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Implications on the Pb bioaccumulation and metallothionein levels due to dietary and waterborne exposures: The *Callinectes danae* case

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ABSTRACT

This study aimed to assess the bioaccumulation of Pb and induction of metallothionein-like proteins (MT) in *Callinectes danae* through single and combined dietary and waterborne exposures. Male *C. danae* individuals were collected in the south area of the Cananéia-Iguape-Peruíbe Protected Area (APA-CIP), in São Paulo State, Brazil. After an acclimatization period, exposure assays were performed during 7 and 14 days, at two Pb concentrations (0.5 e 2.0 μg/g) in 4 treatments: 1) control; 2) contaminated water only; 3) contaminated food only; 4) contaminated water and food. The results indicate that *C. danae* is highly tolerant to Pb exposure at the evaluated concentrations. In gills, Pb bioaccumulation is more dependent of water efflux and time of exposure (higher Pb values). However, pathways act simultaneously in the induction of MT expression in this tissue. The decreases in Pb accumulation in the combined treatments and MT increases after 14 days in gills suggests that these proteins play a detoxification function in the presence of Pb. In hepatopancreas, depending on the predominance of a certain pathway or combined pathways, accumulation occured at different times. For muscle tissue, bioaccumulation was observed due to contaminated water exposure, but not dietary exposure, probably because Pb concentrations were low.

1. Introduction

In Brazil, blue crabs belonging to the *Callinectes* genus are ecologically important, due to the niches these organisms occupy and their contribution to the recycling of organic matter in marine and estuarine environments. Blue crabs also represent an important fishing resource, especially for traditional communities (Severino-Rodrigues et al., 2001; Bordon et al., 2012b; Lavradas et al., 2014). Specifically, *Callinectes danae* Smith, 1869 (Crustacea, Decapoda, Portunidae) is an epibenthic and omnivorous species, feeding on algae, macroinvertebrates such as Mollusca, Polychaeta and other Brachyura; and available organic matter (Branco and Verani, 1997). This species is highly tolerant to salinity changes and can be found in brackish water environments, such as estuaries, and in marine areas up to 75 m in depth (Melo, 1996). *C. danae* is distributed throughout the Atlantic Ocean, from Florida (USA) to the Southern Coast of Brazil (Costa and Negreiros-Fransozo, 1998), being common in coastal regions, including areas impacted by human activities.

Few previous studies have assessed metal concentrations in *C. danae* tissues for biomonitoring purposes (Virga et al., 2007; Virga and Geraldo, 2008; Bordon et al., 2016); some have reported that different metals may present different target tissues and bioaccumulate differently in distinct tissues (Bordon et al. 2012b; Lavradas et al., 2014). The combination of the uptake and depuration processes of each tissue and in the whole organism would lead to the following processes: metals can be efficiently excreted, can accumulate in immobilized form, or can remain active in the tissue and lead to deleterious effects in the animals (Rainbow, 1988, 1998, 2007, 2002). Due to this complexity related to metal exposure and toxicity in aquatic organisms, efforts are required to understand the role of different pathways involved in metal uptake and

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bioaccumulation, which include both dietary and waterborne exposures.

Lead (Pb) is considered a legacy contaminant of great concern, as it is associated to mining activities and industrial pollution sources, and is used as an additive in fuels, among others. In addition, it is also toxic to humans, plants and animals, and has been frequently detected in the environment. The addition of Pb in fuel has strongly contributed to the increase of its occurrence in the atmosphere, and wet and dry deposition into waterbodies, where uptake by aquatic organisms can occur, leading to accumulation in animal tissues (Otitoloju et al., 2009). Kakkar and Jaffery (2005) reported the transfer of Pb between a primary (*Daphnia magna*) and a secondary consumer (*Poecilia reticulum*), showing potential biomagnification and the inherent public health risk of this metal. In fact, environmental Pb exposure has been recognized as a serious public health problem (WHO, 1995). In Brazil, episodes of environmental contamination related to effects on human populations associated with Pb mining and processing were identified at the Santo Amaro da Purificação municipality (BA) (Andrade and Moraes, 2013) and across the Ribeira de Iguape River basin (SP) (Guimarães and Sigolo, 2008a, 2008b; Abessa et al., 2012; Rodrigues et al., 2012).

Based on results reported by Bordon et al. (2012a,b) and Bordon et al. (2016), two main hypotheses were assumed for Pb accumulation in *C. danae*: 1- Pb bioaccumulation may occur in different concentrations according to the type of tissue; and 2 – the uptake pathway (i.e., direct water contact or feeding) interferes directly on bioaccumulation in each tissue.

In this context, the aim of the present study was to assess Pb bioaccumulation in different *Callinectes danae* tissues and detect the induction of depuration responses, by quantifying metalothionein-like proteins (MT), in exposure scenarios considering both dietary and waterborne routes, as well as both combined. The results would also serve to provide further information to subsidize the use of *C. danae* as a biomonitor for environmental contamination.

2. Material and methods

Initially, about 100 L of a commercial substrate formed by seashells were introduced in a 500 L-container. This substrate was applied as a biofilter to remove residual metals from the water, as well as chlorine and other possible residues. The container was then filled up with previously filtered tap water and, subsequently, artificial sea salts (Ocean Fish - Prodac) were added. This water was only used after salinity stabilization (app. 1 day, 25 ppm). Salinity was chosen according to results reported by Miyao et al. (1986) for the sampling area. Recent studies have reported similar salinities close to the sampling site (Godoy et al., 2015; Miyashita and Calliari, 2016; Garcia et al., 2018). It was important to guarantee the adequate acclimation of collected organisms in laboratory, considering that salinity variation and influence were not the aim of this first approach. Subsequently, Pb concentrations were determined in this artificial seawater.

Under proper authorization of the Brazilian federal protected areas agency -ICMBio (SISBIO n. 45881-3), male *C. danae* individuals were collected with commercial traps and trawling nets in the south area of the Cananéia-Iguape-Peruíbe Protected Area (APA-CIP), in São Paulo, Brazil, at 25°2.659'S, 47°55.262'W in Autumn 2015 (Fig. 1).

The choice for male individuals is justified because females migrate to estuarine regions, where salinities are higher. In the field, blue crabs were identified according to Melo (1996) and sexed according to Willians (1974). Maturation stage due to the shape and degree of adherence of the abdomen to thoracic sternites was determined and total weight, carapace length and width were measured. The blue crabs were then transported to the laboratory in 10 L plastic containers filled with local water and mobile artificial aeration. No mortality was observed during the laboratory assays. All males were at the mature stage. Mean (± SD) weight, carapace width and length were 64.39 ± 15.99 g; 7.79 ± 0.65 cm and 4.50 ± 0.36 cm, respectively.

At the laboratory, each blue crab was maintained in a 6 L aquarium, filled with 5 L of clean artificial seawater, for acclimatization, for 5 days. Subsequently, the organisms were separated into four treatment groups and exposed to Pb for **7 or 14 days** considering two Pb test concentrations (0.5 and 2.0 μg/g) as described below (Fig. 2):

1) control treatment: eight crabs maintained in non-contaminated artificial seawater and fed non-contaminated artificial food (**C7, n = 4; and C14, n = 4**);
2) contaminated water: eight crabs maintained in contaminated artificial seawater and fed non-contaminated artificial food (**H_2O 0.5, n = 4; and H_2O 2.0, n = 4**);
3) contaminated food: eight crabs maintained in non-contaminated artificial seawater and fed contaminated artificial food (**F 0.5, n = 4; and F 2.0, n = 4**);
4) combined treatment: eight crabs maintained in contaminated artificial seawater and fed contaminated artificial food (**COMB 0.5, n = 4; and COMB 2.0, n = 4**).

Lead concentrations were chosen based on available data in Brazilian law for water quality (Brazil, 2005) and seafood consumption (Brazil, 1998, 2013). It was decided to create a laboratory environment where bioaccumulation could be tested in all evaluated pathways.

For contaminated water and combined treatments, the stock solution consisted of an ultrapure standard solution (Perkin Elmer, [Pb] = 10,000 μg/mL). Aliquots of this stock solution were added to 5 L of artificial seawater in each aquarium. The contaminated artificial food for dietary exposure was previously developed for this study (unpublished data). Basically, it consisted of a mixture of agarose (KASVI) previously contaminated with a volume of ultrapure standard of Pb (Perkin Elmer, [Pb] = 1000 μg/mL) and mixed with shrimp meat. Food was offered to blue crabs in 1 g portions, 3 times each 7 days (a total of 6 portions in 14 day assays). Previous experiments were performed to guarantee that food would not dissolve in water and assess crabs' acceptance (non-published data). A monitoring period was evaluated, and it was concluded that after 5 min, crabs could eat the food completely. Thus, this monitoring period was checked and applied in all experiments to guarantee that organisms have eaten the entire food.

During the experiments, physical-chemical water parameters in the test-chambers were monitored as follows: mean (± SD) temperatures (°C) were measured using digital thermometers; pH values were determined by colorimetric tests (SERA); salinities were measured by hand refractometers; total ammonia (NH_3-NH_4^+) concentrations were obtained by a colorimetric test (Labcon) and dissolved oxygen levels (mg/L) were measured with an oxygen meter (DO-5519 LUTRON meter). The physico-chemical variables remained within the suitable ranges for the species. The mean (± SD) temperatures (° C) ranged from 19.2 to 24.5 °C, the pH ranged between 7.5 and 8.0; salinities varied from 22 to 26 ppm and DO ranged between 7.5 and 9.9 mg/L. Total ammonia concentrations of ranged from 0 ppm (in water changes) to 3 ppm (corresponding to 0.164 ppm for NH_3, after the longest time of Pb exposure)

After 7 and 14 days of exposure, soft tissues (gills, hepatopancreas, muscle) from four organisms from each treatment group were removed and frozen at −80 °C until the Pb and MT concentration analyses.

For Pb determinations, the acid extraction consisted of 10 mL HNO_3 added to 1 g of soft tissue samples and certified reference material (Mussel tissue – NIST 2976) in microwave vessels (PFA Teflon, fluorocarbon polymer). Digestions were performed in a high-pressure microwave system (CEM Corporation, model MARS 6), After cooling, the extracts were transferred to 50 mL centrifuge vials and the volumes were made up to 25 mL with ultrapure water (Milli-Q).

Pb concentrations were determined by a AAnalyst 800 Perkin Elmer Graphite Furnace Atomic Absorption Spectrometer (GF AAS).

The limit of detection (LOD) was calculated according to INMETRO

Fig. 1. The sampling site (star) located at the South portion of Cananéia municipality, in the São Paulo State (gray), Brazil.

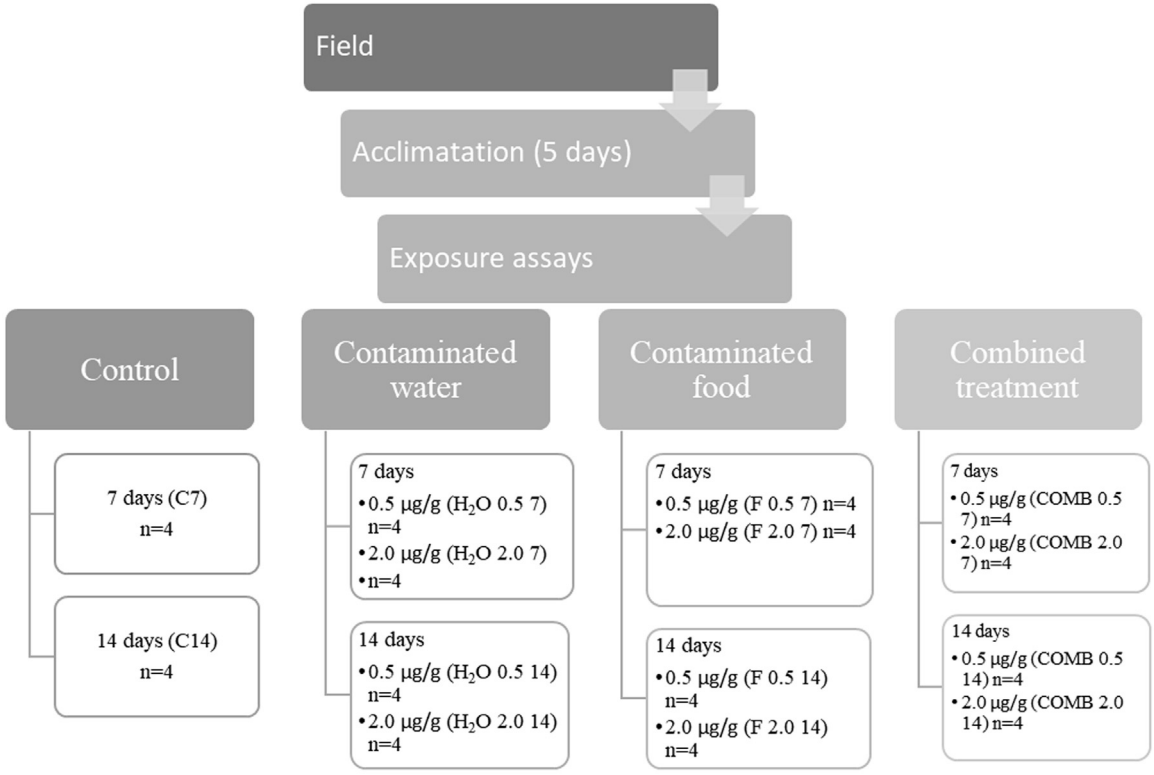

Fig. 2. Experimental design (C = control; F = contaminated food; H_2O = contaminated water; COMB = combined treatment; 0.5 and 2.0 µg/g – 7 and 14 days).

(2011), as described below:

$$LOD = mean + t\ (n - 1;\ 1 - \alpha)\ x\ SD$$

where mean is the mean concentrations measured in 7 sample blanks, *t* is the *t*- Student value according to the degrees of freedom (n-1) and $\alpha = 0.05$, and SD is the Standard deviation of concentrations measured in 7 sample blanks.

The calculated limit of detection for Pb was 0.001 μg/g. The Pb recoveries (%) in water for the certified standard material (Elements in Natural Water, SRM NIST 1640a, certified value = 0.0121 μg/g) and spiked artificial sea water (0.5 μg/g, using an ultrapure standard solution [Pb] = 1000 μg/mL, MERCK) were 98% (0.0119 ± 0.0004 μg/g) and 92% (0.46 ± 0.03 μg/g), respectively. The Pb concentrations in the tap water and the artificial seawater before the assays were below the LD. In addition, the methodological validation of Pb determination in certified mussel tissue material (NIST 2976, certified value = 1.19 μg/g) and artificial foods (0.5 and 2.0 μg/g) were performed. Results were 1.01 ± 0.03 μg/g (Recovery = 85%), 0.53 ± 0.08 μg/g and 2.37 ± 0.21 μg/g, respectively.

Since MT often occur in organs that play detoxification functions (as in the case of gills and hepatopancreas), MT were not determined in muscle tissue. So, for MT quantification in soft tissues (hepatopancreas and gills), the samples were homogenized in 20 mM Tris-HCL buffer supplemented with 0.5 mol/L sucrose, 0.01% β- mercaptoethanol and centrifuged at 15,000 × *g* for 30 min at 4 °C. Subsequently, cold (−20 °C) absolute ethanol and chloroform were added to the supernatants, in order to precipitate high molecular weight proteins. Samples were then centrifuged at 6000 × *g* for 10 min at 4 °C. This supernatant fraction was then acidified with HCl to co-precipitate MT and improve recovery. Before acidification, samples were stored at − 20 °C for 1 h. The samples were subsequently re-centrifuged at 6000 × *g* for 10 min at 4 °C and the obtained MT pellet was resuspended with an ethanol/chloroform/homogenizing Tris–HCl 20 mM buffer solution. Samples were re-centrifuged at 6000 × *g* for 10 min at 4 °C. The obtained MT pellet was resuspended in 0.25 M NaCl solution and then HCl/EDTA was added to remove metal cations still bound to the MT. MT were then quantified using Ellman's reagent after centrifugation at 3000 × g for 5 min at room temperature (Viarengo et al., 1997). The absorbances were recorded at 412 nm.

A two-way analysis of variance (ANOVA) followed by a *Post hoc* Tukey's test was applied to the results, considering exposure treatments (water only, food only, combination of food and water, at each concentration) and time as factors. When interactions were not significant, a one-way ANOVA followed by a *Post hoc* Tukey's test was applied to the treatment data but separated by time. In addition, each treatment was tested for both times of exposure (7 and 14 days) using the *t*-Student test. Significance was detected when $p < 0.05$.

3. Results

In gills, the two-way ANOVA detected statistically significant differences between treatments regarding time (significant interaction, p = 0.0000). Pb bioaccumulation in gills of blue crabs from the H_2O 2.0 treatment occurred effectively after both times of exposure (7 and 14 days), being statistically greater after 14 days of exposure. Regarding the COMB2.0 treatment, Pb concentrations in gills were statistically higher only after 14 days of exposure. In addition, Pb concentrations in gills of individuals from the COMB 2.0 treatment after 14 days and in gills of those from H_2O 2.0 treatment after 7 days were similar but lower than the concentrations determined in gills of blue crabs from the H_2O 2.0 treatment after 14 days (Fig. 3a).

Regarding MT contents in gills, significant differences were observed between treatments as a function of time (significant interaction, p = 0.00003). The MT concentrations in gills of individuals from the combined treatments at 0.5 and 2.0 μg/g after 14 days (COMB 0.5 14 and COMB 2.0 14) were higher than those obtained in gills of blue crabs

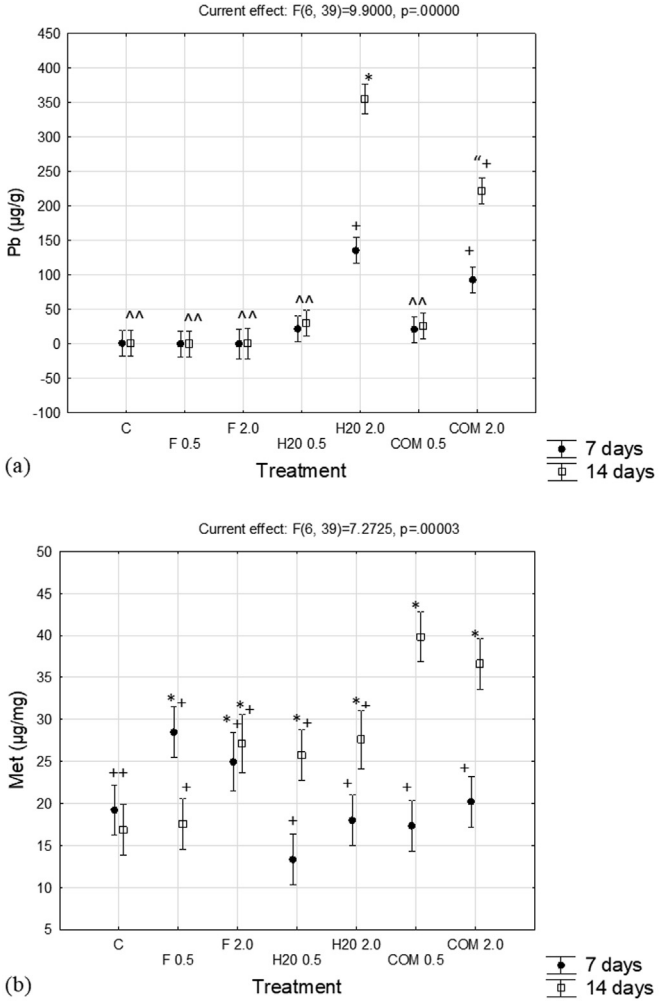

Fig. 3. Concentrations of Pb (a) and Metallothioneins (b) in gills of blue crabs *C. danae* after exposure assays (vertical bars denote +/- standard errors; C = control; F = contaminated food; H_2O = contaminated water; COMB = combined treatment; 0.5 and 2.0 μg/g– 7 and 14 days). Different symbols indicate statistical differences (Tukey test, $p < 0.05$).

from the controls in both times of exposure and some treatments after 7 days (F 0.5 H_2O 0.5 and 2.0 COMB 0.5 and 2.0 all after 7 days) (Fig. 3b). The results obtained for the combined treatments confirmed that the intake of contaminated food plus exposure through contaminated water may be simultaneously inducing the expression of MT.

Pb concentrations in muscle were below the LD in controls and food treatments, for both concentrations and times of exposure (C7 and 14 days, F 0.5 7 and 14 days, F 2.0 7 and 14 days). For the other treatments, no significant interaction (p = 0.21) was detected by the two-way ANOVA, but treatment was significant ($p < 0.0002$). Data were not grouped by treatment since this would unite different times of exposure. Thus, a one-way ANOVA was applied, after separating the data by time of exposure (Fig. 4a,b). After 7 days, Pb bioaccumulation in muscle of individuals from the H_2O 2.0 treatment was statistically higher than that observed in muscle of individuals from H_2O 0.5 and COMB 0.5 treatments (Fig. 4a). After 14 days, Pb bioaccumulation in muscles of blue crabs from the H_2O 2.0 treatment was statistically higher than that observed in muscles of individuals from the COMB 0.5 treatment only (Fig. 4b). When the results obtained for each treatment were compared considering both times of exposure (7 and 14 days), no significant differences were detected.

For hepatopancreas, the two-way ANOVA did not detect any significant interaction (p = 0.10421), but treatment ($p < 0.0001$) and

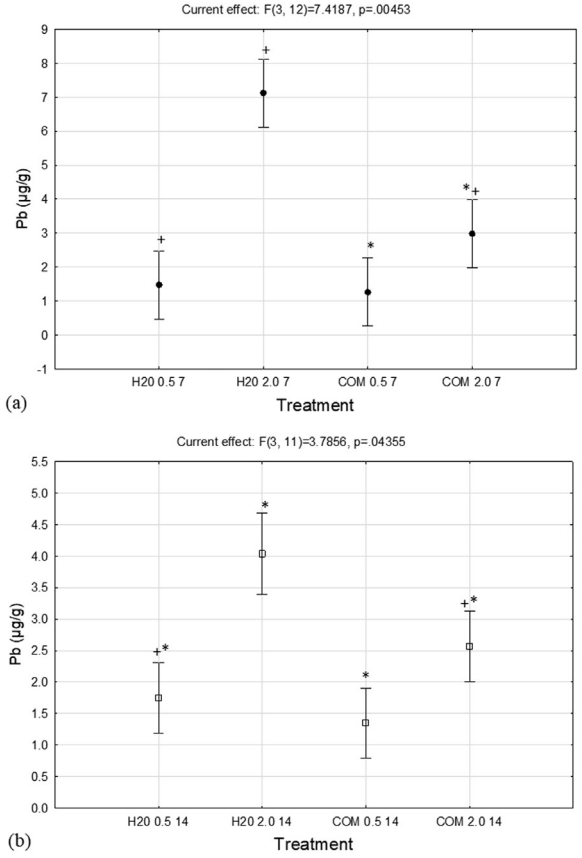

Fig. 4. Concentrations of Pb in muscle tissues of blue crabs *C. danae* after 7 (a) and 14 (b) days of Pb exposure (vertical bars denote +/- standard errors; H_2O = contaminated water; COMB = combined treatment; 0.5 and 2.0 µg/g). Different symbols indicate statistical differences (Tukey test, $p < 0.05$).

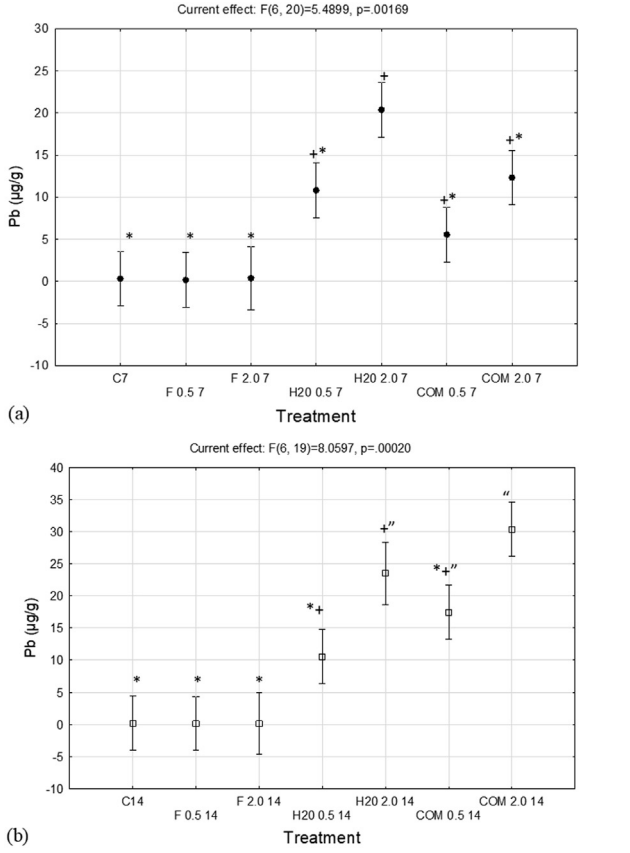

Fig. 5. Concentrations of Pb in hepatopancreas of blue crabs *C. danae* after 7 (a) and 14 (b) days of Pb exposure (vertical bars denote +/- standard errors; C = control; F = contaminated food; H_2O = contaminated water; COMB = combined treatment; 0.5 and 2.0 µg/g – 7 and 14 days). Different symbols indicate statistical differences (Tukey test, $p < 0.05$).

time ($p < 0.0001$) were significant. Again, data of different treatments were not grouped, since this would unite different times of exposure. So, a one-way ANOVA was also applied to treatments, in order to compare the results for time of exposure (Fig. 5a,b). After 7 days, Pb bioaccumulation in hepatopancreas of blue crabs from the H_2O 2.0 treatment was statistically higher than the accumulation in hepatopancreas of individuals from the control treatment and of those exposed to contaminated food at 0.5 and 2.0 (Fig. 5a). After 14 days, Pb bioaccumulation in hepatopancreas of blue crabs from the H_2O 2.0 and to COMB 2.0 treatments was statistically higher than the accumulation in hepatopancreas of individuals from the control treatment and of those exposed to contaminated food at 0.5 and 2.0 (Fig. 5b).

Regarding COMB 2.0 treatment, Pb concentrations in hepatopancreas were higher than Pb concentrations in hepatopancreas of individuals from H_2O 0.5 treatment. The *t*-Student's test was applied to evaluate each treatment after both times of exposure (7 and 14 days), and only Pb concentrations in hepatopancreas of blue crabs from the COMB 2.0 after 14 days were higher than the concentrations obtained after 7 days ($p < 0.05$) (Fig. 6)

Although variations were observed, the two-way ANOVA did not detect any differences in MT contents between treatments as a function of the time of exposure (or interaction) ($p = 0.15327$) (Fig. 7).

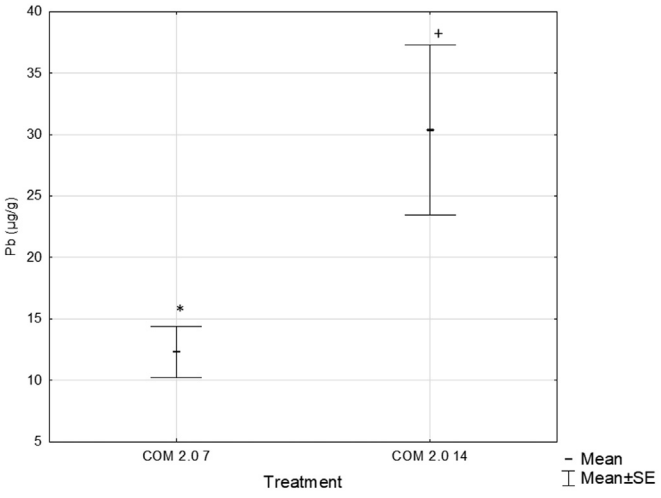

Fig. 6. Concentration of Pb in hepatopancreas of blue crabs *C. danae* from the combined treatment at [Pb] = 2.0 µg/g after 7 and 14 days (vertical bars denote +/- standard errors; COMB = combined treatment; 0.5 and 2.0 µg/g – 7 and 14 days). Different symbols indicate statistical differences (*t*-Student test, $p < 0.05$).

4. Discussion

Some theoretical models used to describe metal uptake in invertebrates (and, consequently, the roles played by MT) have been developed, discussed and reported. One such model was proposed by Rainbow (2002), who stated that crustaceans can: 1) assimilate non-essential metals without excretion, mostly in the detoxified form, mainly binded to MT; 2) assimilate non-essential metals with some excretion; but without varying concentrations, since the excretion rate tends to be the same as the total uptake rate. In a review, Ahearn et al. (2004) emphasized that metals can also be incorporated into insoluble

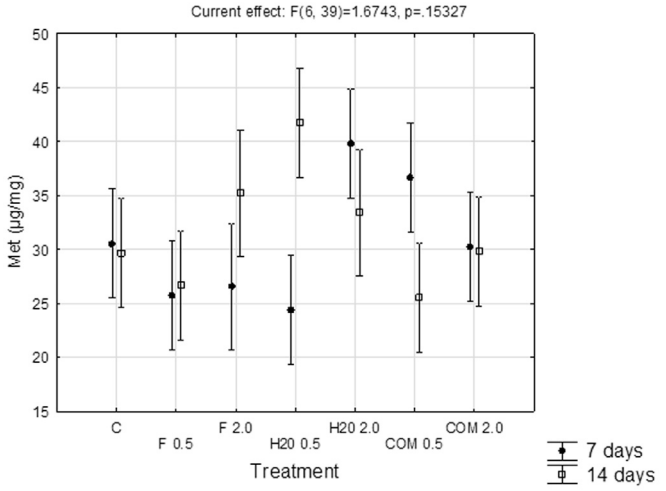

Fig. 7. Metallothioneins levels in hepatopancreas of blue crabs *C.danae* after exposure assays (vertical bars denote +/- standard errors; C = control; F = contaminated food; H_2O = contaminated water; COMB = combined treatment; 0.5 and 2.0 µg/g – 7 and 14 days). Different symbols indicate statistical differences (Tukey test, $p < 0.05$).

metal-rich granules, while Pourang et al. (2004) discussed metal distribution and redistribution (influenced by MT) in shrimp and highlighted the influence of growth and molting in the distribution of metals between soft tissues and the exoskeleton. In waterborne exposure settings, Bondgaard and Bjerregaard (2005) reported an increased cadmium uptake performed by apical gill epithelium Ca^+ channels in female post-moult shore crabs (*Carcinus maenas*), in comparison to crabs in the inter-moult stage.

For crustaceans, Rainbow (2007) highlighted that metal toxicity in a metal-exposed organism is related to differences between metal uptake and metal excretion and combined detoxification rates, and not to total accumulated metal concentrations. If the uptake rate (which accounts for both dissolved and ingested metals) is less than the combined rate of detoxification and excretion, the accumulated metal will not be available and toxic effects are not expected. More recently, Rainbow and Luoma (2011) suggested that, during an increasing efflux, new metals will bind not only to MT inside cells but also to other sites not commonly used in this sense, such as organelles and heat-sensitive proteins. Thus, beyond long-term metal granules and metals bound to MT (which are inert), crustaceans could maintain metals that could be available only in some circumstances, such as during metabolic needs or available in MT sites. The gill Na^+/K^+-ATPase activities activity were shown to be increased in *Uca rapax* collected in a chronically metal-contaminated area as a compensatory mechanism, underpinning osmoregulatory ability (Capparelli et al., 2016). Based on the gill Na^+,K^+-ATPase model reported for *C. danae* (Masui et al., 2005) to excrete NH^{+4} ions, this species may use the same mechanism to excrete excess Pb.

Many studies have also described the bioavailable, stored and detoxified fractions of metals in bivalves and polychaetes, assessing uptake not only from solutions but also sediment and diet, and strategies to maintain homeostasis (Rainbow et al., 2009; Wallace et al., 2003; Campana et al., 2015). However, these organisms do not present strategies previously reported for crustaceans, demonstrating that the metal uptake in the latter is significantly more complex.

Although some bioaccumulation was observed in this study, the results indicate that total Pb concentrations did not lead to lethality, suggesting that Pb did not reach the threshold concentration mentioned by Rainbow (2007) and that relatively efficient detoxification could be observed in all tissues in the dietary exposure treatments.

It is important to point out that water efflux through gills and the contact of the whole body with water are continuous in these organisms, unlike food exposure. Thus, Pb bioaccumulation reached higher concentrations in gills, being higher after 14 days due to contaminated water exposure at 2 µg/g. A significant contribution of Pb from artificial food was observed in the combined treatment at 2.0 µg/g only after 14 days. However, this concentration was lower than that observed in gills exposed to contaminated water at the same concentration and after the same time of exposure. The increasing of MT expression in gills from both combined treatments (at 0.5 and 2.0 µg/g) after 14 days indicated that the waterborne and dietary exposures acted simultaneously in MT induction. In addition, the decreasing Pb concentrations in these treatments comparing concentrations in gills exposed to contaminated water 2.0 µg/g only after 14 days suggest that MT play an important detoxification function in gills.

According to Rainbow (1988) and Bordon et al. (2016), metals absorbed by gills and/or the epidermis can only be proportionally transferred to muscle tissue if in excess, depending on the pattern of accumulation of each species and chemical properties of the considered element. Thus, metals absorbed by gills should not be directly and proportionally transferred to muscle tissue. The results for Pb bioaccumulation in *C. danae* muscle exposed to contaminated water and combined treatment corroborate these previous reports. Bioaccumulation in these muscle samples occurred only due to the excess of Pb in the gills, which was probably re-located to other tissues, via hemolymph. In addition, it seems that dietary exposure only contributes to Pb bioaccumulation in muscle tissue at concentrations higher than those applied in this study.

In *Penaeus monodon* shrimp hepatopancreas, Vogt and Quinitio (1994) suggested that Pb is aggregated as deposits (or granules) of non-soluble Pb and excreted after lysosomal autolysis, a process that may involve MT. Núñez-Nogueira et al., (2010, 2012) suggested a partial Pb regulation by the *Penaeus vannamei* shrimp, since most Pb was detected as non-soluble deposits in hepatopancreas. Rainbow (1998) pointed out that isopods accumulate metals in granules, with rapid Pb elimination in comparison to other metals. Some authors (Viarengo et al., 1985; Poirier et al., 2006) have suggested that MT induction is an intermediate step before the formation of these insoluble granules. In this study, Pb bioaccumulation was observed in hepatopancreas exposed to contaminated water at 2.0 µg/g after 7 and 14 days; and in combined treatment at 2.0 µg/g only after 14 days. In addition, a significant increase in Pb concentrations could be detected in the combined treatment at 2.0 µg/g, after both times of exposure (7 and 14 days). Although MT concentrations were detected in the hepatopancreas, no statistically significant increase was detected. It seems that, at the tested concentrations, Pb did not compromise the threshold MT level and/or the evaluated times of exposure were not adequate to assess any modification in MT levels.

An increasing awareness of investigating toxicological effects in environmentally realistic experiments is preponderant, since organisms in natural environments may be potentially exposed to mixtures of different contaminants, and, thus, uptake by different pathways would occur (Dang et al., 2012). Studies have, thus, focused on evaluating diet metal uptake in decapods, using organisms from contaminated sites (Reichmuth et al., 2009, 2010; Rainbow et al., 2006a, b; Rainbow and Smith, 2013) or artificially contaminated food (Torres et al., 2014). Since metal concentrations in prey are not under researcher control, producing an artificial food in laboratory is more adequate, since the metal delivery can be tested and controlled. Our results were robust enough to confirm that Pb was maintained inside the food at the chosen concentrations, so bioaccumulation or detoxification could be observed and effectively confirmed.

5. Conclusions

C. danae is highly tolerant to Pb exposure at the evaluated concentrations. The water contamination treatment was more efficient in increasing Pb concentrations in gills, hepatopancreas and muscle

tissues. The combined treatment was efficient in altering the amounts of MT, especially in gills.

In gills, both the waterborne and dietary exposures acted simultaneously to induce MT expression. Compared to Pb bioaccumulation in gills exposed to contaminated water, the decreases in Pb accumulation in the combined treatments and MT increases after 14 days suggest that these proteins play a detoxification function in the presence of Pb in this tissue.

In the hepatopancreas, depending on the predominance of isolated or combined pathways, Pb bioaccumulation occured at different times of exposure. No statistical differences in MT contents between treatments as a function of the time of exposure (or interaction) were detected in this tissue.

For muscle tissue, Pb bioaccumulation was observed due to contaminated water exposure, but not dietary exposure, probably because the chosen Pb concentrations were low and because muscle tissue may not be a target tissue for this element.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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