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Study of biochemical biomarkers in freshwater prawn *Macrobrachium borellii* (*Crustacea*: *Palaemonidae*) exposed to organophosphate fenitrothion

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ABSTRACT

Several agrochemicals like organophosphates are extensively used to control pests in agricultural practices but they also adversely affect non-target fauna. The effect of organophosphorous fenitrothion on the prawn *Macrobrachium borellii* was evaluated. The 96-h LC_{50} was determined. Activity of superoxide dismutase, catalase, glutathione-*S*-transferase and lipid oxidation levels, were evaluated in the hepatopancreas from adults exposed to sublethal fenitrothion concentrations for 1, 2, 4 and 7 days. In addition, superoxide dismutase mRNA expression, acetylcholinesterase inhibition and haemocyte DNA damage were determined. The 96-h LC_{50} was 4.24 μg/l of fenitrothion. Prawn exposed to sublethal FS concentrations showed an increase of both catalase and superoxide dismutase activities, mainly after 2 and 4 days exposure and an increase of glutathione-*S*-transferase activity from day 2 to day 7 while lipid oxidation levels increased mainly on day 1. Superoxide dismutase transcripts were significantly higher in fenitrothion -treated prawns, indicating an induction mechanism. Hemolymph analysis showed that while acetylcholinesterase activity decreased after 2 days, haemocytes displayed most DNA damage after 7-day exposure to fenitrothion.

These results indicate that prawn enzymes are highly sensitive to fenitrothion exposure, and these biological responses in *M. borellii* could be valuable biomarkers to monitor organophosphorous contamination in estuarine environments.

1. Introduction

Freshwater systems are often exposed to pollution due to the use of insecticides in agricultural practices. Organophosphorous (OP) are one of the most utilized insecticides, but this also results in non-target organisms exposed to either lethal or sublethal concentrations of these contaminants (García-de la Parra et al., 2006). Crustaceans exposed to OP show metabolic alterations in physiological processes such as enzymatic activities, oxidative metabolism, oxygen consumption, energy metabolism, osmoregulation and immunological responses (Chang et al., 2013). It is well-known that OP acts *via* the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) which blocks nerve transmission at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine (Kennedy, 1991). Thus, AChE inhibition induces nerve tissue disruption. In invertebrates, this inhibition leads to the typical pattern of poisoning by producing restlessness, hyperactivity, tremors, convulsions and paralysis (Ware, 1989).

Despite the fact that AChE inhibition is a widely used biomarker for OP contamination, other oxidative stress-related parameters have been used as xenobiotic indicators in aquatic invertebrates as well (Favari et al., 2002; Monserrat et al., 2003; Dorval et al., 2005). Organic contaminants like the OP are oxidatively biotransformed by mixed-function oxidases (MFO) inducing an increase of the reactive oxygen species (ROS) which in term might produce oxidative stress (Porte et al., 1991). Subsequently, the antioxidant defense system is activated to avoid ROS harmful effects on biomolecules. These systems include several enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathion peroxidase as well as non-enzymatic compounds as reduced glutathion (GSH), ascorbic acid, uric acid, vitamin E and β-carotene (Correia et al., 2003). SOD converts O_2^- to H_2O_2, while CAT is an essential enzyme to promote the degradation of H_2O_2, a precursor of the hydroxyl radical that induces DNA damage, protein degradation and lipid peroxidation (Di Giulio et al., 1995).

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In turn, glutathione *S*-transferases (GSTs, EC 2.5.1.18) are the major phase II-related enzymes, conjugating electrophilic compounds with GSH. This renders OP less toxic and more water soluble so that they can be easily excreted from cells after further metabolism (Crane et al., 2002). Besides, an increased lipid peroxidation (LPO) is one of the major contributors to the loss of cell function on oxidative stress situations. LPO determination has also been successfully employed in aquatic animals to indicate oxidative stress induced by organic contaminants, including organophosphorus pesticides (Monserrat et al., 2007).

It has been observed that penaeid prawns are generally more sensitive to most pesticides than fish and mollusks and they have been proposed as indicators of estuarine health due to their world-wide distribution (García-de la Parra et al., 2006). Also, crustaceans represent an advantageous tool to monitor environmental contamination as their communities are numerous and easily cultivated in the laboratory (Fossi et al., 2000; Gerhardt et al., 2002; Shigehisa and Shiraishi, 1998).

The Rio de La Plata river basin is an intensive agro ecosystem where pesticides are largely used. To evaluate the ecotoxicological action of pesticides in this area, we selected the crustacean decapod palemonidae, *Macrobrachium borellii* which represents the high trophic level within the community, and is taxonomically related to aquacultured species. Fenitrothion (FS, *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate) is a powerful insecticide that has been widely used in the area for agricultural purposes and is currently used for mosquito control (Delatte et al., 2008). Our group has previously reported the effect of FS on some physicochemical parameters in *M. borellii* (Garcia et al., 2002, 2004,2005). Therefore, the aim of the present work was: (a) to determine LC_{50} of FS on the freshwater prawn *M. borellii* and (b) to assess the effects of sublethal concentrations of FS in this species using a variety of potential biomarkers including SOD, CAT, GST, and AChE activities, LPO levels and DNA damage.

2. Materials and methods

2.1. Sample collection

M. borellii adults were collected in a 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 ($CaCO_3$ hardness, 160 mg/l, pH between 6.6 and 6.9, and dissolved oxygen between 4.5 and 5 mg/l) at 22 ± 2 °C, and 14:10 h L:D photoperiod for at least a week before the experiments. Animals were fed only during acclimation with the recommended diet for *M. borellii* (Collins and Petriella, 1996).

2.2. Toxicity tests

Prawns were collected in late winter during the pre-reproductive season, the selected animals were in intermolt.

2.2.1. Determination of 96-h LC_{50}

Experimental animals were randomly placed at each of five FS (purchased from GLEBA S.A., La Plata, Argentina) concentrations and one control. A stock solution of 100 g/l FS was prepared in absolute ethanol. The subsequent working stock solutions were obtained by diluting the main stock in absolute ethanol to the concentrations for exposures where 1- 2- 4- 8- 12- 16 μg/l FS using Triton X-100 as solvent (final concentration 8 μg/l) plus a control group held without FS but with ethanol and Triton X-100, and another water control group (Gonzalez-Baró et al., 1997).

For each experiment, groups of six adults (mean wet weight 1.05 g) were placed into glass flasks containing 2 l of test solution and constant aeration. For each concentration, 2 flasks were used and FS solution was replaced daily for 4 days. Before each daily medium changed, mortality was recorded and dead shrimps were removed. Temperature, pH, and dissolved oxygen were measured in all containers. Experiments were done in triplicate, without feeding, at 20–22 °C and a 14-h light: 10-h dark cycle.

2.2.2. Sublethal assays

To evaluate the effect on the selected biomarkers, prawns were exposed to different sublethal FS concentrations below NOAEL concentration: 0.2, 0.8 and 1.4 μg/l for 1, 2, 4 and 7 days, and then the selected biochemical parameter measured. No mortality was observed under the experimental conditions employed. For each group six replicates were made. The prawns were exposed at the same conditions as in the previous assays.

Because FS could be extremely labile in experimental conditions (Leboulanger et al., 2011) their concentrations were measured at the beginning and 24 h after exposition, when the medium was replaced.

FS was serially extracted with methylene chloride, the extract was dried and exchanged into hexane according to the EPA method 3510C. FS concentrations were determined by gas chromatography (Agilent 6890 equipped with a nitrogen-phosphorous detector), using a capillary column (Hewlett Packard 5 percent phenyl methyl siloxane) of 30 m × 0.25 mm and 0.25 mm film thickness. Helium was used as a carrier and nitrogen as an auxiliary gas.

2.3. Preparation of total cellular homogenate

After exposure to FS, animals were anesthetized on ice for about 5 min, hepatopancreas was removed, weighed and cooled on ice and then stored at −70 °C until used.

Hepatopancreas were pooled (two prawns each) and homogenized (1:9 w/v) in 125 mM Tris-base cold buffer solution, 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 antioxidant activities and LPO levels. Total protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Hemolymph extraction

Hemolymph was aseptically withdrawn from the pericardial sinus using a 1-ml syringe with a 1.5″, 23 gauge needle rinsed with crustacean anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid and 20 mM EDTA, pH 4.5) (Sierra et al., 2001). Hemolymph samples were pooled (3 specimens each) and centrifuged at 10 000 × *g* for 10 min at 4 °C to remove haemocytes; the resulting supernatant was stored at −70 °C for acetylcholinesterase assay. For the comet assay, the pooled fresh hemolymph was mixed with either an equal volume of crustacean anticoagulant and haemocytes were washed twice. The haemocytes viability was assessed by their ability to exclude Trypan blue (0.2 percent, w/v, final concentration) (Phillips, 1973), using a Neubauer chamber with an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems Wetzlar, Germany).

2.5. Comet assay

The comet assay was performed according to the method of Singh et al. (1988) with minor modifications (Tice et al., 2000). Briefly, conventional slides were covered with a layer of 180 μl 0.5 percent (w/v) normal agarose (GIBCO-BRL). An amount of 75 μl of low melting point agarose (0.5 percent, w/v) (GIBCO-BRL) was mixed with approximately 1×10^4 cells suspended in 15 μl; the mixture was then layered onto the slides, and immediately overlaid with coverslips. After agarose solidification at 4 °C for 10 min, the coverslips were removed and the slides were immersed overnight in fresh lysing solution at 4 °C (2.5 M NaCl; 100 mM Na2 EDTA, 10 mM Tris, pH 10 containing 1 percent Triton X-100 and 10 percent dimethyl-sulphoxide, added just before use). The slides were equilibrated in alkaline solution (1 mM Na_2 EDTA, 300 mM NaOH, pH > 13) for 20 min. Electrophoresis was carried out at 25 V and 250 mA for 15 min. After this, slides were neutralized by washing them three times with Tris buffer (pH 7.5), 5 min each, and finally with distilled water; slides were then stained with 1/1000 Sybr Green I.

Fluorescence microscopy observations were performed with an Olympus BX51 microscope (Tokyo, Japan) equipped with a U-WIBA filter cube (excitation filter 460–490 nm, barrier filter 515–550 nm) for image analysis. Individual cells were analyzed and photographed using an Olympus DP70 digital camera and the ImagePro Plus (IPPTM) v5.1 image analysis software (Media Cynernetics, Silver Spring, MD).

Based on the extent of strand breakage, cells were classified according to their tail length into five categories (Crespo et al., 2011). Quantification of DNA damage was calculated using the damage index (DI) parameter defined by Güerci et al. (2008) expressing the degree of cell damage cumulatively for each treatment evaluated.

2.6. Acetylcholinesterase (AChE) activity

AChE activity was determined according to a modified version of the colorimetric technique described by Ellman et al. (1961). The reaction mixture was prepared in 50 mM of potassium phosphate buffer pH 7.7 containing *S*-butyrylthiocholine iodide and 5,5′-dithiobis-2-nitrobenzoic acid at final concentrations

of 6 mM and 0.25 mM, respectively. Ten microliters of hemolymph were added to start the reaction, which was followed spectrophotometrically at 405 nm and 25 °C. AChE activity was expressed as enzyme units/mg protein. One AChE unit was the amount of enzyme that hydrolyzed 1 nmol of acetylcholine/min.

Total protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.7. Antioxidant enzyme analyses

SOD activity was determined according to Misra and Fridovich (1972). This method is based on the inhibition of the auto-oxidation of epinephrine (2 mM, pH 2). The reaction was carried out in 50 mM glycine buffer (pH 10.2), and the absorbance was measured at 480 nm. Results were expressed as units of SOD per mg of proteins. One SOD unit was considered as the amount of enzyme necessary to inhibit 50 percent rate of autocatalytic adrenochrome formation/min.

CAT activity was determined by following the decomposition of H_2O_2 espectrophotometrically at 240 nm, in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7) and 10 mM H_2O_2 (Aebi, 1984). Results were expressed as pmol CAT per mg of proteins. One CAT unit was the amount of enzyme required to catalyze 1 pmol of H_2O_2/min.

GST activity was assayed as described by Habig et al.(1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, the final reaction mixture contained 1 mM CDNB and 1 mM reduced glutathione (GSH). Results were expressed as units of GST per mg of proteins. 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.

2.8. Lipid oxidation

The LPO level was measured according to Buege and Aust (1978) using the formation of thiobarbituric acid-reactive substances (TBARS). Hepatopancreas homogenates were added to the reaction mixture (trichloroacetic acid 15 percent (w/v), 2-thiobarbituric acid 0.375 percent (w/v), and butylhydroxytoluene 0.147 mM) at a ratio of 1:20 (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 measured spectrophotometrically at 535 nm. LPO was expressed as nmol TBARS hydrolyzed per mg of wet weight.

2.9. Sod expression analysis

Total RNA of hepatopancreas 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 1 percent (w/v) agarose gel. The expression pattern of *sod* was analyzed as previously described (Lavarías et al., 2011). Briefly, qReal Time-PCR was carried out with 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 fenitrothion treatment). 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 calibration curve method was used for data analysis. Actin was chosen as housekeeping gene, using the following primers: 5′-ACGAGGCCCAGAGCAAGAGA-3′ and 5′-GTTGCCCTTGGGGTTGAGTG-3′. The gene-specific primers of SOD were: 5′-CCGCCGACTTCCACCTTCTATC-3′ and 5′-GGCTCGCTGAACAGTGATGCTG-3′ (Lavarías et al., 2011). The assay was performed in duplicate for each of the three independent biological replicates performed.

2.10. Statistical analyses

Median lethal concentrations (LC_{50}) with 95 percent confidence limits were determined by PROBIT analysis program version 1.5 (US, EPA). LC_{50} was calculated for each experiment and then averaged. In the sublethal pesticide bioassay, the results are shown as mean ± standard deviations (SD) and different treatments were compared using a one-way ANOVA after checking for normality and homogeneity of variances. Significant differences ($p < 0.05$) were compared using Tukey post hoc test. Data were analyzed using Instat v. 3.01.

3. Results

3.1. Determination of LC_{50}

The 96-h LC_{50} determined for *M. borellii* was 4.24 μg/l of FS with 95 percent interval from 3.18 to 5.68 (Fig. 1). This result allowed us to select the appropriate FS concentrations for the biomarker studies.

As the effective concentration of FS at the beginning of exposure was reduced to 48.2 ± 2.6 percent after 24 h, the test solution was replaced daily.

3.2. AChE activity

Although hemolymph AChE activity of exposed prawns showed a decreasing tendency from day 2 on, it significantly decreased compared to the control ($p < 0.02$) only on day 7 (Fig. 2). Thus, after 7 days of exposure to 0.2, 0.8 and 1.4 μg/l of FS hemolymph AChE activity decreased by 42 percent, 59 percent and 57 percent, respectively, as compared to controls.

3.3. Sublethal effects of FS on enzymatic antioxidant defense

The effect of FS on the enzymatic antioxidant defense was evaluated in prawn hepatopancreas. SOD activity was affected only on day 2 and day 4 at higher concentrations of FS when compared to control (Fig. 3A). On day 2 this activity significantly increased ($p < 0.02$) 57 percent at 1.4 μg/l treatment while on day 4 this activity significantly increased by 19 percent and 25 percent at 0.8 μg/l and 1.4 μg/l of FS, respectively.

Fig. 3B shows that CAT activity significantly increased ($p < 0.02$) mainly on 2-day treated organisms as compared with controls by

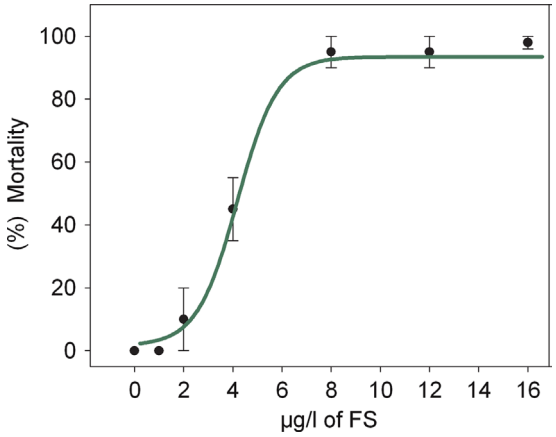

Fig. 1. Percentage of mortality of *Macrobrachium borellii* exposed to FS. Values represent the mean of tree determinations ± SD.

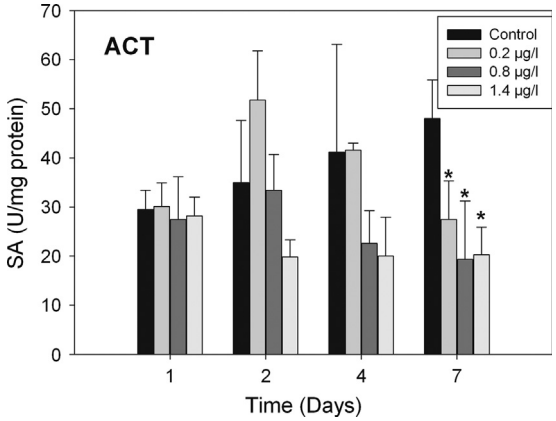

Fig. 2. Effect of 1-day to 7-day exposure to sublethal concentrations of Fs on hemolymph AChE activity of *M. borellii*. Values represent the mean of six determinations ± SD. * Significant differences from the control ($p < 0.02$). SA: specific activity.

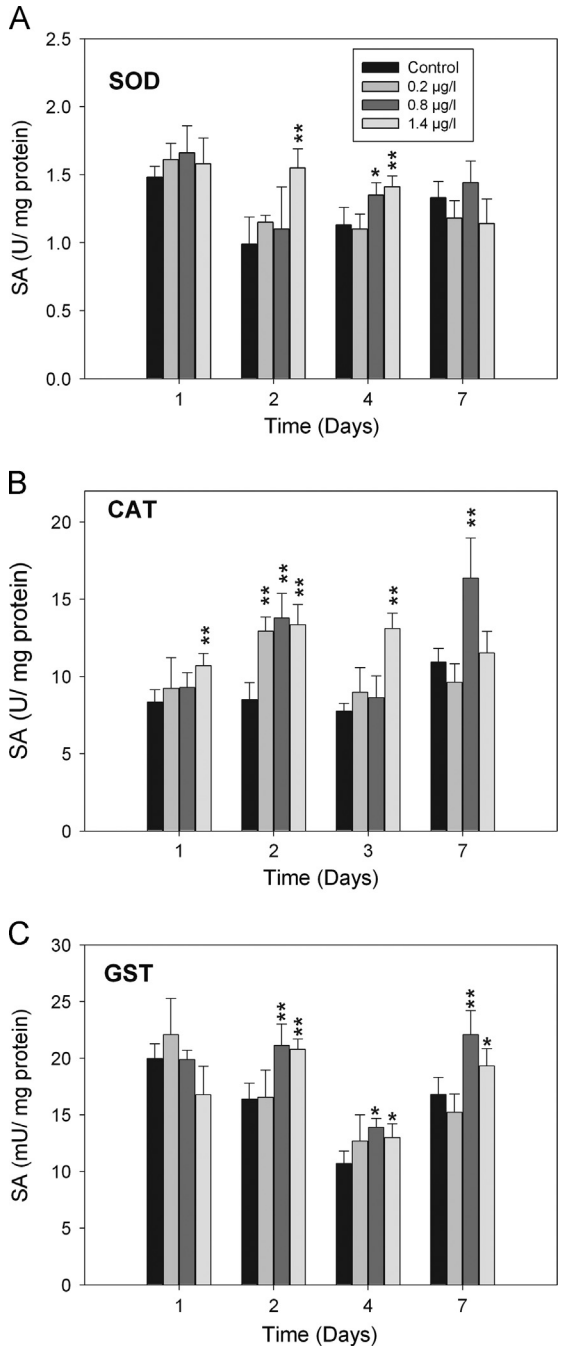

Fig. 3. Effect of 1-day to 7-day exposure to sublethal concentrations of FS on enzymatic activity of Superoxide dismutase (A), Catalase (B) and glutathione-*S*-transferase (C) in *M. borellii* hepatopancreas. Values represent the mean of six determinations ± SD. * Significant differences from the control ($p < 0.02$); ** ($p < 0.001$). SA: specific activity.

52 percent, 62 percent and 57 percent at 0.2, 0.8 and 1.4 µg/l of FS, respectively. On days 1 and 4 the increase was at 1.4 µg/l of FS (28 percent and 69 percent, respectively), but on day 7 only 0.8 µg/l of Fs showed very high activity (50 percent).

GST activity in hepatopancreas significantly increased ($p < 0.05$) at the highest FS concentrations, on day-2 by 29 percent and 27 percent at 0.8 and 1.4 µg/l of FS, respectively. The increase on day-4 was 30 percent and 21 percent at 0.8 and 1.4 µg/l FS, respectively, and on day-7 by 31 percent and 15 percent at 0.8 and 1.4 µg/l FS, respectively (Fig. 3C).

3.4. Lipid oxidation

LPO levels were significantly higher ($p < 0.02$) in prawns exposed to different FS mainly on day 1 regardless of the concentration assayed. At longer exposure times, LPO level increased only at 0.8 µg/l of FS on days 2 and 4 (Fig. 4).

3.5. Evaluation of viability and DNA damage in haemocytes

As the most noticeable changes in enzymes activities were observed mainly after the first day and at higher concentrations of exposure, prawns were exposed to 0.8 and 1.4 µg/l FS for both 2 and 7 days, and compared to controls.

As shown in Table 1, no significant differences in haemocyte population density (number of haemocytes/µl hemolymph) or percent mortality between haemocytes of controls and treated with different concentration of FS were observed, regardless of exposure time. However, FS caused DNA damage in prawn haemocytes after a 7-day exposure. The two highest FS concentrations showed a significant increase ($p < 0.05$) in the number of cells with DNA damage. Moreover, the DNA damage index (that represents the level of DNA damage) significantly increased in all FS-treated organisms, compared with the control ones in these experimental conditions.

3.6. Effect of FS on sod expression in hepatopancreas

In order to examine whether the *sod* gene was induced by FS, its expression pattern was analyzed in prawns exposed to 0.8 and 1.4 µg/l FS for either 2 or 7 days, and compared to controls. The expression of actin was used as an internal control (housekeeping gene). As shown in Table 2, the level of expression was affected by both, concentration and treatment time. A significant *sod* induction (1.6 ± 0.2 fold) was observed in prawns exposed to 1.4 µg/l FS after 2-day exposure, and an even higher expression level was observed after 7 days of exposure, with 1.9 and 2.7 fold induction over controls ($p < 0.02$) in prawns exposed to 0.8 and 1.4 µg/l FS, respectively. At the lowest dose tested, a significant ($p < 0.05$) induction was observed between 2- and 7- day treatments.

4. Discussion

M. borellii was found to be very sensitive to FS toxicity in comparison with other crustacean species like *Orconectes limosus* and *Callinectes sapidus* (Lignot et al., 1998). However, within *Macrobrachium* genus, FS was less toxic to *M. borellii* than to other species such as *M. lamerii* (Avelin Mary et al., 1986) and

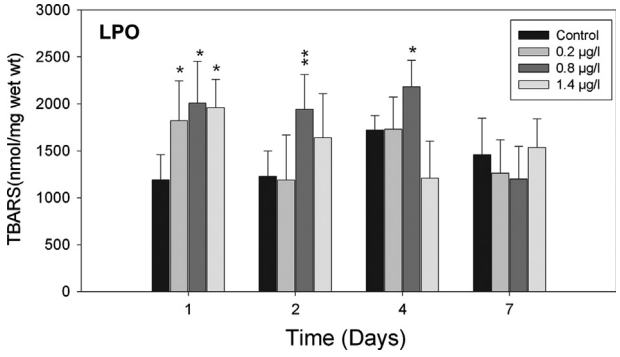

Fig. 4. Effect of 1-day to 7-day exposure to sublethal concentrations of FS on lipid oxidation in *M. borellii* hepatopancreas. Values represent the mean of six determinations ± SD. * Significant differences from the control ($p < 0.02$); ** ($p < 0.001$). SA: specific activity.

Table 1
Effect of 2-day and 7-day exposures to sublethal concentrations of FS in *Macrobrachium borellii* hemolymph cells.

Fenitrothion (μg/l)	Number of haemocytes/μl hemolymph[a]		Cell mortality[a] (%)		Cells with DNA damage[b](%)		DNA damage index[b]	
	2-day	7-day	2-day	7-day	2-day	7-day	2-day	7-day
Control	321 ± 40.1	217 ± 45.6	8.3 ± 1.3	11.7 ± 4.6	54.1 ± 11.7	30.9 ± 7.0	195 ± 47.2	130 ± 11.0
0.2	219 ± 28.1	177 ± 35.6	12.4 ± 5.2	19.2 ± 7.1	68.1 ± 12.7	47.5 ± 7.8	264 ± 52.4	171 ± 7.0*
0.8	533 ± 161.7	143 ± 15.3	10.9 ± 3.3	9.9 ± 0.5	41.5 ± 1.2	60.5 ± 12.4*	171 ± 4.7	215 ± 24,5*
1.4	396 ± 139.6	167 ± 8.9	15.7 ± 4.7	11.6 ± 2.2	57.7 ± 5.8	59.7 ± 7.5*	212 ± 22.9	219 ± 10**

[a] Mean ± SEM, $n=3$ pools of 4 prawns each.
[b] Mean ± SEM, $n=3$ replicates of 200–300 cells each.
* Significant differences from the control ($p<0.05$).
** ($p<0.001$).

Table 2
Induction of SOD mRNA expression in *M. borellii* hepatopancreas exposed to FS for 2 and 7 days.

Time of exposure	FS μg/l	
	0.8	1.4
2 days	0.7 ± 0.3	1.6 ± 0.2*
7 days	1.9 ± 0.4*	2.7 ± 0.8*

Values represent the mean of three determinations ± SD.
* Significantly different ($p<0,02$).

M. kistnensis (Pawar and Katdare, 1984) with 96-h LC_{50} values of 0.6 and 0.9 μg/l, respectively.

Although FS is extremely labile under experimental conditions (the effective concentration of FS was reduced 49.1 ± 2.9 percent), field doses may be higher than 500 g/ha, and may reach concentrations of 80 μg/l in the water body (Leboulanger et al., 2011). These water concentrations are certainly much higher than those of the LC_{50} of many of the organisms living in freshwater systems, including *M. borellii*.

Given the high sensitivity of *M. borellii* to FS, the effect of sublethal concentrations of this pesticide on several biomarkers was studied. The relationship between the presence of OP in the aquatic environment and AChE activity in tissues has been widely studied and utilized as biomarker in invertebrates (Escartín and Porte, 1996; Dias Bainy, 2000; Crane et al., 2002; Devi et al., 2005; Monserrat et al., 2007; Xuereb et al., 2007; Domingues, et al., 2008). In *M. borellii* only the hemolymphatic AChE was affected by FS treatment, while the muscle isoform showed no significant alteration (data not shown). Likewise, *Macrobrachium rosenbergii* exposed to the OP trichlorofon also showed a similar behavior to that of *M. borellii* because of the two AChE isoforms studied, only the hemolynphatic one significantly decreased (Yeh et al., 2005). Nevertheless, other decapod crustaceans exposed to FS showed a significant decrease of AChE activity in muscle (Escartín and Porte, 1996; Lignot et al., 1998).

Although OP mainly affects AChE, the inhibition of other enzymes and the stimulation of oxygen consumption have also been observed after the exposure to these pesticides (Day and Scott, 1990). Porte and Escartín (1998) demonstrated that the hepatopancreas of the crayfish *Procambarus clarkii* is able to metabolize FS which is transformed into oxidized products as fenitrooxon after the oxidative biotransformation process by the MFO system, which generates harmful ROS. In fact, there is evidence that the hepatopancreas is the major ROS-producing tissue in crustaceans causing lipid peroxidation and the induction of antioxidant enzyme activities in this organ (Bianchini and Monserrat, 2007). In *M. borellii*, FS significantly increased LPOs in the hepatopancreas even on the first day exposure, when the antioxidant defense enzymes SOD, CAT and GST were not activated yet. However, TBARS generally decreased after the second day of exposure and different treatments. Other authors have observed that OP markedly increase LPO levels in other crustaceans (Bianchini and Monserrat, 2007), however in *M. borellii* those metabolites measured by TBARS could be metabolized and/or excreted underestimating LPO. In fact, malondialdehyde is the precursor of fluorescent pigments such as lipofusin, a small and hydrosoluble molecule which could be released into water, resulting in a temporary accumulation in the organism, and producing an alteration in time concentration (Sukhotin et al., 2002). Another possibility to account for the diminution of TBARS levels would be the increased SOD and CAT activities between the second and fourth day of FS treatment, which would be enough to remove and avoid ROS accumulation. In contrast, prawns exposed for 7 days, displayed a decrease of SOD and CAT activities to values near the control, together with a decrease of LPO levels, suggesting that as a whole, the antioxidant system was able to cope with the stress. In *Daphnia magna* exposed to endosulphan pesticide, CAT activity was not altered while SOD activity was inhibited by the treatment (Barata et al., 2005). Also, in *M. rosenbergii* the oxidative stress produced by OP trichlorofon promoted the inhibition of SOD with the resulting accumulation of O_2^- (Chang et al., 2006).

In addition, FS exposure increased GST activity in *M. borellii* at the highest concentrations from the second day of exposure until the end of the treatment. A similar effect was noticed in hepatopancreas of the crayfish *P. clarkii* exposed to the same pesticide (Blat et al., 1988). These results suggest that GST activity would be strongly involved in the detoxification of FS products during its metabolization.

Although Bianchini and Monserrat (2007) reported a significant increase of the antioxidant enzymes CAT and GST in hepatopancreas of *Chasmagnathus granulatus* exposed to OP methyl-parathion, another OP, the histological damage found in these crabs clearly indicated that the capacity of the antioxidant system was surpassed during the exposure. In *M. borellii*, ROS resulting from FS exposure might produce oxidative stress, as indicated by the DNA damage in haemocytes. It is worth recalling that DNA strand breaks are produced either by apoptosis or necrosis and secondarily by ROS interaction or by inactivation of DNA-reparing enzymes. Such DNA lesions are usually evaluated by comet assay (Lee and Steinert, 2003), a valuable tool to determine the action of genotoxic compounds that induce genetic alterations and serious ecological consequences (Monserrat et al., 2007). The incremental damage of DNA in haemocytes of *M. borellii* exposed to FS for 7 days clearly indicated that the antioxidant defense system was not able to neutralize all ROS action. Thus, ROS not only damaged haemocyte DNA but also inhibited CAT and SOD enzymes in hepatopancreas, which seemed more vulnerable to the toxic than to GST. These results may help to understand the decreased TBARS levels at longer exposure times, masking lipid susceptibility to oxidation. However, the oxidative stress induced by FS in *M. borellii* was not enough to cause changes in haemocyte viability.

Although environmental contaminants have been reported to reduce haemocyte counting in crustaceans (Smith and Johnston, 1992; Victor et al., 1990), this parameter was found to be unaltered either in *M. borellii* after FS treatment or in *M. rosenbergii* exposed to OP trichlorofon (Yeh et al., 2005).

Recently there has been an increasing interest in the use of differential gene expression as a biomarker, though this technique has seldom been used in studies on pesticide pollution. As far as we know, this is the first report on the transcriptional levels about stress caused by organophosphates in aquatic invertebrates. Using RT-PCR we were able to confirm that the increased SOD activity in FS-exposed prawn on day 2 was correlated with an increase of its mRNA expression, clearly indicating an induction. Similar results were reported in the same prawn exposed to hydrocarbon contamination (Lavarías et al., 2011). After 7 days exposure, prawns exposed to FS showed a dose-dependent increase in SOD mRNA expression, while SOD activity was not modified with respect to control, suggesting a compensatory mechanism to overcome the enzymatic inactivation induced by ROS with an increase of enzyme synthesis. SOD transcription was also induced in the liver of fish *Oryzias javanicus* after exposure to the OP pesticide Iprobenfos (Woo et al., 2009). Likewise, exposure to the herbicide Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) induced oxidative stress and increased SOD mRNA expression in liver and ovary of zebrafish (*Danio rerio*), though the mRNA induction patterns were not in accordance with those of antioxidant enzyme changes (Jin et al., 2010). The arthropod *Bombus ignitus* exposed to paraquat (methyl viologen), also up-regulated SOD mRNA expression in the fat body (Choi et al., 2006).

5. Conclusions

This study provides evidence on FS toxicity in *M. borellii* that is caused by OP-induced oxidative stress, indicating that the biological response to sublethal FS exposure in *M. borellii* could be a valuable tool for monitoring OP contamination in freshwater environments. Particularly, the following combination of biomarkers and exposure times could be suggested: GST activity and mRNA *sod* levels regardless of exposure conditions, SOD and CAT activities at short exposure times and AChE inhibition and DNA damage at long exposure times.

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