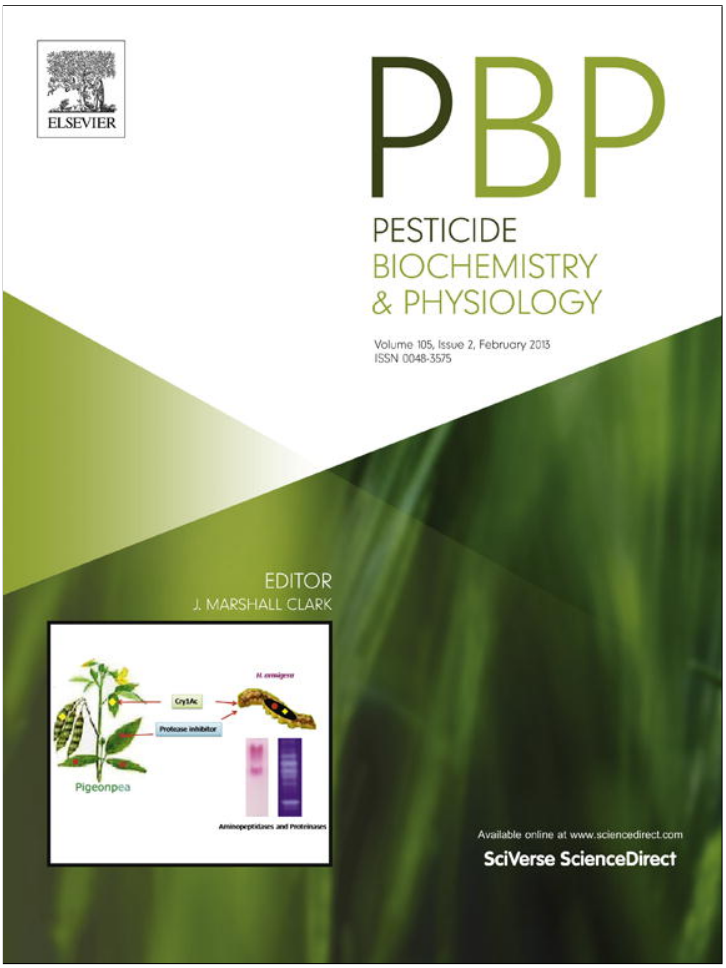

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Oxidative stress and genotoxicity in the South American cichlid, *Australoheros facetus*, after short-term sublethal exposure to endosulfan

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ABSTRACT

Short-term responses at the antioxidant enzymatic systems, together with genotoxic effects were studied in the freshwater fish *Australoheros facetus*, exposed to endosulfan (ES) (0.02, 0.5, 5, 10 μg/L) for 24 h. Brain was the most responsive organ, showing inhibition of the enzymatic systems together with an increase of hydrogen peroxide (H_2O_2) content. Concentration-dependent inhibition was observed for superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) with IC_{25} values of 0.012, 0.017, 0.018 μg/L, respectively. In liver, a similar behavior was observed for SOD with IC_{25} values of 2.22 μg/L. In addition, increased thiobarbituric acid reactive substances (TBARs) at 5 μg/L and H_2O_2 at 5 and 10 μg/L were observed. No effects were evidenced on ethoxyresorufin O-deethylase (EROD), glutathione-S-transferase (GST), GR and CAT activities. In gills, only H_2O_2 decreased at 0.5 and 5 μg/L ES. Genotoxic effects were detected by the increase of the frequency of both, nuclear abnormalities (NA) at 0.02 μg/L and micronucleus (MN) at 5 μg/L. Environmentally realistic concentrations of ES exerted toxic responses in *A. facetus*, encouraging the further field validation of the observed pattern (tissue specificity, sensitiveness and concentration-response relationship) as a potential suit of biomarkers for assessing acute sublethal effects in *A. facetus* under short-term pulsed exposure to ES.

1. Introduction

The Cichlidae is one of the richest families of Perciformes in freshwater worldwide [1]. However, the number of ecotoxicological studies on this group of fishes is comparatively poor. In particular, *Australoheros facetus* is a native cichlid from Argentina, Brazil, Uruguay and Paraguay, representative of freshwater ecosystems [2]. This species is easy to rear and breed under laboratory conditions and has demonstrated to be suitable for ecotoxicological studies [3]. In particular, *A. facetus* inhabit vegetated pond and streams of the Pampas region, the main agriculture district of Argentina.

Previously studies showed that small ponds and streams are particularly susceptible to be impacted by agrochemicals [4]. These compounds reach the aquatic ecosystems by drift or run off, showing short-term concentrations pulses in surface water and sediment after spraying and rain events that take place during the crop season [5]. Aquatic biota, including *A. facetus*, is then periodically exposed under this kind of scenario and little is known on the specific responses triggered in this species under this particular sceneries.

Among agrochemicals, endosulfan (ES) is an organochlorine insecticide widely used for pest control in agriculture [6]. In Argentina, this pesticide is broadly used and it is able to reach the aquatic ecosystem [7–9]. Despite it has been added to the Convention of Stockholm since 2011 [10], several countries including Argentina extended its use for additional times [11]. It has been reported that the run-off from fields treated with this insecticide can contain high concentrations of ES, ranging from 0.004 to over 2 μg/L in Argentina [12,9,13], and reaching values higher than 100 μg/L another countries [14,15]. Endosulfan is highly toxic and potentially bioaccumulative for fish [16,17]. In particular, the reported 24 h LC50 for the cichlid *Cichlasoma dimerus* was 13.6 μg/L [18]. Potential toxic effects of ES on the cichlid African tilapia have been evaluated on the reproductive behavior [19], thyroid hormones metabolism and EROD activity in relation to liver histology [20,21], antioxidant and AChE enzymatic activity [22], respiratory

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behavior [23], and hematological and immunological parameters [24–26]. Recently, some studies have been also reported on the South American species *C. dimerus*, assessing effects on reproductive endocrine system [27], biochemical disruption, histological alterations [28,29,18]. Endosulfan induced genotoxicity and oxidative stress in fish, and its effects on various antioxidants have been reported in recent studies [28,30,31].

The understanding of the toxic response at sub-individual level (molecular, biochemical, physiological, histological) is useful for identifying early "warning signals" that can be used as biomarkers of exposure or effect to assess pollution-induced stress before environmental "damage" become irreversible [32].

In an environmental context, they offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed among tissues, and are eliciting a toxic effect at critical targets. In the present work, early responses at the detoxifying and antioxidant enzymatic systems, in relation to oxidative stress and genotoxic effects, were studied in the South American Cichlid, *A. facetus*, in order to identify toxic effects induced by ES after short-term exposure scenarios to environmentally realistic sublethal concentrations and define a suit of biological responses that could be valuable as early warning signals.

2. Materials and methods

2.1. Fish exposure

Adult fish were collected in non-anthropized freshwater bodies around Mar del Plata city (Buenos Aires Province, Argentina, 37° 53′ South, 57° 59′ West) and acclimatized for 2 months to laboratory conditions in 140 L tanks. Healthy specimens with approximately the same size (mean total length (±SD): 10.1 ± 1.4 cm; mean weight (±SD): 24.1 ± 11.4 g) were selected.

Experiment was set up using a single-factor fixed-effect model. The factor ES was tested using four exposure levels: 0.02, 0.5, 5 and 10 μg /L ES during an exposure time of 24 h. A negative control (Co-) in tap water with DMSO at 0.004% (same concentration of DMSO used in ES treatments) was included. In addition, one positive mutagenic control group (Co+) was added exposing fish for 24 h to 10 mg/L of methyl methanesulfonate (MMS, CAS 66-27-3) in tap water. ES concentrations were established taking into account reported values for Argentina and other parts of the world [14,9] as well as the maximum permitted quantities (MPQ) for protection of aquatic life in superficial freshwater of 0.02 μg/L [33,34].

Considering that commercial formulations of endosulfan (6, 7, 8, 9, 10, 10 – hexachlor - 1, 5, 5, 6, 9, 9- hexahydro - 6,9 – metane - 2,4,3 –benzo (e) dioxatiepin -3–oxide) consist in a mixture of α- and β- isomers (70:30), we used this mixture throughout the experiment. Thus, we prepared two stock solutions by diluting α- endosulfan (Riedel-de Haën, CAS 959-98-8) (0.04 g in 25 mL in dimethyl sulfoxide- DMSO, Mallinckrodt) and β-endosulfan (Riedel-de Haën, CAS 33213-65-9) (0.04 g in 50 mL in dimethyl sulfoxide-DMSO, Mallinckrodt). Afterwards, we prepared the appropriate exposure medium by diluting different amounts of both α- and - β endosulfan (70:30) in tap water.

The experiments were conducted in glass tanks containing six fish ($n = 6$ per treatment) in tap water. The experimental room was illuminated with fluorescent lamps with 12:12 light: dark periods. Mar del Plata city tap water was used for the experiments. Experiments were carried out under the following conditions: temperature 18 °C, pH 8.2 ± 0.2, mean total hardness 270.2 mg/L $CaCO_3$ and mean alkalinity of 160 mg/L $CaCO_3$. In addition, four glass tanks containing the ES solutions plus the negative control, but without fish were used to evaluate the chemical stability of ES in the exposure media. Water samples were collected from both tanks with and without fish, after 30 min and 24 h using 250 mL pre-cleaned glass bottle with Teflon lined caps. Samples were stored at 4 °C temperature until ES analyses. Laboratory glassware used during sampling and analysis were washed with appropriate solvents to avoid interferences in the chromatographic analysis. All experiments were carried out simultaneously.

All fish were sacrificed after an exposure period of 24 h through by transecting the spinal cord, using a fresh razor blade. Liver, brain and gills were dissected, weighed, immediately frozen using liquid nitrogen, and stored at −80 °C until analysis. For the different determinations each organ was analyzed as an independent sample. The blood of each animal was obtained through heart puncture with heparinized tips; and peripheral blood smears, two per fish, were immediately made by applying a drop of blood on clean slides, fixed in absolute methanol for 15 min, and air dried.

2.2. Measurement of exposure concentrations of endosulfan

Water extraction: The extraction and clean-up was according to Gonzalez et al., 2012. Thus, 100 mL of water was taken in amber-glass bottle and spiked with 20 ng of PCB#103 as internal standard. Liquid–liquid extraction was done by adding 60 mL of a mixture hexane:dichloromethane (1:2). Endosulfan isomers (α- and β-) and its metabolite endosulfan sulfate were identified and quantified by Gas Chromatography (Shimadzu 17-A gas equipped with a ^{63}Ni Electron Capture Detector) (GC-ECD), according to Miglioranza et al. [35]. Laboratory and instrumental blanks were analyzed to ensure the absence of contaminants, or interference arising from samples or laboratory handling. Recoveries, calculated from a spiked matrix, were greater than 90%. Detection limits (LOD), ranged between 0.08–0.1 ng/mL in agreement with values reported in the literature [36].

2.3. Extract preparation and measurement of enzyme activities

Ethoxyresorufin *O*-deethylase (EROD) activity in liver was determined as described by Scholz and Segner [37]. The amount of produced resorufin was determined at 25 °C using special microliter plates (Microfluor®) in a fluorimeter (Synergy HT, BioTek) at an excitation wavelength of 530 nm and at the emission wavelength of 590 nm. Each sample was analyzed in triplicate.

The extraction of antioxidant cytosolic enzymes in liver, gills and brain was done according to the method described by Wiegand et al. [38], with modifications of Cazenave et al. [39]. Enzymatic activities were determined by spectrophotometry. The activity of the soluble (cytosolic) glutathione-S-transferase (GST) was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate, according to Habig et al. [40]. Glutathione reductase activity (GR) was assayed according to Tanaka et al. [41], CAT activity according to Claiborne [42] and SOD was assessed by the inhibition of nitro blue tetrazolium (NBT) reduction [43].

The total protein content for each sample was assessed spectrophotometrically by means of the method of Bradford [44], using bovine serum albumin solution as standard.

Enzymatic activities are reported in nano katals per milligram of protein (nkat/mg prot), where 1 katal correspond to the conversion of 1 mol of substrate per second. Each enzymatic assay was carried out by triplicate.

2.4. Non-enzymatic parameters

Hydrogen peroxide content (H_2O_2) was quantified in liver, gills and brain according to Bellincampi et al. [45]. The content of H_2O_2 was calculated based on a standard curve. Lipid peroxidation (LPO) was determined in liver and gills by measuring the formation of thiobarbituric acid reactive substances (TBARs), according to the procedures of Oakes and Van Der Kraak [46]. The supernatant

containing was measured spectrophotometrically (λ_{abs}: 532 nm). The content of TBARs was expressed as nanomoles per mg of fresh tissue using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Brain tissues were not analyzed for TBARs because the sample was not enough to prepare the extract.

2.5. Genotoxicity parameters

Twenty-four hours after the fixation the material was stained with 15% Giemsa solution for 10 min. Two thousand erythrocytes, 1000 per slide, were analyzed from each animal under 1000 X magnification to determine the micronuclei and other nuclear abnormalities frequency. Coded and randomized slides were scored using a blind review by a single observer. Only cells with intact cell and nuclear membranes were scored. The micronuclei (MN) were always considered separately from the other nuclear abnormalities (NA) following Carrasco et al. [47] and both were expressed as number per 1000 cells (‰). Four types of abnormalities were analyzed: (i) MN were only non-refractory particles, with the same colour as the nucleus cell and with round or ovoid shape, (ii) "*blebbed nuclei*" presents a relatively small evagination of the nuclear envelope, which seemed to contain euchromatin, (iii) "*lobed nuclei*" presenting evaginations larger that the blebbed nuclei and (iv) "*notched nucleus*" with an appreciable depth into a nucleus that not contain nuclear material.

2.6. Calculations and statistics

Normality and homogeneity of variances were verified by Shapiro–Wilk and Levene tests, respectively. For most of the studied parameters, a one-way ANOVA test was applied followed by the post hoc LSD test. The non-parametric Kruskal–Wallis or Mann–Whitney U tests were apply in those cases the assumption of homogeneity of variance was not meet [48]. Differences in all tests were considered significant at $p < 0.05$.

The inhibitory concentration 25 or 50: IC_{25} and IC_{50} were estimated using a 4-parameter logistic mathematical model (4PL) [49]. The equation of the 4PL was: $F(Y) = ((a-d)/(1+((X/c)^b))) + d$, where "Y" is the response and "X" is the concentration. The lower asymptote is "a", the bottom of the curve or lower plateau (commonly referred to as the min); and the upper asymptote is "d", the top of the curve or upper plateau (commonly referred to as the max). The steepness of the linear portion of the curve is described by the slope factor, "b". The parameter "c" is the concentration corresponding to the response midway between "a" and "d" (relative IC50 value). The modification $d = 100/(1 + d')$ was introduced to the model in order to obtain inhibition values between 0 and 100%. The parameters a, b, c, and d were estimated fitting the 4PL to the experimental data by nonlinear regression. Inverse regression was then used to estimate the absolute ICx values.

3. Results

3.1. Measurement of exposure concentrations of endosulfan

The nominal and measured concentrations of ES in each exposure tank are showed in Table 1. In tanks without fish, water concentrations of α- and β- endosulfan after 30 min of exposure were between 75 and 100% of the nominal concentrations. After 24 h 4–12% of the nominal concentrations were found at higher exposure levels. At the lowest concentration (0.02 μg/L) there were no changes in the levels of ES measured at 30 min and 24 h. (Table 1). Levels of endosulfan sulfate were below the detection limit (LOD) in all water samples. On the other hand, in tanks containing fish, a similar trend was observed. Thus, at 30 min of exposure the measured concentration of α- and β- endosulfan were between 34 and 60% of the nominal concentrations. At 24 h of exposure there was a drop of the ES levels to 2–14% of the nominal concentrations. At the higher concentrations (5 and 10 μg/L) endosulfan sulfate was detected at 30 min as well as at 24 h of exposure.

3.2. Biochemical responses

3.2.1. Liver

The phase I enzyme EROD was not significantly affected by the short term sublethal exposure to ES (Table 2). However, the enzyme of the antioxidant system, SOD, was significantly inhibited in this organ (Table 2) following a clear concentration–response relationship (Fig. 1). Maximum inhibition was observed at the highest exposure concentration (10 μg/L), showing 60% of the SOD activity observed in the negative control. Experimental data satisfactorily fitted to the four-parameter logistic model. The estimated parameters together with the IC_{25} and IC_{50} values are shown in (Table 3). On the other hand, the other assessed enzymes of the

Table 1
Nominal and measured concentrations (μg/L) of endosulfan (ES) in the experimental solutions after 30 min and 24 h.

Nominal concentration		Measured concentration			
		Without fish		With fish	
		30 min	24 h	30 min	24 h
0.02	α-ES	0.021 ± 0.005	0.0214 ± 0.004	0.010 ± 0.001	0.012 ± 0.003
	β-ES	0.007 ± 0.003	0.006 ± 0.003	0.002 ± 0.001	0.004 ± 0.001
	ES sulfate	<LOD	<LOD	<LOD	<LOD
	Total ES	0.027 ± 0.002	0.027 ± 0.001	0.015 ± 0.004	0.012 ± 0.002
0.50	α-ES	0.303 ± 0.065	0.019 ± 0.003	0.168 ± 0.118	0.054 ± 0.017
	β-ES	0.073 ± 0.031	0.003 ± 0.001	0.048 ± 0.040	0.015 ± 0.007
	ES sulfate	<LOD	<LOD	<LOD	<LOD
	Total ES	0.376 ± 0.096	0.022 ± 0.003	0.216 ± 0.158	0.069 ± 0.024
5.00	α-ES	4.630 ± 0.192	0.591 ± 0.027	1.663 ± 0.077	0.189 ± 0.031
	β-ES	0.635 ± 0.695	0.043 ± 0.016	0.431 ± 0.032	0.035 ± 0.007
	ES sulfate	<LOD	<LOD	0.001 ± 0.000	0.035 ± 0.004
	Total ES	5.265 ± 0.503	0.634 ± 0.044	2.095 ± 0.109	0.259 ± 0.036
10.00	α-ES	9.832 ± 0.330	1.373 ± 0.421	2.864 ± 0.749	0.181 ± 0.066
	β-ES	3.162 ± 0.194	0.154 ± 0.046	0.529 ± 0.366	0.026 ± 0.026
	ES sulfate	<LOD	<LOD	0.004 ± 0.003	0.036 ± 0.035
	Total ES	12.994 ± 0.137	1.528 ± 0.467	3.397 ± 1.105	0.243 ± 0.127

Data are expressed as mean ± 1 standard deviation, $n = 3$. Means and standard deviation are calculated from triplicates. <LOD: below detection limit.

Table 2
Enzyme activities (nkat/mg protein), hydrogen peroxide (mmol/mg FW) and thiobarbituric acid reactive substances (nmol/mg FW) contents in liver of *Australoheros facetus* exposed to endosulfan (ES).

Treatments	EROD	GST	GR	SOD	CAT	H_2O_2	TBARs
Control	0.99 ± 0.45 A	6.50 ± 0.30 A	0.27 ± 0.06 A	659.00 ± 128.59 A	2152.96 ± 67.64A	0.94 ± 0.04A	0.10 ± 0.03A
0.02 μg/L ES	0.71 ± 0.29 A	5.65 ± 0.66 A	0.25 ± 0.07 A	609.53 ± 66.81 A	1945.75 ± 54.55A	0.99 ± 0.05A	0.09 ± 0.02A
0.50 μg/L ES	0.43 ± 0.19 A	5.35 ± 0.99 A	0.25 ± 0.04 A	572.50 ± 78.27 A	1910.67 ± 298.06A	1.79 ± 0.03B	0.13 ± 0.05A
5.00 μg/L ES	1.08 ± 0.68 A	7.37 ± 1.51 A	0.26 ± 0.03 A	483.83 ± 97.54 B	2162.05 ± 81.10A	1.99 ± 0.07B	0.2 ± 0.04B
10.00 μg/L ES	0.70 ± 0.34 A	6.56 ± 0.92 A	0.31 ± 0.08 A	397.55 ± 46.25 B	1917.94 ± 47.53A	2.16 ± 0.07B	0.15 ± 0.04A

Values are expressed as mean ± 1 standard deviation. Significant differences from the controls indicated by different letters ($p < 0.05$). EROD: Ethoxyresorufin *O*-deethylase, GST: glutathione-S-transferases, GR: glutathione reductase, SOD: superoxide dismutase, CAT: catalase, H_2O_2: hydrogen peroxide, TBARs: thiobarbituric acid reactive substances.

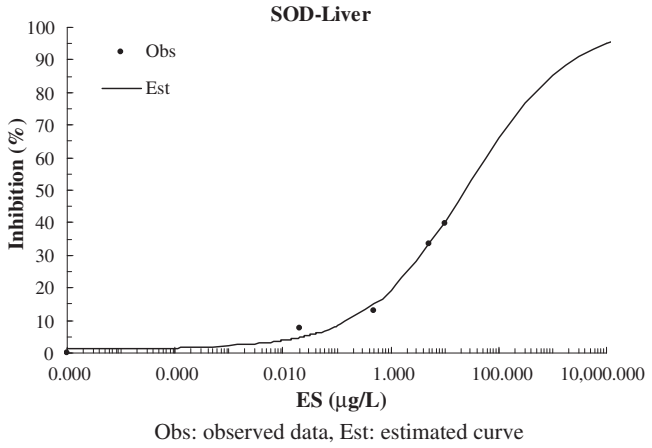


Fig. 1. Inhibition curve of superoxide dismutase (SOD) in liver of *Australoheros facetus* exposed to endosulfan (ES).

Table 3
Parameters estimated from 4PL model for the IC_{25} and IC_{50} in liver and brain of *Australoheros facetus* exposed to endosulfan (ES).

Parameter estimates	SOD Liver	SOD Brain	CAT Brain	GR Brain
a	1.230	0.000	0.000	0.000
b	0.460	2.805	4.343	4.203
c	26,983	0.0124	0.0165	0.0172
d'	0.000	0.841	1.136	1.217
d	100.0	54.3	46.8	45.1
r^2	0.994	0.998	0.990	0.951
IC25 (μg/L)	2.22	0.0117	0.0171	0.0181
IC50 (μg/L)	25.6	0.03	NC	NC

SOD: superoxide dismutase, CAT: catalase, GR: glutathione reductase, NC: non calculated. IC25: inhibitory concentration 25. IC50: inhibitory concentration 50.

antioxidant system, CAT, GR and GST were not significantly affected by the exposure, but the levels of H_2O_2 were significantly increased at 0.5, 5 and 10 μg/L ES (Table 2). TBARs levels were only significantly increased at 5 μg/L ES. A concentration-dependent increase of H_2O_2 levels was observed, reaching a value of 129% above of the control at the highest ES concentration. In a different way, maximum concentrations of TBARs were observed in fish at 5 μg/L and then decreased in fish exposed to 10 μg/L with values 95 and 51% above of the control group, respectively.

3.2.2. Brain

A generalized inhibition of the enzymatic activity was observed in the brain, except for GST values that were not significantly different than in the control group (Table 4). In particular, the reduction of the activity was statistically significant for the studied enzymes of the antioxidant system: SOD, GR and CAT. For all of them a marked reduction of the activity from the lowest ES concentration tested was observed. Experimental data of the percentage of inhibition was satisfactorily fitted to the four-parameter concentration-response logistic model, showing for the three enzymes maximum inhibition values around 50% (Fig. 2). The estimated parameters of the model together with the IC_{25} (for SOD, GR and CAT) and IC_{50} (only for SOD) are shown in Table 3. A very steeply slope was estimated for the three enzymes changing from 1 to 99% of the maximum inhibition in less than 0.05 μg/L ES span. Accordingly with the IC values, the responsiveness order of the studied enzymes to ES exposure was: SOD > GR > CAT. The opposite pattern was observed for H_2O_2. For this parameter, the levels were increased from controls to 0.5 μg/L and then reached a plateau with values around 70–100% higher than those in controls (Table 4).

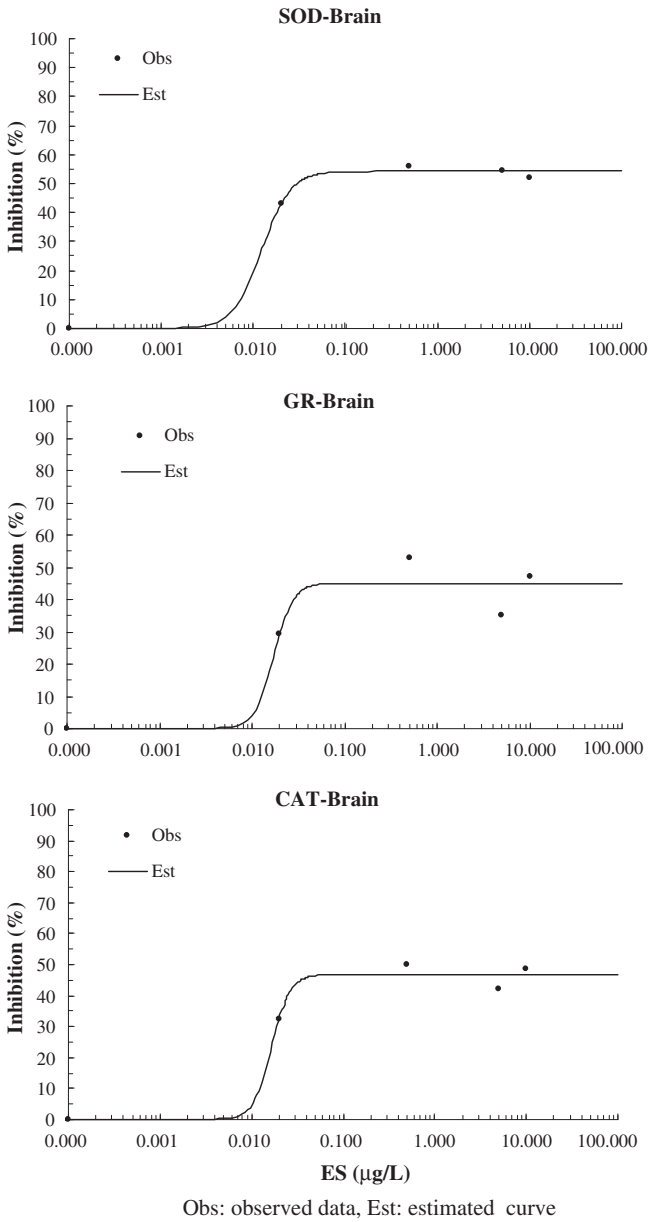
3.2.3. Gills

In the gill, no significant effects on the studied enzymes (GST, SOD, CAT and GR) were induced by the short-term exposure to sublethal concentrations of ES (Table 5). On the other hand, a significant response was detected in the H_2O_2 levels, though the response did not show a classical concentration-response pattern (Table 5). Concentrations were significantly reduced in fish exposed to 0.5 and 5 μg/L, but control values were observed in fish exposed to 10 μg/L. In addition, the average concentrations of the TBARs at all ES concentrations were not statistically different with respect to controls (Table 5).

Table 4
Enzyme activities (nkat/mg protein), hydrogen peroxide (mmol/mg FW) contents in brain of *Australoheros facetus* exposed to endosulfan (ES).

Treatments	GST	GR	SOD	CAT	H_2O_2
Control	3.04 ± 0.37 A	0.34 ± 0.05 A	1377.25 ± 135.41 A	209.03 ± 86.94 A	1.07 ± 0.04 A
0.02 μg/L ES	2.88 ± 0.30 A	0.25 ± 0.05 B	783.72 ± 139.99 B	140.88 ± 55.97 B	2.13 ± 0.05 B
0.50 μg/L ES	2.63 ± 0.47 A	0.16 ± 0.07 B	604.53 ± 128.76 B	104.99 ± 51.44 B	2.16 ± 0.05 B
5.00 μg/L ES	2.90 ± 0.27 A	0.23 ± 0.07 B	625.06 ± 107.41 B	120.84 ± 41.15 B	1.74 ± 0.03 B
10.00 μg/L ES	2.69 ± 0.18 A	0.18 ± 0.06 B	658.33 ± 68.80 B	107.84 ± 23.98 B	1.79 ± 0.02 B

Values are expressed as mean ± 1 standard deviation. Significant differences from the controls indicated by different letters ($p < 0.05$). GST, glutathione-S-transferases; GR, glutathione reductase; SOD, superoxide dismutase; CAT, catalase; H_2O_2, hydrogen peroxide.



Obs: observed data, Est: estimated curve

Fig. 2. Inhibition curve of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) in brain of *Australoheros facetus* exposed to endosulfan (ES).

3.3. Genotoxicity parameters: micronucleous and other nuclear abnormalities in blood

In blood smears, the mature erythrocytes were large and oval nucleated cells. The nucleus was clearly structured and had a well-defined boundary, allowing the easy identification of micronucleus in the cytoplasm. We found four main types of nuclear abnormalities as described in Carrasco et al. [47] (Fig. 3).

The observed frequencies of MN and the other NA are shown in Table 6. In addition, significant effects were induced by ES on both MN and NA, but while a monotonous concentration-response increase of the MN was observed, a bell-shaped (Gaussian) response was observed for NA. Increased frequency of MN was significant at 5 and 10 µg/L ES compared to the negative control while the other NA resulted increased at all tested concentrations ($p < 0.05$).

4. Discussion

4.1. Concentrations of endosulfan

Results shows that there is an important drop in ES levels compared with the nominal concentrations at 30 min as well as 24 h. It is known that hydrolysis of ES is affected greatly by pH, being susceptible to rapid hydrolysis in alkaline waters [50,51]. The changes observed in the ES concentrations over the exposure period without fish, could be attributed to the alkalinity of the water used in the bioassay, which is similar to that in freshwater environments like Los Padres Lake where *A. facetus* inhabits [52]. On the other hand, in the exposure tanks (with fish) the diminished concentrations observed at 30 min with respect to the tanks without fish, could be attributed to the quick uptake of the pesticide by the organisms. This is in agreement with the rapid response observed in the biomarkers of oxidative stress and genotoxicity. The presence in water of endosulfan sulphate at 24 h could be explained by the fish metabolism and subsequent excretion, since it is recognized as the main metabolite in fish [53].

4.2. Biochemical responses

The main response observed at the biochemical level in of *A. facetus* after the short-term exposure to ES was characterized by a general inhibition of the antioxidant enzymatic system together with the increase of oxidative stress parameters either in the brain and the liver. Oxidative stress effect was characterized by the increase of H_2O_2 and TBARs. This pattern constitutes a typical case of damage where pro- oxidants are able to overcome antioxidant defenses such as, in this case, SOD activity. The depressed SOD catalytic activity may result from damage elicited to the active site of the enzyme by the overproduction of H_2O_2, as it was observed by Modesto and Martinez [54]. In addition, the generation of hydroxyl radicals through the Haber- Weiss reaction would also be favored by the excess of H_2O_2 induced by ES and then the lipid peroxidation process could be consequently enhanced, leading to the significant increase of TBARs content. Previous reports have shown that concentrations of 1–2 µg/L ES also increased TBARs levels in the liver of the freshwater fish *Prochilodus lineatus* [29] and *Jenynsia multidentata* [28]. In general terms, peroxidized membranes become rigid and lose permeability and integrity. Cumulative effects

Table 5
Enzyme activities (nkat/mg protein), hydrogen peroxide (mmol/mg FW) and thiobarbituric acid reactive substances (nmol/mg FW) contents in gills of *Australoheros facetus* exposed to endosulfan (ES).

Treatment	GST	GR	SOD	CAT	H_2O_2	TBARs
Control	2.04 ± 0.08 A	1.21 ± 0.17 A	531.69 ± 108.85 A	351.81 ± 86.27A	0.76 ± 0.03 A	0.49 ± 0.09 A
0.02 µg/L ES	2.41 ± 0.32 A	1.24 ± 0.27 A	639.14 ± 111.20 A	389.90 ± 112.73A	0.79 ± 0.03 A	0.53 ± 0.12 A
0.50 µg/L ES	2.08 ± 0.20 A	1.17 ± 0.04 A	542.53 ± 57.62 A	346.02 ± 75.52A	0.56 ± 0.07 B	0.62 ± 0.13 A
5.00 µg/L ES	2.40 ± 0.25 A	1.15 ± 0.12 A	583.83 ± 112.68 A	299.90 ± 84.71A	0.57 ± 0.07 B	0.65 ± 0.13 A
10.00 µg/L ES	2.32 ± 0.46 A	1.12 ± 0.19 A	557.92 ± 47.57 A	345.06 ± 57.04A	0.77 ± 0.04 A	0.59 ± 0.07 A

Values expressed as mean ± 1 standard deviation. Significant differences from the controls indicated by different letters ($p < 0.05$). EROD: Ethoxyresorufin *O*-deethylase, GST: glutathione-S-transferases, GR: glutathione reductase, SOD: superoxide dismutase, CAT: catalase, H_2O_2: hydrogen peroxide, TBARs: thiobarbituric acid reactive substances.

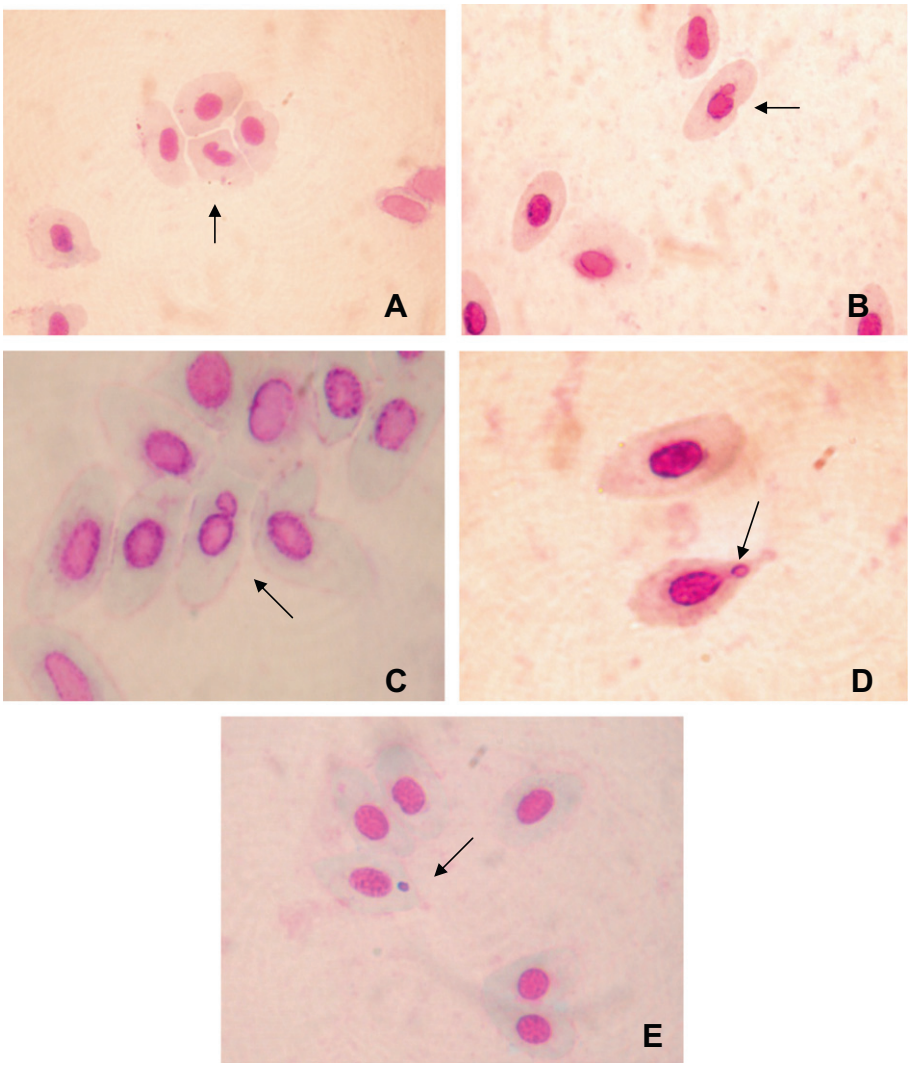


Fig. 3. Photomicrographs of peripheral erythrocytes from *Australoherus facetus* exposed with endosulfan (ES), showing A: "notched nuclei, B: "blebbed nuclei", C: "Lobed nuclei" and D, E: micronuleous. Giemsa-stained blood smear.

Table 6
Micronuclei and other nuclear abnormalities in erythrocytes of *Australoheros facetus* exposed to endosulfan (ES).

	Micronuclei (MN/ ‰)	Other Abnormalities (NA/ ‰)
Control (−)	2.70 ± 0.91 A	3.80 ± 1.40 A
Control (+)	10.90 ± 2.88 B	19.60 ± 13.96 B
0.02 μg/L ES	1.25 ± 0.69 A	24.00 ± 7.71B
0.50 μg/L ES	2.42 ± 0.86 A	102.33 ± 59.44 B
5.00 μg/L ES	6.50 ± 2.24 B	24.66 ± 4.97 B
10.00 μg/L ES	8.25 ± 2.36 B	16.41 ± 10.67 B

Control (−): negative control, control (+): positive control (10 μg/L methyl methanesulfonate). Significant differences from the negative control indicated by different letters ($p < 0.05$).

of lipid peroxidation have been implicated as underlying mechanisms in numerous pathological conditions such as hemolytic anemia, ischemia, etc. in several organisms [55]. The aldehydes malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) produced by lipid peroxidation are not only biomarkers to prove that lipid peroxidation has occurred, but also they play a direct role in the toxicity of oxidant xenobiotics, even implicated in cancer formation [56]. Then, *A. facetus* environmentally exposed to relatively low concentrations of ES could be under risk of develop several diseases.

Differential patterns and sensitivity levels were observed among the studied tissues in the oxidative stress response induced by ES in *A. facetus*. Despite the high rate of ROS production, as a consequence of a high rate of oxidative metabolism, and the abundance of polyunsaturated fatty acids in cell membrane, brain has a relatively low antioxidant defense system [57]. Thus, brain resulted particularly susceptible to oxidative damage in *A. facetus* since all the enzymatic systems studied were inhibited. Depression of the CAT activity was previously reported in the freshwater fish *Channa punctatus* exposed to ES and it was attributed to the flux of superoxide radicals [31]. Due to CAT activity catalyze the conversion of H_2O_2 in H_2O and O_2, its inhibition in *A. facetus* could explain directly the 2-fold increase of H_2O_2 observed. In addition, the IC_{25} and IC_{50} estimated for SOD in brain were two orders of magnitude lower than in liver, showing that this organ is more sensitive than liver in *A facetus*. Similar results has been reported by Song et al. [58] using the organochlorine hexachlorobenzene. Then, its consideration in future biomarker evaluations is suggested.

Although fish gills constitute a direct exposure route for environmental waterborne pollutants like ES, the absence of induction or inhibition of the enzyme systems analyzed could reflect a

hypothetic compensatory response at 24 h since antioxidant enzymes have been described to respond at very little short exposure times such as 4 h [59]. Ballesteros et al. [28] reported induction of glutathione peroxidase (GPx) and no changes in TBARs in gills of the fish *J. multidentata* exposed to 0.29 μg/L ES during 24 h. Since GPx catalyses the reduction of H_2O_2 and lipid hydroperoxides, a possible induction of this enzyme would explain the very low levels of H_2O_2 observed in *A. facetus* but further studies are necessary to confirm this hypothesis.

In humans organochlorine pesticides such as ES are recognized to induce both CYP1A and CYP2B [60]. In humans endosulfan sulfate formation was mainly correlated with the activity of the CYP2B6 and CYP3A mixed function oxidase isoforms [61], although, in fish the induction of the CYP1A isoform (EROD activity) by ES has been reported "*in vivo*" in liver microsomes of *Oncorhynchus mykiss* intraperitoneally exposed during 24 h [62], and *Salmo salar* dietary exposed during 34 d [63]. These results suggest that CYP1A-mediated hydroxylation may be important for the metabolic transformation of ES in fish. However, no significant response to ES exposure was observed in the hepatic EROD activity of *A. facetus*. Assuming, like in other fish species, CYP1A1 should be induced by ES, a possible explanation is feasible in relation to the toxicokinetics of the ES and the length of the experiment. Under waterborne exposure, 24 h could not be enough time to let ES to reach effective concentrations in the liver to induce the CYP1A1, but further studies will be necessary to gain insight on this specific aspect.

4.3. Genotoxicity parameters

DNA in cellular nuclei is a key cellular component that is particularly susceptible to oxidative damage by ROS. The formation of MN in dividing cells is the result of chromosome breakage due to unrepaired or misrepaired DNA lesions, or chromosome malsegregation due to mitotic spindle malfunction. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell-cycle checkpoint and/or DNA repair genes, and by deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation [64]. The occurrence of MN represents an integrated response to chromosome-instability phenotypes and altered cellular viabilities caused by genetic defects and/or exogenous exposures to genotoxic agents [64]. The insecticide ES has been shown to be aneunogenic in plants [65], similar to other cyclodiene pesticides such as heptachlor, aldrin and dieldrin [66]. It is noteworthy that an increased frequency of MN was observed as early as 24 h after exposure to ES. However, this fact has been previously detected in other fish species with other pesticides [67,68]. Micronuclei frequency observed in *A. facetus* shows its genotoxicity, although the specific mechanism of formation in this species is unknown. Previous studies in tadpoles showed a quite small MN increases after ES treatment (5 and 10 μg/L), although statistically significant compared to the negative control at 48 and 96 h [69]. In our study, however, the MN at the same exposure concentrations were similar to the positive control, which added to the significant increase of NA, denotes a clear damage. The decreased enzymatic antioxidant capacity observed in the organs of *A. facetus* could be an important factor that may contribute to the DNA damage induced by ES. On the other hand, Carrasco et al. [47] found that micronuclei correlated with both blebbed and lobed nuclei but in other work [70] induction of NA was recorded even if micronuclei were not, leading to not clear evidences of common origin between both types of nuclear lesions. In *A. facetus* the NA were much more common than were the MN, similarly to observations in white croaker and minnows exposed to different mutagenic compounds [47,70], although its significance for the health of the organism is unknown.

4.4. Feasibility of using the parameters studied for the detection of environmental concentrations of endosulfan

In the present study, biochemical and genetic parameters were simultaneously assessed for the first time, under ES exposure. A good correspondence was obtained between oxidative stress and genotoxic effects sublethally induced in fish by the insecticide. Although it has been established that the biomarker should respond in a dose or time-dependent manner to the toxicant [71] and in *A. facetus* was not that the case for all parameters, the observed increase of lipid peroxidation and the general inhibition of the enzymes allowed the identification of oxidative stress and encourage to develop a more deep toxicological mechanistic study. In most cases, xenobiotics are absorbed and distributed throughout the body, where they cause either general systemic effects or effects that occur predominantly in certain specific target organs. Such a target-organ-selective toxicity, often independent of the route of uptake, is called organotropic toxicity [56], and it is the case of ES in *A. facetus* because brain was the most sensitive organ. These results encourage the further field validation of the observed response pattern as a potential suit of biomarkers to assess acute sublethal effects induced in feral fish by short-term-pulsed exposure to ES, similar to those observed in the field after spraying events.

5. Conclusions

The present study demonstrates that a set of sublethal effects are induced in the Neotropical fish *A. facetus* after very short-term exposures to environmentally realistic concentration of ES. In particular, these effects are characterized by a general pattern of inhibition of the antioxidant enzymatic system together with the increase of H_2O_2, lipid peroxidation and DNA damage. The brain is the most sensitive organ regarding the oxidative stress response, followed by the liver and then the gill. Micronucleous and nuclear abnormalities in erythrocytes sensitively point out genotoxic effects. Based on the results of this study and previous data, we conclude that environmentally relevant concentrations of ES that exceed the maximal concentration allowed for freshwater environments constitute a risk for aquatic organisms. Moreover, the use of a battery of biomarkers is needed to better understand the effects of ES in fish as well as its potential application in biomonitoring programs.

Finally, observed responses encourage further studies toward understanding the potential long-term biological consequences to the fish associated with repeated short-term pulsed scenarios.

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