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Biochemical biomarkers and hydrocarbons concentrations in the mangrove oyster *Crassostrea brasiliana* following exposure to diesel fuel water-accommodated fraction

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ABSTRACT

Understanding the toxic mechanisms by which organisms cope to environmental stressful conditions is a fundamental question for ecotoxicology. In this study, we evaluated biochemical responses and hydrocarbons bioaccumulation of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four sublethal concentrations of diesel fuel water-accommodated fraction (WAF). For that purpose, enzymatic activities (SOD, CAT, GPx, GR, G6PDH, GST and GGT), HSP60 and HSP90 immunocontent and lipid peroxidation (LPO) levels were determined in the gill and digestive gland of oysters and related to the hydrocarbons accumulated in the whole soft tissues. The results of this study revealed clear biochemical responses to diesel fuel WAF exposure in both tissues of the oyster. The capacity of *C. brasiliana* to bioaccumulate aliphatic and aromatic hydrocarbons in a dose-dependent manner is a strong indication of its suitability as a model in biomonitoring programs along the Brazilian coast, which was also validated by the response of the antioxidant defenses, phase II biotransformation and chaperones. HSP60 levels and GGT activity were the most promising biomarkers in the gill, while GST and GR activities stood out as suitable biomarkers for the detection of diesel toxicity in the digestive gland. The decrease of SOD activity and HSP90 levels may also reflect a negative effect of diesel exposure regardless the tissue. The present results provide a sound preliminary report on the biochemical responses of *C. brasiliana* challenged with a petroleum by-product and should be carefully considered for use in the monitoring of oil and gas activities in Brazil.

1. Introduction

The recent discovery of potentially massive oil reserves in Brazilian offshore waters has projected the country into becoming one of the world's largest producers of crude oil (Wertheim, 2009). There is, therefore, a need for monitoring of petroleum industry activities and the possibility of marine contamination. Oil exploration, production and transport potentially expose marine organisms to petrochemical compounds; hence, a pollution-monitoring program is a priority to assess the effects of petroleum by-products on such organisms. It may also enhance any decision-making in the public and private sectors on the environmental effects of accidents, such as the recent BP oil spill in the Gulf of Mexico.

Filter-feeding mollusks, such as oysters, play a significant role as sentinel organisms in monitoring programs due to their capacity to bioaccumulate environmental contaminants as well as to respond to their presence (Bebianno and Barreira, 2009; Solé et al., 2007). Moreover, they are sessile, globally distributed and economically important. Chemical analysis of contaminants in bivalve tissues has been recommended for biomonitoring (Solé et al., 2007), which gives an indication of the bioavailable fraction of environmental contamination and of direct exposure to chemicals. However, they do not necessarily reveal potential biological effects of the contaminants (Baumard et al., 1999) and biomarkers have been developed to detect and evaluate the effects of exposure to contaminants in the aquatic environment (Richardson et al., 2008).

Brazilian oyster production has expanded considerably over the past decade, and the native mangrove oyster *Crassostrea brasiliana* has become prevalent in oyster farms in Brazil (Pie et al., 2006). In addition, this species occurs naturally along the entire

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Brazilian coast in environments known to be exposed to petroleum by-products, such as diesel fuel, the most commonly used boat fuel in the world (Kennish, 1992).

Petroleum by-products and their metabolic products are able to cause a range of biochemical responses involved in the biotransformation of xenobiotics, the antioxidant defense system and general cellular metabolism in marine bivalves (Altenburger et al., 2003; Banni et al., 2010; Bebianno and Barreira, 2009; Lima et al., 2007). Under these conditions, a metabolic impairment might result in the formation of excessive amounts of ROS (reactive oxygen species) that can lead to oxidative stress. As a result, the normal intracellular reducing environment is compromised, damaging proteins, nucleic acids and lipids. Lipid peroxidation (LPO) is considered a major mechanism by which oxyradicals can cause injury, impairing cellular function and ultimately resulting in the failure of normal cell function (Livingstone, 2001). Links between hydrocarbon exposure and increased levels of lipid peroxidation in bivalves have been shown by Bebianno and Barreira (2009) and Lima et al. (2007).

However, to protect against the deleterious effects of ROS, cells contain a complex network of antioxidant defenses, composed of both enzymatic and nonenzymatic antioxidants (Halliwell and Gutteridge, 2007). The antioxidant system involves enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD dismutates the superoxide anion radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2), which is degraded by CAT and GPx (Halliwell and Gutteridge, 2007). Ancillary enzymes, such as glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH), recycle GSH and NADPH, respectively, contributing to the maintenance of an optimal intracellular redox environment for proper function of cellular proteins (Circu and Aw, 2010). Among the nonenzymatic defenses, glutathione (GSH), the most abundant intracellular nonprotein thiol, participates in many important biological processes including protection against toxic compounds. Different enzymes mediate the metabolism of glutathione and γ-glutamyl transpeptidase (GGT) is involved in glutathione synthesis, exerting an essential role in oxidant-challenged cells (Liu et al., 1998).

Enzymes implicated in the elimination of by-products of ROS play an important role as indirect antioxidants (Boutet et al., 2004), such as glutathione *S*-transferase (GST). GSTs are a group of multifunctional enzymes catalyzing the conjugation of a broad range of electrophilic substrates, generally produced during phase I of biotransformation of organic compounds, to endogenous glutathione (Storey, 1996). These enzymes are involved in the cellular detoxification and excretion of many xenobiotics, being ideal as biomarkers of polycyclic aromatic hydrocarbon (PAH) exposure in marine bivalves (Bebianno and Barreira, 2009). Moreover, the heat shock proteins (HSPs) have also been considered a potential petroleum hydrocarbon target (Downs et al., 2001; Wolfe et al., 1999). Ubiquitous in nature, HSPs play a role as molecular chaperones, being able to protect cells against the toxic effects of xenobiotics (Kalmar and Greensmith, 2009). HSPs also play a role in a number of other cellular processes that occur during and after exposure to oxidative stress (Kalmar and Greensmith, 2009), although their exact role in protection from the effects of petroleum hydrocarbons is not fully understood.

Despite the existence of several studies that evaluate the impact of petroleum by-products in the field using oysters, relatively few have focused on the effects of complex mixtures of water-accommodated hydrocarbons from the petroleum by-products under laboratory exposure. Furthermore, the combination of chemical analysis with biochemical biomarker assessment has not been applied along the Brazilian coast. In line with this approach, the present work integrates for the first time the assessment of a set of putative biochemical biomarkers of hydrocarbons exposure in the mangrove oyster *C. brasiliana* with the characterization of the water-accommodated hydrocarbons derived from diesel fuel (oil N°2).

2. Materials and methods

2.1. Experimental design

Adult mangrove oysters, *C. brasiliana*, of similar shell length (4–5.5 cm) were obtained from an oyster farming area at the Laboratório de Moluscos Marinhos (UFSC) in Florianópolis, southern Brazil, and immediately transported to the laboratory for an acclimation period of 7 d. Under laboratory conditions, the oysters were held in aerated 0.45 μm-filtered seawater at a constant temperature (21 °C) and salinity (25 ppt), and fed on microalgae (*Chaetoceros muelleri* and *Isochrysis* sp.) twice a day at a density of 3.3×10^6 cells mL^{-1} and 2.2×10^6 cells mL^{-1}, respectively.

Groups of 10 oysters were exposed for 96 h to four concentrations (2.5%, 5.0%, 10.0% and 20.0%) of diesel fuel water-accommodated fraction (diesel WAF), with seawater used as the control group. Diesel fuel was purchased at a Petrobras petrol station and WAF was obtained according to Singer et al. (2000) with minor modifications. Briefly, one part (1 L) of fresh diesel fuel was diluted with nine parts (9 L) of the 0.45 μm-filtered seawater (salinity 25 ppt) in a sealed 14 L glass flask protected from light, in order to minimize evaporation and degradation of the fuel components. The diesel–water mixture was stirred for 23 h with the tissue homogenizer Glas-Col at 1600 rpm at a constant temperature of 21 °C. The mixture was then made to stand for 1 h before the lower layer of water (diesel WAF) was transferred into the aquaria. The four concentrations of diesel WAF were prepared through dilutions of the WAF with the control seawater. The animals were not fed during the exposure period and the experiment was carried out in duplicate. No mortality was observed in both the control and treated groups.

After the 96 h experimental period, three oysters from each aquarium, totalizing six animals from each experimental group, were killed, pooled, wrapped in aluminum foil and immediately frozen at −80 °C for further chemical analysis. For biochemical parameters, six oysters from each aquarium, totalizing 12 animals from each experimental group, were killed and the gill and digestive gland were immediately excised, frozen in liquid nitrogen and stored at −80 °C until preparation for analysis.

2.2. Chemical analysis

The procedure for the analysis of aliphatic hydrocarbons (AH) and polycyclic aromatic hydrocarbons followed that described in MacLeod et al. (1985) with minor modifications. Briefly, five grams of wet tissue was extracted, after the addition of anhydrous Na_2SO_4, with hexane/dichloromethane 50% (v/v) using Soxhlet apparatus for 8 h. Before extraction, *n*-hexadecene, *n*-eicosene, d_8-naphthalene, d_{10}-acenaphthene, d_{10}-phenanthrene, d_{12}-chrysene, and d_{12}-perylene were added to all samples, blanks and reference material as surrogates. Aliphatic hydrocarbons were eluted in a partially deactivated (5%) silica:alumina column chromatography with 40 mL of *n*-hexane (F1) and PAHs with 45 mL of a 1:1 mixture of *n*-hexane and methylene chloride (F2). The PAH fraction was further purified by high-performance liquid chromatography (HPLC) to remove lipids and finally concentrated to a volume of 1 mL in hexane and internal standards tetradecene and d_{12}-benzo(b)fluoranthene were, respectively, added to F1 and F2 before gas chromatographic analysis. Aliphatic hydrocarbons were analyzed by gas chromatography using a flame ionization detector (FID). PAHs were quantitatively analyzed by gas chromatograph

coupled to a mass spectrometer (GC/MS) in a selected ion mode (SIM).

2.3. Tissue preparation for biochemical analyses

Gill and digestive gland of each oyster were individually weighed and homogenized in 1:4 (w/v) chilled buffer (20 mM Tris–HCl buffer, pH 7.6, containing 0.5 M sucrose, 1 mM DTT, 1 mM EDTA, 0.15 M KCl and 0.1 mM PMSF) using the tissue homogenizer Tissue-Tearor™. The homogenates were centrifuged at 9000 × *g* for 30 min at 4 °C, followed by a second centrifugation of the supernatants at 37,000 × *g* for 74 min at 4 °C to obtain the cytosolic fraction. The resulting supernatants were used for measurements of antioxidant enzyme activity and HSP90 immunocontent. The pellets from the first centrifugation were used for measurement of γ-glutamyl transpeptidase activity, HSP60 immunocontent and end products of lipid peroxidation. Total protein levels were quantified in both supernatant and pellet according to Peterson (1977) using bovine serum albumin as standard.

2.4. Enzyme assays

The cytosolic copper/zinc superoxide dismutase activity was determined by an indirect method through the inhibition of cytochrome *c* reduction in the presence of hypoxanthine/xantine oxidase $O_2^{\bullet -}$ generator system at 550 nm (McCord and Fridovich, 1969). Catalase activity was measured by the decrease in absorbance at 240 nm by H_2O_2 decomposition, according to Beutler (1975). Glutathione peroxidase activity was measured indirectly by monitoring the NADPH oxidation rate at 340 nm according to Wendel (1981) using *tert*-butylhydroperoxide (*t*-BOOH) as substrate. Glutathione reductase activity was quantified by the NADPH oxidation rate at 340 nm (Carlberg and Mannervik, 1985). Glucose-6-phosphate dehydrogenase activity was determined following the method of Glock and McLean (1953), which evaluates the increase in absorbance at 340 nm, caused by the reduction of $NADP^+$ to NADPH. Glutathione *S*-transferase activity was assayed by increasing absorbance at 340 nm, using 1-chloro-2,4 dinitrobenzene (CDNB) as substrate (Keen et al., 1976). γ-glutamyl transpeptidase activity was determined using commercially available kit (Biotecnica Ltda). All enzyme assays using visible wavelengths were carried out by use of 96 wells plates reader (Spectramax 250, Molecular Devices, Sunnyvale, CA), while CAT activity was assayed in a PerkinElmer Lambda Bio20 UV/visible spectrophotometer (PerkinElmer, Cambridge, UK).

2.5. HSP immunodetection

Samples of equal total protein content (30 μg of protein) were separated by SDS-PAGE using 10% gels under denaturant conditions and transferred to nitrocellulose membrane for 90 min using 400 mA current. The membranes were blocked for 1 h with 5% (w/v) nonfat powered milk in TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5). Membranes were then washed three times with TBS (10 mM Tris, 150 mM NaCl, pH7.5) and incubated with primary antibody. For HSP detection, rabbit polyclonal antibody SPA-805 (1:10,000) (StressGen), anti-insect HSP60, and mouse monoclonal antibody AC88 (1:2000) (Calbiochem), anti-mouse HSP90, were used as primary antibodies. NA934 (1:1000) donkey anti-rabbit IgG peroxidase-linked (Amersham) and NA931 (1:1000) sheep anti-mouse IgG peroxidase-linked (Amersham) were used as the secondary antibodies for detecting both isoforms. Because only one immunoreactive band was observed on the western blots for both antibodies, a dot-blot procedure was used for quantification of samples. A Bio-Dot® Microfiltration Apparatus (Bio-Rad) provided a reproducible method for binding HSP proteins onto the nitrocellulose membranes. Immunodetection of HSP60 and HSP90 were completed on the membrane in an identical manner to the western blots. Immunoblotting was developed using the enhanced chemiluminescence (ECL) system (Amersham, São Paulo) and HSP60 and HSP90 expression was quantified by densitometric analysis of the immunoreactive dots using the Scion Image® software.

Table 1
Aliphatic hydrocarbons levels (μg g^{-1}) on a wet weight basis detected in whole soft tissues of oysters from both control and diesel WAF exposed groups.

	Control	2.5% WAF	5% WAF	10% WAF	20% WAF
Pristane	0.174	0.320	0.374	0.725	1.84
Phytane	<0.085	0.139	0.255	0.400	1.15
Pristane/phytane	NA	2.3	1.5	1.8	1.6
Total *n*-alkanes	1.5	2.2	2.9	3.4	9.9
UCM	<5.34	56.2	98.3	64.5	201
∑AH	43.8	95.9	154	119	292

UCM, unresolved complex mixture; ΣAH, total aliphatic hydrocarbons; NA, not analyzed.

2.6. Lipid peroxidation

Oxidative stress damage was measured in terms of lipid peroxidation determination, according to Hermes-Lima et al. (1995) with minor modifications. Cumene hydroperoxide was used as a standard.

2.7. Statistical analysis

Differences in mean values were analyzed by one-way ANOVA followed by complementary Tukey's test with significance level established at $p < 0.05$. Normality (Shapiro–Wilks test) and homogeneity of variances assumptions were previously checked (Bartlett's test) and logarithmic transformation was applied when necessary (Zar, 1999). Outliers were excluded according to the Grubbs test. Spearman Correlation matrix was also calculated to study the relationships between the biochemical biomarkers measured. Statistical analyses were performed with the software GraphPad 5.0.

A principal component analysis (PCA) on correlation matrix was used to explore and describe the relationship between the biochemical biomarkers and the concentration of WAF for each tissue separately. PCA was applied on the media of all samples for each biochemical response and treatment. The data was previously log-transformed ($\log_{[x+1]}$) and the analyses were performed with the software CANOCO.

3. Results

3.1. Aliphatic and polycyclic aromatic hydrocarbon concentrations

Aliphatic and polycyclic aromatic hydrocarbon concentrations were determined in the whole soft tissues of oysters from both control and diesel WAF exposed groups. The concentrations of different types of aliphatic hydrocarbons, total *n*-alkanes and isoprenoids (pristane and phytane), and total aliphatic hydrocarbons (∑AH) are shown in Table 1. The ∑AH levels varied among experimental groups, ranging from 43.8 μg g^{-1} in the control group to 292 μg g^{-1} in the 20% diesel WAF group. Oysters from the control group accumulated only biogenic *n*-alkanes, characteristic of both phytoplankton (C_{15}, C_{16} and C_{17}) and superior plants (C_{23}–C_{33}) (data not shown). In oysters exposed to diesel WAF, *n*-alkanes from both biogenic and petrogenic sources were detected (data not shown). Oysters exposed to diesel presented a ratio of pristane/phytane ranged from 1.5 to 2.5 (Table 1), values similar to

the diesel WAF itself used in the exposures (data not shown). The unresolved complex mixture (UCM) levels were also determined in the oysters (Table 1). The UCM is an indicator of petrogenic sources and is composed by both ramified and cyclic aliphatic hydrocarbons (Readman et al., 2002). UCM levels were above the detection limits only in the exposed groups, where oysters from 2.5%, 5%, 10% and 20% diesel WAF accumulated 56.2 $\mu g\,g^{-1}$, 98.3 $\mu g\,g^{-1}$, 64.5 $\mu g\,g^{-1}$ and 201 $\mu g\,g^{-1}$, respectively (Table 1).

The levels of individual and total polycyclic aromatic hydrocarbons ($\sum$PAH) accumulated in the oysters are summarized in Table 2. The $\sum$PAH concentrations increased in a dose-dependent manner along the experimental range, from 2.6 $\mu g\,g^{-1}$ in the control group to 115 $\mu g\,g^{-1}$ in the 20% diesel WAF group (Table 2). Oysters exposed to diesel WAF accumulated only low molecular weight PAHs, with 2 or 3 aromatic rings, the most water soluble PAHs. Individual parental PAH determination showed that biphenyl, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene were detected in all the exposed groups (Table 2), with phenanthrene being the most abundant, accounting for 3.7% of total PAHs. Among the non-parental PAHs, the sum of trimethylnaphthalenes (52.5%), followed by the sum of dimethylnaphthalenes (26.7%) were the most abundant of all the accumulated PAHs.

3.2. Biochemical biomarkers

Fig. 1 presents the activity of antioxidant enzymes in the gill and digestive gland of oysters exposed to different diesel WAF concentrations. Antioxidant enzymes SOD, CAT and GPx showed the same pattern of response in both tissues. SOD activity showed a decreasing trend response with a significant difference in oysters exposed to 20% WAF compared to control [$F(4,53) = 3.171$; $p < 0.05$ for gill and $F(4,51) = 2.941$; $p < 0.05$ for digestive gland] (Fig. 1A). Unlike the SOD activity, the levels of CAT and GPx activity did not differ between the experimental and control groups ($p > 0.05$) (Fig. 1B and C, respectively).

Ancillary enzyme GR presented a distinct pattern between tissues. GR activity differed among experimental groups only in digestive gland with significantly higher activities in oysters exposed to 5%, 10% and 20% diesel WAF when compared to the control [$F(4,51) = 11.52$; $p < 0.001$] (Fig. 2A). G6PDH however, did not differ among the experiment groups in both tissues ($p > 0.05$) (Fig. 2B).

GGT activity also presented a different response pattern regarding the tissues. In gills, GGT activity was significantly induced in oysters exposed to 2.5% and 20% WAF. In digestive gland, no significant differences were observed ($p > 0.05$) (Fig. 2C). GST activity was significantly higher in the digestive glands of oysters exposed to 10% and 20% diesel WAF than the control [$F(4,50) = 4.532$; $p < 0.001$] (Fig. 2D). In gills, GST activity remained unchanged in the exposed oysters compared to unexposed ones ($p > 0.05$) (Fig. 2D).

The immunocontent of HSP60, HSP90 and the levels of lipid peroxidation in both gill and digestive gland are shown in Fig. 3A, B and C, respectively. HSP60 levels of gill were significantly higher in the 2.5% and 20% groups, when compared to control [$F(4,48) = 6.098$; $p < 0.001$]. In the digestive gland however, the HSP60 levels were similar among all the experimental groups ($p > 0.05$) (Fig. 3A). Opposite to HSP60, the levels of HSP90 exhibited similar trends between tissues and were significantly lower in exposed groups compared to control [$F(4,51) = 7.733$; $p < 0.001$ for gill and $F(4,52) = 5.979$; $p < 0.001$ for digestive gland] (Fig. 3B).

Significant correlations between the investigated biomarkers were found in both tissues. In gill, CAT activity was positively correlated with SOD ($r = 0.45$; $p < 0.001$), GPx ($r = 0.40$, $p < 0.01$), GR ($r = 0.60$; $p < 0.01$) and G6PDH ($r = 0.68$, $p < 0.001$) activities. In the same tissue, GST was positively correlated with GR ($r = 0.43$; $p < 0.01$) and GPx activities ($r = 0.61$; $p < 0.01$). Significant negative correlations were found among LPO levels and GPx ($r = -0.35$, $p < 0.05$) and GST activity ($r = -0.28$, $p < 0.05$) and HSP60 levels ($r = -0.30$, $p < 0.05$) in the gill.

In digestive gland a positive correlation was found between GST and GR activities ($r = 0.76$; $p < 0.001$), which was also correlated with GPx ($r = 0.62$; $p < 0.001$). Significant negative correlations were identified among HSP90 and GR ($r = -0.47$; $p < 0.01$), GST ($r = -0.48$; $p < 0.01$) and G6PDH ($r = -0.32$; $p < 0.05$) activities. LPO levels were negatively correlated with GR activity ($r = -0.42$; $p < 0.01$) and positively correlated with HSP90 ($r = 0.40$; $p < 0.01$).

3.3. Relationship between aliphatic and polycyclic aromatic hydrocarbons concentrations and biochemical parameters

The relationship between aliphatic and polycyclic aromatic hydrocarbons accumulated in oyster whole soft tissue and the biochemical parameters either in the gill or in the digestive gland of *C. brasiliana* was assessed by principal components analysis. Fig. 4 presents the PCA ordination output for both tissues (Fig. 4A and B for gill and for digestive gland, respectively).

From the gill plot, the two main components explained 92.3% of the total variation. Component 1 explained 66.7%, while component 2 explained 25.6%. The ordination diagram presented a clear difference between treatments along component 1. The biochemical parameters HSP60 levels, GST and GGT activities showed a positive correlation with component 1, which were higher in the highest diesel WAF concentration (20%) (Fig. 4A). On the other hand the same figure shows that the contents of HSP90 and LPO together with the activities of SOD and CAT presented a negative correlation in component 1, being higher in control and lower in 20% diesel WAF concentration. Component 2 was positively correlated with G6PDH and GPx activities, which were higher in the 2.5% diesel WAF concentration (Fig. 4A).

From the digestive gland plot (Fig. 4B), the two main components explained 90.6% of total variation, where component 1 explained 78.2% and component 2 explained 12.4%. A similar pattern to the gill plot was noted in the digestive gland plot with a clear relationship between treatments and component 1. The biochemical parameters that showed a positive correlation with component 1 were mainly GR, GST and GPx activities, which were higher in the 20% diesel WAF concentration (Fig. 4B). A negative correlation with component 1 was observed mainly to SOD activity and LPO and HSP90 levels that were higher in the control treatment. Component 2 showed a positive correlation with CAT activity and HSP60 levels, which were higher in the 10% diesel WAF concentration (Fig. 4B).

4. Discussion

Oysters accumulated both aliphatic and aromatic hydrocarbons in a dose-dependent manner reaching large values in the highest diesel WAF concentration (20%). Such an increase reflects their ability to bioaccumulate petrogenic hydrocarbons as previously reported in *Crassostrea virginica* (Noreña-Barroso et al., 1999; Sanders, 1995) and *Crassostrea gigas* (Bado-Nilles et al., 2010). Bivalves accumulate PAHs directly from the water-phase and food, and the organisms readily accumulate the more-soluble PAHs than the less-soluble and heavier PAHs (Neff, 2002). In fact, the total PAHs accumulated in oyster tissues were mostly due to the uptake of the 2 ring PAH non-parental naphthalene and the 3 ring PAHs phenanthrene. This can be explained by the increase of K_{ow} of the alkyl PAHs, which is also often associated with an increased trend to bioaccumulate when compared to parent compound PAHs (Irwin et al., 1997). Thus, the accumulation of low molecular weights PAHs

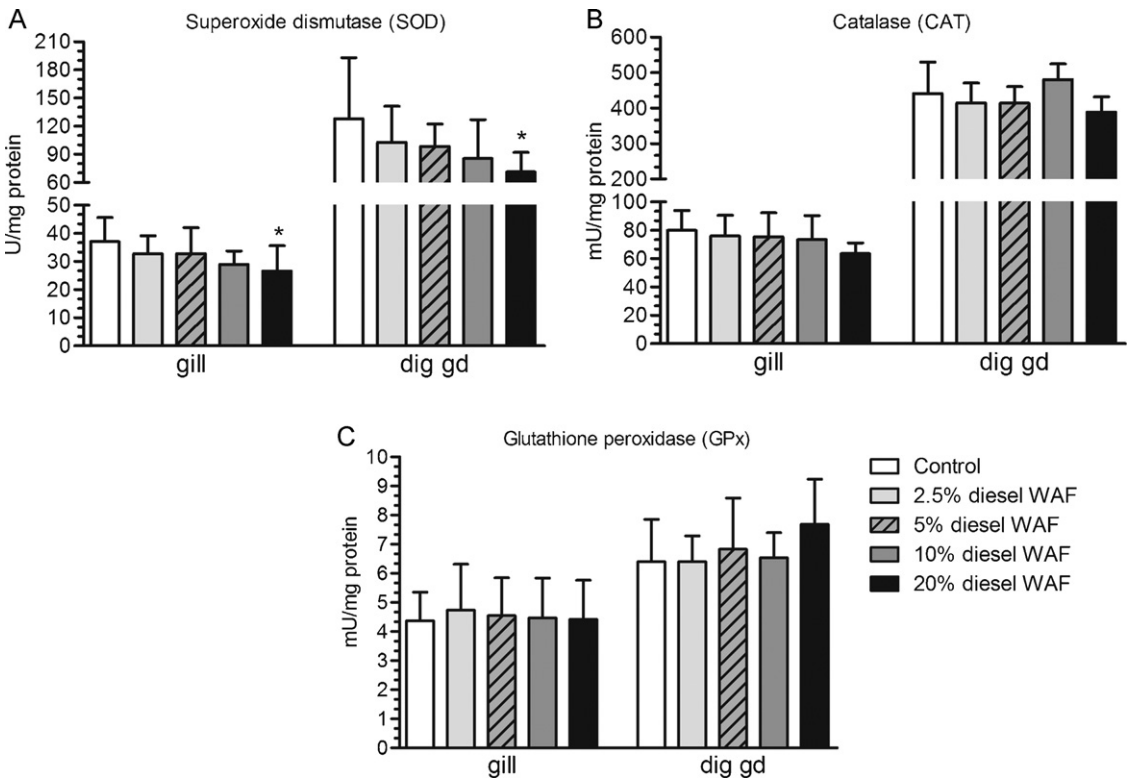

Fig. 1. Superoxide dismutase (A), catalase (B) and glutathione peroxidase (C) enzymes activity in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean ± S.D. (n = 9–12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. $^{*}p < 0.05$ when compared the diesel WAF exposed groups to the control for a given tissue.

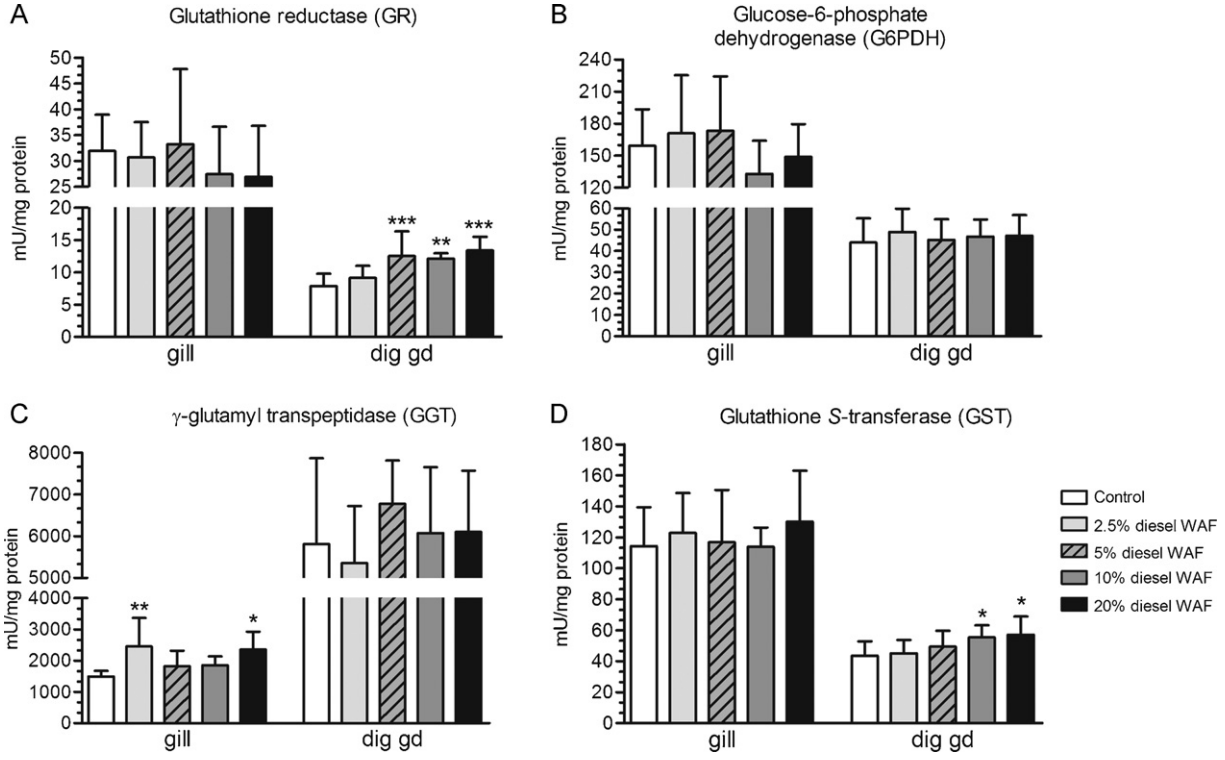

Fig. 2. Glutathione reductase (A), glucose-6-phosphate dehydrogenase (B), γ-glutamyl transpeptidase (C) and glutathione *S*-transferase (D) enzymes activity in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean ± S.D. (n = 9–12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ when compared the diesel WAF exposed groups to the control for a given tissue.

Table 2
Aromatic hydrocarbons levels (ng g^{-1}) on a wet weight basis detected in whole soft tissues of oysters from both control and diesel WAF exposed groups.

	Control	2.5% WAF	5% WAF	10% WAF	20% WAF
∑Methylnaphthalene	62.9	1053	279	1164	4446
Biphenyl	39.8	152	145	373	1273
∑Ethylnaphthalene	<4.71	213	391	1108	3494
∑Dimethylnaphthalene	92.4	1738	3441	10,862	33,364
Acenaphtene	21.9	53.4	109	320	862
∑Trimethylnaphthalene	1404	2164	10,386	27,098	57,751
Fluorene	147	114	303	872	2005
Dibenzothiophene	54.0	21.6	68.0	167	444
Phenanthrene	14.4	226	598	1673	4350
Anthracene	<3.46	8.30	31.4	73.8	150
∑Methyldibenzothiophene	4.56	29.7	162	339	663
∑Dimethylphenantrene	<5.85	228	1253	2841	5553
Fluoranthene	<2.45	9.40	41.1	77.0	148
Pyrene	<5.08	19.0	92.5	186	369
Benz[a]anthracene	<3.52	<3.52	<3.52	<3.52	<3.52
Chrysene	<7.04	<7.04	<7.04	<7.04	<7.04
Benzo[b]fluoranthene	<2.53	<2.53	<2.53	<2.53	<2.53
Benzo[k]fluorantene	<2.40	<2.40	<2.40	<2.40	<2.40
Benzo[e]pyrene	<2.56	<2.56	<2.56	<2.56	<2.56
Benzo[a]pyrene	<2.03	<2.03	<2.03	<2.03	<2.03
Perylene	<1.21	<1.21	<1.21	<1.21	<1.21
Indeno [1,2,3-cd]pyrene	<1.41	<1.41	<1.41	<1.41	<1.41
Dibenzo[ah]anthracene	<2.56	<2.56	<2.56	<2.56	<2.56
Benzo(ghi)perylene	<10.3	<10.3	<10.3	<10.3	<10.3
∑PAH	2596	6029	17,301	47,153	114,872

∑PAH, total polycyclic aliphatic hydrocarbons.

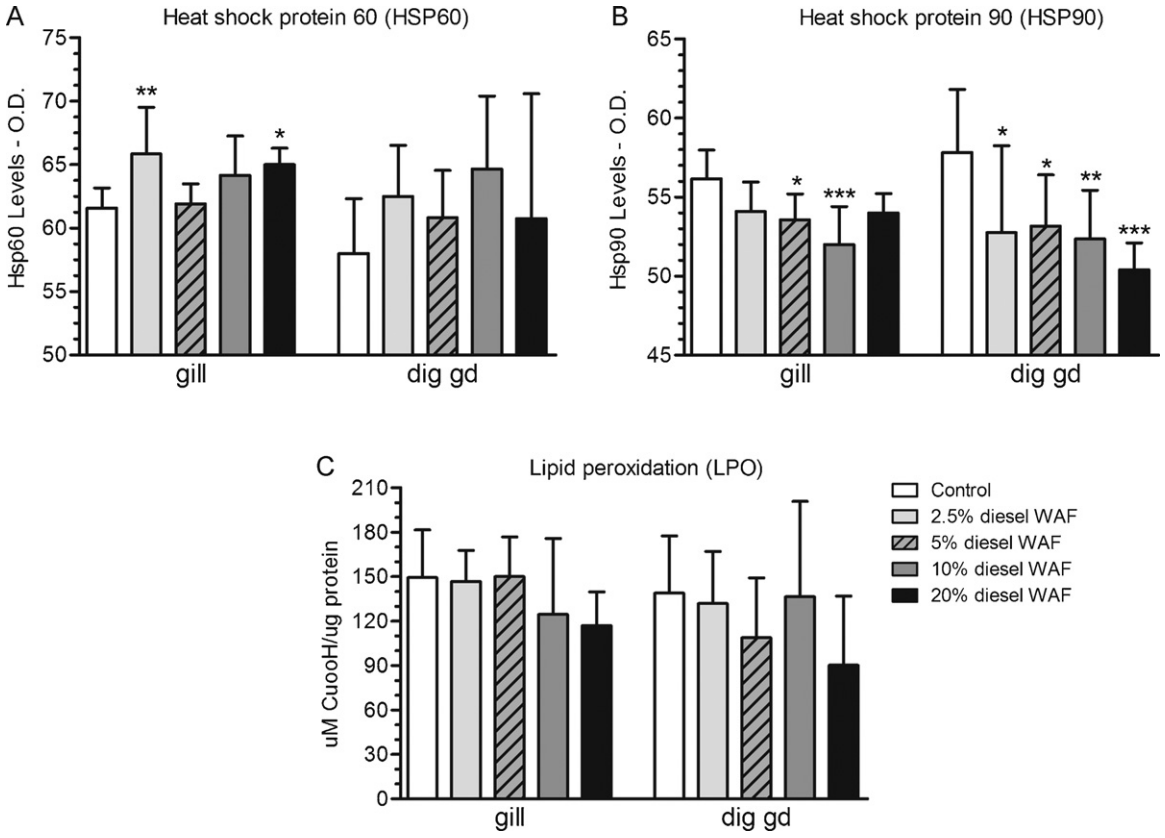

Fig. 3. Immunocontent of heat-shock proteins HSP60 (A) and HSP90 (B) and lipid peroxidation levels (C) in the gill and digestive gland (dig gd) of the mangrove oyster *Crassostrea brasiliana* exposed for 96 h to four concentrations (2.5%, 5%, 10% and 20%) of diesel fuel water-accommodated fraction (diesel WAF), or seawater as the control group. Data are presented as mean ± S.D. (n = 9–12 animals per group). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ when compared the diesel WAF exposed groups to the control for a given tissue.

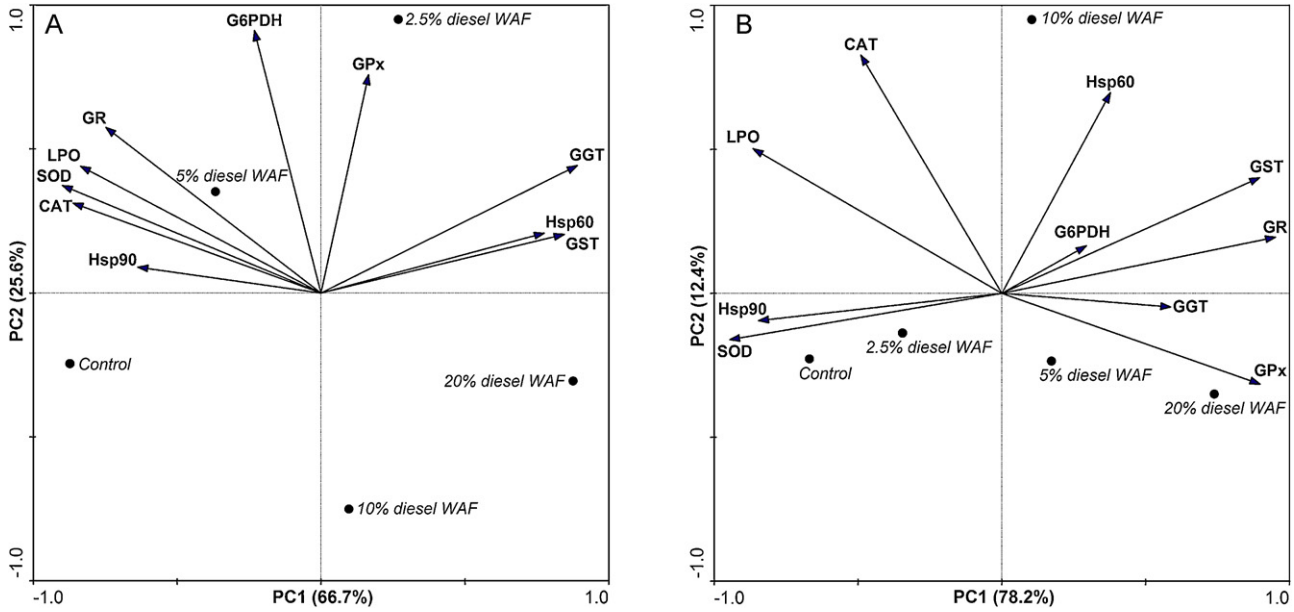

Fig. 4. Biplot from principal components analysis of all biochemical parameters related to the diesel WAF exposure in both gill (A) and digestive gland (B). In gill, the first axis explained 66.7% of overall variation and the second 25.6%. In digestive gland, the first axis explained 78.2% of overall variation and the second 12.4%.

reflects not only the chemical nature of diesel fuel itself, but also the uptake pathway for hydrocarbon accumulation by *C. brasiliana*.

The production of contaminant-stimulated reactive oxygen species (ROS) and their resulting oxidative damage seems to be the main mechanism of petroleum PAHs toxicity in marine bivalves (Banni et al., 2010; Bebianno and Barreira, 2009; Frouin et al., 2007). To cope with this pro-oxidative challenge, bivalves generally produce increased SOD activity as the first line of enzymatic antioxidant defense (Ansaldo et al., 2005; Cheung et al., 2004; Lima et al., 2007). However, this inducibility should not be considered as a general rule since results have not always been consistent and no-effect or decreased SOD activity has been reported in mollusks exposed to environmental stressors (Cossu et al., 1997; Livingstone, 2001; Verlecar et al., 2008). Indeed, in the present study SOD activity of gill and digestive gland decreased with a marked difference in the highest diesel WAF concentration. According to Escobar et al. (1996), SOD activity can be inhibited by ROS, which could be a part of the diesel toxicity response in *C. brasiliana*.

CAT and GPx are known to serve as protective responses to scavenge the generation of ROS and enhanced activities represent an important adaptation to pollutant-induced stress (Cheung et al., 2001; Cossu et al., 1997; Richardson et al., 2008). However, the results obtained here show that diesel WAF did not elicit the expected effects on these enzymes in both tissues. Although not statistically significant, CAT activity in the gill followed the same decreased SOD activity trend for the highest diesel WAF concentration. This result offers evidence toward the functional linkage between SOD and CAT, which is further supported by the positive correlation found between their activities in the gill. On the other hand, digestive gland CAT and GPx activities of both tissues remained unchanged regardless the diesel concentration. Absence of induction of CAT and GPx activities has previously been reported in the mussel *Mytilus galloprovincialis* exposed to B[a]P for 72 h (Banni et al., 2010) and *Perna viridis* treated with mercury chloride for 15 d (Verlecar et al., 2008). On the other hand, GPx had already been found to be a susceptible antioxidant in the freshwater bivalve *Unio tumidus* exposed to environmental contaminants (Cossu et al., 1997) and is considered an efficient protective enzyme against lipid peroxidation in aquatic organisms (Winston and Di Giulio, 1991). Together these results suggest that (i) despite the decrease in SOD and CAT activities together with the unchanged activity of GPx, antioxidant systems of *C. brasiliana* remained active enough to prevent lipid peroxidation and (ii) the oysters also used other approaches than enzymatic antioxidants to cope with the toxic challenge in consequence to accumulated hydrocarbons.

GST activity was significantly increased in digestive gland of oysters exposed to diesel WAF. GST is involved in phase II of the biotransformation process by conjugating reduced glutathione to different electrophilic compounds leading to their detoxification (Storey, 1996). GST is also thought to play a peroxidase activity, exerting a significant supplementary antioxidant role in the cell (Barata et al., 2005; Bebianno and Barreira, 2009), and previous studies have associated its induction to petroleum exposure in bivalves (Banni et al., 2010; Boutet et al., 2004; Lima et al., 2007; Silva et al., 2005; Solé et al., 2007). From the data obtained in this study, it appears clear that GST activity of the digestive gland was effective in preventing damage on lipid membranes of exposed oysters, verified as such by the unchanged LPO levels. A significant negative correlation between GST and LPO was also found in the gill, reinforcing the protective role of GST in *C. brasiliana* following diesel exposure.

This putative GST protection could be mediated by the increased GR activity seen in the digestive gland of exposed oysters. GR is a NADPH-dependent enzyme that plays an essential role in the maintenance of GST activity through the regeneration of GSH from GSSG (Verlecar et al., 2008). Hence, GR induction becomes important during stress conditions, which is seen here through the significant positive correlation between GR and GST activities in both the gill and digestive gland. This finding is consistent with recent reports carried out with mussels challenged with PAHs and other pollutants (Akcha et al., 2000; Gamble et al., 1995; Porte et al., 1991; Sáenz et al., 2010), indicating that GR activity, along with GST, could be sensitive indicators of hydrocarbon exposure in *C. brasiliana*. However, to keep GR activity, a constant NADPH-generating capacity is required within the cell. Such a feature can be achieved by the activity of G6PDH, an ancillary enzyme of the antioxidant defense system (Bainy et al., 1996). Although G6PDH activity did not differ among the experimental groups, a significant positive

correlation was found between G6PDH and GST and GR activities in both tissues, suggesting that G6PDH, together with GR, is involved in the maintenance of reduced GSH levels, which in turn is used as substrate by GST (Sáenz et al., 2010).

GGT activity was the only enzyme of the glutathione pathway induced in the gills following the diesel WAF exposure. GGT is important for oxidant-challenged cells to maintain the intracellular GSH concentration, and an increase in such activity may represent a higher availability of cysteine residues to GSH formation (Liu et al., 1998). GSH comprises the main non-protein antioxidant defense against oxidative injuries (Farid et al., 2009; Halliwell and Gutteridge, 2007; Hannam et al., 2010), and from the data obtained in this study we can hypothesize that the enhanced GGT activity kept the cellular redox balance in the gill of oysters exposed to diesel WAF. The enhanced gill GGT activity together with the unchanged GR and GST activities suggests that oyster tissues differ in the maintenance of intracellular GSH levels.

Diesel fuel WAF was also able to induce a significant increase of HSP60 immunocontent in the gill. Although poorly understood in marine invertebrates in terms of pollutant response, HSP60 is thought to be strongly induced by petroleum hydrocarbons (Snyder et al., 2001; Wheelock et al., 2002), as previously observed by Downs et al. (2001), Oberdörster et al. (1999) and Wolfe et al. (1999). Differential expression of HSP60 in response to diesel WAF indicates a particular physiological condition for the mitochondria. According to Downs et al. (2001), HSP60 is a biomarker for the rate of protein mitochondrial turnover, which is expected to be higher under cellular stress, especially in the presence of ROS. Increased levels of HSP60 suggest that protein denaturation, protein import and synthesis increased in the gill cells of exposed oysters. Moreover, these results indicate that the stress response of oysters in terms of HSP induction in gill is a rapid reaction to short term events whereas in hepatic tissues there is an accumulation of proteotoxic chemicals following chronic exposure, as already observed in fish (Triebskorn et al., 2002; Webb and Gagnon, 2009). Hence, HSP60 induction seems to be an adaptive mechanism of this tissue against diesel generated toxicity, and is suggested as a sensitive biomarker for mitochondrial protein turnover in *C. brasiliana* when the stressor is diesel fuel.

Inversely, diesel fuel lead to a significant dose-dependent inhibition of HSP90 levels in both tissues, with a marked response in the digestive gland. This was a surprising result in our work, since HSP90 is a ubiquitous multifunctional chaperone that is involved in protein folding, cytoprotection, as well as in number of cellular regulatory pathways following a stressor challenge (Li et al., 2009). Based on our data, we can speculate that diesel WAF inhibited the synthesis of HSP90 by some alteration on a transcriptional or translational level, or by the inhibition of the signal-transduction cascade for induction of HSP90 (Downs et al., 2001). Moreover, we cannot discount the idea that the cells from both gill and digestive gland are protected by the action of the remaining HSP90 or other proteins, precluding the need for protein chaperoning in the cytoplasm.

Our results also corroborate the different mechanisms in mollusk tissues that cope with the stress from hydrocarbons exposure (Banni et al., 2010). According to the PCA results, the gill and digestive gland of *C. brasiliana* show some differences in terms of biochemical response when exposed to diesel fuel WAF. The differences relate to the putative regulation of the glutathione pathway, which in gill it is achieved through an induced GGT activity, and in digestive gland through enhanced GR activity. Moreover, gill and digestive gland also differ regarding the chaperone role and the phase II biotransformation process, shown here as HSP60 levels and GST activity. Regardless the tissue, PCA also showed that 20% diesel WAF was indeed the concentration with more influence on the biochemical responses elicited in *C. brasiliana* after 96 h of exposure.

5. Conclusions

In conclusion, the mangrove oyster *C. brasiliana* exposed to four sublethal concentrations of diesel fuel water-accommodated fraction (WAF) is able to bioaccumulate both aliphatic and aromatic hydrocarbons in a dose-dependent manner in a short period of time. In this process, toxic effects were identified in terms of biochemical biomarker responses, which lead to an induction of protective phase II, and ancillary GST and GR enzymes in the digestive gland, and an elevation of HSP60 levels and GGT activity in the gill tissue. These results indicate that different tissues react differently during a chemical exposure, which might reflect the mechanism in which the tissues cope with the stress. The gill appears to be a target tissue for identifying short-term events while the digestive gland may be of greater value to determine the oyster's stress response under chronic exposure to stressors in the environment. Moreover, this study provided evidence toward the suitability of using a set of putative biochemical biomarkers in the oyster *C. brasiliana* for the monitoring of oil and gas activities in Brazil.

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