Bull. Environ. Contam. Toxicol. (2002) 68:809–816

DOI: 10.1007/s00128-002-0027-4

Bulletin of Environmental Contamination and Toxicology

Biochemical Responses of Bluegill Sunfish (*Lepomis macrochirus*, Rafinesque) to Atrazine Induced Oxidative Stress

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Received: 20 July 2001/Accepted: 31 December 2001

Atrazine, a commonly used herbicide in North America, is a contaminant of surface and ground waters with high mobility through soils. The maximum concentration level (MCL) for atrazine in water should be 3 μg/L (US EPA 1991), but it has been found in the environment in much higher concentrations: up to 65 μg/L in groundwater and up to 70 mg/L in pools and soil in a loading and rinsing area in Iowa (Ritter 1990). The possible effects of atrazine on aquatic ecosystems have stimulated studies to understand the mechanisms and measurements of the toxic effects of it to aquatic organisms. Thus, several studies have been undertaken to report the levels of atrazine having lethal or sublethal effects on fish. The lethal concentrations ranged from about 3 to 45 mg/L of atrazine for certain fish. Concentrations are generally in function of the species of fish and of the experimental conditions of exposure (Bathe et al. 1973; Gunkel and Kausch 1976; Neškovic' et al. 1993). Sublethal effects may occur at levels of exposure less than 2 mg/L of atrazine during a long term exposure with biochemical and histopathological alteration of fish tissues (Neškovic' et al. 1993).

Atrazine (Stolze and Nohl 1994) and other herbicides, such as paraquat (Winston and Di Giulio 1991), are known to exert their effects by inducing oxidative stress in tissues of mammals and fish. Malondialdehyde (MDA) is a main oxidation product of peroxidized polyunsaturated fatty acids and increased MDA level is an important index of lipid peroxidation. To prevent oxidation-induced damage, there must be effective antioxidation systems in organisms. Some components of these systems involve reduced glutathione (GSH) and certain antioxidant enzymes including free radical scavenging enzymes such as glutathione peroxidases (GPx Se-dependent, EC 1.11.1.9, or GPx Se-independent enzyme, EC 2.5.1.18), glutathione reductase (GR, EC 1.6.4.2), superoxide dismutase (SOD, EC 1.15.1.1) and catalase (EC 1.11.1.6). Other associated enzymes are the glyoxalase I (GI, EC 4.4.1.5), glyoxalase II (GII, EC 3.1.2.6) and glutathione S-transferases (GST, EC 2.5.1.18). GST are involved in the general detoxification process of atrazine. The major phase I metabolic reaction in plants and mammals appears to be a cytochrome P450-mediated N-dealkylation, while the main phase II reaction is the GST-catalyzed conjugation with GSH. The presence of GST isoenzymes that metabolize atrazine have been demonstrated in many organisms including plants, fish, insects and mammalian species (Egaas et al. 1993).

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The present study examines the changes in the levels of certain metabolites that serve as markers for oxidative stress processes, and also the changes in the activities of some of the enzymes associated with elimination of reactive oxygen species ($O_2^{\bullet-}$ or H_2O_2) in fish that were oxidatively stressed by sub-acute atrazine exposure for 96 hr. Thus, the effects of atrazine on MDA, GSH, GSSG, GR, GST, G I and G II, GPx's, SOD, and catalase were examined following the atrazine exposure.

MATERIALS AND METHODS

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) was purchased from Ciba-Geigy (North Carolina, U.S.A.). Atrazine stock solutions, and all other dilutions, were prepared in ethanol with final concentrations of 0.001% (HPLC grade).

Bluegill sunfish of mixed sex (mean total length 12.2 ± 1.1 cm; mean weight 30.42 ± 8.59 g), were obtained from a fish hatchery in Dublin, Texas, U.S.A. The fish were acclimated to laboratory conditions for 10 days before starting the static renewal toxicity experiments. A 96 hr LC_{50} of 16 mg/L atrazine for bluegill had already been determined (Bathe et al. 1973) and was used as the basis for choosing the nominal concentrations of atrazine used in this study. The fish were divided in four groups (n = 6) and exposed to specific nominal atrazine concentrations (0, 3, 6, 9 mg/L) for 96 hr. The experiment was conducted in glass aquaria (50x20x25 cm), containing 45 L of dechlorinated and gentle aerated tap water: pH 7.97; total hardness 120 mg/L as $CaCO_3$; dissolved oxygen 8.4 mg/L; conductivity 305 μmhos/cm and temperature 20.5 °C. The water with herbicide was changed every 24 hr by adding the fresh atrazine solution in order to counterbalance decreasing herbicide concentrations because of its hydrolysis in water. No mortality occurred following the exposure to atrazine during the experiment. After 96 hr of exposure the fish were killed by severing the spinal cord. The liver and the gills were removed, rinsed with ice-cold saline, blotted dry and divided into three portions which were immediately stored at –80 °C until used. The gill filaments of both sides were trimmed from the gill arches and the arches were discarded. The weighed tissues were used for the determination of GSH, GSSG, MDA, and for the enzymatic analyses.

The MDA concentrations, an index of lipid peroxidation, were measured after incubation at 95°C with thiobarbituric acid. The pink colour produced by these reactions was measured at 532 nm to measure MDA levels (Ohkawa et al. 1979). The GSH and GSSG levels in gill and liver tissues were determined by the method of Hissin and Hilf (1976). This method measures the fluorescence emission of the o-phthalaldehyde (OPA) reaction product with these metabolites. Fluorescence measurements were compared to a standard curve constructed from fluorescence values obtained for known concentrations of GSH or GSSG. Recovery studies were done on gill and liver tissues by spiking the samples with fixed concentrations of GSH or GSSG. The recoveries of GSH and GSSG ranged from 90 to 95%.

The homogenization for the enzymatic assay was performed with a Polytron homogenizer in 10 vol of 100 m*M* TRIS buffer pH 7.8, containing 100 μ*M* phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged at 4 °C at 100,000 *g* for 60 min and the sediment was discarded. The supernatant was used for the enzymatic activities determination.

All the assay conditions of GPx's, GR, GI, GII and GST enzymatic activities have been detailed elsewhere (Elia et al. 2000). Glutathione peroxidases (GPx's, Se-dependent and Se-independent) activities toward cumene hydroperoxide, and H_2O_2 as substrates were determined by the coupled enzyme method with GR. The oxidation of NADPH was followed at 340 nm (ε = - 6.22 m$M^{-1}cm^{-1}$) and the rate of the non-enzymatic reaction was subtracted from the total rate. GR activity was determined by following the decrease in absorbance at 340 nm (ε = - 6.22 m$M^{-1}cm^{-1}$) due to the oxidation of NADPH. GI activity was determined by following at 240 nm (ε = 3.37 m$M^{-1}cm^{-1}$) the formation of SLG from the hemimercaptal adduct of methylglyoxal (MG) and GSH. GII activity was determined at 412 nm (ε = 13.6 m$M^{-1}cm^{-1}$) by monitoring GSH formation in a presence of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). GST activity was measured at 340 nm (ε = 9.6 m$M^{-1}cm^{-1}$) following the formation of the conjugate of GSH with 1-chloro-2, 4-dinitro-benzene (CDNB). SOD activity was determined by the method of McCord and Fridovich (1969) involving the cytochrome c reduction by $O_2^{\bullet-}$ generated by the xanthine oxidase/hypoxanthine system. Catalase activity was measured in according to Greenwald (1985), following the decrease in absorbance at 240 nm due to H_2O_2 consumption (ε = - 0.04 m$M^{-1}cm^{-1}$). Protein concentration was determined according to Lowry et al. (1951), employing bovine serum albumin (BSA) as a protein standard.

Unless otherwise noted, all results are reported as averages of the activities obtained. Prior to statistical analysis each data set was evaluated for normality and homogeneity of variance. In those cases where the data were determined to be both normal and homogeneous the statistical analyses used were ANOVA and Dunnett's test if the number of replicates were equal or ANOVA and Bonferroni's t-test if the number of replicates were unequal. If the data were determined to be either non-normal or heterogeneous Steel's Many-one Rank test was used for data sets with an equal number of replicates, while Wilcoxon's Rank Sum test was used for those data sets with an unequal number of replicates. In the majority of cases the data were normal and homogeneous. In all cases statistical significance was declared at $p < 0.05$.

RESULTS AND DISCUSSION

The exposure to nominal sub-acute atrazine concentrations stimulated lipid peroxidation in bluegill liver, as evidenced by the increase of MDA level in liver of the herbicide exposed fish with respect to the control values (about + 40% at the highest concentration). Exposure of fish to atrazine did not affect the MDA levels in the gill (Table 1). Many findings have demonstrated enhancements of lipid peroxidation and oxidative stress in tissues of different species of aquatic

Table 1. MDA, GSH, and GSSG levels in liver and gill tissues of *Lepomis macrochrius* exposed to atrazine (mean ± SE; n=6; values as nmol/mg protein).

Nominal Atrazine (mg/L)	MDA	GSH	GSSG
Liver			
0	0.80 ± 0.06	5.25 ± 0.55	0.36 ± 0.03
3	0.68 ± 0.05	5.93 ± 0.56	1.46 ± 0.11*
6	1.21 ± 0.61*	10.30 ± 0.70*	1.17 ± 0.17*
9	1.13 ± 0.08*	10.28 ± 1.04*	1.38 ± 0.06*
Gill			
0	2.15 ± 0.14	5.05 ± 0.46	0.40 ± 0.06
3	2.47 ± 0.14	5.77 ± 0.38	0.56 ± 0.03
6	2.65 ± 0.17	4.29 ± 0.32	0.49 ± 0.04
9	2.62 ± 0.24	5.14 ± 0.35	1.39 ± 0.19*

* statistically significantly different from control $p \leq 0.05$.
MDA = malondialdehyde; GSH = reduced glutathione; GSSG = glutathione disulphide.

organisms, which occurs as consequence of exposure to environmental stressors (Winston and Di Giulio 1991). However, it is a not general rule that the exposure of pollutant induces MDA level (Winston and Di Giulio, 1991; Hai et al. 1997), as some authors have shown a lower MDA level in fish sampled in contaminated site of metals and organic contaminants (Rodríguez-Ariza et al. 1993), or no responses for fish exposed to azinphosmethyl and 2,4-D (Oruç and Üner 2000). In all probability, the different responses are a function of the time of exposure and the concentration of stressors.

GSH and GSH-dependent enzymes represent an important antioxidant and detoxification defense in fish. Increased GSH levels in the liver have been found in fish exposed to several pollutants (Winston and Di Giulio 1991; Hai et al. 1997; Elia et al. 2000). Under our experimental conditions the hepatic GSH levels increase of about + 95%, at 6 mg/L and 9 mg/L herbicide, was statistically significant when compared to the controls. In the gills the GSH levels were unchanged after 96 hr herbicide treatment. The levels of GSSG in exposed fish showed significant increase (about 3x) in liver tissue at all atrazine concentrations used, while in the gills they increased significantly (about 3x) only at 9 mg/L of atrazine, when compared to the control. In the liver and in the gills of unexposed fish, the ratio GSH/GSSG was about 15 and about 13, respectively. In the liver and the gills, this ratio decreased at all concentrations of herbicide to which the fish were exposed. Usually, healthy cells maintain low GSSG levels compared to the GSH levels and only in stressed cells does the ratio GSH/GSSG decrease. The very low GSH/GSSG ratio found in the gills of fish exposed to 9 mg/L atrazine is probably reflective of a very high oxidative stress state. The constant value of GSH and the markedly increased value for GSSG in gills can possibly be

explained by an increase in the rate of synthesis of GSH under stress conditions.

It is known that different toxic compounds cause oxidative stress in organisms and the reduction of such stress may be due to the action of some antioxidant enzymes (Winston and Di Giulio 1991). Significant differences were observed for the glutathione peroxidase activity (Se-dependent and Se-independent enzymes) in the liver of treated fish (Table 2). The Se-dependent enzyme exhibited a statistically significant decrease in activity at all exposure concentrations. The Se-independent enzyme showed significant increases of about + 50% at 6 mg/L and 9 mg/L of atrazine. In the gills, neither of the GPx's showed significant changes in activity following exposure to atrazine. The decreased activity of hepatic Se-dependent enzyme showed a reduced antioxidant defense at the cellular level, probably related to the high oxidative stress condition in these fish. No significant changes in GR activity (measured only in the liver) were observed following the exposure of the fish to atrazine. GR enzyme has an important role in cellular antioxidant protection because it catalyzes the regeneration of GSH from GSSG. In fact, a decrease in this enzymatic activity may result in GSH depletion if compensatory synthesis of GSH cannot occur to maintain its redox status. The activity of SOD increased markedly in the liver at 9 mg/L of atrazine to about 1.7 times that of the control values, while no statistically significant differences were detected in the gills (Table 2). As shown in the Table 2, catalase activity was unchanged in both tissues during the atrazine treatment. Similar to the present observation, the hepatic catalase activity was not affected by azinphosmethyl and 2,4 D (Oruç and Üner 2000), and picloram in fish (Gallagher and DiGiulio 1991).

Table 2. Enzyme activities in liver and gill tissues of *Lepomis macrochrius* exposed to atrazine (mean ± SE, n=6).

Nominal Atrazine (mg/L)	GPx-H_2O_2[1]	GPx-CuOOH[1]	GR[1]	SOD[2]	Catalase[3]
Liver					
0	102.1 ± 4.4	135.3 ± 13.6	6.3 ± 1.1	9.1 ± 0.9	15.1 ± 1.7
3	71.9 ± 5.3*	159.8 ± 15.5	5.3 ± 0.6	11.4 ± 1.1	15.3 ± 1.3
6	67.1 ± 7.4*	212.8 ± 19.0*	3.6 ± 0.9	9.0 ± 1.3	13.0 ± 1.0
9	57.1 ± 11.8*	199.1 ± 9.0*	4.8 ± 0.8	15.7 ± 0.7*	15.7 ± 2.3
Gill					
0	20.5 ± 0.8	44.0 ± 5.4	ND	2.5 ± 0.5	5.6 ± 0.3
3	19.8 ± 1.2	25.9 ± 2.2	ND	1.8 ± 0.2	5.3 ± 0.3
6	22.1 ± 1.2	26.7 ± 2.1	ND	2.7 ± 0.3	6.0 ± 0.4
9	27.1 ± 2.3	45.1 ± 4.0	ND	2.7 ± 0.2	5.1 ± 0.2

The enzyme activities are reported in [1]nmol/min/mg protein; [2]U/mg protein; [3]μmol/min/mg protein; ND, not detected. * statistically significantly different from control $p \leq 0.05$. GPx-H_2O_2 = glutathione peroxidase Se-dependent; GPx-CuOOH = glutathione peroxidase Se- independent; SOD = superoxide dismutase; GR = glutathione reductase.

Moreover, as reported by Oruç and Üner (2000), GR and GPx activities increased in liver of fish exposed to azinphosmethyl and 2,4-D, while SOD activity decreased in those treated with azinphosmethyl and increased in those exposed to 2,4-D.

To our knowledge, there are no previous data concerning the glyoxalase system enzymes in atrazine-exposed fish. In liver tissue, G I was significantly increased by + 30% at 6 and 9 mg/L atrazine concentrations and was also significantly increased by + 30% at 9 mg/L atrazine in gills (Table 3). The specific activity of G II increased significantly in the liver and in gills (about 2x and 4x, respectively) in treated fish at 9 mg/L of atrazine compared to the control (Table 3). The specific activity of G I was 1.7 and 13 times higher than the activity of G II, in the liver and gills of unexposed fish, respectively. The increased level of G I in atrazine-exposed fish could be related to its detoxification role for α-ketoaldehydes that are formed during oxidative cellular process. The increased activity of G II in treated fish could also be related to the increased level of SLG produced by the reaction catalyzed by G I.

In our investigation, GST activity, in both studied tissues of exposed fish, increased significantly during atrazine exposure (Table 3). In the liver, exposure to both 6 and 9 mg/L atrazine caused statistically significant increases while in the gills only 9 mg/L caused a statistically significant increase. The results also showed lower GST activity in gills of control fish compared to the levels in the livers of control fish. It was reported that GST enzyme, under different experimental conditions, did not show variation in activity in the liver of exposed fish to atrazine (Egaas et al. 1993), and to azinphosmethyl and 2,4-D (Oruç and Üner 2000).

Table 3. Enzyme activities in liver and gill tissues of *Lepomis macrochirus* exposed to atrazine (mean ± SE; *n*=6; values as nmol/min/mg protein).

Nominal Atrazine (mg/L)	G I	G II	GST
Liver			
0	204.9 ± 14.2	117.6 ± 4.9	114.8 ± 10.3
3	243.3 ± 12.1	137.8 ± 16.4	109.3 ± 4.7
6	277.2 ± 14.7*	197.9 ± 29.0*	181.5 ± 13.7*
9	266.3 ± 15.9*	238.4 ± 31.3*	201.7 ± 11.7*
Gill			
0	82.9 ± 3.5	6.3 ± 0.9	5.5 ± 0.3
3	60.8 ± 3.6	4.9 ± 0.5	3.9 ± 0.5
6	79.7 ± 7.9	8.1 ± 0.4	4.4 ± 0.3
9	109.5 ± 7.8*	26.9 ± 0.8*	7.8 ± 0.7*

* statistically significantly different from control p≤ 0.05.
GI = glyoxalase I; GII = glyoxalase II; GST = glutathione S-transferases.

The results of these studies evidence that the biochemical responses are dependent on specie, as well as on stressors type, its concentration, and exposure time. Furthermore, the herbicide may lead to the occurrence of transformation products in water with a potential or actual similar or higher toxicity than their parent (Tchounwou et al. 2000). Metabolites/breakdown products of atrazine potentially could have affected the biochemical measurements in exposed bluegill fish. Thus, further studies are required to determine these biochemical changes after the exposure of fish to the atrazine and its transformation products.

The observations of increased antioxidant enzyme activity of SOD, of GSH and GSSG content, and the increase of MDA levels in liver of exposed fish, as well the decreased GPx Se-dependent activity, support the hypothesis that the subacute atrazine concentrations induce oxidative stress in the liver of bluegill fish. In addition, the increased enzyme activities of G I, G II and GST in both liver and gills also provide evidence of stress. These results suggest that the selected markers are sensitive to herbicide concentrations and highlight the utility of biochemical responses as tools for assessing contaminant exposures and atrazine cellular toxicity in fish.

Acknowledgments. This research was supported by a grant from the Consiglio Nazionale delle Ricerche, Italy (CNR, 1995) "Programma CNR- Enti di ricerca italiani ed Istituzioni di ricerca straniere: mobilità di breve durata – short-term fellowships". We thank Dr. Farideh Sabeh for her assistance in this work.

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