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Effect of low dose diuron in oxidative state on the gilthead sea bream *Sparus aurata*

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Abstract: This study investigated effect of repeated exposure to low doses of diuron in gilthead sea bream by measuring the activities of the following antioxidant enzymes: Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GST), and DT-diaphorase (DTD). Examination of the lipid peroxidation (malondialdehyde content- MDA) and glucose 6-phosphate dehydrogenase (G6PDH) activity in these fish was also done. The results show that diuron exposure induces effects that are evident after 15 days of treatment. Almost all tissues are affected by treatment with diuron. MDA value of muscle and liver can be used as a biomarker of alterations in the oxidative state and therefore of the health of the fish.

Key Words: Health, marine pollution, oxidative stress, *Sparus aurata*

Introduction

The permissive limits of many pollutants are well established, and their effect in mammalian birds and other species are well known. Nevertheless, in the main data base of pesticides (PPDB: University of Hertfordshire 2013) the information about fish is reduced to LC-50 or LD-50 at 24 or 96 hour for some species of fish as an example of effect on aquatic biota, but there aren't data about the doses that affect the production of fish. Fish farming is important and involves many species of fresh water and marine organisms. The effects of pesticides on farmed fish should be studied carefully, because it affects the economy, the health and properties of fish as human food.

Presence of pollutants, such as herbicides and other pesticides, in the surrounding areas can affect water quality. Contaminants in the water may induce the formation of reactive

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oxygen species (ROS), and these highly reactive substances cause oxidative deterioration of protein, lipid and DNA (Avery, 2011). Studies related to oxidative stress in animals provide information about their health and welfare. Oxidative stress can occur when there is an imbalance between oxidative factors and antioxidant defence systems (Sies, 1985). This situation may be influenced by external factors, such as pollutants, xenobiotics, nutritional and environmental factors, which all trigger a classical response to stress that sensitises the animal and induces alterations in its antioxidant response mechanisms (Davies, 2000; George *et al*., 2000; Martínez-Álvarez *et al*., 2002).

Diuron [(1,1-dimethyl, 3-(3′,4′-dichloro-phenyl) urea)] is a broad-spectrum residual herbicide and algaecide that is used for the pre- and post-emergent control of broadleaf and grass weeds in agriculture (APVMA (Australian Pesticides and Veterinary Medicines Authority), 2005). Additionally, it is used to control weeds and algae in and around bodies of water and is a component of marine antifouling paints (Call *et al*., 1987). Due to its relatively high solubility and high application rates, diuron is considered one of the most harmful of the agrochemical pollutants (Haynes and Michalek-Wagner, 2000; William, 2001; Negri *et al*., 2005). The highest concentrations of diuron have been detected in marine sediments (Haynes *et al*., 2000; Kumar *et al*., 2010). It has been estimated that the maximum diuron concentration in the marine environment is 3.46 μg/L in Australian marine environment (APVMA, 2005). The concentration of this herbicide in Seto Inland Sea, Japan, was found to be 0.01–0.062 μg/L, 0.01–0.09 μg/g dry weight (dw) and 0.075–0.45 μg/g dw in seawater, sediments and planktons respectively (Balakrishnan *et al*., 2012). Usually, the sub-toxic exposure of farmed fish is caused by the periodic application of the algaecide at low doses. In addition, the liberation of toxins from antifouling paint contributes to this effect.

Diuron blocks plastoquinone from binding to the cytochrome b6f complex of the PSII reaction center, which results in a blockage of electron flow that can ultimately results in ROS production causing oxidative stress (Halliwell, 1991).

Studies on effects of diuron revealed that it is lethal to 10 to 50% of warm water fingerlings at a concentration of 2.8 to 31 mg/L after 1 to 4 days of exposure (Tooby *et al*., 1980). Other investigators suggest that a concentration of 1.4 to 7.7 mg/L diuron is lethal to half of salmon fingerlings after a 4-day exposure (Johnson and Finley, 1980; Mayer A and Ellersieck, 1986). However, the minimum concentration that diuron begins to have some negative effects on fish is not established. In an earlier histological study of fish that were exposed to various diuron concentrations, Kokuricheva (1967) recommended that the maximum concentration of diuron in a reservoir should not exceed 0.03 mg/L, nevertheless

Kumar (1992) considers that the dose of 0.3-0.5 mg/L can reduce the number of fish to a considerable extent in nursery and rearing ponds.

Saglio and Trijasse (1998) reported that short-term exposure of goldfish to a relatively low concentration (5 µg/L) of diuron affects various behaviours in goldfish by altering directly and indirectly the chemical perception of natural substances of ethological importance. Furthermore, inhibition of acetylcholinesterase (AChE) activity has been shown in response to sublethal concentrations of diuron (Murphy, 1980).

The culture of gilthead sea bream are of great importance for aquaculture, with an average cultured production of 140000 tons in 2010 (FAO (Food and Agricultural Organisation), 2013) However, not much is known about the effect of exposure to low doses of pollutants on biology and production of *S. aurata*. Therefore it is necessary to analyse the possible adverse effects of low doses of different pollutants in fish production. In this study, the effects of repeated exposure to low doses of diuron in water, on the oxidative status of gilthead sea bream (*Sparatus aurata*) was investigated by determining the activity of the following antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GST) and DT-diaphorase (DTD). The lipid peroxidation (MDA content) and glucose 6-phosphate dehydrogenase (G6PDH) activity was also investigated.

Materials and Methods

Experimental animals

Immature gilthead sea bream (*Sparus aurata*; n=100) with an initial weight of 21.0±3.5 g, were obtained from a local fish farm (Predomar S.L., Carboneras, Almería, Spain) and transported to the University of Almería where they were placed in 4 tanks (250 L), containing sea water at a flow rate of 10.4 L/h. Oxygen was measured every 10 minutes by a probe connected to an alarm system; NH_3 and NO_2 were measured weekly. The fish were maintained at their natural temperature (20 ºC) and a 12L:12D photoperiod. After one week of acclimatization to laboratory conditions, the fish were weighed, measured and randomly distributed in four tanks (25 fish per tank): 2 tanks each were used for control (C) and fish treated with diuron (D).

The chemical stress was induced by exposure to a low dose of diuron, ($C_9H_{10}Cl_2N_2O$, 3-[3,4-dichlorophenyl]-1,1-dimethylurea), 15 times less than the LC50 (96h) indicated for fish (*Oncorhynchus mykiss*) in the instructions from the company that manufactures the chemical (Giacomazzi and Cochet, 2004). Diuron was added to a concentration of 0.20 mg/L and the water flow was stopped during the exposure time to assure diuron effect on fish (1 day per

week, during 8 weeks). To avoid the negative effect of NH_3 fish were sampled after 5 days to exposure with diuron, the treatment was applied 4 hours before feeding, during 24 h of exposure to diuron, the fish were not fed and the fish (control and those in diuron) were kept at low density (2.5 Kg/m^3).

The maintenance, handling and sacrificing of the fish conformed to the Royal Decree1201/2005 on animal protection (B.O.E. 2005). All procedures were conducted according to the guidelines of Council Directive 86/609/EEC (European Economic Communities, 1986) on the protection of animals used for experimental and other scientific purposes.

Throughout the experimental period (60 days), fish (control and experimental) were fed *ad libitum*, (2.1% of biomass), once per day at 10 a.m. with the same commercial diet that is given to fish in farms (Mistral-12, Biomar-Proaqua) which has the following macronutrient composition: 44% protein, 12% lipids, 28% carbohydrates and 16% fibre.

Sampling

Samples (5 fish/tank; 10 fish/treatment) were obtained at 15 days and at the end of the experiment (60 days). The times were established to study effect of diuron in different time exposure and if the effect were proportional to the exposure time.

The animals were anesthetized with essence of clove and liver, digestive tract, brain, gills and a portion of the white muscle from the last dorsal region were removed immediately *in situ*. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

A portion of each sample was homogenized in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA and 0.1% triton X-100 (v/v), pH 7.8) at a ratio of 1:9 (w/v). Homogenates were centrifuged at 30,000×g for 30 min in a Centrikon H-401 centrifuge. After centrifugation, the supernatants were collected and stored at −80 °C until analysis.

Analytical methods

All enzymatic assays were carried out at 25±0.5 ºC in duplicate in 96-well microplates (UVStar®, Greiner Bio-One, Germany) using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The enzymatic reactions were started by the addition of the tissue extract, with the exception of the SOD reaction in which xanthine oxidase was used. The specific assay conditions were as follows;

Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the decrease in H_2O_2 concentration at 240 nm, using methods described by Aebi (1984). The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0) and freshly prepared 10.6 mM H_2O_2.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured spectrophotochemically using the ferricytochrome c method (Mc Cord

and Fridovich, 1969) and xanthine/xanthine oxidase was used as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c and 0.024 IU ml^{-1} xanthine oxidase. One activity unit is defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome c reduction rate measured at 550 nm.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured following the method described by Flohé and Günzler(1984). A freshly prepared glutathione reductase solution (2.4 U ml^{-1} in 0.1 M potassium phosphate buffer, pH 7.0) was added to 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 1 mM sodium azide, 0.15 mM NADPH and 0.15 mM cumene hydroperoxide. After the addition of 1 mM GSH (reduced glutathione), the NADPH-consumption rate was monitored at 340 nm.

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed using methods described by Calberg and Mannervik (1975), with some modifications, by measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH, and 0.15 mM GSSG.

Glutathione transferase (GST, EC 2.5.1.18) activity was determined using the method described by HABIG *et al*. (1974) that was adapted to microplates. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH and a 1.23 mM solution of 1-chloro-2,4- dinitrobenzene in ethanol that was prepared just before their use in the assay. GST activity was monitored at 340 nm by measuring the formation of the glutathione-CDNB-conjugate.

DT-diaphorase (DTD, EC 1.6.99.2) activity was measured using methods described by Sturve *et al.* (2005). The reaction mixture contained 50 mM Tris-HCl (pH 7.3), 50 µM DCPIP (2,6-dichlorophenol indophenol) and 0.5 mM NADH. The control reaction contained distilled water instead of the sample extract. DTD activity is defined as the rest between the sample and the control in the DCPIP reduction reaction.

Glucose-6-phosphate dehydrogenase (G6P DH, 1.1.1.49) activity was determined by measuring the reduction of NADP+ at 340 nm. The assay mixture consisted of 50 mM imidazole–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM NADP+, and 1 mM glucose-6 phosphate.

With the exception of SOD, for which the arbitrary units have already been defined, one unit of activity is defined for all enzymatic activities as the amount of enzyme required to transform 1 µmol of substrate/min under the above assay conditions.

The protein content of the supernatant solutions was determined using the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Lipid-peroxidation levels were determined according to Buege and Aust (1978) by quantifying the concentration of thiobarbituric-acid-reacting substances (TBARS) and expressed as nmol malondialdehyde (MDA)/g of tissue.

All biochemicals, including the substrates, the coenzymes, and the purified enzymes, were obtained from Roche (Mannheim, Germany) or Sigma Chemical Co. (USA). All other chemicals were obtained from Merck (Darmstadt, Germany) and were reagent grade.

Statistical analysis

All results are expressed as mean ± SE. Mean and SEM was determined for results in each tissue of fish under the same experimental condition (10 fish each for control and diuron for 15 days, , and 10 fish for control and diuron for 60 days). For each sampling time (15 or 60 days) one-way analysis of variance (ANOVA) was used to study any effect of diuron treatment for each enzyme and tissue.

Significant differences among the means ($p= 0.05$) in the same tissue were determined using the Duncan's multiple-range test (Duncan, 1955). For the analysis of the dependence between two variables, the Pearson correlation coefficient was estimated and the data were adjusted using a linear regression. The data analyses were performed using SPSS version 13.0 for Windows software package.

Results

The results revealed that the SOD (Fig. 1A) activity in stressed animals decreased after 15 days of exposure in all tissues with the exception of the digestive tract tissue. The decrease was significant in the muscle and brain tissues. After 60 days, significant differences between the experimental and control groups were found in the digestive tract, showing a significant decrease in SOD activity and increase of this activity in muscle and gill. There were no significant differences in CAT activity (Fig. 1B) between the experimental and control groups during the experimental period (15 and 60 days).

The GPX (Fig. 1C) activity in the control and experimental groups showed a significant increase in muscle of fish that were exposed to diuron after 15 and 60 days. The experimental group exhibited a lower GR activity (Fig. 1D) in the muscle (after 15 days) and the digestive tract (after 15 days and 60 days) for diuron treatment. The GST activity (Fig. 2A) in fish exposed to diuron decreased in the liver and in the digestive tract after 15 days. A significant decrease in the DTD activity of the digestive tract (Fig. 2B) was found in the experimental fish after 15 days of treatment with diuron. Digestive tract and liver also showed a significant decrease in activity after 60 days. The gills of the fish exhibited a significant increase in DTD activity after 60 days of diuron

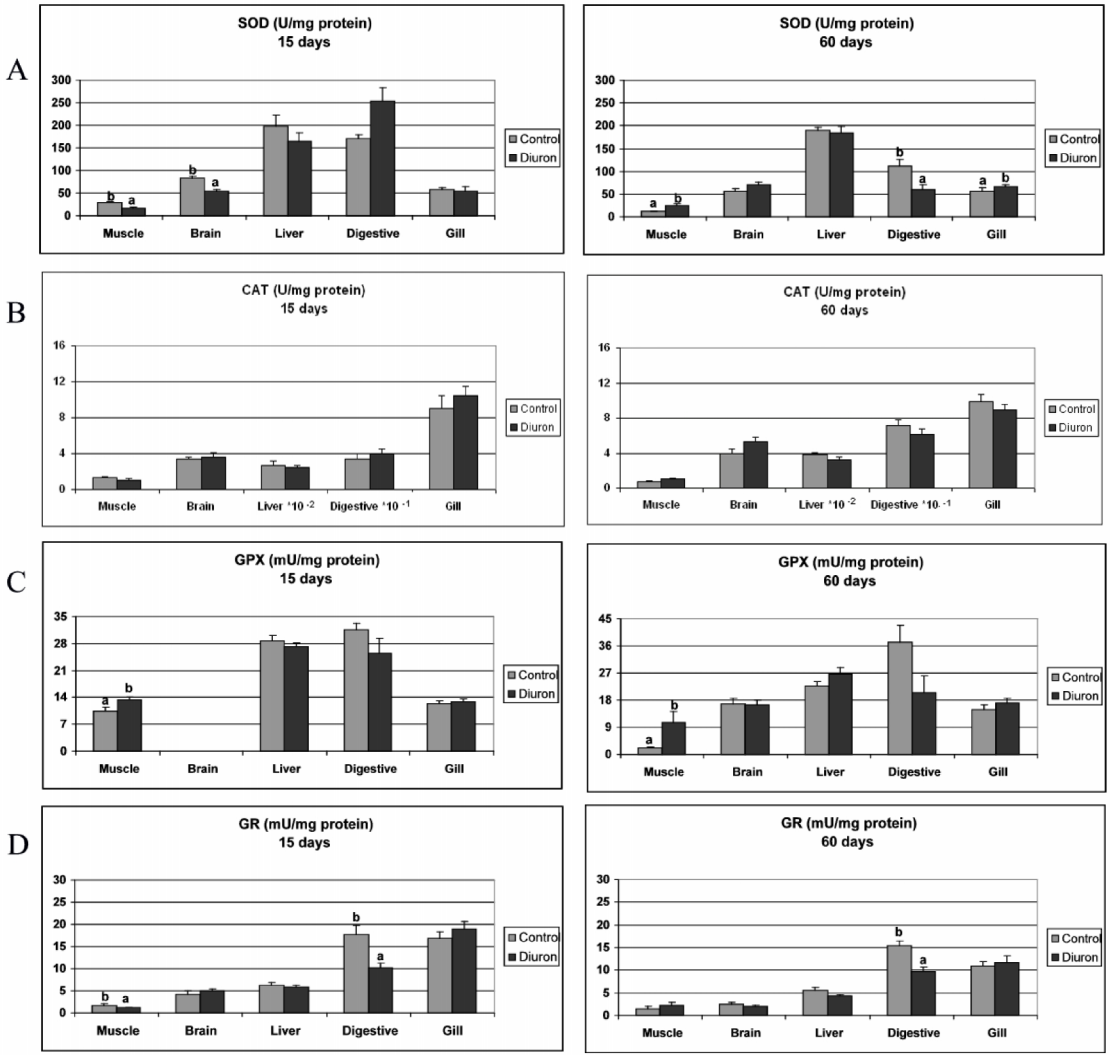

Fig. 1: Effects of diuron exposure (0.20 mg/ L, 24 hours per week) on the antioxidant activity of SOD, CAT, GPX, and GR in tissues of sea bream at 15 and 60 experimental days. Values are expressed as mean ± SEM (n= 10). [a,b]Significant differences between control and chemically stressed groups in the same tissue.

treatment. The G6PDH activity (Fig. 3) of diuron-treated fish exhibited a significant increase in the muscle and the digestive tract tissues after 15 days of exposure. On day 60, these significant differences disappeared. inally, an increase in MDA values (Fig. 4) in the muscle (after 15 and 60 days) and in the digestive tract (after 60 days) in the experimental animals was observed.

On the other hand there was positive correlation between SOD and CAT activities in control (Pearson correlation coefficient: 0.768, $p < 0.01$) and fish exposed to diuron (Pearson correlation coefficient 0.577, $p < 0.01$);

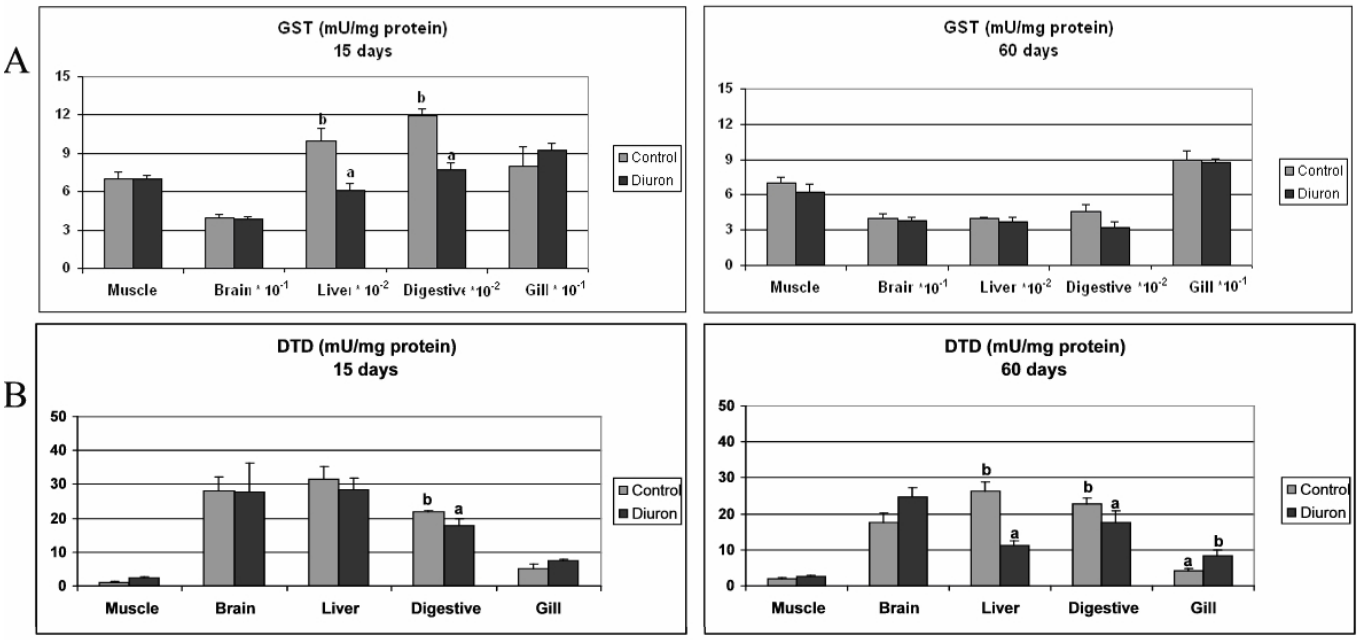

Fig. 2: Effects of diuron exposure (0.20 mg/ L, 24 hours per week) on the antioxidant activity of GST and DTD in tissues from sea bream at 15 and 60 experimental days. Values are expressed as mean ± SEM (n= 10). [a,b]Significant differences between control and chemically stressed groups in the same tissue.

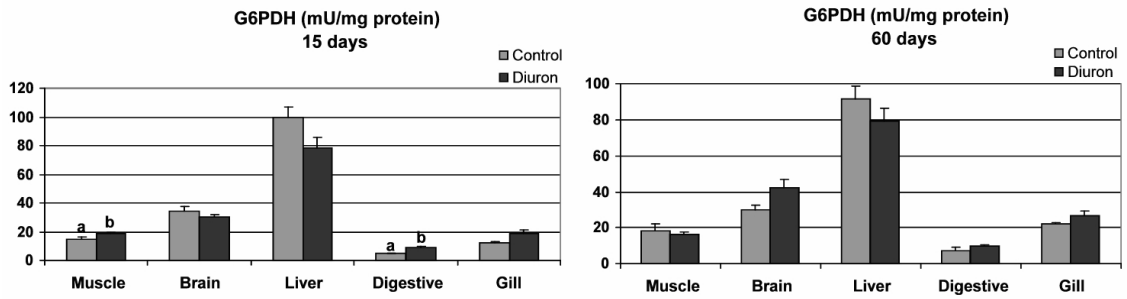

Fig. 3: Effects of diuron exposure (0.20 mg/L, 24 hours per week) on the activity of G6PDH in tissues from sea bream at 15 and 60 experimental days. Values are expressed as mean ± SEM (n= 10). [a,b]Significant differences between control and chemically stressed groups in the same tissue.

between GPX and GR (Pearson correlation coefficient: 0.545, $p < 0.01$) and GPX and G6PDH (Pearson correlation coefficient: 0.434, $p < 0.01$) in fish from control. In addition, a positive correlation was also found between GPX and SOD (Pearson correlation coefficient: 0.736, $p<0.01$ and 0.665, $p < 0.01$) in control and treated fish respectively. There was positive correlation between GPX and CAT (Pearson correlation coefficient 0.368, $p < 0.05$

and 0.597, $p < 0.01$) in control and treated fish respectively. There was positive correlation between GPX and GST (Pearson correlation coefficient 0.702, $p < 01$ and 0.654, $p < 0.01$), in control and treated fish respectively and between GPX and DTD (Pearson correlation coefficient 0.742, $p < 0.01$ and 0.462, $p < 0.01$) in the control and in diuron-exposed fish. There was a negative correlation between G6PDH and MDA (Pearson correlation coefficient: 0.405, $p < 0.01$).

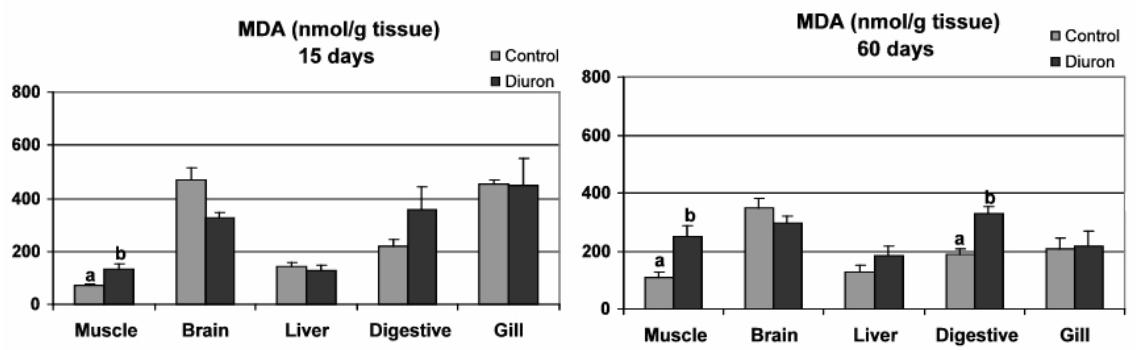

Fig. 4: Effects of diuron exposure (0.20 mg/L, 24 hours per week) on lipid peroxidation (MDA content) in tissues from sea bream at 15 and 60 experimental days. Values are expressed as mean ± SEM (n= 10). [a,b]Significant differences between control and chemically stressed groups in the same tissue.

Discussion

The alteration of the process of oxidative stress is a typical response to toxicity induced by xenobiotics (Lemaire and Livingstone, 1993; Di Giulio *et al*., 1995; Sheehan and Power, 1999; Hai *et al*., 1997; Pena-Llopis *et al*., 2003). This fact may change the dose considered as sub-toxic (non-lethal) of many contaminants and be a sensitive indicator of exposure to these agents. In addition, all this can help establish real safety limits for the production and the health of the fish.

In this experiment, we studied the oxidative status of gilthead sea bream that were exposed to a low dose (0.20 mg/L) (15 times less than the LC50 (96h) indicated for fish) of the herbicide diuron 1 day per week, during 8 weeks. This diuron concentration doesn't lead to mortality of fish or reduction of growth (weight and length), which was similar to studies done by Sánchez-Muros *et al*. (2013).

The results of this study demonstrate that the gilthead sea bream liver has the highest antioxidant capacity, this fact would be corroborated by the high production of FRAP found in this tissue (Sanchez-Muros *et al*., 2013). Similar data were obtained in our

studies with trout (*Oncorhynchus mykiss*) and sturgeon (*Acipenser naccarii*) (Trenzado *et al*., 2006).

The fish liver is an aerobic tissue with a high potential for ROS generation, which is efficiently counterbalanced by powerful protective mechanisms that detoxify and repair damaged tissues. Moraes *et al.* (2007) suggested that the oxidative stress generated by herbicide-containing water suppresses the activity of antioxidant defence enzymes due to oxidative damage and loss of the compensatory mechanisms. Glusczak *et al.* (2007) found that silver catfish (*Rhamdia quelen*) exposed to low doses of the herbicide Roundup had a decrease in antioxidant enzymes. This fact appears to be related to their inactivation by an excess of ROS. An increase of ROS production in the liver and muscle has been described in gilthead sea bream subjected to treatment with diuron (Sánchez-Muros *et al.* 2013).

Tab. 1: Effects of diuron exposure (24 hour exposure each week to 0.20 mg/L) on antioxidant status of gilthead sea bream at 15 and 60 experimental days.

	Tissue									
	Muscle		**Digestive**		**Brain**		**Gill**		**Liver**	
	15 days	60 days	15 days	60 days	15 days	60 days	15 days	60 days	15 days	60 days
SOD	*			*	*	*				
CAT										
GPX	*	*								
GR	*		*	*						
GST			*						*	
DTD			*	*				*		*
G6PDH			*							
MDA	*	*		*						

***Significantly different from the corresponding control group, P≤0.01. n=10**

The results of enzymes activities (Fig. 1, 2 and 3) showed a significant decrease and at times an increase in antioxidant enzymes were found in fish exposed to diuron (Fig. 1. 2 and 3). These changes occur in the digestive tract and the muscle due to the activities of SOD, GPX GR and G6PDH and in liver, digestive and gill due to GST and DTD activities (Tab. 1). Sanz *et al*. (2012) found similar enzymatic behaviour, increases and decreases of several

antioxidant enzymes in different tissue of gilthead sea bream subjected to handling stress. An increase in fish antioxidant enzyme activity in response to environmental stress has been noted in many reports (Winston, 1991; Valavanidis *etaAl*., 2006; Craig *et al*., 2007). Increased GST activity has been demonstrated in the liver of various species of fish following exposure to PCBs (Pérez- Lòpez *et al*., 2002; Schmidt *et al*., 2004), PAHs (Noble *et al*., 1998; Henson *et al*., 2001), and some other pesticides. There are also studies showing a decrease of antioxidant enzyme activities under conditions of oxidative stress. Studies have demonstrated that SOD is inhibited by hydrogen peroxide (Bray *et al*., 1974; Liu and Wu, 2006), catalase is inhibited by superoxide anion and hydrogen peroxide (Bray *et al*., 1974; Liu and Wu, 2006), and GST is sensitive to products of the Haber-Weiss reaction (Hermes-Lima and Storey, 1993). More precisely, Lushchak *et al*. (2009) found that exposure to Roundup (2.5–20 mg/L) substantially decreased SOD, GR and G6PDH activities in the brain, the liver and the kidney of goldfish (*C. auratus*) and GST activity were reduced only in the liver.

The results show a decrease in most enzymatic activities in fish exposed to diuron. The interpretation of this behaviour without taking into account oxidative damage is impossible, since it is difficult to determine if the decrease or no change in enzymatic activity is related to the absence of antioxidant requirement or to enzymatic exhaustation/inhibition associated with oxidative stress. Therefore the results of this experiment, same as those reported by other authors, indicate that an alteration, increase or decrease, of antioxidant enzymes activities could be used as stress indicator.

The lipid peroxidation (MDA content) in different tissues (Fig. 4), showed a significant increase in the digestive tract tissue after 60 days exposure and in the muscle tissue after 15 and 60 days exposure in the diuron-treated fish compared with the non-treated control fish. In addition, the biggest enzymatic changes were seen in these tissues (Table 1). These results also suggest that an excess of formation of oxidative agents due to exhaustion and/or inhibition causes the decrease in antioxidant enzyme activities.

On the other hand, a significant positive correlation between the activities of the most studied enzymes (SOD and CAT , GPX and SOD, GPX and CAT, GPX and GST, GPX and DTD in control and fish exposed to diuron; between GPX and GR and GPX and G6PDH in fish control) was found. On the other hand, there was a negative correlation between G6PDH and MDA which would indicate the importance of the enzyme as a reducing agent. G6PDH is a cytosolic enzyme of the pentose phosphate pathway that is involved in different metabolic processes and catalyses the conversion of glucose-6-phosphate to 6-phosphogluconate

with the generation of NADPH from $NADP^+$. NADPH is a molecule that helps the antioxidant system to regenerate reduced glutathione (GSH) through the activity of GR.

These results indicate that antioxidant enzymes work together to prevent oxidative imbalance. The MDA rates and enzyme activities show that in muscle and digestive tract of fish exposed to diuron, a major imbalance occur and consequently oxidative stress. Digestive system and gills are the organs more exposed to the environment, and interestingly the activities of detoxifying enzymes GST and DTD undergo significant changes in these tissues in animals subjected to diuron. This could have prompted the protection of gill tissue oxidation. Also it should be noted that brain tissue seems to be protected from the toxicity of diuron as the only difference found with respect to the fish brain controls has been an alteration of SOD activity only after 15 days of treatment with diuron (Table 1, Fig. 1 and 2).

In summary, the results show that exposure of gilthead sea bream to low concentrations of diuron (0.20 mg/L, 1 day per week, during 8 weeks), induces effects that can be seen after 15 days of treatment. The antioxidant enzymes response varied depending on the enzyme and on the tissue. The digestive tract tissues were more affected by diuron treatment at 30 and 60 days. The brain, gills and liver were affected to a lesser degree. These results highlight the need for further study on the dose that is actually harmless. On the other hand, the muscle and liver MDA concentration can be used as biomarker of alterations in the oxidative status of fish and therefore a tool for assessing health and understanding of welfare in fish farming.

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