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Shell regeneration in juveniles pearl oyster *Pinctada imbricata* (Röding, 1798) as a physiological index of the sublethal effect of cadmium

Regeneración de la concha en juveniles de la ostra perla *Pinctada imbricata* (Röding, 1798) como índice fisiológico del efecto subletal del cadmio

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ABSTRACT

The shell regeneration of juveniles pearl oyster *Pinctada imbricata* exposed to sublethal levels of Cadmium (Cd), was analyzed as a physiological index. Two bioassays were performed; the first to select the time and effectiveness of shell regeneration according to the zone (posterior, ventral, anterior) after removing pieces of 3-4 mm of shell. The second was carried out to determine the effect of Cd on shell regeneration, RNA / DNA index, respiration rate and mass of body tissues. Exposure to Cd was carried out during 96 h, in sublethal concentrations (0, 0.2, 0.4, 0.8 ppm) or lethal (1.6 ppm). No regeneration was observed in the anterior zone of the shell. At 72 h, the regeneration of the shell in the ventral area (0.78 ± 0.083 mm) was significantly greater compared to the posterior one (0.51 ± 0.044 mm); at 96 h, this difference was not significant. At the first trial, the percentage of individuals involved in shell regeneration was not significantly different at both 72 and 96 h. The second assay showed a clear negative trend in shell regeneration with the increase in Cd concentration. However, no significant relationship was found with the other indices studied. The results show the feasibility of using the regeneration of the ventral zone of the shell as a physiological index to evaluate the effect of Cd on juveniles of the pearl oyster *Pinctada imbricata*.

Key words: Median lethal dose, sea pollution, ecotoxicity.

RESUMEN

Se analizó la regeneración de la concha como índice fisiológico, en juveniles de la ostra perla *Pinctada imbricata* expuestos a niveles subletales de Cadmio (Cd). Se realizaron dos bioensayos; el primero para seleccionar el tiempo y la efectividad de regeneración de concha según la zona (posterior, ventral, anterior) luego de remover trozos de 3-4 mm de la misma. El segundo se realizó para determinar el efecto del Cd sobre la regeneración de la concha, índice de ARN/ ADN, tasa de respiración y masa de los tejidos corporales. La exposición al Cd se realizó durante 96 h, en concentraciones subletales (0; 0,2; 0,4; 0,8 ppm) o letales (1,6 ppm). No se observó regeneración en la zona anterior de la concha. A las 72 h, la regeneración de la concha en la zona ventral (0,78 ± 0,083 mm) fue significativamente mayor comparada a la posterior (0,51 ± 0,044 mm); a las 96 h, esta diferencia no fue significativa. En el primer experimento, el porcentaje de individuos involucrados en la regeneración de concha no fue significativamente diferente tanto a las 72 como 96 h. En el segundo ensayo, se observó una clara tendencia negativa en la regeneración de concha con el incremento en la concentración de Cd. Sin embargo, no se encontró relación significativa con los demás índices estudiados. Los resultados muestran la factibilidad de usar la regeneración de la zona ventral de la concha como índice fisiológico para evaluar el efecto del Cd en juveniles de la ostra perla *Pinctada imbricata*.

Palabras Clave: Dosis letal media, contaminación marina, ecotoxicidad.

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INTRODUCTION

Pollution of aquatic ecosystems has mainly originated by the exacerbated industrial development and rapid growth of the regions in their vicinity, with the consequence that some chemical products, like polycyclic aromatic hydrocarbons, polychlorinated compounds and heavy metals, are drained daily at different coastal areas. Due to this phenomenon, interest has increased in recent years to study the effect of pollution in the marine environment, being the heavy metals some of the most important and better studied pollutants (Dahms, 2014), among them Cadmium, since it is one of the most toxic metals that shows a high incidence in marine-coastal zones and estuaries (Yuan *et al*., 2004; Meng *et al*., 2008; Yu and Chu, 2006).

Bivalves have been widely used to evaluate environmental quality of aquatic ecosystems. Due to their filtering capacity of the surrounding environment, they can bioaccumulate contaminants; furthermore, their sessile condition makes them useful as evidence of events that occurred in the inhabited areas (Tynan *et al*., 2005). The pearl oyster, *Pinctada imbricata,* is a bivalve of the family *Pteriidae* with a broad distribution along the Venezuelan and the Caribbean Sea coasts, forming oyster banks with economic importance. It is a cosmopolitan species of tropical and subtropical seas; hence, it has been suggested as a model species in toxicity tests (Lodeiros, 2011).

The measurement of shell regeneration after its traumatism, evaluated in short periods of time (4 days), is shown as an index for an easy determination of the effect of xenobiotics, which gives it a character of universal application, being already a standard test used with *Crassostrea virginica* (US-EPA, 1996). Villegas *et al*. (2015), in a study of the effect of Cd on the ontogeny of *P. imbricata* (adults of different sizes since early reproductive stages) concluded that the shell regeneration could be a suitable index to evaluate the Cd effect in a short period of time in juveniles, however, due to the discontinuity of shell regeneration in their results, he, recommends studies to select the zone of shell more suitable for its regeneration.

The purposes of the current study were to select the best zone of shell regeneration and later to evaluate the regeneration of the shell in juveniles of the pearl oyster, *P. imbricata,* exposed to sublethal or lethal doses of Cadmium in order to validate the shell regeneration as an evaluative parameter in toxicity assays.

MATERIALS AND METHODS

Bioassays

Bioassays were performed in plastic containers of 2 L capacity, with 1.5 L filtered sea water (36 UPS), treated with UV light and continuously aerated with an air flow of 100 ml/min. Juveniles of *P. imbricata* (20-25 mm dorso-ventral length) came from culture baskets and were acclimated during seven days at laboratory conditions (22°C, luminosity of 200 lux with a 12:12 h photoperiod) and fed daily with 10.000 cel/mL of the microalga *Tetraselmis chuii.*

A first trial was conducted in order to select the best zone of shell regeneration; for this, 3-4 mm shell pieces were removed in *P. imbricata* juvenils with an electric saw (Dremel) in three different zones (anterior, posterior and ventral). The individuals (two replicates of 20 juveniles for each treatment) were placed under the laboratory conditions mentioned before, during 96 h, without feed. Five individuals from each replicate were removed daily and the shell regeneration of each one was determined (generated lamellae), by measuring the recently formed lamellae using an image analyzer incorporated to a stereoscopic microscope.

A second bioassay was carried out in order to evaluating the effect of Cd on regeneration of the shell. To select the sublethal dose of Cd in juveniles of *P. imbricata*, it was determined the Median Lethal Dose (DL_{50}), following the criteria suggested by international organizations like ASTM and FAO (Nascimento *et al*. 2002). In this sense, 1.791 g $CdCl_2.H_2O$ (Sigma-Aldrich, 98%) was dissolved in 1000 mL of deionized water to prepare a stock solution (1000 ppm). From this solution, the required metal concentrations were prepared. The organisms (15 per replicate), previously acclimated to the laboratory conditions described before, were exposed, by triplicate, to 0.0, 0.6, 1.2, 1.8 and 2.0 ppm of cadmium. Exposure to the toxic metal lasted for 96 h, with a renovation of the medium

at 48 h and counting the dead animals every 24 h. For the determination of death, the criterion of valve post-relaxation of adductor muscle was used, stimulating the reaction with a glass bar. The $DL_{50\%}$ and its 95% confidence intervals were determined from data on mortality.

Taking the $DL_{50\%}$ of Cd as starting point, and the time and zone of shell regeneration obtained in previous tests, 3-4 mm shell pieces were removed in *P. imbricata* juvenils and exposed, by triplicate (25 juveniles) during 96 h, to 3 sublethal concentration of Cd (0.2; 0.4; 0.8) with a control lethal concentration (1.6 ppm) and a control non-lethal concentration (without Cd).

Determination of oxygen consumption, dry biomass of soft tissues and ratio RNA/DNA

At the end of the experiment, the oxygen consumption of all organisms in each replicate was determined using a hermetic chamber with seawater saturated with oxygen, after 20 min of respiration, using oxygen meter YSI 20. The oxygen consumption was expressed in ml/L/g of dry mass of soft tissues.

The determination of dry biomass of soft tissues at the end of the test was evaluated in groups of 10 individuals from each replicate, using an analytical balance with precision 0.0001 g, after a dehydration process of the animals in an oven at 60 °C during 48 h, following Lucas and Beninger (1985). The analyses of nucleic acids were made in groups of 5 individuals from each replicate, following Canino and Calderone (1995) and using 10-20 mg biomass of adductor muscle. With the data on concentrations of RNA and DNA the ratio RNA/DNA was calculated.

Data analysis

The shell regeneration results were analyzed through a two-way analysis of variance (ANOVA II) using as factors time and the place of shell regeneration. The Probit test was used in the bioassay of the lethal effect of Cd to determine the Mean Lethal Dose ($DL_{50\%}$) and its confidence interval (US-EPA, 1993).

The differences in oxygen consumption and dry biomass of the soft tissues were contrasted using a one-way analysis of variance (ANOVA I), using the concentration of Cd as factor and Duncan *a posteriori* test. To estimate the ratio RNA/DNA, the data was transformed calculating the arc cosine. Since the data distribution still deviated from normal, the differences were analyzed through a Kruskal-Wallis test. All tests used a significance level of P=0.05.

RESULTS AND DISCUSSION

The shell of *P. imbricata* did not regenerate in the anterior zone, in contrast with the ventral and posterior zones where regenerative growth was observed after 72 h (Figure 1a). The largest growth was recorded in the ventral zone (0.68±0.19 mm), although at the end of the bioassay there were

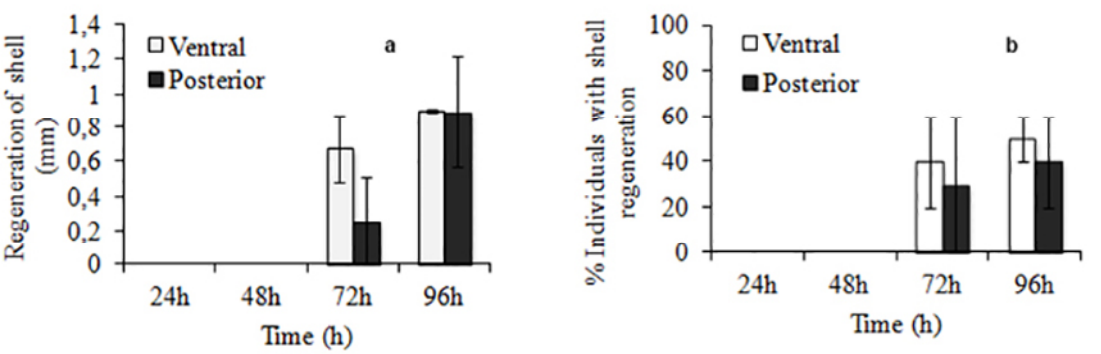

Figure 1. a) Amount of shell regeneration (mm) and b) % individuals that regenerated the shell, by time after breakage of shell and zone of regeneration in juveniles of *Pinctada imbricata*. Bars show the standard error.

not significant differences (P>0.05) between the ventral and posterior zones, reaching average values 0.91±0.011 and 0.89±0.323 mm of shell regeneration, respectively (Figure 1a).

The percentage of individuals that regenerated the shell did not show significant differences between regeneration zones and time after breakage of shell (P>0.05). In average, 50% of the individuals regenerated the ventral zone of the shell at 96 h (Fig. 1b). The dispersal indices in the treatments of this experiment were high, with greater variability in the posterior zone. These results allowed the choosing of the ventral zone and an experimental time of 96 h for further bioassays. The time of exposure to a toxic agent and the zone of the shell where the measurements are made, are important factors to consider when setting up a toxicity assay using juveniles of *P. imbricata.*

During the first 48 h of the control assay, with no toxic agent in the environment, no shell formation was observed. However, after 72 h the growth of the shell was evident and at 96 h there was a greater growth as well as a larger percentage of individuals regenerating the shell (50% in the ventral zone: 40% in the posterior zone). There were no significant differences between the zones where regeneration was actually observed, but there were high levels of dispersion in the replicate samples of the posterior zone; hence, the ventral zone was chosen for further bioassays.

Considering that only 50% of the tested individuals regenerated the shell 96 h after the initiation of the assay, it is suggested that this period should be the minimum duration of assays for protocols that use shell regeneration as an index.

The only published reference found about the selection of the regeneration zone of the shell in bivalve mollusks for toxicity assays, is the one performed by the Environmental Protection Agency of the USA (US-EPA, 1996), in which the ventral zone of the American oyster, *Crassostrea virginica*, was used, which supports the observations made in the present study.

None of the individuals with the shell broken in the anterior section regenerated the shell during the experiment, so this section was discarded for further testing. There are not reported evidences to support this difference with the other sections of the shell, but it may be related to the anatomy and function of this zone. The anterior zone is close to the gland that segregates the byssus, where there is an invagination of the shell with little evidence of lamellae or growth border, in comparison with the ventral and posterior zones. Thus, the anterior zone of the shell would have a smaller ability of segregating shell since in the surrounding areas to the byssus gland there is no mantle, which is the tissue in charge of producing the shell.

During the determination of the Cd concentrations that would be used, it was observed that after exposing juveniles of *P. imbricata* to several concentrations of Cd (0, 0.6, 1.2, 1.8 and 2 ppm), no mortality was observed at 0 ppm and 0.6 ppm, but this parameter increased proportionally as concentration of Cd raised in the environment, reaching a demise of 86.7% at a concentration of 2 ppm. The $DL_{50\%}$ was established at 1.68 ppm (95% C.I.: 1.49-1.85). These results suggested that for to determine the sublethal effect of Cd, the concentrations 0; 0.2; 0.4; 0.8 should be used. Low values were also obtained by Villegas *et al.* (2000) in juveniles of the same species (0.63 mg $^{L-1}$). The effect of Cd in other species has been observed at higher concentrations, for example in *C. virginica*, the value of $DL_{50\%}$ (at 72 h) is 24.87 mg $^{L-1}$ (Barrera, 2006). These differences suggest a high sensibility of the pearl oyster to Cd and possibly to other xenobiotics, supporting its selection as a model species for toxicological studies (Lodeiros, 2011).

The amount of shell regeneration showed significant differences (P<0.05) among the different concentrations of Cd, with a trend inversely proportional to the concentration of the metal. No shell formation was observed in the highest Cd concentration (1.6 ppm; Figure 2a). Individuals in the control group (with no Cd) showed a shell growth significantly higher (2.47 ± 0.678 mm, P<0.05) than oysters in the other treatments. Shell growth in the treatment of 0.8 ppm of Cd (0.47 ± 0.327 mm) did not show significant differences with that of the treatment of 0.4 ppm Cd (0.59 ± 0.485 mm), but they were significantly different to the one observed in the treatment of 0.2 ppm Cd (1.11± 0.820 mm).

Likewise, significant differences were observed with the percentage of individuals that regenerated the shell, forming the same groups

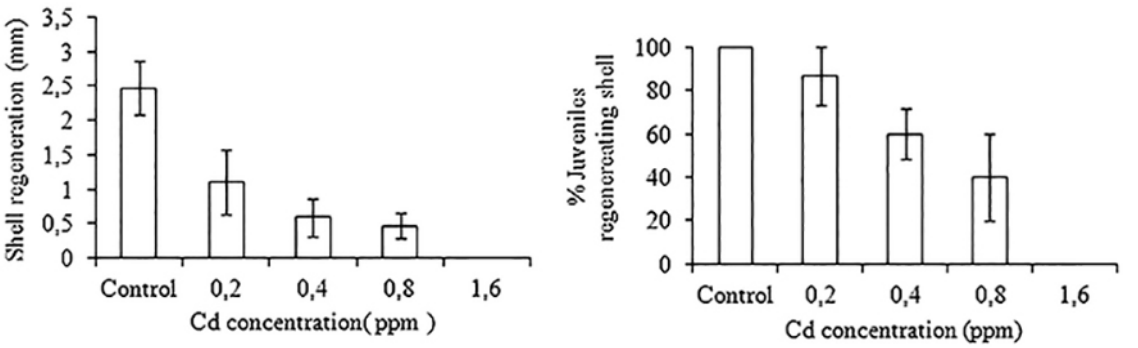

Figure 2. a) Amount of shell regeneration (mm) and b) % individuals that regenerated the shell, among juveniles of *Pinctada imbricata* exposed during 96 h to sublethal doses of Cd. Bars show the standard error.

of significance described for the amount of shell regenerated. All individuals in the control group regenerated the shell while those in the group exposed to 0.2 ppm Cd responded in 86.7%; those exposed to 0.4 ppm Cd in 60% and those exposed to 0.8 ppm Cd in 40%. None of the individuals in the group exposed to 1.6 ppm Cd showed any regeneration of the shell (Figure 2b).

The shell regeneration and the percentage of individuals that regenerated the shell was affected by the amount of metal in the environment; thus, 100% of the individuals in the control group without Cd regenerated the shell while the most affected group was the one exposed to the highest dose of Cd, which did not regenerated the shell and had a mortality rate of 6.7%. It has been shown that Cd can replace calcium in cell processes and it is possible that it affects the mechanism of incorporation calcium in the shell. In the other hand, it has been established that Cd induces a general internal acidosis, a release of calcium and inhibition of reabsorption of calcium affecting the biomineralization of the shell (Faubel *et al.,* 2008).

These results agree with those obtained by Villegas *et al.* (2015) who argued that such differences were due to the response of shell reconstruction, where the processes of antitoxic protection lead to a reduction of the speed of shell deposition, assuring the distribution of the accumulated energy for the basic physiological processes. Borthwick and Patrick (1982) obtained shell deposition in 50% of the individuals of the american oyster *C. virginica* exposed to a concentration of creosote of 0.7 mg/L (96 h) and Steven *et al.* (1977) found an inhibition in the deposition of shell by the american oyster exposed to 3.1 µg/L of the insecticide toxaphene. In the other hand, Lowe *et al.* (1971) also obtained a smaller growth of the American oyster when exposed to toxaphene (1 µg/L) during 12 weeks. Finally, the shell deposition was slower when the american oyster was exposed to concentrations of the chlorinated organic insecticides chlordane of 4.7 µg/L and 4.9 µg/L of endrin (Parrish *et al.,* 1976; Schimmel *et al.,* 1975).

There were no significant differences (P>0.05) in the oxygen consumption, ratio RNA/DNA or total dry biomass among the sublethal concentrations of Cd to which the juveniles of *P. imbricata* were exposed, in spite of the observed increasing trend in the oxygen consumption and decreasing trend of the ratio RNA/DNA as Cd concentration raised (Figure 3).

Even though the differences in oxygen consumption in the individuals exposed to the different concentrations of Cd were non-significant, an increasing trend was observed in the consumption of oxygen directly proportional to the concentration of the contaminant in the environment. This can be associated to the stress generated by the contaminant, which induces a greater antitoxic metabolism. The results of Villegas *et al.* (2015) in a study made with *P. imbricata* agree with those obtained in the present study. In a similar way, Barrera (2006) reported for the American oyster *C. virginica,* that exposure to Cd significantly altered the respiratory rate, depending on time and magnitude of exposure to the metal. In the

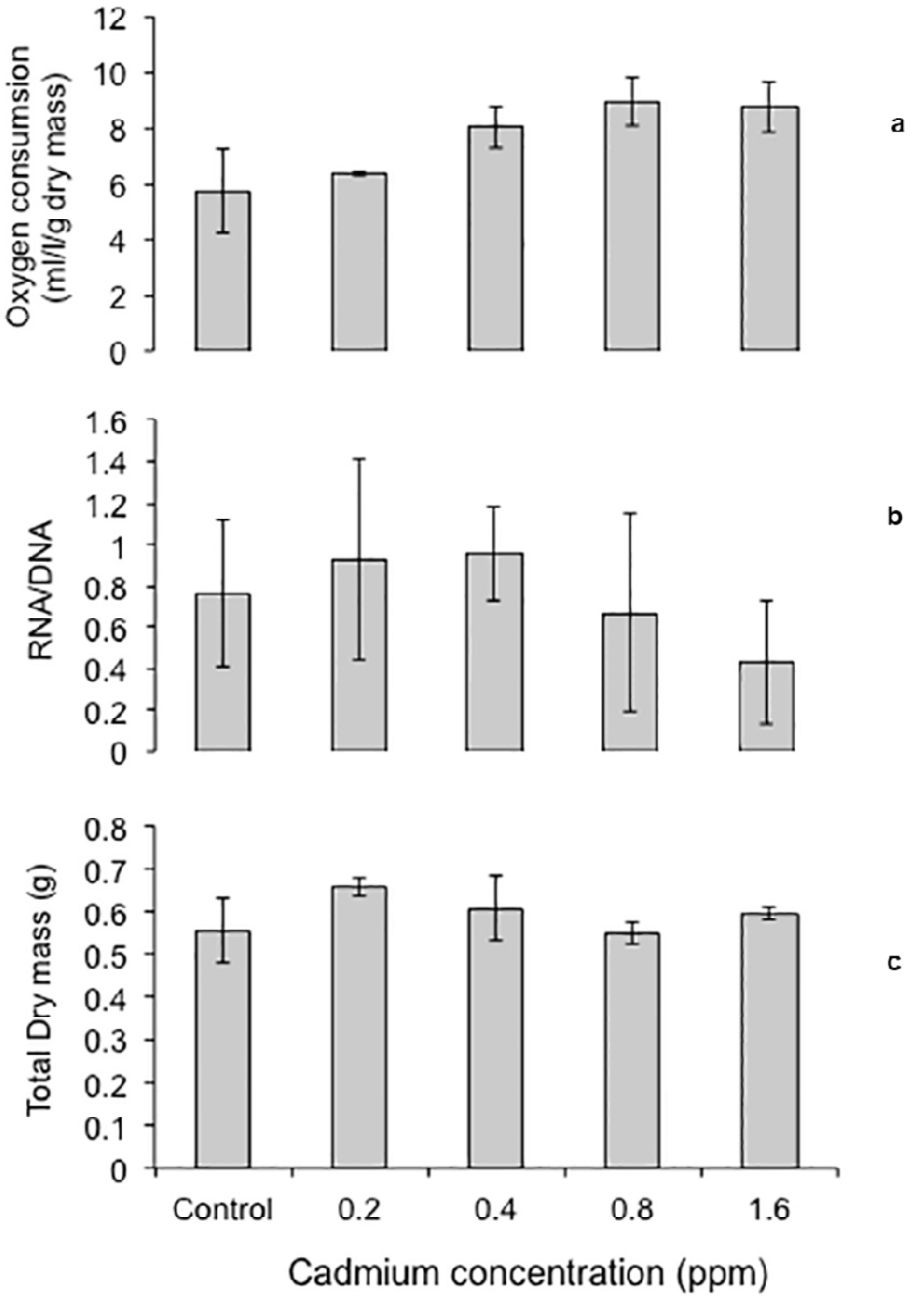

Figure 3. a) Oxygen consumption, b) Ratio RNA/DNA and c) Total dry mass of juveniles of *Pinctada imbricata* exposed to different concentrations of Cd. Bars show the standard error.

case of mollusks, the increase in the respiratory rate has been related to the deterioration of the osmoregulatory processes and the inhibition of Ca transport (Sadiq, 1992). However, it cannot be discarded that there are also energetic requirements that favor its increment. Thus, the increase of the respiratory rate of individuals of *P. imbricata* when exposed to Cd can be due to the energetic costs associated to the detoxification mechanisms and cell protection produced by the toxic effect of the metal, both in the synthesis of metallothioneins (MTs) and the oxidative stress registered in short time exposures (Lemus *et al.,* 2012).

Glutathione (GSH) seems to play an important role in the mantle, increasing as a function of Cd concentration. This tissue has as main functions to segregate the shell and participate in respiratory activities. The perturbing effect of Cd in the electron transport chain has been clearly evidenced (Faubel *et al. 2*008); the toxic effect of this metal produces the release of cytochrome c and the formation of free radicals, which will also promote an increase of MTs (Chelomin *et al.* 2005; Buico *et al.* 2008). Since mantle is a tissue with high vascularization, it possibly plays an active metabolic role synthesizing MTs and other proteins in the presence of Cd, as is also found in the hepatopancreas (Faubel *et al.,* 2008). In order to study the effect of Cd and other xenobiotics on MTs of *P. imbricata* it would be necessary to verify the aforementioned hypothesis.

The results did not show significant differences in the index RNA/DNA in individuals exposed to different concentrations of Cd. The great variability and high dispersal indices in each treatment made evident the different responses of enzymatic activation in the metabolism. Lodeiros *et al.* (1996) indicate that such variability limits the usefulness of this index to predict the growth in the scallop *Euvola ziczac* in sizes where reproduction as a physiological process is implicated; nevertheless, since in the present study juvenile individuals were used, the variability of this index in the pearl oyster could be associated to the influence of metabolic processes other than reproduction. Antón *et al*. (2008) show that the reduction of the index RNA/DNA in *Donax denticulatus* exposed to Cd was associated to a decrease in the levels of RNA due to a decline in the synthesis of these biomolecules. Many biomolecules such as glutathione, metallothionein and proteins with capacity to inactivate the metal requires energy for their synthesis. Likewise, those proteins related to the antioxidant activity of the body could be activated as a consequence of the capacity of Cd and other metals to induce oxidative stress in the organisms (Nusetti *et al.,* 2001; Risso *et al.,* 2004).

In the experiment of sublethal effects of Cd, the dry mass of the pearl oyster did not show significant differences among the tested concentrations of the metal. It is possible that, regardless of the starvation stage of the individuals, the time of exposure was not long enough for a detrimental effect on the dry mass of the tissues to become evident. These results agree with those of Villegas *et al.* (2015) in the exposure to Cd in three sizes of *P. imbricata* (including juveniles). Likewise, in a study on exposure and accumulation of Cd in the Green mussel, *Perna viridis*, during a longer period (7 days), the dry mass of the tissues was not an adequate index to detect effects of the xenobiotic agent on these species (Narváez *et al.,* 2005).

CONCLUSION

Shell regeneration in the ventral zone can be an adequate index to observe sublethal effects of Cd in *P. imbricata*. The use of shell regeneration could be suggested as a simple index in toxicological tests with bivalve mollusks, although feasibility tests should be made with other xenobiotics and others species.

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