SETAC – Brazil

J. Braz. Soc. Ecotoxicol., v. 1, n. 1, 2006, 31-35

JBSE

N. 6 Fuel Oil Effects on Antioxidant Enzymes and Immunological Responses in the Fish *Thalassophryne maculosa* (Pisces: Batrochoididae)

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ABSTRACT

With the aim of investigate the acute effects of two concentrations (25 and 100%) of N. 6 fuel oil-water-accommodated fraction (OWAF) on antioxidant enzyme activities and immunological responses, fishes *Thalassophryne maculosa* were sampled from the La Restiga Lagoon, Margarita island, in eastern Venezuela. The antioxidant enzymes, catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) were measured in liver and gills. The macrophagic phagocytosis and antibacterial lysozyme activity of anterior kidney were the immune parameters examined. Increases in gill GST activity and hepatic GR activity were observed after the exposure to 25 and 100% OWAF, respectively. GPX, CAT and immune responses were not affected by the experimental treatments. Our results demonstrated that among the antioxidant enzymes tested, GR and GST activities were the most sensible to the acute treatment with fuel oil, indicating changes in the redox cellular state that may result in oxidative stress in *T. maculosa*.

Key words: antioxidant enzymes, immunological responses, fuel oil, *T. maculosa*.

RESUMO

Efeitos do óleo combustível N. 6 sobre as enzimas antioxidantes e respostas imunológicas em peixe *Thalassophryne maculosa* (Pisces: Batrochoididae)

Com o objetivo de investigar os efeitos agudos de duas concentrações (25% e 100%) de fração óleo-água (OWAF) de óleo combustível N. 6 em atividades de enzima antioxidante e as respostas imunológicas, foram coletados peixes *Thalassophryne maculosa* da Lagoa de La Restiga, Ilha de Margarita, Venezuela. As enzimas antioxidantes, catalase (CAT), glutationa peroxidase (GPX), glutationa redutase (GR) e glutationa-S-transferase (GST) foram medidas no fígado e nas brânquias. A atividade de fagocitose macrofágica e antibacteriana dos lisossomos foram as respostas imunológicas examinadas. Os aumentos na atividade da brânquia GST e na atividade hepática de GR foram observados após a exposição a 25% e a 100% de OWAF, respectivamente. GPX, CAT e as respostas imunológicas não foram afetadas pelos tratamentos. Os resultados demonstraram que entre as enzimas antioxidantes testadas, as atividades de GR e GST foram as mais sensíveis ao tratamento agudo com o óleo combustível, indicando mudança no redox celular, que pode resultar no estresse oxidativo em *T. maculosa*..

Key words: enzimas antioxidantes, respostas imunológicas, óleo combustível, *T. maculosa*.

INTRODUCTION

Aquatic life is currently being exposed to chemical contamination by increasing variety of anthropogenic activities that can induce many different mechanism of toxicity, each contributing to varying degrees of deleterious effects (Correia *et al*., 2003). Consequently, in environmental disturbance assessment, the integration of chemical data with biological responses (so-called biomarkers) is strongly recommended in order to assess effects of pollutants on the organisms (Besten *et al*., 1998; Cajaraville *et al*., 2000).

Many studies showed that oxidative stress may be a common pathway of toxicity, induced by several contaminants, often through metabolic activation, rendering organisms susceptible to environmental stressors (Viarengo *et al*., 1991; Di Giulio *et al*., 1995; Solé *et al*., 1995a; Livingstone *et al*., 2000; Shaw *et al*., 2004). Among the chemicals that may cause a rise in the production of reactive oxygen species (ROS) are

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quinones, bipyridyls, aromatic nitrocompounds, hydroxylamines and heavy metal which can react and damage cellular macromolecules (Stephensen *et al.*, 2002). A key mechanism by which these compounds enhance cellular production oxyradical is referred to as redox cycling (Di Giulio *et al*., 1995). To minimize oxidative damage to cellular components produced by xenobiotics, organisms have developed antioxidant enzymatic defense system: superoxide dismutase (SOD, EC 1.15.1.1), converts superoxide anion to hydrogen peroxide, which is subsequently detoxified by catalase (CAT, EC 1.11.1.6) or glutathione peroxidase (GPX, EC 1.11.1.9). Glutathione-S-transferase (GST, EC 2.5.1.18) catalyze conjugation reaction with reduced glutathione (GSH) being the most important phase II enzymes and, glutathione reductase (GR, EC 1.6.4.2) contribute to the maintenance of cellular redox state (Cavaletto *et al*., 2002).

Recent studies for toxicological assessments have also considered the immune system as a target of toxicant insult after exposure to some chemical compounds. The immune defense mechanisms are important for studying toxic effects of chemical exposure which may result increased host susceptibility to disease (Anderson & Zeeman, 1995). The immunity of aquatic organisms could be adversely affected by development of oxidative stress associated with xenobiotic pollution, and hence decrease the fitness of natural populations. In this sense, the potential use of antioxidant enzymes and immune responses as biochemical markers to assess early detrimental effects of chemical contaminants in many different aquatic species have been published (Winston & Di Giulio, 1991; Anderson & Zeeman, 1995; Marcano *et al.*, 1997; Regoli *et al.*, 1998; Canesi *et al.*, 1999; Livingstone *et al.*, 2000; Regoli *et al.*, 2000; Nusetti *et al.*, 2001; Geracitano *et al.*, 2002; Manduzio *et al.*, 2004). However, few investigations have been direct toward assessment of the effects of complex xenobiotic mitxure (such as petroleum derived products) on benthic biota, mainly in Caribbean species. These studies should be important in countries with intensive petroleum industrial development, such as Venezuela and others countries, due to eventually oil-spill may occur causing perturbation in the aquatic ecosystem.

The aim of present study was to evaluate antioxidant enzyme activities and immunological responses in the fish *Thalassophryne maculosa* (Pisces: Batrochoididae) after exposure to a water-accommodated fraction of N. 6 fuel oil, which is widely utilized as fuel in electricity-generated plant. The industrial products represent a source of environmental contamination, since that chemical composition include, asphaltenes, aromatic resins, aromatic polycyclic hydrocarbons and heavy metals (AMOP, 2000). *T. maculosa* is a benthic fish with wide distribution in the Caribbean regions that live associate with sediments give a reference and therefore this organism is an appropriate biological indicator for assessment of environmental risk.

MATERIAL AND METHODS

Fish and bioassays

Adult specimens of *T. maculosa*, immature sexually were collected from La Restinga Lagoon, Margarita Island, in eastern Venezuela. The fishes were maintained indoors in aerated aquaria at 24 ± 1°C, containing seawater (36‰, pH 7.8) from the collection site for 15 days prior the experimental bioassays. Two groups of 10 organisms (two replicate) were exposed at 25 or 100% of OWAF during 7 days. The oil mixture (1 g/L) was prepared by adding 1 g of fuel oil to 1 L volumes of Millipore (0.45 μm) filtered seawater, mixing with constant stirring for 5 min in a closed glass vessel. After standing for 20 min, the aqueous layer was drawn off (100%), and further diluted to 25 percent in filtered seawater. The exposure system was renewed every 3 days to minimize accumulation of waste products during the exposure period.

Enzymatic assays

After exposure period, the fishes were immediately sampled; the gills and hepatic tissue were rapidly frozen in liquid nitrogen, and then stored at –70ºC for one week prior to the enzymatic assays. Groups of eight organisms each one; control and experimental, were used for the enzymatic assays. The tissues were homogenized in (1:4) 20 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, 1 mM DTT, 0.5 M sucrose, 0.15 M KCl, and 0.2 mM PMSF. The homogenates were centrifuged at 5,000 and 12,000xg at 4ºC for 20 min to obtain the supernatant samples for the enzyme assays.

Antioxidant enzymes activities were measured by spectrophotometry at 25ºC following the protocol described in Nusetti *et al*. (2001). The incubation mixtures provided optimal conditions for measuring reaction rates proportional to the quantity of enzyme extracts. GPX activity was recorded at 340 nm with 0.19 mM NADPH (extinction coefficient: 6.2 mM^{-1}. cm^{-1}), 3.4 mM reduced glutathione, 0.017 $U.ml^{-1}$ glutathione reductase, 17 mM H_2O_2, and 40 mM sodium azide in 100 mM potassium phosphate buffer, pH 7.5. GR activity was measured at 340 nm with 0.14 mM NADPH and 3.4 mM oxidized glutathione (GSSG) in 100 mM potassium phosphate buffer, pH 7.5. GST activity was assayed at 340 nm with 0.5 mM 1-chloro-2,4-dinitrobenzene (extinction coefficient: 9.6 $mM^{-1}.cm^{-1}$) and 2 mM GSH in 100 mM potassium phosphate buffer, pH 6.5. CAT activity was determined at 240 nm with 0.6 M H_2O_2 (extinction coefficient: 40 $M^{-1}.cm^{-1}$) in 50 mM sodium phosphate buffer, pH 7.0. The total enzyme activity was expressed in terms of units (μmoles of substrate converted to product min^{-1}).g^{-1} of wet tissue.

Lysozyme activity

The lysozyme activity was determined by the method of Osserman & Lawlor (1986). Aliquots of 40 μl of the extract from anterior kidney were dispensed into 5-mm diameter wells

in 1% agarose in 5-cm diameter Petri dishes, containing 67 mM phosphate buffer (pH 6.2) and *Micrococcus lysodeikticus* (0.6 mg freeze-dried cell.ml^{-1}) as substrate. After incubation for 48 h at 24°C, the diameters of the bacterial lyses zones were measured and the lysozyme concentration was determined by reference to a calibration curve with hen egg-white lysozyme (5 mg.ml^{-1} in 100 mM phosphate buffer pH 6.2; HEL, Sigma Chemical Co.). The results were presented as HEL-equivalent (µg.ml^{-1}) activity, calculated by the following regression model: HEL-equivalent (µg.ml^{-1}) = antilog$_{10}$ {a + b·(diameter, mm)}.

Macrophage collection

The fishes were intraperitoneal injected with 2 ml of sterile seawater. The abdominal region was massaged for 5 min and then, macrophages were extracted using a heparin impregned-5 ml syringe (20 gauge). Then the samples were centrifuged in eppendorf tubes at 200xg for 5 min at room temperature. The pellets were resuspended in 100 µl of sterilized seawater containing 5 mM EDTA, pH 7.8, followed by centrifugation, this process was repeated several times for cellular washing. The viability and the total number of cells were measured based on the trypan blue (0.4% in sterile seawater) coloration, prior to phagocytosis test.

Phagocytosis

Heat inactivated commercial yeasts was used as antigen for the phagocytes. The phagocytic activity was measured in an incubation of suspension of 100 µl peritoneal cells (1×10^6 cells. ml^{-1}) and 100 µl of yeasts (5×10^6 cells.ml^{-1}) for 24 hours at 4ºC. Afterwards, the preparations were centrifuged at 900xg for 5 minutes. To quantify the phagocytic activity, an aliquot of 100 µl of the cell suspension was mixed with 100 µl of 0.4% crystal violet solution and 100 cells were count in a hemocytometer chamber to a magnification of the 400X in a microscope. The cells that showed at least a yeast cell clearly visible in the cytoplasm were scored as phagocytics.

Statistical analyses

Statistic differences between experimental and control groups were examined using a one-way ANOVA. Significant differences among pairs of groups were tested by the Least Significant Differences (Zar, 1984) with significance level of 5% ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Following 7 days of exposure to 25 and 100% N. 6 fuel oil-OWAF, a significant increase ($p < 0.001$) in gill GST and hepatic GR activities was observed, whereas GPX and CAT were not altered with the experimental treatments (Tables 1, 2). The fuel oil-induced increase in GR and GST activities may be related with the induction of an adaptive response of GSH dependent reactions, mediating the phase II of xenobiotic metabolism and antioxidant defense pathway.

The role of GR is to maintain the cytosolic level of GSH involved in a variety of detoxification reactions, to assure the maintenance of a balance between production and removal of endogenous ROS and other pro-oxidants. This pro-oxidant/antioxidant balance and detoxification of potentially damaging ROS is crucial for cellular homeostasis (Di Giulio *et al*., 1995; Livingstone, 2001; Correia *et al*., 2003). GSH acts directly as a free radical quencher and as an antioxidant enzyme cosubstrate. GST catalyze conjugation reaction of GSH with xenobiotic metabolites derived of phase I reactions (NADPH cytochrome P450 reductase), transforming them into water soluble and easily excretable products. GST also functions as an antioxidant enzyme by conjugating breakdown products of lipid peroxides to GSH (Di Giulio *et al*., 1995; Stephensen *et al*., 2002).

Table 1 — Antioxidant enzyme activities (U/g wt mass) in liver of *Thalassophryne maculosa*, after 7-days exposure to 25 and 100% of OWSF. Values are presented as means ± S.D (n = 8).

Enzymes	Controls	25%	100%
GST	7.78 ± 3,23	12.26 ± 5,37^{NS}	10.43 ± 3.94^{NS}
GPX	21.47 ± 5.49	17.45 ± 2.91^{NS}	18.84 ± 4.02^{NS}
GR	0.86 ± 0.20	0.90 ± 0.27^{NS}	1.29 ± 0.27*
CAT	17.85 ± 4.04	26.50 ± 26.50^{NS}	23.91 ± 14.79^{NS}

* $p < 0.05$; NS: $p > 0.05$ respect control group.

Table 2 — Antioxidant enzyme activities (U/g wt mass) in gills of *Thalassophryne maculosa*, after 7-days exposure to 25 and 100% of OWSF. Values are presented as means ± S.D (n = 8).

Enzymes	Controls	25%	100%
GST	4.91 ± 0.67	$16.94 \pm 3.07^{***}$	5.72 ± 2.13^{NS}
GPX	32.95 ± 2.07	28.55 ± 3.53^{NS}	31.64 ± 2.29^{NS}
GR	1.38 ± 0.33	1.42 ± 0.20^{NS}	1.26 ± 0.29^{NS}
CAT	0.39 ± 0.29	0.35 ± 0.12^{NS}	0.34 ± 0.10^{NS}

***$p < 0.001$; NS: $p > 0.05$ respect control group.

Table 3 — Immunological parameter in the fish *Thalassophryne maculosa*, after 7-days exposure to 25 and 100% of OWSF. Values are presented as means ± S.D (n = 8).

Parameters	Controls	25%	100%
Viability (%)	92.4 ± 2.9	91.8 ± 2.38^{NS}	92.6 ± 4.09^{NS}
Cellular count (cell/ml)	$2.2 \times 10^6 \pm 1.0 \times 10^6$	$1.3 \times 10^6 \pm 1.6 \times 10^{5\,NS}$	$1.1 \times 10^6 \pm 2.8 \times 10^{5\,NS}$
Phagocytosis (%)	14.0 ± 3.4	10.6 ± 2.4^{NS}	12.4 ± 2.30^{NS}
Lysozyme (μg/ml)	12.8 ± 4.4	8.25 ± 2.82^{NS}	8.77 ± 2.15^{NS}

*** $p < 0.001$; NS: $p > 0.05$ respect control group.

Several studies on the antioxidant enzyme responses to xenobiotics that enhance oxyradical flux, suggested that ROS increases can induce antioxidant enzymes, reflecting an adaptational and protective responses against oxidative stress. Nevertheless, individual and inter specific differences in the induction response have been reported in fishes (Winston & Di Giulio, 1991; Stephensen *et al*., 2002). Our finding agrees with the induced GR and GST activities in rainbow trout treated with different organic toxicants (Petrivalsky *et al*., 1997; Machala *et al*., 1997; Stephensen *et al*., 2002). Channel catfish exposed to contaminant effluents, showed hepatic CAT activities with dose-and time-dependent increases, but no other index of oxidative stress (malonaldehyde or methemoglobin) displayed consistent responses (Winston & Di Giulio, 1991). Moreover, Steadman *et al*. (1991) reported a dose and time dependence of xenobiotic metabolism in rainbow trout exposed to No. 2 fuel oil. Following 3 days of exposure of adult rainbow trout to sublethal level of the fuel oil observed a 25 to 50 percent depletion of glutathione. By 7-day exposure, cellular glutathione concentrations exceeded those of control by 50 to 100 percent which persisted throughout the exposure period (14 days). Increased hepatic of GSH have also been reported in cadmium-and fuel oil-exposed striped mullet (*Mugil cephalus*) (Thomas *et al*., 1982; Thomas & Wofford, 1984).

In contrast to the antioxidant enzyme results, the immunological responses were not significantly ($p > 0.05$) affected by the acute OWAF exposure (Table 3), suggesting that the phagocytic function of macrophages and antibacterial activity of lysozyme of anterior kidney were not adversely altered. Probably the immune system in *T. maculosa* is less susceptible to toxicant compounds that GSH dependent antioxidant enzymes.

In conclusion, our results indicate that among the antioxidant enzymes tested, GR and GST activities were the most sensible to the acute treatment with N. 6 fuel oil, indicating that may be useful for detecting changes in the redox cellular status that may result in oxidative stress in *T. maculosa*. These finding should be considered as a contribution for further research on toxicological risk of petroleum contamination to marine benthic fishes.

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