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Antioxidant response and oxidative stress levels in *Macrobrachium borellii* (Crustacea: Palaemonidae) exposed to the water-soluble fraction of petroleum

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ABSTRACT

The aim of the present work was to evaluate the effect of the water soluble fraction of hydrocarbons (WSF) on the antioxidant status of the freshwater prawn *Macrobrachium borellii*. First, seasonal variations were studied in a non-polluted area. Hepatopancreas and gills showed season-related fluctuations in catalase (CAT), glutathione-S-transferase (GST) activities and in lipid peroxidation levels (LPO), but not in superoxide dismutase (SOD). Then, adults were exposed semi-statically to sublethal doses for 7 days. CAT, SOD, GST, and glutathione peroxidase (GPx) activities and LPO, reduced glutathione (GSH) and protein oxidation (PO) levels were determined. Exposed individuals showed significant increases in CAT, SOD, and GST activities in hepatopancreas and CAT activity in gills. GPx activity did not vary in either tissues. While LPO levels increased, GSH levels decreased significantly in hepatopancreas of exposed animals, but PO levels showed no variation. Induction of SOD was also assessed by Real-time PCR mRNA expression in hepatopancreas. The non-enzymatic antioxidant activity was also tested; ABTS 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) was higher in hemolymph of treated-prawns compared to controls, but ferric reducing activity of plasma assay (FRAP) values did not change. Taken together, the present results indicated that the antioxidant defenses of *M. borellii,* mainly in hepatopancreas, were significantly affected by aquatic hydrocarbon contamination, regardless of the season.

1. Introduction

Taking into account the numerous forms of environmental pollution, the impact of anthropic hydrocarbons on aquatic and terrestrial ecosystems is likely to be the one that has caught most public attention (Mendes et al., 1997). Oil spill in freshwater is the consequence of many oil-related activities and it is critical to study the impact of petroleum spills in freshwater habitats (Bhattacharyya et al., 2003), though this topic has not been given the attention it deserves. La Plata River estuary (Argentina) is an area which is exposed to several xenobiotics, and it is the most contaminated region of the country in terms of hydrocarbons (FCS, 1994). Thus, it is important to evaluate the ecotoxicological effects of hydrocarbons on the species of this community. One such species, representative of the benthic community of the La Plata River area, is the autochthonous freshwater prawn *Macrobrachium borellii* selected for the present study. Freshwater crustaceans are currently employed to monitor environmental pollution status because of their advantageous characteristics (for example they are often the major component of ecosystems, their populations are usually numerous and they are easily cultured in the laboratory) (Fossi et al., 2000; Gerhardt et al., 2002; Shigehisa and Shiraishi, 1998). Regardless of the importance of oil spills in freshwater environments, *M. borellii* is one of the few crustaceans whose metabolic alterations by hydrocarbon pollution have been studied (Lavarías et al., 2005a, 2006, 2007). Remarkably there is little information available in the literature about the effect of the WSF from crude oil. This WSF is mainly composed of single ring aromatic hydrocarbons (toluene, benzene, xylene and di- and trimethylbenzenes) and very low concentrations of C_{12}–C_{24} n-alkanes, closely resembling the fuel diesel oil. These low boiling aromatics are the primary toxic agents for aquatic organisms (Heras et al., 1992; Lavarías et al., 2004). At present, most of the work regarding the effect of oil discharges on crustacean metabolism has been done in marine milieu, while there is a general lack of knowledge on freshwater environments.

Reactive oxygen species (ROS) are the most studied biomarkers to evaluate the biochemical alterations by organic contaminants on aquatic organisms. This is a general pathway of toxicity induced by many redox cycling chemicals such as hydrocarbons and many other compounds leading to a condition of oxidative stress. ROS include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OHs). The organisms have developed antioxidant defense in order to minimize oxidative damage to cellular

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components as lipids, proteins and DNA. The most important antioxidant enzymes are: superoxide dismutase (SOD, EC 1.15.1.1 converts O_2^- to H_2O_2), catalase (CAT, EC 1.11.1.6 reduces H_2O_2 to water) and glutathione peroxidase (GPX, EC 1.11.1.9 detoxifies H_2O_2 or organic hydroperoxides produced, for example, by lipid peroxidation (LPO) (Di Giulio et al., 1995; Halliwell and Gutteridge, 1989)). Glutathione S-transferases (GST, EC 2.5.1.18) catalyze the conjugation of glutathione (GSH) with various electrophilic substances, and they play a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Ketterer et al., 1983).

Organisms can be adapted to the increasing ROS production by up-regulating antioxidant enzymes (Livingstone, 2003). Failure of antioxidant defense to detoxify the excess of ROS production can lead to significant oxidative damages including enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Di Giulio et al., 1995). In particular, alterations in the activity of specific enzymes such as GSH and non-enzymatic free radical scavengers such as albumin, ascorbic acid, α-tocopherol, β-carotene, uric acid, bilirubin, and flavonoids, involved in the metabolism and elimination of complex pollutant mixtures such as crude oils, have been employed as subcellular biochemical biomarkers (Reid and Mac Fralane, 2003). Moreover, it has been suggested that antioxidant status must be studied to build up a more comprehensive picture of toxic effect of contaminants (Niyogi et al., 2001b).

Studies on biotransformation in a wide range of xenobiotics of some aquatic invertebrate species revealed differences in metabolism between different groups of toxicants. For instance, aliphatic and aromatic hydrocarbons are metabolized more slowly than xenobiotics already containing functional groups. Comparing the biotransformation capacity among invertebrates, crustaceans metabolized both components faster than mollusks (Livingstone, 1998).

These biomarkers are known to be sensitive to petroleum compounds and in particular to polycyclic aromatic hydrocarbon (PAHs) (de Knecht et al., 2001; Morales-Caselles et al., 2008; Niyogi et al., 2001a; Orbea et al., 2002).

The aim of the present work was to evaluate the effect of the water soluble fraction of hydrocarbons (WSF) on the antioxidant status of the freshwater prawn *M. borellii* using a battery of biomarkers. Seasonal variations were also studied. The present study enlarges the knowledge on the biomarker responses in crustaceans.

2. Materials and methods

2.1. Sample collection

M. borellii adults were sampled in an uncontaminated watercourse close to the Rio de La Plata river, Argentina (20 km SW from La Plata city). They were taken to the laboratory and kept in dechlorinated tap water at 22 ± 2 °C, and 14:10 h L:D photoperiod for at least a week before experiments. Adult prawns were collected once a month for one year (2007–2008); their hepatopancreas, gills and hemolymph were immediately removed and frozen until used in order to evaluate basal levels in the antioxidant defense mechanism as well as those variations produced by seasonal changes.

2.2. Preparation of the WSF of crude oil

Punta Loyola light crude oil, obtained from Santa Cruz, Argentina, stored at 4 °C, was used to prepare the water-soluble fraction. It was stirred in a 10 L stainless steel mixing vessel equipped with a mechanical stirrer, a bottom drain, and kept in a cold room at 4 °C. Crude oil and freshwater in a ratio of 1:100 (v/v) were stirred at low speed for 24 h and allowed to settle for additional 48 h (lower settling times produced a fraction with dispersed oil droplets) (Heras et al., 1992). WSF was collected daily using the bottom drain. During experiments, fresh WSF batches were prepared in several 10 L vessels every 2 days.

2.3. Toxicity tests

Exposure assays were performed using sublethal concentrations of WSF as determined previously for this prawn (Lavarías et al., 2004). Prawns were collected at the end of winter, when they are in the prereproductive season. Groups of 12 adults were exposed to a sublethal level of WSF (0.6 ppm) at 22 ± 2 °C and a 14-h light:10-h dark cycle. These prawns are normally exposed to sublethal hydrocarbon concentration in their environment, but after an oil spill, concentrations can reach several times in this level (Lavarías et al., 2004). Control groups were kept in clean water. Prawns were held individually in 0.5 L flasks with the lid sealed to avoid hydrocarbon loss and cannibalism, and exposed to WSF for either 4 or 7 days, with a daily change of media and no feeding. Temperature, pH, and dissolved oxygen were measured in the control containers (Lavarías et al., 2006).

2.4. Preparation of total cellular homogenate

After exposure to WSF, the animals were anesthetized on ice for almost 5 min, and hepatopancreas and gills were removed, weighed and cooled on ice, then stored until used.

Hepatopancreas (1:9 w/v) and gills (1:5 w/v) were homogenized in a cold buffer solution of 125 mM Tris-base, pH 6.8 containing 1 mM 2-mercaptoethanol and 0.1 mM PMSF (Vijayavel et al., 2004). Homogenates were centrifuged at 10,000 × *g* at 4 °C for 10 min and the supernatant used for determining enzyme activity, LPO, PO and GSH levels. Total protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.5. Hemolymph extraction

Hemolymph was extracted by dorsal hemolymphatic cavity puncture, then samples were pooled (three specimens each), and 1:10 sodium citrate 0.01 N was added as anticoagulant (Karim-Malka et al., 1983). Centrifugation was carried out at 7500 *g* for 10 min in order to remove hemocytes, and cell-free supernatant was stored at −70 °C.

2.6. Enzyme activity assays

The SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GST (EC 2.5.1.18) and GPx (EC 1.11.1.9) activities were determined using the spectrophotometric methods described by Misra and Fridovich (1972), Aebi (1984), Habig et al. (1974) and St-Clair and Chow (1996), respectively. Specific enzyme activity was calculated considering the total protein content of the supernatant; results were expressed as enzyme units/mg protein. One CAT unit was the amount of enzyme required to catalyze 1 pmol of H_2O_2/min measured at 240 nm. One GST unit represented the amount of enzyme required to conjugate GSH with 1 μmol of 1-chloro-2,4-dinitro-benzene/min, determined at 340 nm. GPx units are expressed as pmol NADPH consumed/min. One SOD unit was considered as the amount of enzyme necessary to inhibit 50% the rate of autocatalytic adrenochrome formation/min measured at 480 nm.

2.7. Lipid and protein oxidation

The LPO level was measured according to Buege and Aust (1978) using the formation of thiobarbituric acid reactive substances (TBARS). Hemolymph and homogenates were added to the reaction mixture (trichloroacetic acid 15% (w/v), 2-thiobarbituric acid 0.375% (w/v), and butylhydroxytoluene 0.147 mM) at a ratio of 1:10 (v/v). The mixture was vigorously shaken, maintained in boiling water for 60 min, and immediately cooled at 4 °C for 5 min (Ohkawa et al., 1979). Then it was centrifuged at 5000 *g* for 10 min, and the supernatant was measured spectrophotometrically at 535 nm.

The PO level was evaluated as previously described (Ansaldo et al., 2007) by measuring the formation of protein hydrazones resulting from the reaction of dinitrophenyl hydrazine (DNPH) with protein carbonyls. The carbonyl content was measured at 375 nm.

2.8. Hemolymph non-enzymatic antioxidant levels

The GSH level was determined by the method of Moron et al. (1979). After deproteinization with trichloroacetic acid (TCA 50%), free endogenous GSH was determined using 0.5 mM of 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB). The absorbance was read at 412 nm. GSH was used as standard to calculate the concentration expressed in nmol/mL.

The ferric reducing activity of hemolymph was estimated by FRAP (Ferric Reducing Antioxidant Power) measuring the combined reducing power of the electron donating antioxidants present in hemolymph (Benzie and Strain, 1996). Stock solutions consisted of a 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tris(2-pyridyl)-*s*-triazine) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$. Reagent was prepared on the day of assay by mixing 25 mL acetate buffer with 2.5 mL TPTZ solution and 2.5 mL $FeCl_3$ $6H_2O$. The assay was performed adding 800 μL of FRAP reagent to a 25 μL sample and its reduction to the ferrous form (Fe^{+2}) monitored at 593 nm over 20 min. The change in absorbance was related to the absorbance changes of a standard solution of ascorbic acid tested in parallel. Results were expressed as μmol of ascorbic acid equivalents/mg protein.

The antioxidant capacity of hemolymph was also determined by TEAC assay (scavenging of the radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6 sulfonic acid, ABTS) (Re et al., 1999) with some modifications. $ABTS^+$ radical was generated by reacting 7 mM of ABTS solution in water with 2.45 mM of potassium persulfate in the dark for 12–16 h. Absorbance at 734 nm of the reactant was then adjusted to 0.700 ± 0.02 with PBS. Radical scavenging reaction was started by adding 10 μL of appropriately diluted hemolymph to 990 μL of the $ABTS^+$ solution. The absorbance of the mixture was recorded at 734 nm for 20 min after the addition of the sample. The change in absorbance was related to the absorbance changes of a standard solution of ascorbic acid tested in parallel. Results were expressed as μmol of ascorbic acid equivalents/mg protein.

2.9. Gene expression analysis

Total RNA was extracted using an RNeasy Mini kit (Qiagen), with on-column DNA digestion. RNA was quantified by Quant-iT RNA assay kit (Invitrogen) and integrity was assessed on a 1% (w/v) agarose gel. We attempted to amplify cDNA fragments of SOD, CAT, and GST in *M. borellii* to design specific primers to be used in real-time PCR analysis. With this aim, gene information of the closest crustacean species available was employed, namely *Macrobrachium rosenbergii* for SOD (GenBank accession no. DQ073104), *Litopenaeus vannamei* (AY518322) and *Fenneropenaeus chinensis* (EU102287) for CAT, and *L. vannamei* (AY573381) and the copepod *Tigriopus japonicus* (DQ088365) for GST. A 455 bp fragment with 92% homology with the cytosolic Mn-SOD of *M. rosenbergii* was amplified (Lavarías, unpublished data). No amplification was obtained for CAT and GST genes.

Two-step RT real-time PCR was carried out with an iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad). Amplification was performed in a Stratagene Mx3000P QPCR System (Stratagene) employing 20 ng reverse transcribed total RNA for each sample (control and WSF treatment). The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with 3-segment amplification (30 s at 95 °C for denaturation, 30 s at 56 °C for annealing, and 30 s at 72 °C for polymerase elongation). To confirm that only single products were amplified, a temperature melting step was then performed. The calibration curve method was used for the analysis of data obtained from the RT PCR system. Actin was chosen as the control gene, using the following primers: 5′-ACGAGGCCCAGAGCAAGAGA-3′ and 5′-GTTGCCCTTGGGGTTGAGTG-3′. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The gene-specific primers of SOD were: 5′-CCGCCGACTTCCACCTTCTATC-3′ and 5′-GGCTCGCTGAACAGTGATGCTG-3′. The assay was performed in duplicate for each of the three independent biological replicates performed.

2.10. Statistical analyses

Data were analyzed using Instat v. 3.01 by Student *t*-test and ANOVA and Tukey multiple comparison. Results were considered significant at 5% level.

3. Results

3.1. Seasonal variations of antioxidant enzyme activities and oxidative stress in M. borellii

Seasonal differences in the antioxidant defense and oxidative stress index in gills and hepatopancreas of *M. borellii* were studied monthly for one year (mid 2007 to mid 2008). Changes of water pH in the sampling site varied between 6.7 and 6.9, whereas temperature changed between 10 °C in winter and 25 °C in summer.

The SOD activity was similar in both tissues and did not show significant differences among seasons (Fig. 1A). In gills, CAT activity was significantly higher in spring and early summer than in winter, whereas in hepatopancreas the activity did not show significant differences among seasons (Fig. 1B). GST activity was similar in both tissues, showing a significant decrease during warm months compared with the cold ones, except in May-2008 (Fig. 1C). LPO levels in hepatopancreas were also significantly higher from mid-spring to the beginning of autumn (Fig. 1D).

3.2. Effect of WSF

3.2.1. Antioxidant defense

The activities of some enzymes belonging to the antioxidant defense system were evaluated in adult prawns exposed to 0.6 mg/L of WSF for 4 and 7 days. SOD activity in hepatopancreas showed a significant increase in activity (43%) only after 7-day exposure to WSF, while the activity in controls did not change between 4 and 7 days (Fig. 2A). In gills, SOD activity was not altered after WSF exposure with values of 0.74 ± 0.20 (controls) and 0.92 ± 0.39 U/min·mg protein (treated).

CAT activity significantly increased in hepatopancreas on 4-day and 7-day treated organisms as compared with controls (Fig. 2B). A direct relationship was observed between CAT activity and the number of exposure days. In gills, a significant increment ($p<0.05$) was observed after a 7-day exposure (8.3 ± 4.6 and 15.0 ± 4.1 U/min·mg protein for controls and WSF-exposed, respectively), which was 45.5% lower than that in hepatopancreas.

Fig. 2C shows that GST activity in hepatopancreas was higher (78%) on day 7 ($p<0.05$) in WSF-exposed prawns as compared with controls. Interestingly, GST activity in both control and treated prawns exposed for 7 days significantly diminished ($p<0.01$) as compared with those exposed for 4 days to WSF (Fig. 2C). In gills, enzymatic activities were not affected after a 7-day treatment, showing values of 35.5 ± 4.6 and 31.8 ± 4.2 mU/min·mg protein for controls and WST-treated prawns, respectively. This represents a 7-fold higher activity in gill than in hepatopancreas of control prawns ($p<0.0001$).

No significant differences were observed in GPx activity between treated organisms and controls. Values were 43.2 ± 15.8 (control) and 43.7 ± 12.0 U/min·mg protein (treated) in hepatopancreas, and 43.5 ± 23.9 (control) and 46.8 ± 17.6 U/min·mg protein (treated) in gills. No differences were found between tissues either.

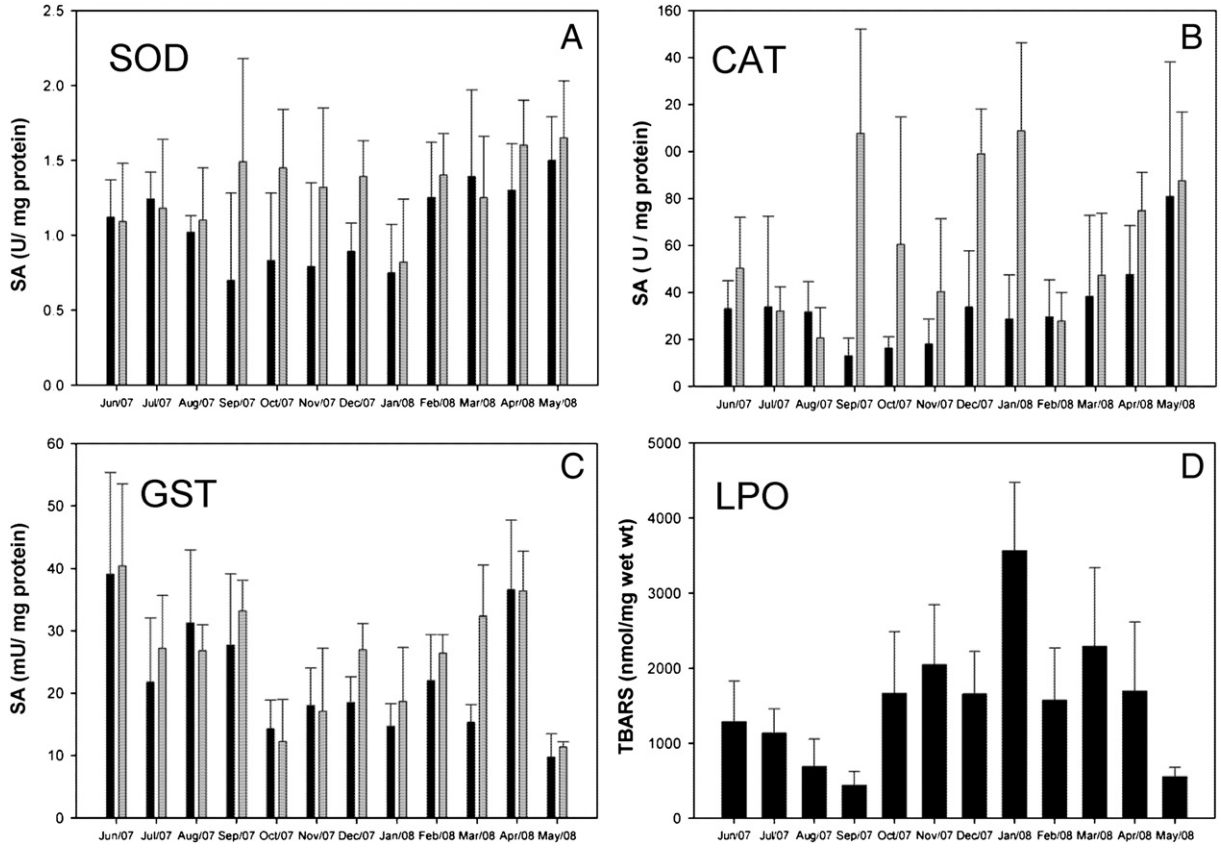

Fig. 1. Seasonal variation on some parameters of the antioxidant defense system in *M. borellii*. Values represent the mean of eight determinations ± SD. Superoxide dismutase (A), Catalase (B), Glutathione-S-transferase (C) lipid peroxidation levels LPO (D). SA: specific activity. ■ Hepatopancreas □ Gills.

3.2.2. Lipid and protein oxidation

The effect of WSF on the oxidation of both lipids and proteins from different prawn tissues is shown in Table 1. LPO significantly increased in hepatopancreas of treated prawns, but unexpectedly gills and hemolymph showed a significant decrease of lipid peroxidation. PO was not affected, although the highest level was detected in hepatopancreas.

3.2.3. Non-enzymatic antioxidant defense system

The GSH levels registered in hepatopancreas decreased from 4.03 ± 1.11 to 2.95 ± 0.88 GSH nmol/mg wet mass for controls and treated prawns, respectively. The other tissues analyzed did not show significant changes (39.3 ± 12.8 to 33.4 ± 3.80 GSH nmol/mL for control and treated hemolymph, respectively), and from 3.75 ± 0.71 to 2.90 ± 1.26 GSH nmol/mg wet mass (control and treated gills, respectively).

Table 2 shows the hemolymph total antioxidant capacity measured by ABTS and FRAP methods (see Materials and methods for details). A significant increase in ABTS values was observed in hemolymph from WSF-treated prawn as compared with controls. When the antioxidant capacity was studied by reducing FRAP reagent, hemolymph showed no significant changes between WSF-treated and control prawns.

3.2.4. Effect of WSF on SOD expression in hepatopancreas

The induction of SOD genes was studied by real-time quantitative PCR analysis. Primers were synthesized according to the available gene sequences from a related crustacean species. We obtained a 209 bp amplicon for SOD. Prawns exposed to WSF for 7 days had significantly higher ($p<0.0001$) levels of SOD mRNA than their corresponding controls, showing a similar response to that observed for enzyme activity after WSF treatment.

4. Discussion

4.1. Seasonal variation of the antioxidant defense status

It is known that aerobic organisms have an antioxidant defense system for reaching a degree of equilibrium between production and removal of endogenous ROS and other pro-oxidants. Thus, it is of utmost importance to study the antioxidant system in those organisms that could be useful for monitoring environmental conditions. Nevertheless, the comparison of the antioxidant system enzyme activities in invertebrates is limited partly due to the scarce information on the seasonal effects and other factors such as genus and age. For this reason, a study on seasonal variations in some biochemical biomarkers from *M. borellii* was carried out. Sampling was performed in a stream which was known to be virtually free from anthropogenic hydrocarbon (Lavarías et al., 2005b). It was observed that LPO levels in hepatopancreas were significantly higher in summer and autumn, in agreement with a report on the shrimp *Aristeus antennatus* (Anto et al., 2009) and on the amphipod *Hyalella curvispina* (Dutra et al., 2008). In contrast, the barnacle *Balanus balanoides* evidenced an opposite trend (Niyogi et al., 2001b).

Seasonal variations of enzyme activities were greater in gills than in hepatopancreas. In particular, the gill CAT activity markedly increased in summer. Crustacean hepatopancreas has been characterized as the main site for toxicant metabolism and biotransformation of ROS (Livingstone, 1998). For this reason, most studies on the antioxidant responses were performed in this organ. Interestingly, no

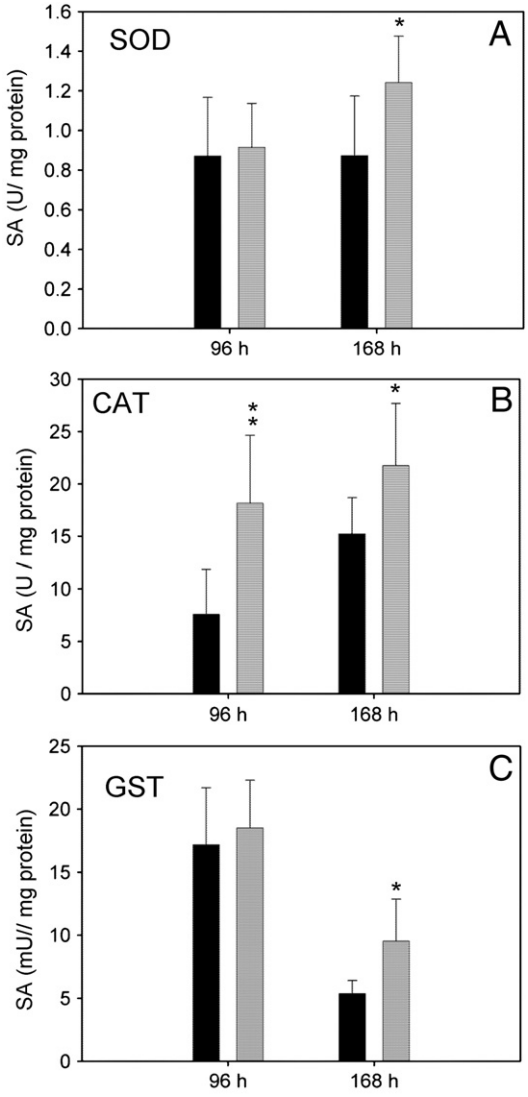

Fig. 2. Effect of 4-day and 7-day exposures to 0.6 mg/L WSF on enzymatic activity of Superoxide dismutase (A), Catalase (B) and Glutathione-S-transferase (C) in *M. borellii* hepatopancreas. Values represent the mean of twelve determinations ±SD. * Significant differences ($p<0.05$); ** ($p<0.01$). SA: specific activity. Control WSF.

clear seasonal trend was observed in the CAT, SOD and GST activities in *M. borellii* hepatopancreas. Results contrast with those observed by Niyogi et al. (2001a) in barnacles, where these enzymes exhibited a distinct seasonal pattern with a maximum activity in summer followed by a gradual decrease in winter in a reference site and in *A. antennatus* which displayed a marked increase of CAT and GST activities in autumn (Anto et al., 2009). Taken together, these studies suggest that the results dealing with the seasonal variation of the antioxidant defense system in invertebrates are currently very few to draw a general conclusion.

Table 1
Effect of a 7-day exposure to 0.6 mg/L WSF on protein and lipid oxidation in tissues of *M. borellii*.

	Protein oxidation (PO)			Lipid peroxidatio (LPO)		
	Control	WSF	*p*	Control	WSF	*p*
Gills	2.57 ± 0.99	2.49 ± 1.02	NS	76.3 ± 3.9	61.7 ± 2.4	**
Hepatopancreas	6.39 ± 3.85	7.39 ± 4.54	NS	117.1 ± 25.8	156.7 ± 13.7	*
Hemolymph	1.83 ± 0.67	1.90 ± 0.94	NS	1.63 ± 0.23	1.35 ± 0.12	**

Values represent the mean of twelve determinations ±SD. PO: [Carbonyl] (nmoles/mg·protein). LPO: TBARS (nmol/mg wet mass) in gills and hepatopancreas, and TBARS (nmol/mL) in hemolymph. ** Significant difference ($p<0.01$). * Significant difference ($p<0.05$).

Table 2
Changes of the total antioxidant activity in hemolymph of *M. borellii* after 7-day exposure to 0.6 mg/L WSF.

	Control	WSF	*p*
ABTS	23.9 ± 1.97	27.0 ± 0.77	*
FRAP	231.0 ± 48.40	275.9 ± 45.51	NS

Values represent the mean of twelve determinations ± SD, expressed as equivalents of ascorbic acid/mg protein. * Significantly different ($p<0.05$).

4.2. Effect of WSF exposure on the antioxidant defense system

When the organisms are exposed to contaminants such as hydrocarbon, an increase of ROS production is likely to be produced, leading to an increment in the enzyme activity of the antioxidant system. For instance, work carried out by Niyogi et al. (Niyogi et al., 2001a, 2001b) evidenced an induction of CAT and SOD antioxidant enzymes in the digestive gland of the barnacle *B. balanoides*, and an increased activity of CAT, SOD, GPx, as well as a microsomal lipid peroxidation in the mollusc *Saccostrea cucullata,* collected from an estuary contaminated with PAH. Other authors have also found increments of CAT and SOD activities in bivalves exposed to PAH, with a positive correlation between these enzyme activities and PAH tissue concentration (Porte et al., 1991; Solé et al., 1996). Similarly, the exposure of *M. borellii* to WSF caused a significant increase of CAT and SOD in hepatopancreas. On the contrary, *M. borelli* GPx activity did not show any significant difference.

As mentioned above, the hepatopancreas is the major site for toxicant uptake and oxyradical-generating biotransformation enzymes. In *M. borellii* however, the activity was also significant in gills. A similar trend is observed in other prawns of the same genus as *Macrobrachium malcolmsonii* and *M. lamarrei lamarrei*, were the highest activities of CAT, SOD and GPx were observed in hepatopancreas but also followed by gill (Arun and Subramanian, 1998a). It is worth noticing that Livingstone et al. (1992) indicated that while GPx plays a significant role in the H_2O_2 detoxification in vertebrates, CAT would fulfill this role in invertebrates, though with large interspecific variability.

GST is one of the most studied enzymes in crustaceans concerning the metabolism of contaminants of Fase II (Arun and Subramanian, 1998a, 1998b; Ishizuka et al., 1998; Saravana Bhavan and Geraldine, 2001). However, its role as potential biomarker in ecotoxicological studies has recently arisen in this taxon (Barata et al., 2005; Gowland et al., 2002). GST activity is generally found in crustacean gills and hepatopancreas, which have the highest metabolic rates and directly interact with the external environment. It is interesting to note that though *M. borellii* showed much higher levels of GST activities in gills compared with hepatopancreas, WSF exposure induced a significant increase of GST activity especially in hepatopancreas. The decrease of GSH could be explained either because it is used for biotransformation through conjugation of WSF or a metabolite of WSF (see below), or another possibility could be that induction of GST occurs because of the oxidative stress that causes an excess of hydroperoxides produced by the increase in LPO. These results suggest that hepatopancreas is the main organ for hydrocarbon detoxification. This agrees with reports in other freshwater crustaceans, showing higher levels of GST activity such as *M. malcolmsonii* treated with endosulfan (Saravana Bhavan and Geraldine, 2001), the crayfish *Eriocheir japonicus* contaminated with PAH and polychlorinated biphenyls (Ishizuka et al., 1998) and the marine cladoceran *Daphnia*, exposed to WSF from No 20 diesel oil (Zhang et al., 2004). These reports would favor the

utilization of GST as a complementary biomarker together with more specific biomarkers.

4.3. Effect of WSF exposure on the non-enzymatic antioxidant defense system

The characterization of antioxidant defense in aquatic organisms can also be evaluated by measuring the total antioxidant capacity. This procedure provides a good understanding and predicting capacity of the effects of environmental stressors on the redox status of the organisms and/or on their susceptibility to oxidative stress (Livingstone, 2001; Camus et al., 2002). In the present study, two methods namely ABTS and FRAP were employed to evaluate the total antioxidant capacity in hemolymph. The first one determines the overall antioxidant capacity of free radicals scavenging, whereas the second one shows the Fe^{+3} reducing capacity of the sample. Although both methods evidenced an increase due to WSF exposure, only ABTS showed significant results. This different sensitivity was probably due to the fact that the reaction with different antioxidants was slightly different (Benzie and Strain, 1996; Rice-Evans and Miller, 1997) measuring quantity but not quality of antioxidants (Romay et al., 1996). Likewise, in gills of the crab *Chasmagnatus granulatus*, there was an increment in the levels of the total antioxidant capacity in response to the oxidative stress induced by microcystine (Vinagre et al., 2003). In spider crab, the hepatopancreas total oxyradical scavenging capacity (TOSC) was not affected by exposure to PHA, but the authors suggested that hemolymph, where hemocytes represent the main defense system against foreign substances, may be more appropriate to measure TOSC (Camus et al., 2002).

In addition, the tripeptide GSH concentration plays a significant role in xenobiotic detoxification as well as an antioxidant. The very low GSH levels in *M. borellii* hepatopancreas after WSF exposure might be due either to the fact that GSH may be conjugated to hydrocarbon metabolites, representing the major detoxification pathway, or to the fact that the prawns were kept without feeding during the experiments. In agreement with the last alternative, the decreased GSH contents in hepatopancreas of the freshwater crab *Sinopotamon yangtsekiense,* fasted during treatment with high doses of Cd, were partially explained by the inhibition of *de novo* synthesis caused by the depletion of limiting amino acid L-cysteine (Wang et al., 2008). Moreover, Ansaldo et al. (2007) observed in the limpet *Nacella concinna* that GSH level for the starved group remained unchanged, explained by peptidase activity on peptides from intracellular pool. The grass prawn *Palaemonetes pugio* exposed to diesel fuel displayed a significant increase of GSH and LPO levels and of SOD activity (Downs et al., 2001).

In the last few years there has been an increasing interest in the use of differential gene expression as biomarker, though this technique has been seldom used in studies of hydrocarbon pollution. Using RT-PCR we were able to confirm that the increase of SOD activity in WSF-exposed prawn was correlated with an increase of its mRNA expression, clearly indicating an induction. Similar results were reported in the copepod *Calanus finmarchicus* exposed to low concentrations of naphthalene (Hansen et al., 2008). Thus, measurement of SOD expression in *M. borellii* could be used as potential biomarker of hydrocarbon exposure if complemented with other specific biomarkers. No products were amplified for CAT and GST, as mentioned in Materials and methods, probably due to the use of crustacean species that are not closely related to *Macrobrachium* in the primer design.

5. Conclusions

We have provided evidence of the seasonal changes of the defense system in *M. borellii*. Enzymatic and non-enzymatic mechanisms appear to be involved in the defense system against the hydrocarbon WSF-mediated stress. The evidence presented here supports the notion that the mode of WSF toxicity in *M. borellii* is evident through both lipid peroxidation and general cellular oxidative stress. These results indicate that antioxidant defense components in *M. borellii,* especially in hepatopancreas are significantly affected by aquatic hydrocarbon contamination. The biological response to sublethal exposure in *M. borellii* could be a valuable tool, in particular, using a combination of GSH, LPO, GST, SOD activities and SOD mRNA levels. These biomarkers could be proposed as a battery to monitor hydrocarbon pollution in freshwater environments.

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