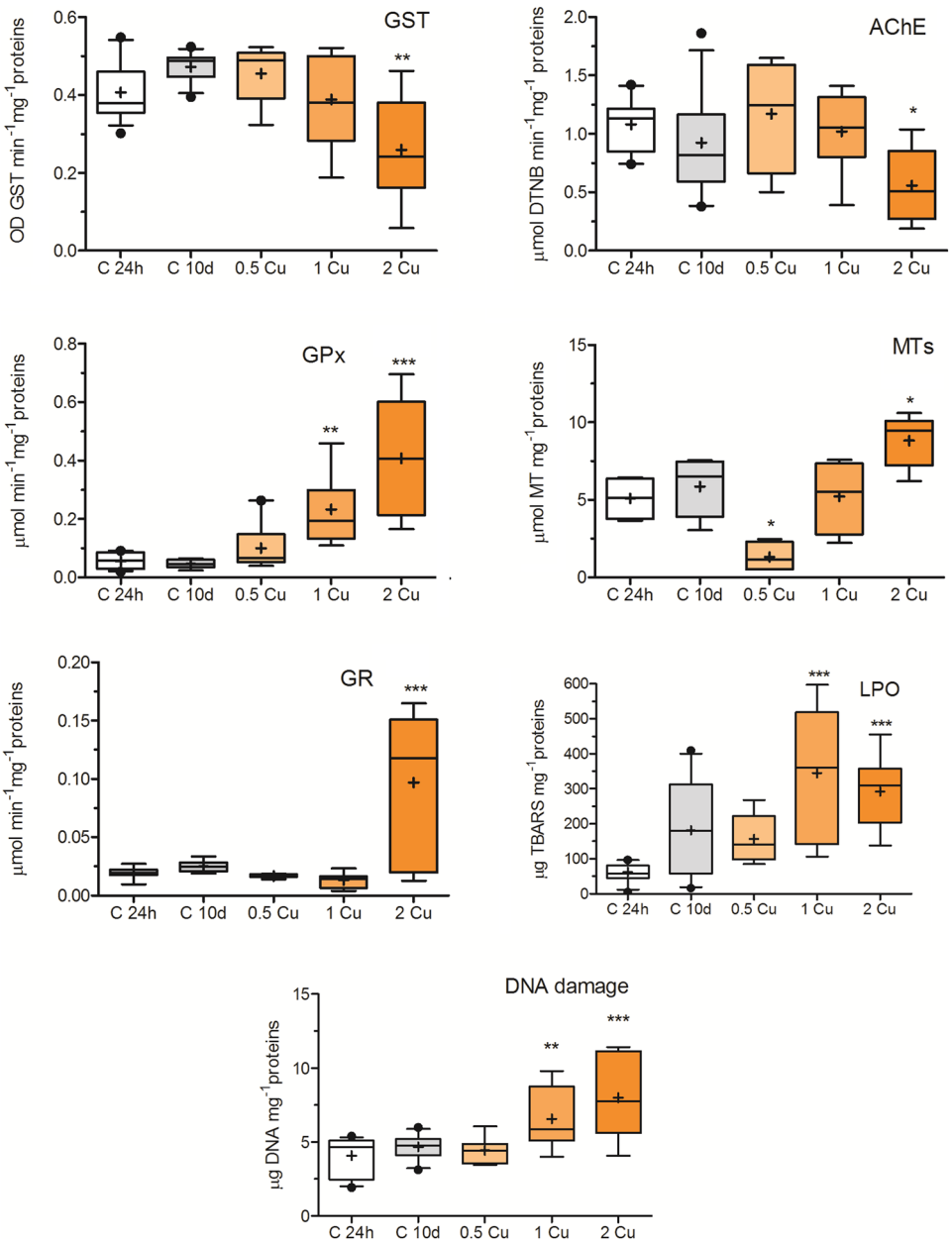

Fig. 1 Box plot representation of different biomarker responses displayed by the Asian clam after 24 h (C24h) and 10 days (C10d) acclimatation after collection and the different responses into the spiked treatments: 0.5, 1, and 2 mg of Cu L^{-1} (0.5 Cu, 1 Cu, and 2 Cu). *Asterisk* represents significance level pointed by the ANOVA respect C24h: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

in clams from the low-exposure treatment (0.5 Cu). Clams exposed to 1 and 2 mg of L^{-1} Cu showed an increase of GPx activity compared with the control, whereas only clams from the 2 mg L^{-1} treatment displayed an increase of GR activity and a decrease of GST ($p < 0.05$).

Doyotte et al. (1997) also pointed changes in GR within 7-day exposure as cellular antioxidant protector to regenerate reduced glutathione, in agreement with findings of this study. Significant reduction ($p < 0.05$) in MT content was detected in clams from the 0.5 Cu tanks and a significant peak ($p < 0.05$) in clams from the 2 Cu tanks, but there was a correspondence between the dose–response of whole tissue of *C. fluminea* exposed for 7 days to the different concentrations (Fig. 1). Similar MT responses in *C. fluminea* and *D. polymorpha* under polymetallic pollution gradient were revealed by Marie et al. (2006) and attributed to an increased protection against toxicity.

Acetylcholinesterase (AChE) assays showed a decreasing activity in clams exposed to 2 mg of Cu^{2+} L^{-1} for 7 days. This enzyme is widely known to be involved in the transmission of nerve impulses, and its inhibition has been used as a specific biomarker for organophosphate (OP) and carbamate (CB) pesticide pollution (Bocquené et al. 1990; Key and Fulton 2002). However, recent findings indicated that some metals and detergents also can inhibit the AChE activity in aquatic organisms at both in vivo and in vitro conditions, but the mechanism of AChE inhibition by metals is still unknown (Kopecka-Pilarczyk 2010). For example, Frasco et al. (2005) analyzed the inhibitory effect of metals on AChE activity and despite the limitations of methods as the possible interference of metals with buffer constituents, Hg, Cd, Zn, and Cu induced inhibition of AChE activity.

Compared with the control group (C24h), LPO quantities were significant difference ($p < 0.001$) in clams from the intermediate and higher concentrations (1 Cu and 2 Cu). This quantity was low ($p > 0.05$) in the tissues of animals kept at lower Cu concentrations (0.5 mg L^{-1}). The variability was very high between clams within 1 mg L^{-1} Cu treatment but significantly different ($p < 0.001$) than the control group. LPO after 6 days of Cu^{2+} exposure was observed in gills and digestive glands of *Mytillus galloprovincialis* (Viarengo et al. 1990).

According to the hypothetical graph of dose–response patterns presented by Depledge (1994), the oxidative stress in xenobiotic-exposed bivalves involves three phases of stress (alarm, compensation, and exhaustion). Therefore, the antioxidant enzymes represented by the GR, GPx, and GST might be indicative of the alarm phase. In their turn, the oxidative damage, represented by the LPO and DNA damage, would represent the exhaustion phase.

The linear regression analysis of averaged biomarker responses with the different Cu concentrations evidenced induction for MTs, GPx, and DNA damage and inhibition for AChE and GST (Table 1). In contrast, LPO neither displayed a good regression fit ($R^2 = 0.159$), not a clear tendency for GR. Additionally, this might be explained by a hormesis event, followed by an inducted activity for greater Cu concentration (2 Cu).

Summarized equations in the Table 1 might be used to obtain predictive enzymatic results when exposing the Asian clam to a determinate concentration of copper and vice versa. However, this is a simplistic model, because it only provides approaches under laboratory conditions and demonstrates the trend (activation/inhibition) of single biomarker to individual contamination of Cu.

The biomarker results were presented as an integrative response according to the Integrated Biological Response developed by Sánchez et al. (2013), based on a modification of the classical IBR proposed by Beliaeff and Burgeot (2002). This second version brings two main advantages: (1) it eliminates the dependency on the arrangement of the biomarkers on the star plot; and (2) it allows discriminating induction and inhibition for each biomarker.

As the first IBR has been widely used, this study also presents the results as proposed by the classical IBR for comparison with other studies. The novel version is based on the principle of the reference deviation. The first reference treatment (C24h) was established as basal line. The set of measured biomarkers (AChE, MTs, GST, GPx, GR, LPO, and DNA damage) in *C. fluminea* were represented against these basal values. When comparing the initial reference values (C24h) against the second reference treatment (C10d), it is possible to observe that the IBRv2 provides a robust basal value along the time.

The IBRv2 draws an integrated response of biomarkers as reflected in Fig. 1. It provides information of the inhibitory activity of GST and AChE with the increasing concentration of Cu, and an induction or activation of LPO, GPx, and DNA strand damage. Several biomarkers exhibit a response that might be induced or inhibited according to contamination degree. This is reflected by the IBRv2, which displayed an increasing value in presence of dissolved Cu: 4.54 for 0.5 Cu, 6.58 for 1 Cu, and 12.5 for 2 Cu.

Glutathione activity (GSH) plays a fundamental role in redox reaction as transportation of amino acids and detoxification of many toxic agents; this is the first line of defense against cell lesions mediated by oxidants (van der Oost et al. 2003). The decrease in GSH might have impaired the antioxidant capacity of the organisms, leading to an excess of free oxygen species that could cause oxidative damage. The findings of the current work indicate that dissolved Cu inhibits GST activity (Table 1; Fig. 2). In contrast, the defense systems that inhibit oxy-radical formation are active as reflected by the increased

Table 1 Linear modeling based on the average response versus the different treatments

	Equation	Adjust (R^2)	Effect
AChE	µmol DTNB $min^{-1}mg^{-1}$ proteins = $-0.4176 * C_{Cu} + 1.4038$	0.9904	↓
MTs	µmol MT mg^{-1} proteins = $4.7983 * C_{Cu} - 0.4749$	0.9553	↑
GST	OD µmol min^{-1} mg^{-1} proteins = $- 0.1302 * C_{Cu} + 0.5194$	0.9999	↓
GPx	µmol min^{-1} mg^{-1} proteins = $0.2005 * C_{Cu} + 0.0128$	0.9877	↑
GR	µmol min^{-1} mg^{-1} proteins = $0.0577 * C_{Cu} - 0.025$	0.8687	ND
DNA damage	µg DNA mg^{-1} proteins = $1.783 * C_{Cu} + 2.775$	0.9876	↑
LPO	µg TBARS mg^{-1} proteins = $62.539 * C_{Cu} + 208.67$	0.159	ND

↑ induction/increase; ↓ inhibition/decrease

ND not determined, C_{Cu} represents the dissolved concentration of copper

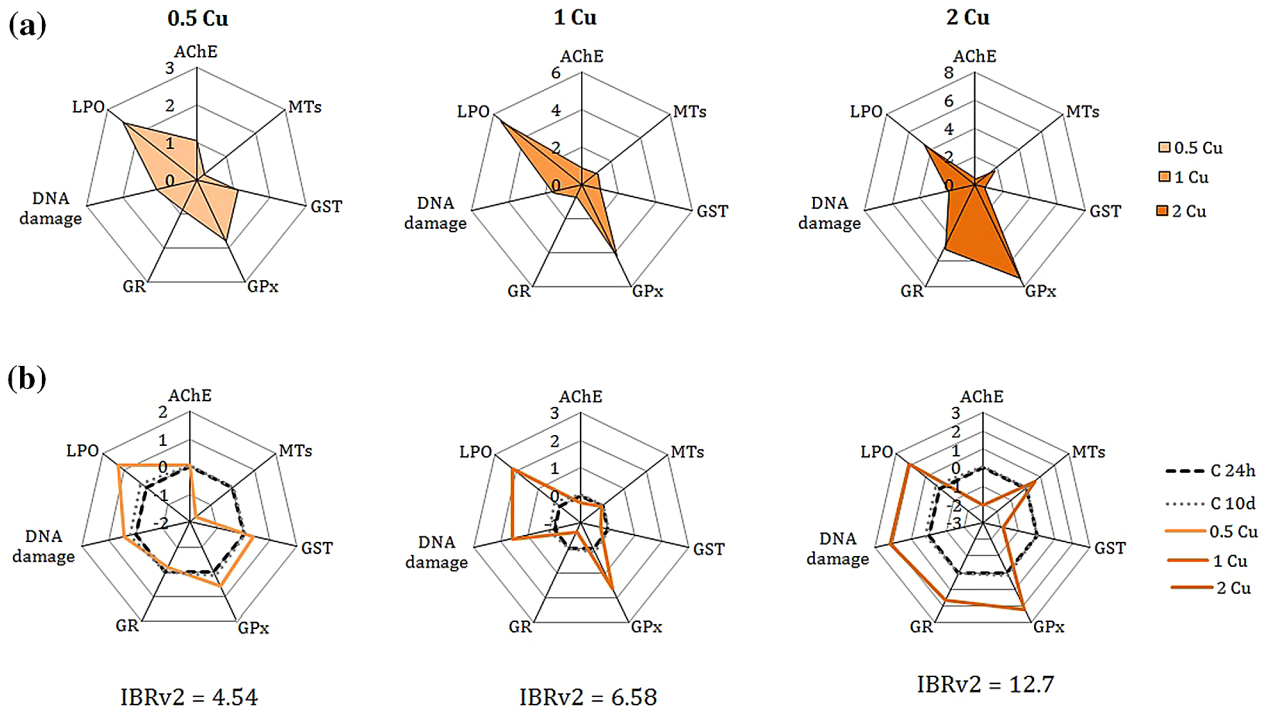

Fig. 2 Theoretical comparison of classical IBR star plot developed by Beliaeff and Burgeot (2002) with the novel version IBRv2 developed by Sánchez et al. (2013). The IBRv2 values are associated to star plots for the three treatments (0.5 Cu, 1 Cu, and 2 Cu) for *C. fluminea* for the different biomarkers

GPx activity. In fact, the exposed organisms exhibited slightly increase levels of LPO characterizing the oxidative stress in presence of Cu >1 mg L^{-1} in water. Overall, metals induce oxidative changes that are evidenced by the build-up of ROS, LPO, and protein and DNA oxidation (Bagchi et al. 1995; Franco et al. 2009), as evidenced in our study. Increased LPO levels lead to membrane integrity loss and, with increased permeability, a change in the flow of ions across the membrane, dysfunction in the transportation of Na^+/K^+, excessive inflow of Ca ions, and activation of enzymes, such as proteases, phospholipases, and nucleases (Meagher and Fitzgerald 2000; Valavanidis et al. 2006). Based on the occurrence of genetic damage of clams after exposure, it is reasonable to suppose that additional effects would occur on other cell components, such as nucleic acids.

There is a positive correlation between the Cu concentrations and the MT contents. MTs constitute a family of low-molecular-weight, cysteine-rich proteins functioning in the regulation of the essential metals Cu and Zn, and in the detoxication of these and other nonessential elements, such as Cd and Hg (Roesijadi and Robinson 1994). The Asian clam seems to be a useful species in metallothionein biomarker testing. Some laboratory experiments have determined induction of MTs over very short time (Barka et al. 2001). The affinity of Cu by the thiol groups of the

MTs of the Asian clam also has been observed for other types of contaminants containing Cu (Capdevila et al. 2012; Oliveira et al. 2014), as in other bivalves species, such as *Mytilus galloprovincialis* (Tsarpali and Dailianis 2012).

The overall biomarkers' behavior suggests that after 7-day exposure, antioxidant enzymes, such as GST and GPx, were induced to avoid oxidative stress caused by Cu. On the other hand, MTs work as detoxification mechanisms; however, this induction is not fast or efficient enough to prevent lipid peroxidation under 1 mg dissolved Cu per liter. These results are in agreement with Gaetke and Chow (2003) findings regarding the Cu-induced oxidative damage associated with abnormal Cu metabolism and neurodegenerative changes to avoid damages. Previous studies support the idea of the Asian clam as a good biomonitor of Cu contaminated environments due to a positive correlation Cu dose-bioaccumulation response (Graney et al. 1983; Bonnail et al. 2016).

Conclusions

This study provides a first appraisal of the amounts of antioxidant enzymes, MTs, LPO, and DNA strand damage in general soft tissue of the Asian clam when exposed to different concentrations of dissolved copper. *C. fluminea* appears to cope effectively with copper toxicity, due to its ability to induce specific cellular defense mechanisms against oxidative stress.

Different behaviors of the biomarker responses were observed related to the dose-concentration. According to the dissolved metal concentration, the biomarker responses observed in this study appear to follow dose-response linear relationship: positive for MT, GPx, and DNA strand damage, and negative for AChE and GST. Nevertheless, this study should be understood as the response at a punctual period of time. Due to that, it is recommended the use of biomarker assays that specifically consider the temporal progression of ecotoxicological responses.

Acknowledgments The first author thanks the International Grant from Bank Santander/UNESCO Chair UNITWIN/WiCop and the Erasmus Mundus Programme for the MACOMA Doctoral funding contract (SGA 2012-1701/001-001-EMJD). We thank the Brazilian National Council for Scientific and Technological Development (CNPq; Grant #479899/2013-4), the São Paulo Research Foundation (FAPESP, Grant #2009/52762-6), and the Brazilian Aquatic Research Foundation (FUNDESPA) for the financial support. Dr. Lucas Buruaem thanks FAPESP (Grant #2013/15482-0, São Paulo Research Foundation) and Dr. Abessa thanks CNPq (Grants 308649/2011-7 and 311609/2014-7). The authors thank Dr. Van Den Brink and the reviewers for their expert guidance that has contributed to the improvement of the manuscript and R. Ojeda for the English language review.

References

Abessa DMS, Gonçalves L, Fernando C, Davanso MB, Guimarães V et al (2014) Sediment geochemistry and climatic influences in a river influenced by former mining activities: the case of Ribeira de Iguape River, SP-PR, Brazil. Open J Water Pollut Treat 1:43–54

Bagchi D, Bagchi M, Hassoun EA, Stohs SJ (1995) In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides. Toxicology 104:129–140

Barka S, Pavillon JF, Amiard JC (2001) Influence of different essential and non- essential metals on MTLP levels in the copepod *Tigriopus brevicornis*. Comp Biochem Physiol C 128:479–493

Bayne BL, Brown DA, Burns K, Dixon DP, Ivanovici A, Livingstone DR, Lowe DM et al (1985) The effects of stress and pollution on marine animals. Praeger Scientific, New York

Beliaeff B, Burgeot T (2002) Integrated biomarker response: a useful tool for ecological risk assessment. Environ Toxicol Chem 21(6):1316–1322

Bocquené G, Galgani F, Truquet P (1990) Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. Mar Environ Res 30:75–89

Bonnail E, Sarmiento AM, DelValls TA, Nieto JM, Riba I (2016) Assessment of metal contamination, bioavailability, toxicity and bioaccumulation in extreme metallic environments (Iberian Pyrite Belt) using *Corbicula fluminea*. Sci Total Environ 544:1031–1044

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 2:248–254

Capdevila M, Bofill R, Palacio O, Atrian S (2012) State-of-the-art of metallothioneins at the beginning of the 21st centuruy. Coord Chem Rev 256:46–62

CONAMA 357/2005. Resoluçao N257, de 17 de Março de 2005. http://www.mma.gov.br/port/conama/res/res05/res35705.pdf

Depledge MH (1994) The rational basis for use of biomarkers as ecotoxicological tools. In: Fossi MC, Leonzio C (eds) Nondestructive biomarkers in vertebrates. Lewis Publishers, Boca Raton, pp 271–295

Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy. http://ec.europa.eu/environment/water/water-framework/index_en.html

Doyotte A, Cossu C, Jacquin MC, Babut M, Vasseur P (1997) Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquat Toxicol 39(2):93–110

Ellman GL, Courtney D, Andres JV, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–96

Franco R, Sánchez-Olea R, Reyes-Reyes EM, Panayiotidis MI (2009) Environmental toxicity, oxidative stress and apoptosis: ménage à trois. Mutat Res 674:3–22

Frasco MF, Fournier D, Carvalho F, Guilhermino L (2005) Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10(5):360–375

Frausto da Silva JJR, Williams JP (2001) Copper: extracytoplasmic oxidases and matrix formation. In: Silva JJR, Williams JP (eds) The biological chemistry of the element, 2nd edn. Oxford University Press, Oxford

Gaetke LM, Chow K (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189:147–163

Graney RL, Donald JS, Cairns CJ Jr (1983) Heavy metal indicator potential of the Asiatic clam (*Corbicula fluminea*) in artificial stream systems. Hydrobiologia 102:81–88

Huggett JR, Kimerle RA, Mehrle PM, Bergman HL (1992) Biomarkers, biochemical, physiological, and histological markers and anthropogenic stress. Lewis, Boca Raton, p 347

Key PB, Fulton MH (2002) Characterization of cholinesterase activity in tissues of the grass shrimp (*Palaemonetes pugio*). Pestic Biochem Physiol 72:186–192

Kopecka-Pilarczyk J (2010) The effect of pesticides and metals on acetylcholinesterase (AChE) in various tissues of blue mussel (*Mytilus trossulus L.*) in short-term in vivo exposures at different temperatures. J Environ Sci Health B 45:336–346

Luza SC, Speisky HC (1996) Liver copper storage and transport during development: implications for cytotoxicity. Am J Clin Nutr 63:812S–820S

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 gradient. Chemosphere 65:609–617

Martín-Díaz ML, Blasco J, Sales D, DelValls TA (2009) The use of a kinetic biomarker approach for in situ monitoring of littoral sediments using the crab *Carcinus maenas*. Mar Environ Res 68(2):82–88

Mason AZ, Jenkins KD (1995) Metal detoxification in aquatic organisms. In: Tessier A, Turner DR (eds) Metal speciation and bioavailability in aquatic systems. Wiley, Chichester, p 679

McFarland VA, Inouye SL, Lutz CH, Jarvis AS, Clarke JU, McCant DD (1999) Biomarkers of oxidative stress and genotoxicity in livers of field collected brown bullhead, *Ameiurus nebulosus*. Arch Environ Contam Toxicol 37:236–241

Meagher EA, Fitzgerald GA (2000) Indices of lipid peroxidation in vivo: strengths and limitations. Free Radic Biol Med 28:1745–1750

Mora P, Fournier D, Narbonne JF (1999) Cholinesterases from the marine mussels *Mytilus galloprovincialis* Lmk. and *Mytillus edulis* from the freshwater bivalve *Corbicula fluminea* Müller. Comp Biochem Physiol C 122:353–361

Morais LG, Perina FC, Davanso MB, Buruaem LM, Rodrigues VGS, Sígolo JB, Abessa DMS (2013) Water and sediment ecotoxicological assessment in a river affected by former mining activities. Pan-Am J Aquat Sci 8(4):327–338

Olive PL (1988) DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. Environ Mol Mutagen 11:487–495

Oliveira LF, Silva SMCP, Martinez CBR (2014) Assessment of domestic landfill leachate toxicity to the Asian clam *Corbicula fluminea* via biomarkers. Ecotoxiol Environ Safe 103:17–23

Paekeall DB, Shugart RL (1993) Research and application in the assessment of environmental health. In: Paekall DB, Shugart RL (eds) Biomarkers: research and application in the assessment of environmental health. NATO ASI Series—Series H: Cell Biology 68. NATO, Texel

Ringwood AH, Conners DE (2000) The effects of glutathione depletion on reproductive success in oysters, *Crassostrea virginica*. Mar Environ Res 50:207–211

Roesijadi G, Robinson WE (1994) Metal regulation in aquatic animals: mechanisms of uptake, accumulation and release. In: Malins DC, Ostrander GK (eds) Aquatic toxicology; molecular biochemical and cellular perspectives. CRC Press, Boca Raton

Sánchez W, Burgeot T, Porcher JM (2013) A novel "Integrated Biomarker Response" calculation based on reference deviation concept. Environ Sci Pollut R 20(5):2721–2725

Stagg RM (1998) The development of an international programme for monitoring the biological effects of contaminants in the OSPAR convention area. Mar Environ Res 46:307–313

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal-ions. Free Radic Biol Med 18:321–336

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

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol Environ Safe 64:178–189

van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Viarengo A, Nott JA (1993) Mechanisms of heavy metal cation homeostasis in marine invertebrates. Comp Biochem Phys C 104:355–372

Viarengo A, Canesi L, Pertica M, Poli G, Moore MN, Orunesu M (1990) Heavy metal effects on lipid peroxidation in the tissues of *Mytilus galloprovincialis* Lam. Comp Biochem Physiol 97C:37–42

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

Viarengo A, Lowe D, Bolognesi C, Fabbri E, Koehler A (2007) The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp Biochem Phys Part C 146:281–300

Wills ED (1987) Evaluation of lipid peroxidation in lipids and biological membranes. In: Mullock B, Snell K (ed) Biochemical toxicology a practical approach. IRL Press, Oxford, pp 127–150