



Evaluation of pollution in Camichin estuary (Mexico): Pro-oxidant and antioxidant response in oyster (*Crassostrea corteziensis*)[☆]



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ABSTRACT

The physiological system of molluscs, particularly pro-oxidant and antioxidant mechanisms, could be altered by pollutants and induce disturbance on health status and productive parameters of aquatic organisms, such as oyster. Therefore, the aim of this study was to evaluate the chemical contamination in water (total metals and polycyclic aromatic hydrocarbons) and oxidative stress parameters in oysters (*Crassostrea corteziensis*) in Camichin estuary, located in Mexican Tropical Pacific. The results obtained showed the presence of arsenic, lead and zinc, as well as naphthalene, pyrene and benzo[a]pyrene in concentrations relatively higher than criteria established by local and international guidelines. Regarding the biomarkers of oxidative stress response (H_2O_2 and O_2^{\bullet} concentration, catalase activity, lipid peroxidation, and hydroperoxide concentration), differences between oyster from estuary and control group were significant. These results indicate that these pollutants could be related with oxidative stress detected in oyster.

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1. Introduction

Aquatic environments are potentially vulnerable to pollution since almost all kind of chemicals used in anthropogenic activities, directly or indirectly, reach the water bodies (Islam and Tanaka, 2004). The presence of pollutants is able to induce an imbalance in the pro-oxidants and antioxidant mechanisms in organisms through the release of reactive oxygen species (ROS), such as H_2O_2 , O_2^{\bullet} , OH^- , and $^{1/2}O_2$. These molecules carry odd numbers of electrons in binding orbital and are able to extract other electrons from biomolecules, causing adverse effects, as lipid peroxidation, proteins and DNA oxidation, altering cell physiology and can induce cell death (Masroor et al., 2000; Galloway and Depledge, 2001; Hermes-Lima, 2004; Basova et al., 2012). However, organisms have enzymatic antioxidant defenses, as the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), detoxify ROS before they cause oxidative damage, and trigger off loss in cellular integrity (Hebbel, 1986; Sies, 1993; Guerra et al., 2012).

Lipid peroxidation is a chain reaction, in which ROS react with membrane phospholipids, inducing the formation of conjugated dienes, lipid hydroperoxides and finally chemical changes in polyunsaturated fatty acids (PUFAs). This may cause a reduction in the fluidity of the membrane as well as cell membrane destruction. In addition, the lipoperoxidation can affect internal membrane systems, such as endoplasmic reticulum and mitochondria (Hermes-Lima, 2004). Although proteins can be altered by ROS, there are few cellular mechanisms involved in protection against protein oxidation, such as the ubiquitin–proteasome pathway (Goldberg, 2003).

Bivalve molluscs such as oysters have been used as sentinel organisms for monitoring pollution in coastal environments. These invertebrate have characteristics such as: wide distribution around the world, sessile nature and filter-feed habits; for these reasons, the bivalve molluscs accumulate large amounts of chemical contaminants (Regoli and Principato, 1995), causing oxidative damage, due to excessive ROS production, that would compromise cellular functioning (Beckman and Ames, 1998).

Chemical-related sources of enhanced ROS formation include exposure to organic contaminants, such as cycling redox compounds (quinines, nitroaromatics, nitroamines, herbicides), polycyclic aromatic hydrocarbons (PAHs), pesticides, dioxins and trace metals (Livingstone, 2001; Regoli et al., 2005). Thus, it has been reported that certain pollutants, such as benzo[a]pyrene, increase ROS production and causes disruption of lysosomal membranes in bivalves (Livingstone et al., 1993). On the other hand, the exposure of molluscs to cadmium inhibits mitochondrial respiration (Alves de Almeida et al., 2007). Moreover, studies

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realized in clam *Megapitaria squalida* have reported a positive correlation between the concentration of oxidized lipids and cadmium levels in tissue (Cantú-Medellín et al., 2009).

The most important site producer of oyster (*Crassostrea corteziensis*) in the Mexican Pacific is the Camichin estuary, which is located in San Pedro-Mezquital basin, at Northwestern Nayarit state. Commercial exploitation of this mollusc is about 1200 t per year. However, the up-basin also carries out agricultural, mining and tourist activities (CONAPESCA, 2008; González-Arias et al., 2010) that can impact the growth and production of oysters; these anthropogenic activities generate multiple stressors, such as urban wastewaters, pesticides, metals, and petroleum by-products (Siung-Chang, 1997; Sokolova and Lannig, 2008).

Therefore the aim of this study was to evaluate the chemical contamination (metals and polycyclic aromatic hydrocarbons) and oxidative stress parameters in oysters (*C. corteziensis*) in Camichin estuary.

2. Material and methods

2.1. Sampling

Three stations were selected in the sampling area from Camichin estuary: station 1 was located downstream and near to mouth of estuary (21°43'58 "N, 105°29'15 .6"W), station 2 was located in the middle of the culture area (21°44'50 .1 "N, 105°29'46 0.3 "W) and station 3 was located towards the head of the estuary (21°45'45 .1"N, 105°29'41 .5"W) (Fig. 1). To determine metals and PAHs concentration, in each sampling station, 3 water samples were collected in 3 standard depths of water column (surface, medium, and bottom).

2.2. Metal determination

The total concentration (dissolved and particulate) of arsenic (As_T), copper (Cu_T), iron (Fe_T), manganese (Mn_T), lead (Pb_T) and zinc (Zn_T) in water was determined by acid digestion followed by flame atomic absorption spectrometry using the direct air-acetylene flame method (APHA, 1998a) with a GBC 932 Plus spectrophotometer. Arsenic was evaluated by electrothermal atomic absorption spectrometry (APHA, 1998b) in a GBC GF3000 graphite furnace system coupled to the spectrophotometer. In all cases GBC certified standards were used. Quality control program was performed as follows: method precision was estimated through the coefficient of variation: $\%CV = (\alpha - 1 \text{ replicates} / \text{mean}) * 100$. Accuracy was determined by the recovery average: $\%R = [(ECA) - (C) / (A)] * 100$; where ECA = the experimental concentration of the spiked sample; C = sample concentration; and A = the concentration of metal used to spike samples (Vega-López et al., 2006).

The observed precision in the assessment of metals had the following values: As_T (1.55%CV), Cu_T (9.79%CV), Fe_T (4.56%CV), Mn_T (11.75%CV), Pb_T (12.13%CV), and Zn_T (1.37%CV).

The accuracy founded in the assessment of metals had the following values: As_T with 102.73%R, Cu_T with 95.04%R, Fe_T with 98.97%R, Mn_T with 97.46%R, Pb_T with 99.34%R, and Zn_T with 97.52%R.

2.3. Polycyclic aromatic hydrocarbons (PAHs) determination

The concentrations of naphthalene, pyrene and benzo[a]pyrene were analyzed according to the Mexican norms (NOM-138-SEMARNAT/SS-2003). Hydrocarbons were extracted in 500 mL of water with

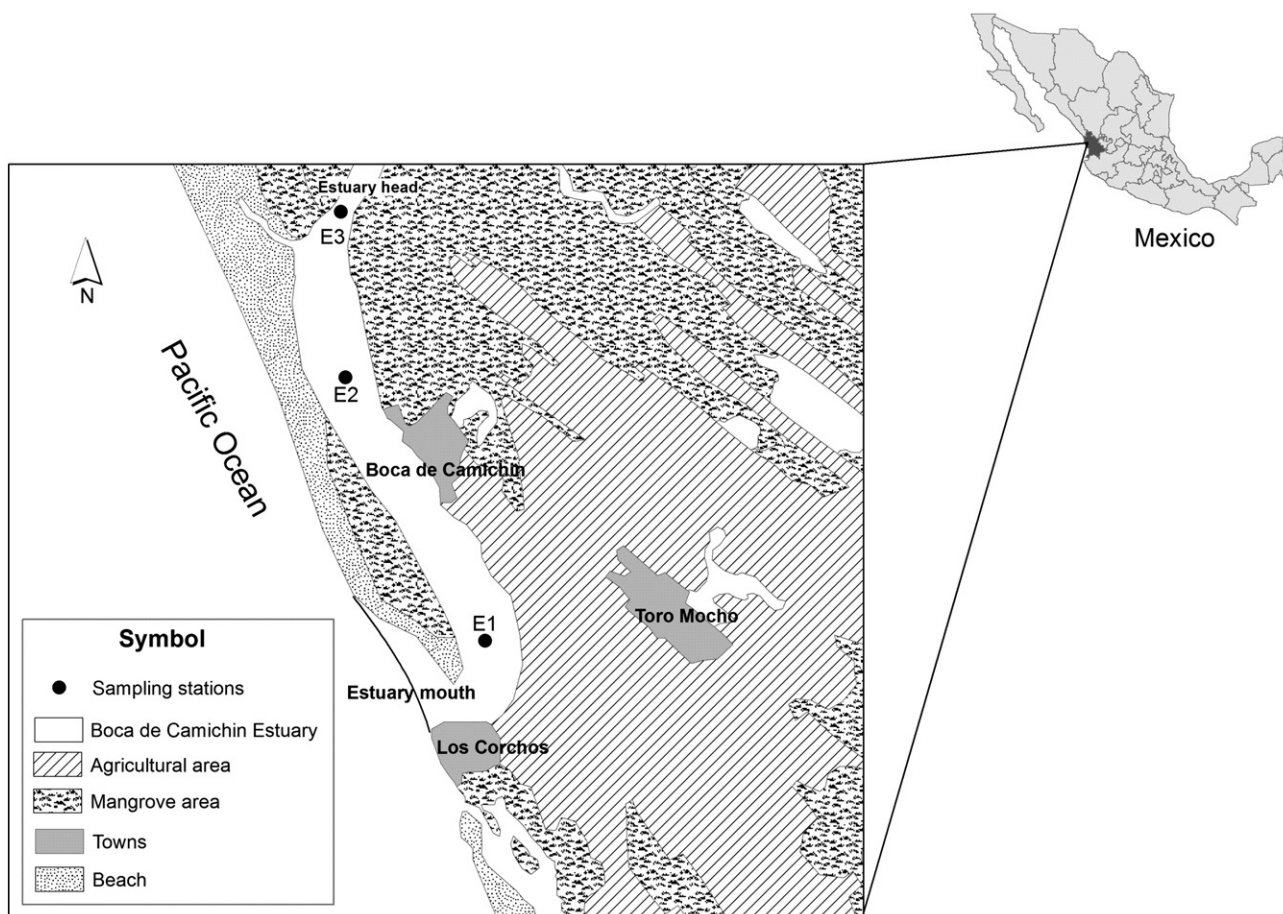


Fig. 1. Geographical localization of Camichin estuary. The map shows the localization of the three sampling stations and areas of natural and anthropogenic influence immediate to study area.

25 mL of dichloromethane HPLC-grade. The sample was mix-shaked with subsequent separation of the aqueous and solvent using a red separating funnel. The extracts were pre-filtered through fiberglass previously rinsed in same-grade dichloromethane and anhydrous sodium sulfate, then purified in C18 column. The PAHs concentrations were quantified in a LS-55 Perkin–Elmer spectrofluorometer according to the following excitation/emission longitudes: naphthalene (273/360 nm), pyrene (327/385 nm), and benzo[a]pyrene (380/430 nm) using a PAHs mix as standard (SULPECO, Cat. 47543-U). Quality control program was performed as previously detailed.

The precision values in the PAHs quantification were: naphthalene (9.52%CV), pyrene (12.56%CV), and benzo[a]pyrene (7.58%CV). The observed accuracy in the evaluation of hydrocarbons had the following values: 98.42%R for naphthalene, 92.77%R for pyrene, and 88.46%R for benzo[a]pyrene.

2.4. Stress oxidative parameters

2.4.1. ROS determination (O_2^{\bullet} and H_2O_2)

Oxidative stress parameters were measured in gills of oyster. In each site sampling was random, selecting 10 organisms, and gills of each one were obtained. The tissue was mechanically homogenate with polytron, and one sub-sample was centrifuged to ROS concentration and CAT activity determination.

A sample of gills from each organism ($n = 10$) was centrifuged at 9000 rpm for 20 min, and 20 μ L of supernatant was mixed with 160 μ L of work solution (6.25 μ M 2',7'-dichlorodihydrofluorescein diacetate and 6.25 μ M dihydroethidium) pH 7.4, and 20 μ L 1.4 mM NADH into ELISA plate. Immediately fluorescence (time 0) and 10 min at 485 nm excitation and 525 nm emission for H_2O_2 , and 530 nm excitation and 624 nm emission for O_2^{\bullet} was assessed. The concentration of ROS was calculated by interpolation on a curve type.

2.4.2. Catalase activity (CAT; EC 1.11.1.6)

A sample of gills from each organism ($n = 10$) was centrifuged at 12,000 g for 20 min, and 20 μ L of supernatant was mixed with 1000 μ L of buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES and 5 mM KH_2PO_4) and 200 μ L of 20 mM H_2O_2 . Immediately the absorbance was evaluated at 240 nm for 2 min every 30 s. The molar extinction coefficient of H_2O_2 was 0.093 $mM^{-1} cm^{-1}$ (Radi et al., 1991). To adjust CAT activity, the protein concentration was determined using the Bradford (1976).

2.4.3. Lipid peroxidation determination

Oyster gills homogenates (100 μ L) were mixed with 500 μ L 150 mM Tris–HCl buffer and pre-incubated during 30 min at 37 °C. Subsequently, 1 mL 0.37% thiobarbituric acid was added. The mixture was heated to boiling for 45 min and cooled on ice. To calculate thiobarbituric acid reactive substance concentration (TBARS), the absorbance was determined at 535 nm, using molar extinction coefficient of thiobarbituric acid 156,000 $mM^{-1} cm^{-1}$ (Buege and Aust, 1972).

2.4.4. Lipid hydroperoxide (ROOH) determination

Oyster gill homogenates (100 μ L) were mixed with 100 μ L of cold methanol, shaken in a vortex, and placed on ice. Then 0.40 μ L 0.25 mM $FeSO_4$, 0.4 μ L 25 mM H_2SO_4 and 0.10 μ L 0.1 mM xylenol orange were added. The mixture was incubated during 24 h at room temperature in the dark. 10 μ L 1 mM of cumene hydroperoxide was added and incubated for 30 min. The absorbance was determined at 580 nm (Jiang et al., 1991).

2.5. Control group

As control group, oysters were maintained under laboratory condition (5 oysters/10 L water) during 30-days in filtered seawater to 25‰ salinity, and light–dark periods 12:12. The average values for

water quality were: temperature 28 ± 2 °C, pH 8.0 ± 0.1 , dissolved oxygen 7.0 ± 0.2 mg L^{-1} , and oxygen saturation $85.4 \pm 2.4\%$. Oysters were fed daily with 8.5 mg L^{-1} of spirulina (ultra PRONAT) dissolved in filtered seawater.

2.6. Data processing

In order to determine pollutant spatial distribution in the estuary, central tendency measured and dispersion were calculated. Distribution maps of chemical contaminants were constructed also using the Surfer v. 8.0 plot program. To compare different parameters, data were analyzed by one-way ANOVA and Tukey tests for all pair wise comparison of the mean responses to the different experimental groups using Sigma Stat 2.0 software. $p < 0.05$ was accepted as statically differences.

3. Results

3.1. Metal concentrations

The total metal concentrations in Camichin estuary had a heterogeneous distribution in the water column, suggesting a distribution pattern according to the possible sources of each metal in the estuary. The highest concentrations of Pb_T (0.017 ± 0.002 mg L^{-1}) and Mn_T (1.71 ± 0.317 mg L^{-1}) were detected in the head of estuary (station 3), which decrease towards the mouth (station 1) of the system. In contrast, the highest concentration of As_T (35.55 ± 5.88 μ g L^{-1}) was registered at station 1, which decreased towards the station 3, while the highest concentration of Fe_T (0.93 ± 0.35 mg L^{-1}) was found at near the bottom water. In contrast, higher concentrations of Zn_T (0.47 ± 0.04 mg L^{-1}) and Cu_T (0.75 ± 0.15 mg L^{-1}) were quantified in surface water (Fig. 2).

3.2. PAHs concentrations

The concentrations of naphthalene, pyrene and benzo[a]pyrene also displayed a heterogeneous distribution in the water column (Fig. 3). However, relative higher concentrations of PAHs were observed in the station 2. The naphthalene concentration were significantly higher ($p < 0.001$) (409 ± 208.2 μ g L^{-1}), followed by pyrene (25.5 ± 13.6 μ g L^{-1}) and benzo[a]pyrene (1.13 ± 0.3 μ g L^{-1}) concentration (Fig. 3).

3.3. Pro-oxidant and antioxidant response

Relative higher concentration of O_2^{\bullet} was detected in oysters from station 1 (0.076 ± 0.024 mM g^{-1} tissue), compared with oysters from station 2 (0.039 ± 0.011 mM g^{-1} tissue) and station 3 (0.062 ± 0.017 mM g^{-1} tissue). However, no statistical differences were observed between control group (0.043 ± 0.032 mM g^{-1} tissue) and oyster from estuary (Fig. 4A).

Similar to O_2^{\bullet} , higher H_2O_2 concentration was detected in oysters from station 1 (0.015 ± 0.008 mM g^{-1} tissue), compared with oysters from station 2 (0.008 ± 0.005 mM g^{-1} tissue) and station 3 (0.0072 ± 0.0029 mM g^{-1} tissue). Nevertheless, concentration in control group (0.013 ± 0.005 mM g^{-1} tissue) was similar to oyster from station 1 ($p > 0.05$), but higher than in oyster from station 2 and 3 ($p < 0.05$) (Fig. 4B).

Regarding the antioxidant enzyme activities, lower CAT activity was detected in oysters from the estuary in comparison with control group ($p < 0.05$) (Fig. 5).

Meanwhile, lipid peroxidation and hydroperoxide (ROOH) concentrations were significantly higher in estuary oyster compared to control group ($p < 0.05$). Statically analyses showed that lipid peroxidation was higher in oyster from station 1 and 2 vs. station 3 (Fig. 6A). Whereas there were no differences observed in ROOH values among different stations (Fig. 6B).

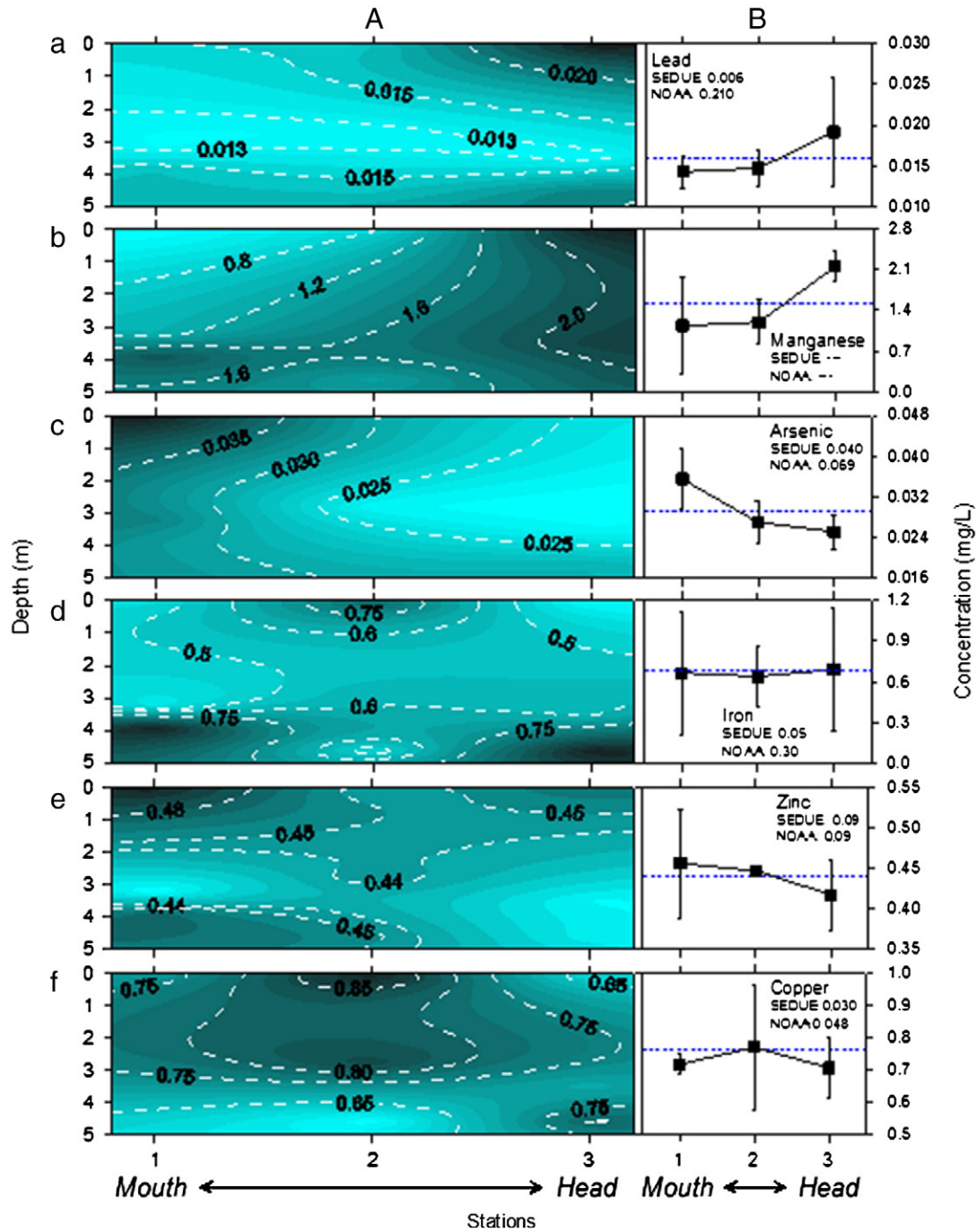


Fig. 2. Spatial distribution (A) and mean \pm SD sampling station (B) concentrations of a) lead b) manganese, c) arsenic, d) iron, e) zinc and f) copper in the water column from Camichin estuary. The dotted line in panel B corresponds to the average concentrations of metal. Values in each graph refer to allowable limits for the protection of marine aquatic life (SEDUE, 1989), and concentrations of acute effect on marine organisms (NOAA, 1999).

4. Discussion

The heterogeneous distribution of metal concentrations observed in the water column allows partially the possible sources of each metal in Camichin estuary during sampling period. Pb_T and Mn_T concentration gradient from the head of the estuary to the mouth was observed; this may suggest that these metals can have a riverine or immediate coastal lagoon origin. In contrast, the As_T concentration distribution suggests a marked influence from adjacent sea, which is an unusual pattern in horizontal gradients of the distribution of metals in coastal aquatic systems (Chester, 2000). On the other hand, the relative high concentrations of Zn_T and Cu_T registered in the surface

water may be associated with point sources or diffuse pollution. The total trace metals registered in Camichin estuary was higher than reported in water of oyster culture areas in Sonora, Mexico (García-Rico et al., 2011).

According to the Ecological Criteria of Water Quality in the Mexican regulations (SEDUE, 1989, CE-CCA-001/89), the average concentrations of Pb_T , Zn_T and Fe_T were relatively higher than permissible limit values established for the protection of marine aquatic life; As_T concentration was below these limits, while Mn_T is not considered in this regulation. In contrast, the average Cu_T concentration was significantly higher than Mexican norms and above the water quality criteria established by the NOAA (1999) for acute effects on marine organisms (Fig. 2B).

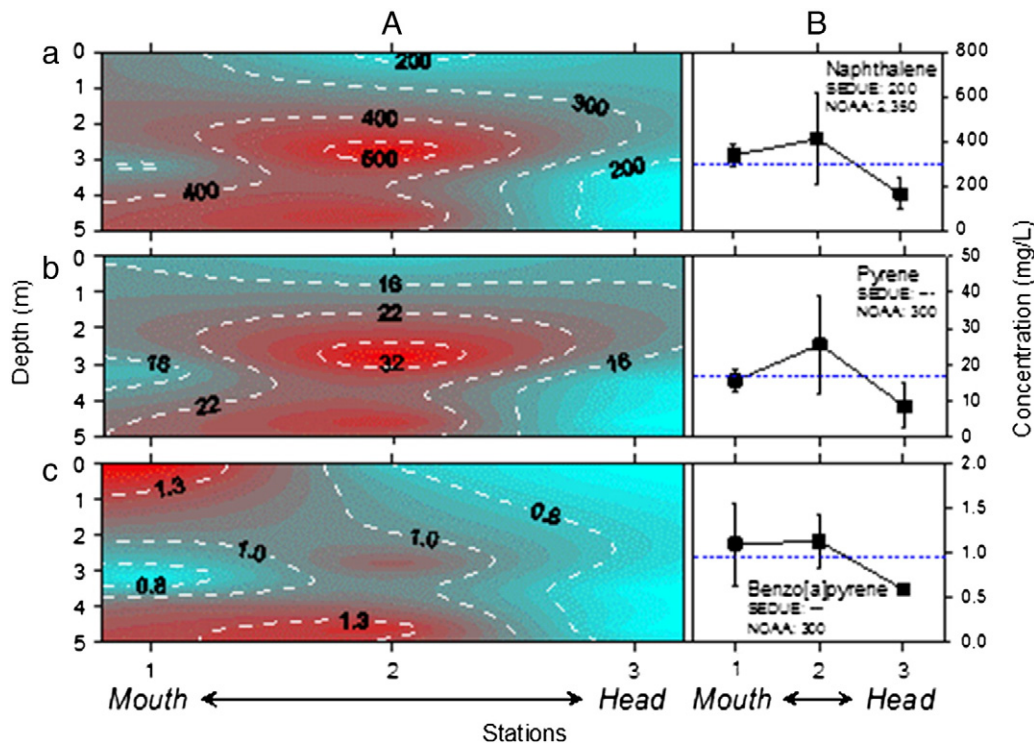


Fig. 3. Spatial distribution (A) and mean \pm SD sampling station (B) concentrations of a) naphthalene, b) pyrene c) benzo[a]pyrene in the water column from Camichin estuary. The dotted line in panel B corresponds to the average concentrations of the PAHs. Values in each graph refer to allowable limits for the protection of marine aquatic life (SEDUE, 1989), and concentrations of acute effect on marine organisms (NOAA, 1999).

The high Cu_T concentrations and its distribution in the water column suggest a point pollution source in the central portion of the study area. One of the main sources of Cu_T globally in coastal systems is attributed to leaching and releasing of anti-fouling paints commonly used on boat ships (Schiff et al., 2004; Singh and Turner, 2009). Although the activity of boat ships in this study area is moderate, it could be a clear possible pollution source, considering that: 1) the highest concentrations of Cu_T were found at station 2, the estuary area with most activity in small boats, and 2) Cu_T concentrations in this station decrease from the surface to the bottom waters, a finding that indicates a typical distribution of Cu_T pollution due to the boat ships activity in semi-enclosed coastal systems (Neira et al., 2009).

Nevertheless, the average concentrations of Fe_T , Zn_T and Cu_T were higher than the criteria established by the NOAA (1999) for acute effects on marine organisms, there was no observed morbidity in oysters or other organisms in Camichin estuary, prior, during and after the study period. This suggests particularly that organisms of this aquatic system during the sampling period could have a strong tolerance to relatively high concentrations of Fe_T , Zn_T and Cu_T . However, the detected concentrations of these elements in the water could potentially have sublethal effects and could alter oysters' health in this system.

The distribution pattern of PAHs concentration was similar to the one of the Cu_T , suggesting that these compounds are dispersed from the central portion of the study area to the rest of estuary. These findings confirm that human activities taking place near the station 2 may be a source of pollution. The average concentration of naphthalene was found considerably above the limits in the Ecological Criteria Marine Water Quality. The permissible limits for pyrene and benzo[a]pyrene are not considered in this regulation (SEDUE, 1989). However, in terms of toxicity, it can be assumed that the water concentrations of naphthalene, pyrene and benzo[a]pyrene do not imply a risk of acute effects on organisms in Camichin estuary, because their concentrations were considerably below the water quality criteria established by the NOAA (1999).

Regarding to the oxidative stress response, ROS production is counterbalanced by antioxidant mechanisms. Some other pollutants can increase the intracellular generation of ROS through the oxidative metabolism of PAHs (Regoli et al., 2002). Benzo[a]pyrene increases ROS production and causes disruption of lysosomal membranes in bivalves (Livingstone et al., 1993). Studies with clams (*Chlamys islandicus*) showed that benzo[a]pyrene exposure (74 and 94 $mg\ kg^{-1}$) caused a decrease in total antioxidant capacity (Camus et al., 2002). Thus, when the balance between ROS/antioxidant defenses is unbalanced, oxidative stress is generated (Alves de Almeida et al., 2007). About the effect of metals, it has been shown that Fe and Cu can generate H_2O_2 and OH^- in the presence of O_2^* , H_2O_2 and O_2 , while Cd has the ability to inhibit mitochondrial respiration, which results in an increased ROS generation. Reports have also documented that acute exposure (120 h) to Cd, Fe, Cu and Pb induced lipid peroxidation and decreased GPx activity in *Perna perna* (Alves de Almeida et al., 2007).

On the pro-oxidants and antioxidant mechanisms in this study, generation of free radicals (O_2^*) had a relationship with lipid peroxidation evaluated as malondialdehyde (MDA) and ROOH (see Figs. 4A, 6A and B), suggesting that the greater amount of ROS, particularly O_2^* , increased oxidative damage in polyunsaturated fatty acids (PUFAs). These data could be explained by inhibition of CAT, although H_2O_2 was similar between control group and oyster from station 1, CAT activity was higher in controls, indicating the involvement of CAT in the detoxification of H_2O_2 in natural conditions; however, under stress conditions depleted CAT activity was found (see Fig. 5). Although there are no previous report in oysters, H_2O_2 may damage the active site of CAT (Kono and Fridovich, 1982; Bairy et al., 1996). Obtained results are in agreement with these finding in oyster from station 1 to 3. As a consequence of these damages, elevated levels of ROOH were noted (see Fig. 6B). In addition, O_2^* can autodismute to H_2O_2 , suggesting that in oysters form Camichin estuary, autodismutation of this radical could induce damage at late stage.

Besides, a factor that was not explored in this study is the possible synergistic effect between the effect of pollutants and environmental

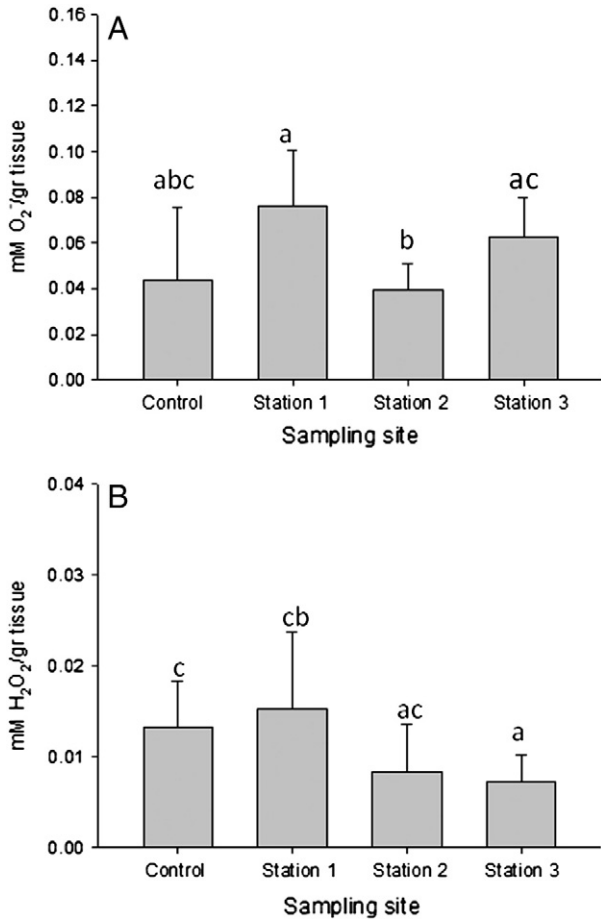


Fig. 4. O₂* (A), and H₂O₂ (B) concentrations in gills of oyster (n = 10) from Camichin estuary and control group. Data are expressed as mean ± SD, one-way ANOVA and Tukey test were applied. Bars with different superscript letters differ significantly (p < 0.05).

variables. Thus Garcia-Sampaio et al. (2010) demonstrated that when *Piaractus mesopotamicus* were exposed to Cu_r, there was a reduction in the activity of CAT and SOD activity, an effect that was more evident when the exposure conditions were performed in water at acid pH. In addition, it was shown that exposure to Cd induced an increase in mitochondrial activity of *Crassostrea virginica*, this phenomenon was more evident when oysters were subjected in warm water (Cherkasov et al., 2006; Lanning et al., 2010).

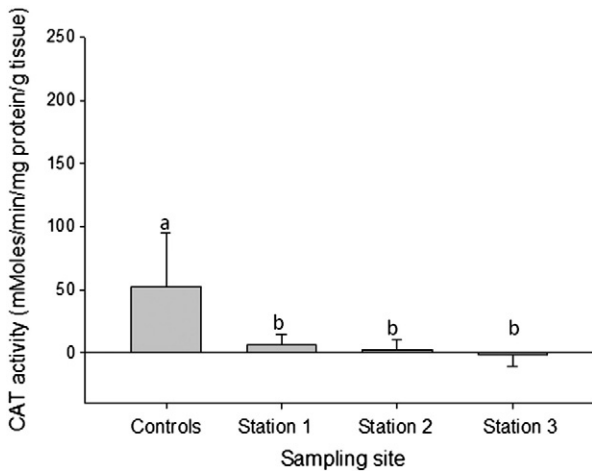


Fig. 5. Catalase activity in gills of oyster (n = 10) from Camichin estuary and control group. Data are expressed as mean ± SD, one-way ANOVA and Tukey test were applied. Bars with different superscript letters differ significantly (p < 0.05).

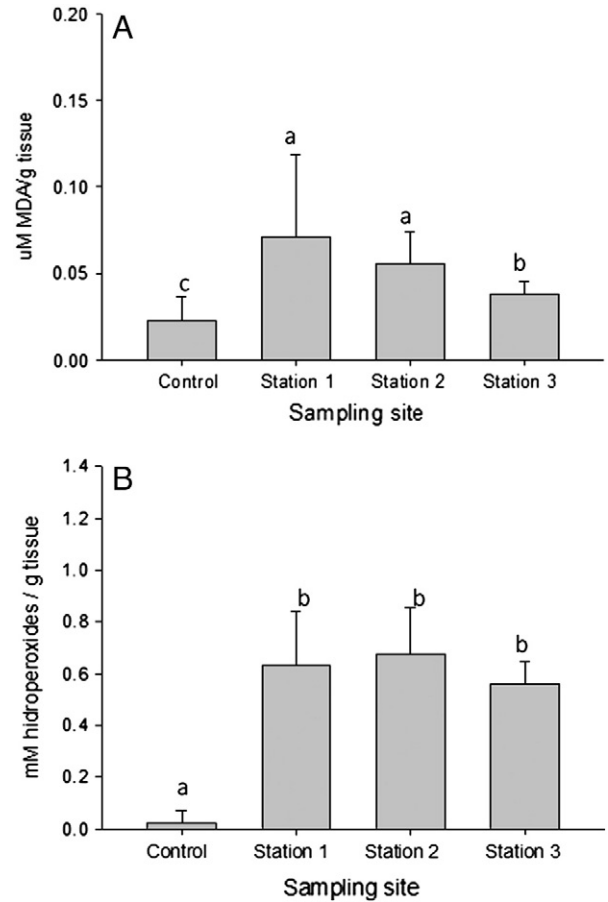


Fig. 6. Lipid peroxidation (A), and hydroperoxides (ROOH) concentration (B) in gills from oyster (n = 10) from Camichin estuary and control group. Results are expressed as mean ± SD, one-way ANOVA and Tukey test were applied. Bars with different superscript letters differ significantly (p < 0.05).

5. Conclusions

It is the first study in this estuarine zone, where it has been determined simultaneous oxidative stress parameters as biomarkers, and pollutants in water. These results indicated high values of metals and PAHs, substances that could induce stress in oyster. Thus, future research is also necessary to evaluate, during a period of time or cycle culture, the environmental variables, and to evaluate the influence of environmental and anthropogenic factors on oxidative stress in oyster with economical and ecological importance in this region.

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