

RESEARCH ARTICLE

Diet with Diphenyl Diselenide Mitigates Quinclorac Toxicity in Silver Catfish (*Rhamdia quelen*)

Charlene Menezes^{1*}, Ignacio Ruiz-Jarabo², Juan Antonio Martos-Sitcha^{2,3}, Jossiele Leitemperger¹, Bernardo Baldisserotto⁴, Juan Miguel Mancera², Denis Broock Rosemberg¹, Vania Lucia Loro¹

1. Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil, 2. Departamento de Biología, Facultad de Ciencias Del Mar y Ambientales, Campus de Excelencia Internacional del Mar (CEI-MAR), Universidad de Cádiz Puerto Real, Cádiz, Spain, 3. Instituto de Ciencias Marinas de Andalucía, Consejo Superior de Investigaciones Científicas (ICMAN-CSIC), Puerto Real, Cádiz, Spain, 4. Departamento de Fisiología e Farmacología, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

*charlenemenezes@yahoo.com.br



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Abstract

In this study, the protective effects of diphenyl diselenide [(PhSe)₂] on quinclorac-induced toxicity were investigated in silver catfish (*Rhamdia quelen*). The fish were fed for 60 days with a diet in the absence or in the presence of 3.0 mg/Kg (PhSe)₂. Animals were further exposed to 1 mg/L quinclorac for 8 days. At the end of experimental period, fish were euthanized and biopsies from liver and gills, as well as blood samples, were collected. The cortisol and metabolic parameters were determined in plasma, and those enzyme activities related to osmoregulation were assayed in the gills. In liver, some important enzyme activities of the intermediary metabolism and oxidative stress-related parameters, such as thiobarbituric acid-reactive substance (TBARS), protein carbonyl, catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), nonprotein thiols (NPSH) and ascorbic acid contents were also evaluated. Compared to the control group, quinclorac exposure significantly decreased hepatosomatic index and increased cortisol and lactate values in plasma. Moreover, the activities of fructose biphosphatase (FBPase), glucose-6-phosphate dehydrogenase (G6Pase), glycogen phosphorilase (GPase) and aspartate aminotransferase (AST) were significantly increased in liver. Quinclorac also induced lipid peroxidation while the activity of SOD, NPSH and ascorbic acid levels decreased in the liver. However, dietary (PhSe)₂ reduced the herbicide-induced effects on the studied parameters. In conclusion, (PhSe)₂ has beneficial properties based on its ability to attenuate

toxicity induced by quinclorac by regulating energy metabolism and oxidative stress-related parameters.

Introduction

Micronutrients are required for the normal life processes of all animals. The micronutrients, including vitamins and minerals, are important for skeletal formation, regulation of acid-base equilibrium, synthesis of hormones and enzymes, being also essential for the antioxidant defense system [1]. On the other hand, their deficiencies can cause biochemical, structural and functional changes on several metabolic parameters [2]. Selenium (Se) is an essential trace mineral for animals. However, there is a fine partition line between deficiency and toxicity. In fact, high Se concentration has been found to induce pro-oxidant effects, mainly due its ability to catalyze thiol oxidation and to generate free radicals [3, 4]. High doses of Se may also cause bioaccumulation in the trophic chain, representing a risk for organisms, but its toxicity depends essentially on its chemical structure [2].

Contrastingly, Se compounds are also able to protect fish from the toxicity of pesticides [5–7]. Organic forms of Se have been suggested as relevant biologic antioxidant agents in different experimental models [4, 6]. Diphenyl diselenide is an organoselenium compound and the simplest of the diaryl diselenides that has numerous pharmacological properties such as anti-inflammatory, antinociceptive, neuroprotective and antioxidant [3, 6, 8]. This compound has been used in fish diet to attempt reduce and/or attenuate the impacts of herbicides [6, 7].

Environmental exposure to herbicides may affect terrestrial and aquatic ecosystems and generate adverse effects in the human health as in aquatic organisms. The quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) is a highly selective herbicide that belongs to a class of quinolone, used in turf fields and rice paddies to control some broadleaved weeds and major grass weeds [9]. This herbicide has a water solubility of 0.065 mg/L, half-life in water of around 21 days and constant of acidic ionization (pKa) of 4.34 [10]. The toxic effects of herbicides are mediated by reactive oxygen species (ROS) which can react with biological molecules and initiate oxidative damage including lipid peroxidation, protein oxidation and metabolic and enzymatic changes. Our group has already demonstrated that quinclorac exposure produces changes in the metabolic state, induces oxidative damage and reduces antioxidant status in tissues of *Leporinus obtusidens* and *Cyprinus carpio* [6, 11]. Each metabolic pathway is regulated continuously within cells in order to maintain homeostasis in the organism. Nonetheless, stressful situations can change intermediary metabolism of fish by altering key enzymes in the metabolism of carbohydrates, lipids and amino acids [12] or even osmoregulatory enzymes [13, 14]. These actions are mediated by

cortisol, which provides the energy necessary to cope with the stressful situation [13].

The silver catfish, *Rhamdia quelen*, is a native freshwater fish from southern Brazil and has great economic importance. Studies have documented the physiological and biochemical responses of this species when exposed to herbicides [15, 16]. These studies showed that several biochemical parameters are altered, such as metabolic parameters, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, antioxidant enzymes and thiobarbituric acid-reactive substance (TBARS). Considering that herbicides may induce metabolic changes and oxidative damage in fish and that silver catfish is a species that presents a significant economic importance for human consumption, the search for potential protective molecules against herbicide-mediated toxicity and their mechanisms of action is of great importance. In this scenario, the beneficial effects of Se compounds as potential antioxidants, make these molecules candidates for the protection against quinclorac-induced toxicity. Thus, this study aimed to verify whether dietary $(\text{PhSe})_2$ protects against the detrimental effects induced by quinclorac exposure in silver catfish in terms of metabolic and oxidative-stress related parameters. To our knowledge, this is the first description of the effects of any micronutrient or herbicide over the gill H^+ -ATPase activity in a teleost fish and the effects of enzymes involved in the metabolism of carbohydrates, lipids and amino acids in silver catfish.

Materials and Methods

Chemicals

The herbicide was obtained commercially from BASF as follows: quinclorac (3,7-dichlor-oquinoline -8-carboxylic acid) (Facet; 50% purity). Malondialdehyde (MDA), 2-thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), 2,4-dinitrophenylhydrazine (DNPH), bovine serum albumin, hydrogen peroxide (H_2O_2) and others reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and commercial kits for metabolites determination from Spinreact (Barcelona, Spain). Diphenyl diselenide [$(\text{PhSe})_2$] was synthesized according to literature methods [17]. Analysis of the ^1H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure.

Fish

Juveniles specimens of silver catfish (16.32 ± 1.87 g body mass, and 11.71 ± 1.53 cm body length; average \pm SEM) were acquired from the fish culture sector and at the Federal University of Santa Maria (UFSM) and randomly used for the experiments. Fish were randomly distributed in 250-L fiberglass boxes and acclimated to laboratory conditions prior to experimentation during 15 days. They were kept in continuously aerated tap water with a static system and with a natural photoperiod (12 h light/12 h dark). The water conditions were set as

temperature: $23.0 \pm 1.0^\circ\text{C}$, pH: 7.2 ± 0.1 , dissolved oxygen: 7.0 ± 0.5 mg/L, non-ionized ammonia: 0.3 ± 0.01 $\mu\text{g/L}$, nitrite: 0.04 ± 0.01 mg/L. During acclimation period the fish were fed once a day with commercial fish pellets (Supra, Brazil). Feces and pellet residues were removed by syphon and filter systems were used to maintain water quality.

Diet preparation and Experimental design

After the acclimation period, fish were divided in two groups: control (fish fed with a diet without $(\text{PhSe})_2$, $n=24$) and Se group (fish fed with a diet supplemented with 3.0 mg/Kg $(\text{PhSe})_2$, $n=24$) and were fed with these diets for 60 days. The experimental diets composition was based in previous studies from our group [6]. Briefly, to obtain the diets containing $(\text{PhSe})_2$, this compound was added to control diet and all the ingredients were completely mixed by adding distilled water before further homogenization. Pellets of approximately 5 mm diameter were formed by grinding the mixture through a meat grinder. Control and $(\text{PhSe})_2$ diet pellets were stored at 4°C until they were used. In this experimental protocol fish were fed 3% biomass per day. The daily ration was divided into two equal meals fed at 09:00 and 16:00 h. The concentration of $(\text{PhSe})_2$ chosen for treating fish was based on previous studies where a dose-response curve with 1.5, 3.0 and 5.0 mg/Kg $(\text{PhSe})_2$ in the diet of silver catfish was performed. We have previously demonstrated that 3.0 mg/Kg $(\text{PhSe})_2$ did not cause overt signals of toxicity and also decreased lipid peroxidation, protein carbonylation, and increased antioxidant defenses (e.g. non-protein thiol and ascorbic acid levels). Water quality parameters were monitored daily and were maintained equal to those registered during the acclimation period.

After feeding period of 60 days, groups were sub-divided and allocated in 45-L boxes of fiberglass into four experimental groups maintained in duplicate tanks ($n=6$ fish per tank, $n=12$ fish per treatment): (1) control group (fish fed with a diet without $(\text{PhSe})_2$), (2) $(\text{PhSe})_2$ group (fish fed with a diet supplemented with 3.0 mg/Kg of $(\text{PhSe})_2$), (3) quinclorac group (fish fed with a diet without $(\text{PhSe})_2$ and exposed to 1 mg/L quinclorac for 8 days), (4) quinclorac + $(\text{PhSe})_2$ group (fish fed with a diet supplemented with 3.0 mg/Kg of $(\text{PhSe})_2$ and exposed to 1 mg/L of quinclorac for 8 days). Herbicide (1 mg/L) was added to the water tanks only at the beginning of the experiment, according with other experimental procedures previously described using this chemical compound [6]. Water quality did not change throughout the experimental period, and water was not replaced to avoid any disturbance, which could affect our parameters in an acute toxicity/stress response. The concentration of herbicide in water was measured at the beginning, middle and final of experimental period by high performance liquid chromatography according to Zanella et al. [18]. The limit of detection (LOD) was 0.05 $\mu\text{g/L}$ and the limit of quantification (LOQ) was 0.1 $\mu\text{g/L}$. The water analysis at the end of experimental period, showed that there was a reduction of approximately 32% in the concentration of quinclorac (0.63 mg/L) in comparison to the initial value (0.93 mg/L).

The experimental procedure was authorized by the board of experimentation on Animals of the Federal University of Santa Maria (UFSM), reference number: 23081.015532/2009-31.

Sample preparation

At the end of the exposure period, the fish were anesthetized with 50 mg/L clove oil for 3 min. After blood collection from caudal vein using heparinized syringes, the plasma obtained by centrifugation (3 min, 10 000 g, 4°C) and stored at -80°C until analysis of cortisol and metabolic parameters (glucose, lactate, triglycerides, protein and amino acids). Fish were then euthanized by spinal section and liver and gills were removed. Liver was weighed for determination of hepatosomatic index (HSI) and immediately frozen in liquid nitrogen. Branchial arches were dried in desiccant paper and also frozen in liquid nitrogen. Liver samples were used for the analysis of enzyme activities and to assess oxidative stress-related parameters and antioxidant enzymes, whereas gills biopsies were utilized for the measurement of Na⁺/K⁺-ATPase and H⁺-ATPase activities.

Biochemical determinations in plasma samples

Cortisol levels were measured by indirect enzyme immunoassay (ELISA) adapted to microplate as described previously [19]. Glucose, triglyceride and lactate were measured using commercial kits from Spinreact (Barcelona, Spain). Total proteins were determined using bicinchoninic acid with a commercial Thermo kit using BSA (bovine serum albumin) as standard. Total α-amino acid levels were assessed colorimetrically using the nynhidrin method of Moore [20] adapted to microplates and using L-alanine as standard. All assays were performed using a Bio-Tek PowerWave 340 Microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft Windows XP.

Intermediary metabolism enzymes quantification

Frozen liver was finely minced in an ice-cold Petri dish, homogenized by ultrasonic disruption with 10 vol of ice-cold stopping-buffer containing: 50 mM imidazole (pH 7.5), 1 mM mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM PMSF and 250 mM sucrose. The homogenate was centrifuged at 10 000 g, 30 min, 4°C and the supernatant was immediately frozen using dry ice and kept at -80°C until enzyme assays. Enzyme activities were determined using a Bio-Tek PowerWave 340 Microplate spectrophotometer. Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (15 μL), at a pre-established protein concentration, omitting the substrate in control wells (final volume of 275–295 μL, depending on the enzyme tested), and allowing the reactions to proceed at 37°C. The specific conditions used for hexokinase (HK), pyruvate kinase (PK), L-lactate dehydrogenase (LDH), glucose-6-phosphate

dehydrogenase (G6PDH), fructose biphosphatase (FBPase), glycogen phosphorylase (GPase), alanine transaminase (ALT), aspartate transaminase (AST), glutamate dehydrogenase (GDH), and glycerol-3-phosphate dehydrogenase (G3PDH) activities were similar to those previously described [14, 21]. All enzyme assays were carried out in duplicate, at linear conditions for time and protein content detected in silver catfish (results not shown). Protein levels were assayed by triplicate as in plasma samples.

Hepatic oxidative damage parameters

Lipid peroxidation was estimated by TBARS production, based in the reaction of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA) [22]. TBARS levels were expressed as nmol MDA/mg protein. Protein carbonyl content was assessed by the method described by Yan et al. [23]. The protein carbonyl was expressed as nmol carbonyl/mg protein. All methods were previously tested and the control values are in agreement with publication of our research group [6].

Hepatic non-antioxidants parameters

Non-protein thiols (NPSH) and ascorbic acid levels were determined according to the method of Ellman [24] and Roe [25], respectively. NPSH levels were expressed as μmol NPSH/g tissue and ascorbic acid levels as μg ascorbic acid/g tissue. These methods were also validated in our previous report [6].

Hepatic antioxidant enzyme activities

Superoxide dismutase (SOD) activity was performed based on inhibition of the radical superoxide reaction with adrenalin as described by Misra and Fridovich [26]. The activity was expressed as U SOD/mg protein. Catalase (CAT) activity was assayed by ultraviolet spectrophotometry [27]. CAT activity was expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein. Glutathione S-transferase (GST) activity was measured according to Habig et al. [28] using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The activity was expressed as μmol GS-DNB/min/mg protein.

Protein determination

Protein was determined by the Comassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm [29].

Gill ATPase activities

Gill Na^+/K^+ -ATPase activity was determined according to Mancera and McCormick [30]. Gill H^+ -ATPase activity was measured in the same manner as used for Na^+/K^+ -ATPase, through from gill homogenates in the presence or absence of the specific inhibitor of the V-type H^+ -ATPase (bafilomycin A1) [31].

Na^+/K^+ -ATPase and H^+ -ATPase activities were expressed as $\mu\text{mol ADP}/\text{mg protein}/\text{h}$.

Statistical analysis

Descriptive statistics are presented as means \pm standard error of mean (SEM). Normality and homogeneity of variances among groups were tested with the Kolmogorov-Smirnov and Levene tests, respectively. Comparisons of means was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Results of all parameters tested from replicate tanks in each experimental condition were analyzed by Student's t-test. All data were analyzed using Statistica software (version 6.0) and the level of confidence was set at 0.05.

Results

Weight, length, HSI, cortisol and plasma metabolic parameters

Differences for weight and length were not detected among the treatments. A significantly lower HSI was observed in fish fed with control diet and exposed to quinclorac compared to non-exposed fish, while no changes were observed in the other groups ([Table 1](#)). Quinclorac exposure produces a 2-fold increase in cortisol levels compared to non-exposed fish, whereas $(\text{PhSe})_2$ was effective to avert the increase of this hormone when quinclorac exposure is applied. There was no difference on plasma glucose, triglyceride and amino acid between all groups tested. However, quinclorac-exposed fish showed increased lactate levels. Protein levels were lower in those specimens maintained under quinclorac + $(\text{PhSe})_2$ treatment, while the others groups did not show any variation for this parameter when compared to control group ([Table 2](#)).

Hepatic intermediary metabolism enzymes

Concerning to the carbohydrate metabolism, the measurement of HK, PK and LDH activities did not reveal significant differences when compared to control. Moreover, the FBPase and GPase activities significantly increased in the following groups: $(\text{PhSe})_2$, quinclorac, and quinclorac + $(\text{PhSe})_2$. Furthermore, the G6PDH activity enhanced in silver catfish specimens exposed to quinclorac and quinclorac + $(\text{PhSe})_2$ groups when compared to control, whereas treatment with $(\text{PhSe})_2$ *per se* did not change the activity of this enzyme ([Table 3](#)).

Regarding with amino acid metabolism, different treatments did not show differences on ALT and GDH activities. On the other hand, fish exposed to quinclorac (with or without $(\text{PhSe})_2$ in the diet) showed increased AST activity compared to control fish ([Table 3](#)).

Related to lipid metabolism, the G3PDH activity was significantly higher in silver catfish submitted to quinclorac + $(\text{PhSe})_2$ group and no significant difference was observed among the other groups ([Table 3](#)).

Table 1. Growth parameters.

	Control	(PhSe) ₂	Quinclorac	Quinclorac+(PhSe) ₂
Weight (g)	24.30 ± 2.50	31.15 ± 2.75	24.27 ± 1.85	32.60 ± 5.09
Length (cm)	14.33 ± 0.44	13.41 ± 0.51	13.08 ± 0.39	12.67 ± 0.40
HSI (%)	1.13 ± 0.07	1.17 ± 0.05	0.87 ± 0.02*	1.03 ± 0.05

Weight, length, and HSI of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to 1 mg/L quinclorac herbicide or to control conditions.

HSI (%) = liver weight/wet weight × 100. Values are means ± SEM, n=12. (*) P<0.05 as compared with the control group.

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Hepatic TBARS and protein carbonyl

Fish fed with the control diet and exposed to quinclorac showed higher TBARS levels, while protein carbonyl did not significantly change (Figure 1A and 1B). Treatment with (PhSe)₂ *per se* decreased TBARS levels and protein carbonyl of silver catfish (Figure 1A and 1B). Furthermore, (PhSe)₂ was effective to prevent the increase of both MDA and protein carbonyl content caused by quinclorac exposure (Figure 1A and 1B).

Hepatic NPSH and ascorbic acid levels

The NPSH and ascorbic acid levels decreased in silver catfish exposed to quinclorac when compared to control group (Figure 1C and 1D). Treatment with (PhSe)₂ *per se* caused a significant increase in NPSH levels and did not change the ascorbic acid levels (Figure 1C and 1D). (PhSe)₂ prevented the decrease on NPSH and ascorbic acid levels caused by quinclorac exposure (Figure 1C and 1D). Furthermore, the quinclorac + (PhSe)₂ group showed an increased ascorbic acid levels as compared to control (Figure 1D).

Table 2. Metabolic parameters.

Parameters	Control	(PhSe) ₂	Quinclorac	Quinclorac+(PhSe) ₂
Cortisol	106.50 ± 15.25	139.81 ± 14.94	198.80 ± 12.53*	100.28 ± 6.87 [#]
Glucose	4.15 ± 0.55	3.74 ± 0.42	3.97 ± 0.36	4.98 ± 0.45
Triglycerides	1.02 ± 0.10	1.20 ± 0.09	1.19 ± 0.10	1.19 ± 0.08
Lactate	1.12 ± 0.28	1.40 ± 0.43	3.26 ± 0.53*	2.20 ± 0.44
Protein	45.02 ± 3.22	38.96 ± 2.18	38.15 ± 3.39	34.78 ± 0.80*
Amino acids	12.31 ± 1.53	11.60 ± 1.68	9.74 ± 1.48	13.14 ± 1.83

Glucose, lactate, triglyceride, protein and amino acids in plasma of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to 1 mg/L quinclorac herbicide or to control conditions.

Values are means ± SEM, n=12. Cortisol is expressed as ng/mL, glucose, triglyceride, lactate and amino acids are expressed as mM and protein as mg/mL. (*) P<0.05 as compared with the control group. ([#]) P<0.05 as compared with the quinclorac group.

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Table 3. Hepatic intermediary metabolism enzymes.

	Control	(PhSe) ₂	Quinclorac	Quinclorac+(PhSe) ₂
<i>Carbohydrate metabolism</i>				
HK	0.20 ± 0.02	0.22 ± 0.02	0.23 ± 0.02	0.18 ± 0.01
PK	4.50 ± 0.36	3.42 ± 0.67	4.46 ± 0.27	4.06 ± 0.67
LDH	0.34 ± 0.07	0.39 ± 0.14	0.32 ± 0.10	0.40 ± 0.10
FBPase	1.42 ± 0.05	2.38 ± 0.38*	2.16 ± 0.24*	2.44 ± 0.17*
G6PDH	4.80 ± 1.68	6.40 ± 1.80	10.35 ± 0.88*	11.43 ± 0.90*
GPase	1.29 ± 0.17	2.43 ± 0.16*	2.04 ± 0.21*	2.19 ± 0.29*
<i>Amino acid metabolism</i>				
ALT	2.60 ± 0.57	4.81 ± 1.33	2.45 ± 0.94	2.43 ± 0.48
AST	22.64 ± 2.35	26.29 ± 3.61	33.45 ± 0.98*	32.59 ± 3.32*
GDH	19.38 ± 2.45	16.76 ± 2.69	14.53 ± 1.96	14.07 ± 2.12
<i>Lipid metabolism</i>				
G3PDH	7.56 ± 0.22	8.35 ± 0.62	9.03 ± 0.45	10.81 ± 0.69*#

Enzyme activities of carbohydrate, amino acid and lipid metabolism in liver of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to 1 mg/L quinclorac herbicide or to control conditions.

Values are means ± SEM, n=12. HK, G6PDH, FBPase, PK, GPase, LDH, ALT, AST, GDH, and G3PDH are expressed as U/mg protein. (*) P<0.05 as compared with the control group. (#) P<0.05 as compared with the quinclorac group.

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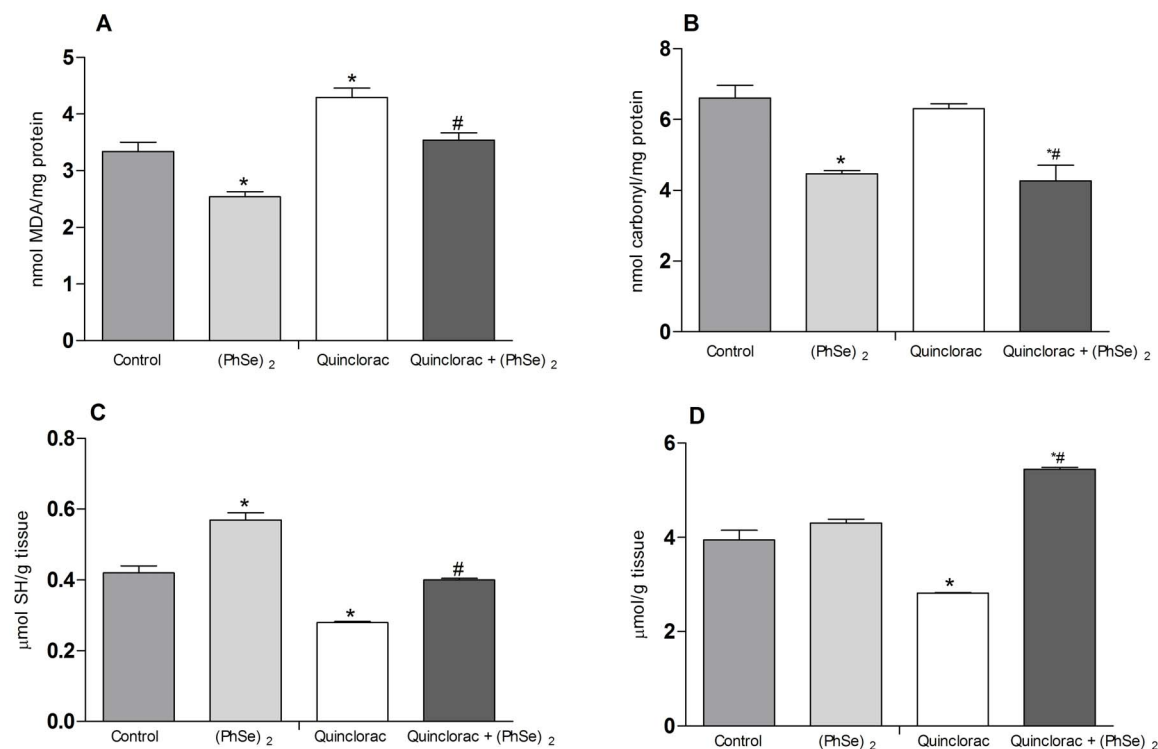


Figure 1. Oxidative parameters and non-enzymatic antioxidants. TBARS (A), protein carbonyl (B), NPSH (C), and ascorbic acid (D) in liver of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to quinclorac herbicide or to control conditions. Data are reported as mean ± SEM (n=12). (*) P<0.05 as compared with the control group. (#) P<0.05 as compared with the quinclorac group.

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Hepatic SOD, CAT and GST activities

SOD activity did not show differences in both groups of fish fed with $(\text{PhSe})_2$ respect to the control group. In addition, this activity decreased in fish exposed to quinclorac when compared to control. Moreover, $(\text{PhSe})_2$ supplementation prevented the inhibition of SOD activity caused by the herbicide ([Figure 2A](#)). CAT activity was not altered by any of the treatments tested in the present experiment ([Figure 2B](#)). Additionally, quinclorac exposure did not modify GST activity, but fish treated with $(\text{PhSe})_2$ *per se* showed an increase on GST activity. However, this enzyme activity was increased in quinclorac + $(\text{PhSe})_2$ group when compared to quinclorac group ([Figure 2C](#)).

Gill ATPase activities

Gill Na^+/K^+ -ATPase activity significantly decreased in the quinclorac + $(\text{PhSe})_2$ group when compared to those fish maintained as control, being also different when compared to quinclorac group ([Figure 3A](#)). Gill H^+ -ATPase activity increased in both groups fed with $(\text{PhSe})_2$, while no differences were found between quinclorac and control groups ([Figure 3B](#)). The raw data obtained from all experimental procedures are shown in Table S1.

Discussion

In the current study, we report the protective actions of $(\text{PhSe})_2$ on the effects promoted by quinclorac, an usual herbicide used on agriculture activities, on metabolic and oxidative stress-related parameters in silver catfish. In general, quinclorac exposure caused hepatic oxidative damage, hepatotoxicity and increased both plasma cortisol and lactate levels. These data strongly suggest that quinclorac provoked stress in silver catfish and that anaerobic glycolysis occurs as a response of quinclorac effects on energy depletion. Considering that catabolism of proteins and amino acids plays a major role in total energy production in fish [32], the decrease of plasma protein levels of fish exposed to quinclorac and previously fed with $(\text{PhSe})_2$ might be due to the increased protein catabolism contributing to the organism defense against herbicide poisoning. In fact, the reduction of plasma proteins could be explained in part as a damage effect of quinclorac on hepatocyte. Importantly, $(\text{PhSe})_2$ attenuated these alterations induced by toxicant, except the increase of AST activity, which could be explained by higher metabolic mobilization produced by quinclorac compound alone as a consequence of stress pathways activation. Similarly to our results of weight and length in silver catfish, other authors also found that diets supplemented with either inorganic or organic Se forms from 1 to 2 mg/Kg levels did not affect the growth of different fish species as Atlantic salmon (*Salmo salar*) [33] or red-tailed Brycon (*Brycon cephalus*) [34]. Since HSI is a general health indicator, reflecting both the metabolic energy demand and changes in the nutritional status the decrease of HSI in fish exposed to quinclorac may result on mobilization of

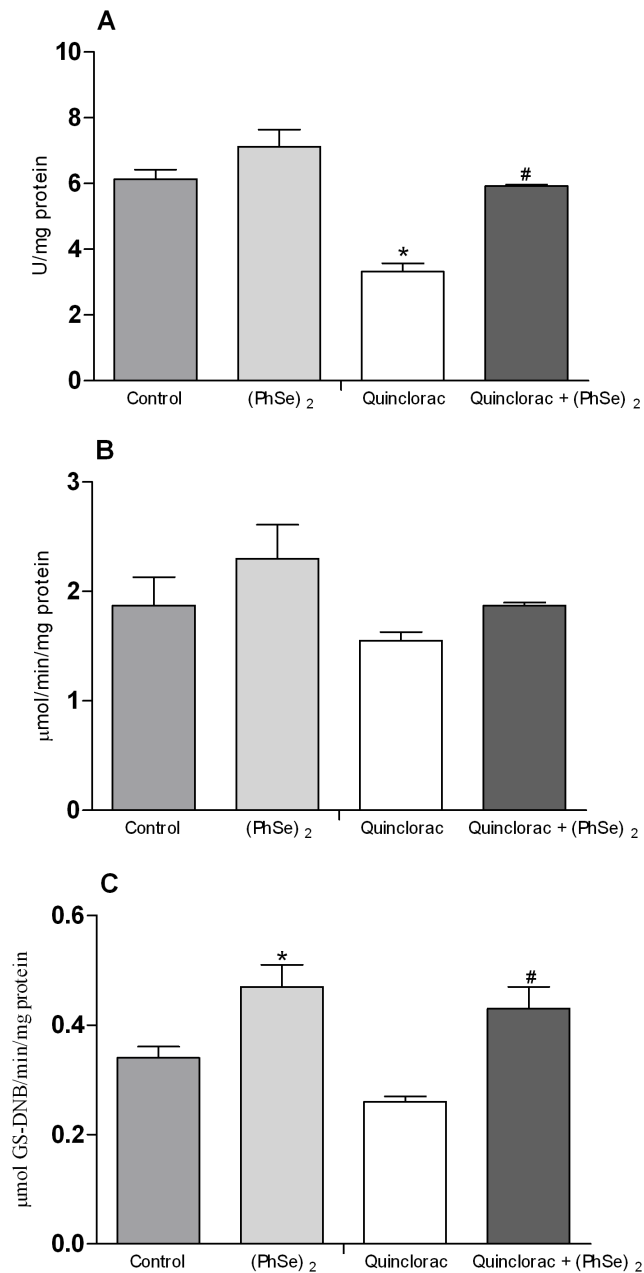


Figure 2. Antioxidants parameters. SOD (A), CAT (B), and GST (C) in liver of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to quinclorac herbicide or to control conditions. Data are reported as mean ± SEM (n=12). (*) $P < 0.05$ as compared with the control group. (#) $P < 0.05$ as compared with the quinclorac group.

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hepatic reserves to maintain homeostasis during stressful conditions. Additionally, this fact could also result from pesticides induced hepatic peroxisome proliferation, depleting the liver relative weight [5]. The maintenance of HSI in

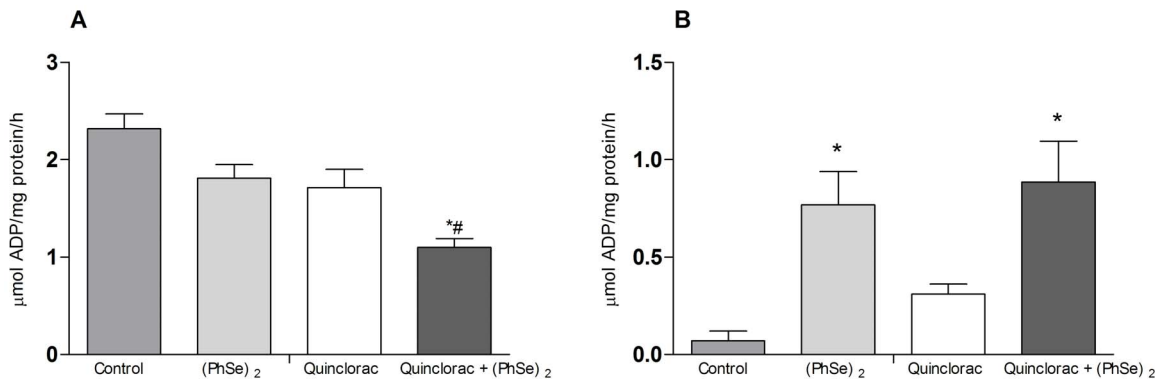


Figure 3. Gill ATPase activities. Na⁺/K⁺-ATPase (A) and H⁺-ATPase (B) activities in gills of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to quinclorac herbicide or to control conditions. Data are reported as mean ± SEM (n=12). (*) P<0.05 as compared with the control group. (#) P<0.05 as compared with the quinclorac group.

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fish exposed to quinclorac and fed with (PhSe)₂ suggests that Se supplementation contributes to hepatic protection.

The liver is a central organ in the metabolic processes that is responsible for various functions associated with the metabolism of contaminants. Additionally, it is also the main detoxifying organ and reacts mainly after stress caused by toxic substances [35]. Liver also has an important role in carbohydrate, protein and lipid metabolism. The activities of key metabolic enzymes can indicate the metabolic status and its ability to modify their activities against changes in environmental and dietary conditions. Due to the alterations observed in liver metabolism, fish exposed to quinclorac and quinclorac + (PhSe)₂ showed increased hepatic gluconeogenesis favored by increased in FBPase activity, and increased power generation by high activity of the pentose phosphate shunt (G6PDH activity). In addition, the glucose produced in liver was apparently exported to other tissues, maintaining plasma glucose levels at the same time. The increased of GPase activity in all experimental groups could be associated with an increase of glycogenolysis helping to maintain glucose homeostasis. Concerning lipid metabolism, we suggest that the increase of G3PDH observed in quinclorac + (PhSe)₂ group could be related with gluconeogenesis from glycerol for glucose production, which presumably is still needed in other tissues.

Stressful situations also induce modifications in the oxidative balance of fish. Quinclorac exposure caused hepatic oxidative damage, as demonstrated by increase in lipid peroxidation. Even so, (PhSe)₂ normalized this parameter and it was also responsible for the reduction of protein carbonyl content exhibit a protective action in fish exposed to herbicide. These results suggest that the antioxidant activity of (PhSe)₂ is involved in protecting against hepatic damage induced by quinclorac exposure in fish. Regarding pesticides, hepatic oxidative damage associated to an increase in lipid peroxidation was previously reported in different fish species as red-tailed Brycon [5] and silver catfish [16]. The biological systems have several mechanisms to counteract the damage caused by reactive

species. Antioxidant enzymes such as SOD, CAT, GPx and non-enzymatic antioxidants such as NPSH and ascorbic acid are the most important antioxidant defenses in biological systems [36]. Importantly, we also showed that (PhSe)₂ restored NPSH and ascorbic acid levels in liver of fish exposed to quinclorac, reinforcing the protective role of this organoselenium compound against the oxidative damage induced by herbicide. Similar results have been also described by our group, which reinforces the idea that (PhSe)₂ could exert protective effects in fish [6].

SOD plays an important role in the dismutation of superoxide radicals to form hydrogen peroxide and molecular oxygen, while CAT is an enzyme that converts the peroxide to molecular oxygen and water. In the present study, we verified an imbalance of SOD/CAT activities induced by quinclorac exposure, which could be associated to an increase in reactive oxygen species culminating in oxidative stress. Although the mechanisms still require to be elucidated, we postulate that the antioxidant properties of (PhSe)₂ may be involved in recovery the liver SOD activity, by increasing activity near to control values. Previous studies showed that Se supplementation increases the activity of some antioxidant enzymes in different fish species as carp or silver catfish [6, 7, 37]. GST is a detoxifying enzyme that catalyzes the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic forms [36]. (PhSe)₂ increased the GST activity in the liver of fish exposed to quinclorac suggesting a plausible mechanism by which (PhSe)₂ acts as a protective agent in the liver of fish. Furthermore, studies have reported that the pharmacological activity of (PhSe)₂ mainly involves its interaction with thiol groups with a generation of intermediate selenol groups [38]. These selenol-selenolate groups can decompose H₂O₂ and lipid peroxides formed during the propagation phase of lipid peroxidation [3]. Our data showed that the dietary (PhSe)₂ prevented the quinclorac-induced decrease in SOD activity and increased GST activity in silver catfish. Since quinclorac exposure did not alter GST, we suggest that the increased enzyme activity in quinclorac + (PhSe)₂ group could be a response to selenium treatment. Previous report demonstrated that juvenile carp fed with low and high selenium commercial diets may present distinct Se levels in liver and kidney, and alterations in enzymatic antioxidant defenses, such as SOD, CAT, GST and GPx in these tissues, depending on the time of treatment [37]. Moreover, studies of our group showed that (PhSe)₂ may play a protective role against quinclorac and clomazone-mediated toxicity in carp and silver catfish by modulating parameters oxidative stress [6, 7]. In the same context, the data presented in the current study reinforce the idea that the protective role of (PhSe)₂ against herbicide-mediated toxicity is attributed at least in part, by regulation of fish antioxidant enzymatic defenses.

Gill H⁺-ATPase acts together with the Na⁺/K⁺-ATPase to maintain the internal homeostasis by the transport of ions across the gills epithelium [39]. The Na⁺/K⁺-ATPase (NKA) is the most important osmoregulatory enzyme in teleost fish [39]. The decreased activity of the NKA in quinclorac + (PhSe)₂ group might be related to the decrease of cortisol release as well as a possible interaction between those two substances in gills, or could be interpreted as a diminished metabolic

resources to cope with ion regulation. As discussed in the present work, the quinclorac + (PhSe)₂ group presented an enhancement on gluconeogenic pathways in order to obtain enough energy to cope with the stressful situation, adversely affecting the energy required to maintain the NKA activity. Otherwise, the pivotal mechanisms of ion uptake in hypo-osmotic environments in the gills are the H⁺-ATPase and a Na⁺/H⁺ exchanger (NHE) among other enzymes [40]. Dietary (PhSe)₂ increased H⁺-ATPase activity in silver catfish, but no effects could be associated to quinclorac using this methodology. The modification of this enzyme in the groups fed dietary Se could be associated to an excess of antioxidant defenses in epithelium cells of the gills, as occurs in the liver of this species when looking at the increased GST activity and NPSH levels or decreased protein carbonyl. This fact could be related to a change in the cytoplasm pH, which will change the substrates of the H⁺-ATPase enzyme.

In conclusion, our findings show the potential mechanisms by which quinclorac induces deleterious effects in fish, mainly by inducing stressful responses, altering the redox profile, and modulating enzymes of energy metabolism. Moreover, we also demonstrate that dietary (PhSe)₂ mitigates the effects of quinclorac exposure, reducing oxidative damage and increases the activities of antioxidant enzymes and ascorbic acid levels. Therefore, diet containing (PhSe)₂ might be beneficial in protecting fish to quinclorac intoxication. However, further studies are needed to elucidate the exact mechanisms by which (PhSe)₂ exerted its antioxidant properties, since this organoselenium compound could be a promising alternative to minimize disorders associated with herbicide exposure in fish.

Supporting Information

Table S1. Raw data of the growth rate and biochemical experiments of silver catfish fed for 60 days with diets containing 0 or 3.0 mg/Kg of (PhSe)₂ and after exposed to quinclorac herbicide or to control conditions.

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Author Contributions

Conceived and designed the experiments: CM IR JAM. Performed the experiments: CM IR JAM JL. Analyzed the data: CM IR BB JMM DR VL. Contributed reagents/materials/analysis tools: BB JMM VL. Wrote the paper: CM IR JAM BB JMM DR VL.

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