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Toxicological responses of *Laeonereis acuta* (Polychaeta, Nereididae) after acute, subchronic and chronic exposure to cadmium

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ABSTRACT

The objective of this study was to analyze the toxicological responses of the estuarine polychaete *Laeonereis acuta* after acute (96 h), subchronic (7 days) and chronic (14 days) exposure to cadmium (Cd). Concentrations of metallothioneins (MT), lipid peroxidation (LPO), total Cd and metal-rich granules (MRG) were evaluated. Seasonal variations of MT and LPO levels in the wild were also measured. Polychaetes were obtained in the Quequén estuary located southeast of Buenos Aires Province, Argentina. For the acute toxicity assay, individuals were exposed to 10; 30, 65; 310; 600; 1300; 2000; 4300; 8100; 16300 μgCd L^{-1}, which included levels of environmental relevance and median lethal concentrations (LC_{50}) for related species of polychaete. Based on 96 h LC_{50} values, polychaetes were exposed to sublethal doses of Cd. The concentrations for both subchronic and chronic assays were: 10; 30; 65; 310; 600; 1300; 2000; 4300 μgCd L^{-1}. The 96 h LC_{50} value was 8234.9 μg L^{-1}, which was within the values reported for other species of polychaete, indicating a high tolerance to Cd. MT induction was not observed for any time exposure. In additoin, LPO levels showed no differences with respect to control levels, which indicated an absence of oxidative damage caused by Cd. However, the total Cd and MRG-Cd concentrations in *L. acuta* in all tested treatments showed significant differences with respect to control levels. *L. acuta* were able to accumulate Cd in their tissues in the form of granules which are the main mechanism of Cd detoxification.

1. Introduction

Cadmium (Cd) is a persistent toxic metal that is ubiquitous in aquatic environments. Sources of Cd to aquatic habitats include mine drainage, wastewater from metal smelting, runoff of agricultural fertilizers, and atmospheric fallout from fossil fuel combustion and refuse incineration (Timbrell, 2001; Martelli et al., 2006). Among marine ecosystems, shallow coastlines and estuaries are most affected by human activities because they are exposed to toxic anthropogenic effluents transported by rivers from remote and nearby urban, industrial and agricultural areas (Kennish, 2002). Sediments in these environments can act as a sink and source of many contaminants including Cd (Förstner and Wittmann, 1983; Dekov et al., 1998). As a result, benthic organisms living in close contact with sediments are particularly exposed to chemical stress.

Polychaetes, the most abundant taxon in benthic communities, are considered a suitable model species for the study of sediment and estuarine pollution (Nusetti et al., 2001; Pérez et al., 2004; Ait Alla et al., 2006; Rhee et al., 2007). In particular, polychaetes are commonly used in ecotoxicological studies due to their abundance, easy capture (Díaz-Jaramillo et al., 2011; Suriya et al., 2012; Won et al., 2012) and assimilation of heavy metals from sediments through their skin and intestine (Durou et al., 2005; Sun and Zhou, 2007). Cd is known to be toxic at low concentrations (Zang and Bolger, 2014). Many studies have reported enhanced lipid peroxidation and DNA damage after Cd treatment (Stohs and Bagchi, 1995; Tandon et al., 2003; Badisa et al., 2007).

Organisms have several defense mechanisms to toxic metals, and these systems can provide suitable biomarkers for the assessment of environmental stress (Won et al., 2012). Biomarkers are biochemical or physiological indicators of either exposure to or effects of environmental contaminants at the suborganism or organism level (Shugart et al., 1992; Livingstone, 1993: Sarkar et al., 2006). For this reason, biomarkers are regarded as early warning signals whose detection can avoid adverse effects (Van der Oost et al., 2003).

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The induction of metallothioneins (MT) in aquatic organisms has been recognized as a potential biomarker of heavy metal toxicity and bioaccumulation (Berthet et al., 2005; Amiard et al., 2006; Perceval et al., 2006; Martín-Díaz et al., 2007; Monserrat et al., 2007; Machreki-Ajmi et al., 2008). MT constitute a family of low molecular weight, cysteine-rich and heat stable proteins (Viarengo et al., 2007). The thiol (–SH) groups in MT enable them to bind heavy metals and sequester them in the organism. They play important roles in the homeostasis of essential metals such as zinc -Zn- and copper -Cu-, as well as detoxification of non-essential metals (e.g., Cd and mercury –Hg-) (Viarengo, 1989; Roesijadi, 1996; Vašák, 2005). MT induction is also considered an indicator of antioxidant processes and free-radical scavenging (Viarengo et al., 2000, 2007). Another mechanism of metal detoxification found in most invertebrates, including annelids, is precipitation into insoluble granules (or metal-rich granules; MRG) (Ng et al., 2008; Eisler, 2010; Khan et al., 2010). These granules are generally found in epithelial cells. Heavy metal cations are removed from the cytoplasm and sequestered within the vacuolar membrane in an insoluble, detoxified form. Subsequent cellular exocytotic events may extrude the granules from the cell followed by organismic excretory mechanisms that deposit the metal back into the environment (Fernandez and Jones, 1989; Ahearn et al., 2004).

Lipid peroxidation (LPO) is considered an important biomarker of cell damage resulting from the interaction of free radicals with membrane lipids (Barata et al., 2005). It has been used extensively to assess the detrimental effects of various pollutants, such as polycyclic aromatic hydrocarbons (Díaz-Jaramillo et al., 2011), fullerene and nanosilver (Marques et al., 2013) and Cd, Cu, Zn, Hg (Geracitano et al., 2002, 2004a, 2004b; Sandrini et al., 2006, 2008; Díaz-Jaramillo et al., 2011, 2013).

The deposit feeding polychaete *Laeonereis acuta* (Polychaeta: Nereididae) is a common infaunal species that has a wide distribution from Connecticut (United States) to the Northern Gulf of Mexico and from Santos (Brazil) to Golfo Nuevo (Patagonia, Argentina) (de Jesús-Flores et al., 2016). L. *acuta* is considered as a key estuarine species due to their influence on sediment properties and local diversity of species that prey on it (Botto et al., 1998; Palomo and Iribarne, 2000). Additionally, this species has been used as a biomonitor of heavy metals (Ferreira-Cravo et al., 2007; Geracitano et al., 2002, 2004a; Sandrini et al., 2006), as well as a model organism in toxicological assays (Geracitano et al., 2002, 2004b; Sandrini et al., 2006, 2008; Ferreira-Cravo et al., 2009; Marques et al., 2013).

Numerous toxicological studies have been carried out in the species, but there are few with regards to Cd (Sandrini et al., 2006, 2008) even though it is an ubiquitous and highly toxic pollutant. The objective of this study was to analyze the toxicological responses of *Laeonereis acuta* after acute, subchronic and chronic exposure of a wide range of Cd concentrations including levels typical of environmental concentrations.

2. Materials and methods

2.1. Sampling site

Polychaetes were obtained from Quequén Grande river (38°33′06.2″S – 58°43′30.5″W), located in the southeast of Buenos Aires province in Argentina. Its water drains into the Atlantic Ocean through an estuary where the Necochea-Quequén harbour is located. Significant urban and industrial activities are concentrated in the area as well as recreational activities. Sediments are composed of sand (70%), coarse silt (10.94%), fine silt (1.56%), clay (17.5%), organic matter (5.0%) (Chiodi et al., 2007), and the waters are alkaline (pH > 8 year round) (Carmona et al., 2011). Previous studies reported low levels of inorganic and organic contaminants in surface sediments (Chiodi et al., 2007; González et al., 2012, 2013) that were below levels established for the protection of aquatic life (Canadian Quality Guidelines for the protection of Aquatic Life –CSQG-, CCME, 2002).

2.2. Animals

Polychaetes (size > 4 cm in length) were manually collected in the estuarine zone at low tide with a shovel (0–25 cm depth) and then transported to the laboratory. Temperature, pH, and salinity were measured in situ by multiparametric monitor (Trademark Horiba, U-10). Individuals were acclimated in sediment for 4 days, and then in the test vessels without sediment for 6 days. During acclimation and bioassays, water was renewed every 48 h, maintaining constant conditions of temperature (18 °C), pH (8), salinity (10) and photoperiod (12:12 h light/dark). Individuals were fed ad libitum with frozen *Artemia* spp after each water renewal.

Animals were maintained in accordance with guidelines of the Institutional Committee for Care and Use of Laboratory Animals (CICUAL, acronym in Spanish) of Mar del Plata University, based on the "Guide for the Care and Use of Laboratory Animals" (2010, 8th Edition, National Research Council, The National Academies Press, Washington DC) and Directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purposes.

2.3. Seasonal variation in MT and LPO

Natural variation in biomarker levels must be understood before they can be used in the laboratory (Mouneyrac et al., 2000). Different biotic and abiotic factors, including reproductive state, age, sex, temperature, salinity, and season may change levels of biochemical biomarkers whatever the contamination of the environment (Amiard et al., 2006). The influence of these factors on MT and LPO levels in polychaetes have been reported (Geracitano et al., 2004a; Gillis et al., 2004; Ait Alla et al., 2006; Díaz-Jaramillo et al., 2011; Gomes et al., 2013). These natural variations may interfere with the estimation of levels induced by pollutants, so seasonal sampling was conducted.

To establish the profile of seasonal variation of MT and LPO levels, the collections were initiated in May 2014 and extended until May 2015. The seasons were defined as spring (September, October and November), summer (December, January and February), fall (March, April and May) and winter (June, July and August). Polychaetes were captured in each season, cleaned (remnants of sediment and mucus), immediately frozen in three pools (n = 15 each) and stored at −80 °C.

2.4. Reagents

The stock solution of Cd (613.2 mgCd L^{-1}) was prepared from cadmium chloride ($CdCl_2$ ≥ 99.99%, Sigma-Aldrich Chemical Corporation USA) and double distilled water (ddH_2O). Each Cd concentration was prepared using a dilution of the stock solution. The analytical Cd concentrations of each treatment were measured by inductively coupled plasma spectrophotometry with an optical resolution (ICP-OES) at the beginning of the experiment (detection limit = 6 μgCd L^{-1}). A cadmium standard of 1000 mgCd ($CdCl_2$ in ddH_2O, Titrisol Merck) was used to prepare the calibration curve.

2.5. Acute toxicity assay

The experimental conditions for the acute assay (96 h) were based on Reish (1980) with water renewal every 48 h. Polychaetes were exposed to the following nominal concentrations of Cd: 10; 30; 65; 310; 600; 1300; 2000; 4300; 8100; 16300 μgCd L^{-1}, which included levels of environmental relevance and median lethal concentrations (LC_{50}) for other related species of polychaetes (EPA, 2001). A total of 10 individuals were used for each treatment and controls. The polychaetes were checked daily, and dead individuals were counted and removed. The absence of response to gentle mechanical stimulus was the criterion for death. Median lethal concentration was determined using the

trimmed Spearman–Karber method (Hamilton et al., 1977). After the 96 h assays, all live polychaetes were divided into three pools, frozen in liquid nitrogen and stored at −80 °C until biomarker analysis.

2.6. Subchronic and chronic toxicity assays

Polychaetes (n = 15 for each treatment) were exposed to sublethal doses of Cd which were established based on the calculated value of LC_{50} for the 96 h assay. The concentrations for both subchronic (7 days) and chronic (14 days) assays were: 10; 30; 65; 310; 600; 1300; 2000; 4300 μgCd L^{-1}. For the subchronic assay, a control with sediment was used to evaluate the possible stress caused by the absence of sediment. At the end of the assays, live polychaetes were divided into three pools, frozen in liquid nitrogen and stored at −80 °C until biomarker analysis.

2.7. Metallothioneins assay and lipid peroxidation

The MT assay was performed according to the spectrometric method described by Viarengo et al. (1997). The absorbance was read at 412 nm, and MT concentration was quantified using reduced glutathione (GSH) as a reference standard. The amount of MT was calculated based on cysteine content in *Perinereis nuntia* (17 cysteines, GenBank accession no. JN579716.1), assuming a similar SH group content in L. *acuta* MT. The MT concentration was reported as μg of MT per gram of wet tissue.

Total LPO was measured according to Oakes and Van Der Kraak (2003) using the formation of thiobarbituric acid reactive substances (TBARS). Fluorescence was measured by excitation at 515 nm with an emission peak at 553 nm. The concentration was expressed as nmol of TBARS per gram of wet tissue, which was calculated from the fluorescence at 553 nm using tetramethoxypropane (TMP) as external standard.

2.8. Total Cd and MRG-Cd determination

For the determination of total Cd in polychaete tissues, samples belonging to subchronic assay were digested according to the method described by FAO/SIDA (1983). The Cd in MRG was extracted from individuals in the chronic assay group using the protocol of Wallace et al. (2003). Briefly, the tissues were homogenized in a buffer solution containing Tris-HCl (20 mM), β-mercaptoethanol (5 mM) and phenylmethanesulphonyl fluoride (PMFS) (0.1 mM) and centrifuged at 1450*g* for 15 min at −4 °C. The pellet was resuspended in 400 μL water. This suspension was heated to 100 °C for 2 min. Then 1 mL sodium hydroxide (NaOH) was added and the solution incubated at 65 °C for 60 min. After the incubation, the samples were centrifuged at 10000*g* for 30 min at 4 °C. The pellet was dissolved in 1 mL 0.5 M NaOH abd centrifuged at 10000*g* for 30 min at 4 °C. The pellet was then resuspended in 100 μL water. Finally, the sample was mineralized using a mixture of concentrated perchloric and nitric acid (0.5:1.5) in a glycerine bath.

Determinations of Cd were performed by ICP-OES. The concentrations of Cd were expressed as μg of Cd per gram of wet tissue, and the detection limit of Cd was 0.006 μg g^{-1}. The analytical quality of the results was validated with Certified Reference Material by the National Research Council from Canada (TORT-2, lobster hepatopancreas). The recovery rates were 93 ± 6% in the reference material.

2.9. Statistical analysis

The data were checked for variance homogeneity by Levene's test and for distribution normality by Shapiro-Wilk's test. Significant differences were assessed by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test if the conditions were met, or with non-parametric tests: U-Mann-Whitney (Zar, 2010). The significance level was $p < 0.05$. All statistical analyses were performed using STATISTICA version 8.0 (Statsoft, Inc.).

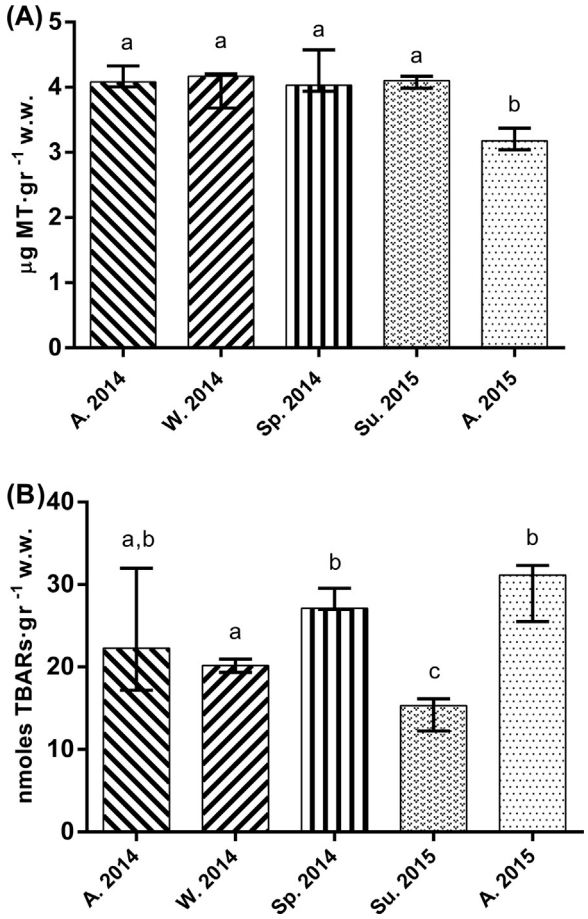

Fig. 1. Seasonal variations in metallothionein (μg MT gr^{-1} w.w., median with min. and max., n = 3) (A) and lipid peroxidation (nmoles TBARS gr^{-1} w.w., median with min. and max, n = 3) (B) levels in *Laeonereis acuta*. Same letters indicate absence of significant differences ($p > 0.05$) between seasons. A: autumn, W: winter, Sp: spring, Su: summer.

3. Results

For all the assays, the analytical values of Cd in solution corresponded to 87–100% of the nominal values ranging from 10 to 16300 μgCd L^{-1}. The measured concentrations (mean ± standard deviation, n = 3) for the ten assay solutions (10; 30; 65; 310; 600; 1300; 2000; 4300; 8100; 16300) were 9 ± 1; 27 ± 2; 61 ± 4; 306 ± 3; 527 ± 5; 1260 ± 17; 1875 ± 15; 4240 ± 24; 8018 ± 27; 16270 ± 21 μgCd L^{-1}, respectively.

3.1. Seasonal variations of MT and LPO levels

The water temperature showed a seasonal variation, with minimum temperatures between autumn and winter (14.5–15.4 °C) and maximum between spring and summer (20.9–21.9). The seasonal variation of pH varied from 7.5 to 9.6 and salinity from 0.3 to 0.7 year round.

The MT levels in polychaetes did not show differences between autumn, winter and spring 2014 and summer 2015 (Fig. 1A). In autumn 2015, the average MT level was significantly lower than in all other seasons (ANOVA with post-hoc Tukey test, $p < 0.05$).

The LPO levels showed seasonal variations (Fig. 1B), although the pattern was not well defined. During autumn and winter 2014, no significant differences were observed (Mann-Whitney test, $p > 0.05$). In spring 2014, a significant increase was observed with respect to winter 2014 (Mann-Whitney test, $p < 0.05$). In summer 2015, a significant decrease (Mann-Whitney test, $p < 0.05$) occurred followed by a

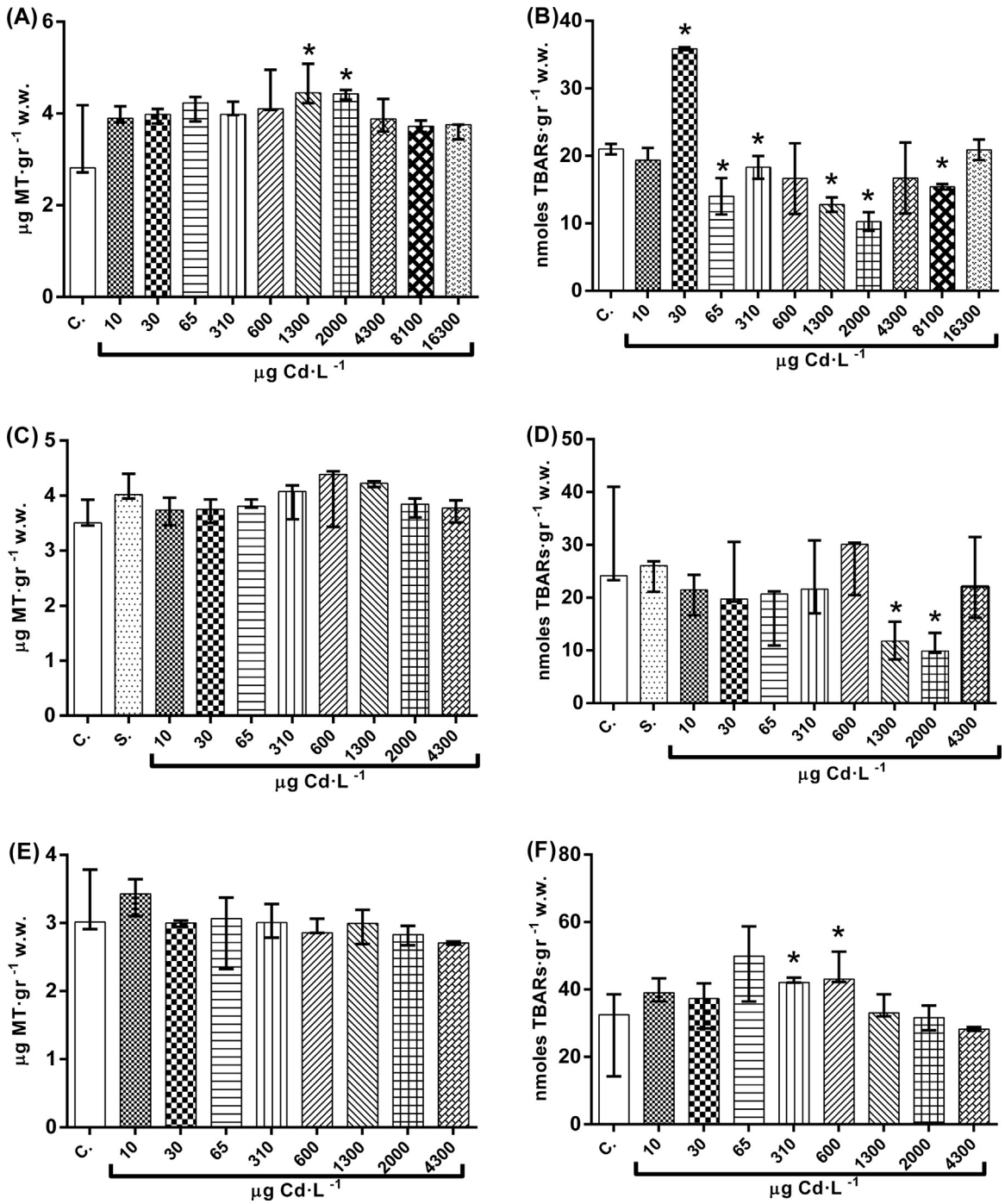

Fig. 2. Metallothionein (µg MT gr $^{-1}$ w.w., median with min. and max, n = 3) and lipid peroxidation (nmoles TBARS gr^{-1} w.w., median with min. and max, n = 3) levels in *Laeonereis acuta* exposed to different concentrations cadmium (µgCd L^{-1}) during 96 h (A, B), 7 days (C, D) and 14 days (E, F). Asterisks indicate statistically significant differences ($p < 0.05$) from control. C: control, S: sediment control.

significant increase during autumn 2015 (Mann-Whitney test, $p < 0.05$). Between autumn 2014 and autumn 2015, no significant differences were observed (Mann-Whitney test, $p > 0.05$).

3.2. Acute toxicity assay (96 h)

The survival of polychaetes at 24, 48 and 72 h was 100% for both control and all treatments (10–16300 µgCd L^{-1}). Similarly, the survival after 96 h was 100% up to 4300 µgCd L^{-1}, 50% at 8100 µgCd L^{-1} and 0% at 16300 µgCd L^{-1}. Throughout the test, neither skin color nor mucus secretion changed with respect to control conditions. Based on mortalities, the 96 h LC_{50} value was 8234.9 µg L^{-1}, and its confidence interval 95% (CI) was 6670.42-10166.23.

MT levels increased significantly only for 1300 and 2000 µgCd L^{-1} in comparision to controls (Fig. 2A) (Mann-Whitney test, $p < 0.05$). In the case of LPO (Fig. 2B), only the 30 µgCd L^{-1} treatment showed a significant increase with respect to controls (Mann-Whitney test, $p < 0.05$). In contrast, for 65; 310; 1300; 2000 and 8100 µgCd L^{-1} treatments, levels decreased significantly respect to controls (Mann-Whitney test, $p < 0.05$). For other treatments (10; 600; 4300; 16300 µgCd L^{-1}), LPO levels showed no significant differences (Mann-Whitney test, $p > 0.05$) compared to controls.

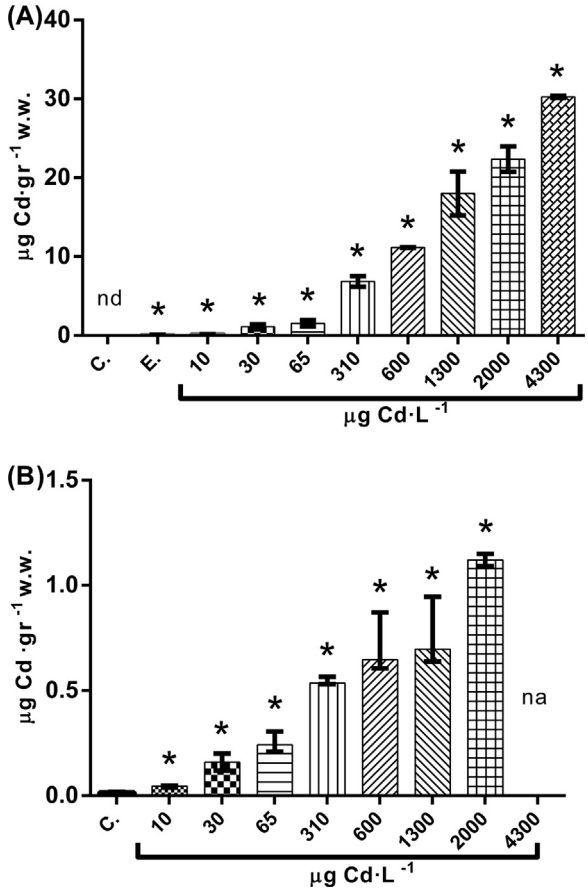

Fig. 3. Concentration of cadmium (μgCd L^{-1} w.w, median with min. and max, n = 3) in *Laeonereis acuta*. A: total cadmium in polychaetes tissues, samples belonging to 7 days assay. B: cadmium in metal-rich granules (MRG), extracted from individuals of 14 days assay. Asterisks indicate statistically significant differences ($p < 0.05$) from control. C: control, E: environmental individuals, nd: not detected, na: not analyzed.

3.3. Subchronic toxicity assay (7 days)

After 7 days of testing, survival was 100% for all treatments including controls except for the 4300 μgCd L^{-1} treatment where survival was 93.3%. Different concentrations of Cd did not produce significant variations in MT levels with respect to controls (Mann-Whitney test, $p > 0.05$) (Fig. 2C), although there was a tendency to increase in sediment controls and the 310; 600 and 1300 μgCd L^{-1} treatments. Similarly, no significant variations in LPO levels were observed for most treatments except for 1300 and 2000 μgCd L^{-1} that were significantly lower compared to controls (ANOVA with post-hoc Tukey test, $p < 0.05$) (Fig. 2D).

The total Cd concentrations in polychaetes exposed for 7 days for all treatments (Fig. 3) showed significant differences respect to controls (Mann-Whitney test, $p < 0.05$), which levels were below the detection limit of the technique (0.006 μg gr^{-1}). The total Cd concentrations in environmental individuals (0.065 ± 0.027 μgCd gr^{-1}) were also significantly higher compared to controls (Mann-Whitney test, $p < 0.05$). All polychaetes exposed exhibited significant accumulation of this element, with reached values for 10 μgCd L^{-1}: 0.16 ± 0.01; 30 μgCd L^{-1}: 1.10 ± 0.18, 60 μgCd L^{-1}: 1.54 ± 0.23; 310 μgCd L^{-1}: 6.85 ± 0.38; 600 μgCd L^{-1}: 11.14 ± 0.02; 1300 μgCd L^{-1}: 18.01 ± 1.61; 2000 μgCd L^{-1}: 22.36 ± 0.93; 4300 μgCd L^{-1}: 30.26 ± 0.06.

3.4. Chronic toxicity assay (14 days)

After 14 days of testing, survival was 100% for controls and the 10; 65; 1300 μgCd. L^{-1} treatments, 80% for the 30 μgCd L^{-1} treatment, 86.6% for the 310; 600 μgCd L^{-1} treatments, 93.3% for the 2000 μgCd L^{-1} treatment, and 33.3% for the 4300 μgCd L^{-1} treatment. Different concentrations of Cd did not produce significant variations in MT levels of *L. acuta* (Mann-Whitney test, $p > 0.05$) (Fig. 2E). In contrast, LPO levels showed an increasing trend for the 65; 310 and 600 μgCd L^{-1} treatments, although it was only significant for 310 and 600 μgCd L^{-1} respect to controls (Mann-Whitney test, $p < 0.05$). Cd concentrations in MRG for all treatments were significantly different from controls (Fig. 3B; Mann-Whitney test, $p < 0.05$), showing a gradual increase of Cd-MRG. Unfortunately, it was not possible to determine Cd-MRG in 4300 μgCd L^{-1} treatment due to lack of samples.

4. Discussion

4.1. Seasonal variations of MT and LPO levels

As mentioned above, both biotic and abiotic factors may influence levels of biochemical biomarkers whatever the contamination of the environment (Amiard et al., 2006). Previous studies have indicated that MT and LPO levels in polychaetes are sensitive to a range of natural variables (Gillis et al., 2004; Ait Alla et al., 2006; Durou et al., 2007; Díaz-Jaramillo et al., 2011; Gomes et al., 2013). Similar to reported for *L. acuta* from the Patos Lagoon estuary (southern Brazil) (Geracitano et al., 2004a), we did not observe seasonality in MT levels. Similar results were observed for *Perinereis gualpensis* (Díaz-Jaramillo et al., 2011) from southern Chile and *Nereis* (= *Hediste) diversicolor* from the Iberian Peninsula (Gomes et al., 2013). In contrast, we observed seasonal variations in LPO, with high values in autumn and spring. The influence of temperature, reproductive period and/or photoperiod on LPO concentrations has been reported for some invertebrate species (Duran-Lizarraga et al., 2001; Fanjul-Moles et al., 2003; Dutra et al., 2008; Sroda and Cossu-Leguille, 2011), increasing LPO levels in parallel with temperature and photoperiod during summer months. However, we found no evidence for this in *L. acuta* similar to previous studies (Geracitano et al., 2004a; Ferreira-Cravo et al., 2007). Similarly, Durou et al. (2007) and Gomes et al. (2013) observed no variations in the levels of this biomarker in *N. diversicolor*. In constrast, Ait Alla et al. (2006) observed variations for *N. diversicolor* with maximum concentrations of LPO in January (winter) and October (autumn), but this was attributed to the release of pollutants through domestic and industrial effluents. A similar situation could occur in the Quequén Grande river estuary, as it has been reported the presence of illegal industrial connections in the stormwater effluents from Necochea and Quequen cities (Polizzi, 2006), which flow into the river. Therefore, the seasonal variations found in this study could be associated with the presence of contaminants in these effluents

4.2. Median lethal concentrations (LC_{50})

The toxicity of Cd for many polychaete and oligochaete species has been well documented (Ahsanullah, 1976; Eisler and Hennekey, 1977; Chapman et al., 1982; Reish and Lemay, 1991), but not for *L. acuta*. For example, the 96 h LC_{50} value of Cd for *Nereis virens*, *Neanthes arenaceodentata* and *Capitella capitata* are 9300; 14100 and 2800 μgCd L^{-1}, respectively (Eisler and Hennekey, 1977; Reish and Lemay, 1991). Likewise, the 96 h LC_{50} of Cd for *Limnodriloides verracosus* and *Hediste diversicolor* are 10000 and 84000 μgCd L^{-1}, respectively (Bryan, 1976; Chapman et al., 1982). The LC_{50} value obtained for *L. acuta* in our study was within values reported for other polychaetes. Despite the great variability in LC_{50} for this biological group, all of them demonstrate tolerance to Cd, where lethal levels are much higher than for most marine invertebrates.

Polychaetes are often the most common invertebrate found in contaminated areas, and many species in this group appear to exhibit extraordinary tolerance to various environmental contaminants (Eriksen et al., 1988). As a result, it has been assumed that polychaetes

have complex and effective detoxification mechanisms to protect them from toxic metals (Bouraoui et al., 2016). In the case of the *L. acuta* population from Quequén Grande estuary, where levels of heavy metals (even Cd) in surface sediments (Chiodi et al., 2007) were below the levels established as safe for the biota (CCME, 2002). As a result, tolerance would not be related to previous exposure.

4.3. Cd accumulation, biomarkers and Cd-MRG

Invertebrates, particularly annelids, are known to have a large capacity to accumulate metals (Nejmeddine et al., 1988; Dallinger, 1994; Demuynck and Dhainaut-Courtois, 1994; Sandrini et al., 2008; Freitas et al., 2012). In *L. acuta*, it was possible to observe the accumulation of Cd in their tissues, even at the lowest concentration exposed (10 μgCd L^{-1}), demonstrating the assimilation of this metal. The same situation was previously observed by Sandrini et al. (2008) in *L. acuta* exposed to 5 μgCd L^{-1}. Many other polychaete species such as *N. virens, H. diversicolor*, *N. diversicolor* show Cd accumulation in their tissues (Ray et al., 1980; Berthet et al., 2003; Geffard et al., 2005; Gomes et al., 2013). The capacity of polychaete to accumulate Cd depends on the concentration of the metal in the surrounding medium (Gomes et al., 2013). Several of them have been shown to accumulate Cd from both dissolved and particulate forms of sediments and water (Zhou et al., 2003; Geffard et al., 2005). Bouché et al. (2000) concluded that accumulation is based on efficient detoxification mechanisms, such as MT binding or the formation of insoluble metallic granules.

As already mentioned, the presence of MT in annelids has been previously reported (Deeds and Klerks, 1999; Berthet et al., 2003; Mouneyrac et al., 2003; Geracitano et al., 2004a; Pérez et al., 2004; Poirier et al., 2006; Gomes et al., 2013). However, there are some discrepancies with respect to its induction. In the present study, no induction of MT at 96 h was observed in *L. acuta*, except for treatments at 1300 and 2000 μgCd L^{-1}. Similarly, no induction was observed at 7 and 14 days for any of the concentrations tested. In studies carried out in *L. acuta* exposed to 100 μgCd L^{-1}, no induction of MT was observed for any exposure time (4, 8, 12 and 24 h) (Sandrini et al., 2006). Mouneyrac et al. (2003) also reported that for *H. diversicolor*, exposure to 100; 178; 316; 562 and 1000 μmolesCd L^{-1} for 21 days caused no alterations in MT level. Similarly, Geracitano et al. (2002) observed that chronic exposure to Cu of *L. acuta* did not alter MT levels. Studies done with the same species in Brazil found higher concentrations of MT in individuals from an impacted site by metals than those corresponding to that without impact, (Geracitano et al., 2004a); while in other field studies of *N. diversicolor,* the pattern was not so clear (Mouneyrac et al., 2003; Pérez et al., 2004; Poirier et al., 2006; Gomes et al., 2013).

The results obtained in this study indicate that there was no *de novo* synthesis of MT to detoxify the assimilated Cd. However, Mouneyrac et al. (2002) and Ng et al. (2007) have reported, for other aquatic invertebrates, an increase in MT turnover cycle in response to high concentrations of Cd, observing at the same time a higher synthesis and degradation. Therefore, concentrations of MT may remain constant during exposure. This situation could be occurring in individuals of *L. acuta*, although, further studies would be necessary to confirm this.

Although there may be no induction of MT in polychaetes, they may respond to exposure to high concentrations of metals by forming metal granules, increased secretion of mucus, and accumulation in specific areas of the body followed by exocytosis (Viarengo and Nott, 1993; Gibbs et al., 2000; Berthet et al., 2003; Mouneyrac et al., 2003). In *L. acuta*, we observed the formation of Cd granules even at the lowest concentration tested (10 μgCd L^{-1}), indicating it as a detoxification mechanism. Similarly, Sandrini et al. (2006), reported in the same species exposed to 100 μgCd L^{-1} for 24 h, Cd granule formed and MT levels remained unchanged. However, Wallace et al. (1998) did observe an increase in MT levels along with granule formation in the marine oligochaeta *Limnodrilus hoffmeisteri* found in areas contaminated with Cd. In the marine polychaete *Neanthes japonica* from environments contaminated with metals, the main mechanism was the formation of metallic granules, while MT represented a smaller fraction of the accumulated metals (Fan et al., 2015). Brown (1982) and Roesijadi (1992) postulated that the formation of metal granules is a mechanism for long-term storage, while the MT is involved in time scales shorter. As a result, the relative importance of these two mechanisms of detoxification varies considerably depending on the species and pollution levels.

Another sublethal effect induced by exposure to metals is the increase in mucus secretion (Lucan-Bouché et al., 1999; Bouché et al., 2000), which is considered as an adaptive response of the animal related to physiological resistance. Mucus secreted by the epidermis has the ability to complex with metals and reduce their availability for uptake (Noël-Lambot, 1981), and is therefore is considered an additional detoxifying mechanism (Bouché et al., 2000; Mouneyrac et al., 2003). Mouneyrac et al. (2003) reported an increase in the amount of mucus in *H. diversicolor* exposed for 21 days to 1780 μmolesCd L^{-1}. However, we observed no mucus secretion in *L. acuta* exposed to Cd.

One consequence of oxidative stress is the presence of LPO. The concentration of this biomarker in *L. acuta* showed no increases at any exposure time, indicating the absence of oxidative damage caused by Cd. Similarly, Sandrini et al. (2008) did not observe an increase of LPO in *L. acuta* exposed to Cd (100 μgCd L^{-1} −7 days), which was associated with an increase in the concentrations of antioxidant enzymes. In the case of this study, the absence of oxidative membrane lipid damage was related to the formation of granules. Although the antioxidant enzymes were not evaluated, they could be also preventing the damage.

5. Conclusions

The results obtained in the present study demonstrated that *L. acuta*, like other polychaetes species, was able to detoxify Cd primarily by forming granules. The absence of LPO indicates the ability of *L. acuta* to cope with the oxidative stress that Cd could generate. However, it will be necessary to quantify MT-RNA messenger to understand the MT response observed in our study.

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