Histol Histopathol (2005) 20: 1017-1020
DOI: 10.14670/HH-20.1017

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Time course variations of antioxidant enzyme activities and histopathology of gilthead seabream gills exposed to malathion

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Summary. In a widely distributed and commercially important fish, gilthead seabream *Sparus aurata L.*, we have studied sublethal effects of malathion in order to identify early warning bioindicators of exposure before irreversible damage occurs.

To achieve this goal, groups of 10 juvenile specimens were exposed for 24, 48, 72 and 96h to a sublethal concentration of malathion (0.4 mg/l). Another group was used as control. The activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and histopathological features from exposed gills were assessed. It should also be mentioned that no mortality was observed during the whole experience.

The activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) were altered significantly from 24 h onward ($p<0.05$). It is of interest to note that catalase activity was decreased after exposure instead of increasing as other antioxidant enzymes assessed. On the other hand, histopathological alterations of the gills were observed as early as at 48 h-exposure, but the most severe damage occurred at 96 h exposure.

The evidence presented here, together with other data from the literature, unequivocally established oxidative-stress-inducing effects of malathion in gilthead seabream *Sparus aurata*. It is also concluded antioxidants employed (SOD, CAT and GPX) changed significantly a long time before histopathological alterations of gills became evident. Consequently, these antioxidant enzymes may be highly recommended as early-warning bioindicators of environmental pollution by malathion in the areas where it is proposed to be used in pest control activities.

Key words: Antioxidant enzymes, Histopathology, Gills, *Sparus aurata*, Malathion

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Introduction

Since the discovery of the importance of radical reactions in biological processes, there has been an explosion of research into pro-oxidant and antioxidant processes, principally in mammalian systems (Caglar et al., 2003; Tsanou et al., 2004). Antioxidant enzymes of fishes, that play a crucial role in maintaining cell homeostasis, have received much attention in ecotoxicology since oxidative damage was considered a mechanism of toxicity in aquatic organisms exposed to environmental contaminants in general (Santos et al., 2004) and organophosphates in particular (Hai et al., 1997). In addition it has been also published that acute exposure to the latter pollutants may lead to histopathological alterations in different organs of exposed specimens (Fanta et al., 2003).

Organophosphate insecticides, especially malathion, are commonly used for mosquito and fruit fly control because of their low mammalian toxicity and relatively short half-life. Further, they have been also used in fish-farming tanks to eliminate aquatic larval stages of insects (Silva et al., 1993). However they are prone to contaminate surface waters that may finally lead to the exposure of non-target organisms such as fish.

Gilthead seabream, *Sparus aurata* (L.), is a high level protein and commercial species much appreciated in the Mediterranean countries where intense fish culture activities are carried out (Ortuno et al., 2000). Accordingly, it has been widely studied not only to improve its farm production but also to assess the influence of some xenobiotics on saltwater fish (Rosety et al., 2001, 2003). Further, this study was focussed on gills because they have been considered one of the main targets for many aquatic pollutants (Rosety-Rodriguez et al., 2002).

For the reasons already mentioned this experimental design was conducted to assess chronologically antioxidant response and histopathological features of gilthead seabream gills after acute exposure to malathion

in order to assess their potential role as early-warning bioindicators.

Materials and methods

A total of 50 juvenile specimens of the teleost gilthead seabream, *Sparus aurata*, L., with a mean ± SE mass of 75±6.3 g were acquired from a commercial hatchery at San Fernando (Cádiz, Spain) and allowed to acclimatise to the dilution seawater used in the tests for 20 days before the experiment. Animals were fed daily with commercial pellets.

After that, fish were randomly allocated to control group and to a sublethal concentration of malathion of 0.4 mg/l for 24 h (Group A), 48 h (Group B), 72 h (Group C) and 96 h (Group D). This concentration was taken as representative of low-level insecticide contamination following literature references (Howard, 1991). Certified solution (96.7% of active ingredient) of malathion (O,O-dimethyl phosphorodithioate of dimethyl mercaptosuccinate) was used as the contaminant (American Cyanamid Co.).

Both control and experimental groups with ten individuals in each one were maintained in 500 l polyethylene tanks with constant aeration and a 12 h light/dark artificial light cycle. The water was changed daily (remaining food, feces and pseudofeces were removed) until the end of the experiments.

At the end of the 24, 48, 72 and 96 h of exposure, fish were sacrificed. To avoid excessive stress during handling fish were introduced for a few minutes into a 20 l container with 10 mg/l of quinaldine sulphate. Then, the anaesthetised fishes were decapitated.

Gill tissues were dissected, washed in physiological saline solution (0.9% NaCl) and frozen at -85°C until required for use. The tissues were homogenized by glass Teflon-homogenizer (Heidolph S01 10R2RO) in 1:10 w/v cold 1.15% KCl solution and then centrifuged at 9500xg for 30 min in a Sorvall RC2B centrifuge at 4°C. Supernatants were used to determine the antioxidant enzyme activities: superoxide dismutase (SOD, E.C. 1.15.1.1) by McCord and Fridovich (1969), catalase (CAT, 1.11.1.6) by Beutler (1975) and gluthathione peroxidase (GPX, E.C. 1.11.1.9) by Floche and Gunzler (1984).

For histopathological assessment, samples were fixed in 10% v/v formol buffered with 0.1 M phosphate buffer, pH 7.2, dehydrated in increasing concentrations of alcohol, cleared with benzol and finally embedded in semisynthetic paraffin wax with a mean fusion point of 54-56°C. Sections were cut at 5 mm. Harris´ hematoxylin and acetic eosin and Harris' hematoxylin-VOF (Gutierrez, 1967) were employed as general stain.

Results were expressed as mean ± SD. The statistical significance of the differences between values of control and at the different days of sampling was evaluated by Student´s t-test for unpaired data by mean of the Statistical Analysis System (SPSS 11.0). The significance of the results was ascertained at $p<0.05$ (Zar, 1999).

Results

Antioxidant activities of SOD, CAT and GPX are listed in Table 1. Significant modifications in gill tissues of all of the above detoxification enzymes were evident from 24-h onward ($p<0.05$). It should also be pointed out that catalase activity was decreased instead of increasing as other antioxidant enzymes assessed. In any case, these alterations were all time-dependent.

Sections of untreated specimens revealed histological patterns similar to that described previously for giltheads (Ribelles et al., 1995) and for other teleost fish (Laurent and Dunel, 1980). In this line, gill arches contain primary lamellae each one with respiratory lamellae (secondary lamellae) lined up along both of its sides, as is usual for teleosts. Primary lamellae are covered by stratified squamous epithelium and they serve more as support for the secondary lamellae than for respiration. The surface of the respiratory lamellae is covered with simple squamous epithelium and blood spaces are delimited by pillar cells separated from the epithelium by a thick basal membrane. In addition the respiratory epithelium contains specialized chloride cells that assist with osmoregulation by excreting chloride, potassium and sodium ions.

Regarding histopathology, the tendency of some secondary lamellae to fuse were found at 48 h of exposure. At 72 h exposure, histological examination revealed the clubbing and fusion of secondary lamellae as well as the hyperplasia of the respiratory epithelium and a thickening of the basal membrane in the secondary lamellae. At 96 h, histopathological features seen before were more generalized. Further, detachment of the epithelial layer caused by strong edema at the primary lamellae and some areas with generalized shrinkage of the lamellar cells at the secondary lamellae were also observed (Fig. 1).

It should also be mentioned no mortality was observed during the whole experience.

Table 1. Antioxidant enzyme activities in gill tissues from gilthead seabream, *Sparus aurata* L. , exposed to a sublethal concentration of malathion (0.4 mg/l) for 24, 48, 72 and 96 h.

	SOD (U/mg Prot)	CAT (U/mg Prot)	GPX U/mg Prot)
Control	0.38±0.04	7.01±0.73	0.10±0.02
Group A	0.47±0.06*	6.54±0.49*	0.19±0.05*
Group B	0.54±0.05*	6.11±0.52*	0.26±0.05*
Group C	0.59±0.07*	5.80±0.33*	0.31±0.08*
Group D	0.62±0.09*	5.67±0.47*	0.36±0.07*

Control: No exposure; Group A: 24 h exposure; Group B: 48 h exposure; Group C: 72 h exposure; Group D: 96 h exposure. Results are expressed as mean ± SD (n=10). *: $p < 0.05$.

Discussion

The majority of research done with organophosphorus pesticides is based on their lethal effects. However, knowledge of the sublethal effects of these compounds seemed to be highly important in order to identify early warning bioindicators of exposure before irreversible damage occurs.

In this line, the redox status of fishes has received much attention in recent years (Pena-Llopis et al., 2003). The findings of the present investigation indicated the possible involvement of free radicals in malathion-induced toxicity and highlight the protective action of antioxidant enzymatic system from tissues such as gills.

Antioxidant enzyme activities of gilthead seabream, such as Sparus aurata, discussed here, could only be compared with other species exposed to organophosphates, since no data were found in the literature for this species. In this respect, increased activities of antioxidant enzymes have been found previously in both freshwater (Hai et al., 1997) and marine fish (Pedrajas et al., 1995) exposed to organophosphates. Regarding Sparus aurata, significant changes in antioxidant enzyme system (SOD, CAT and GPX) have been also described in specimens exposed to cadmium (Vaglio and Landriscina, 1999). Further, it should be noted that comparisons must be taken with caution given that remarkable differences can be obtained depending on species habitat and feeding behaviour (Ahmad et al., 2000). In any case, the data obtained in this study could be useful as reference values for further studies focussed on this topic.

Given its role in the antioxidant defense system, catalase activity was expected to increase as exposure time increased. However, it is interesting to note that catalase activity was not increased as with other examined antioxidants but decreased as was also reported recently by Pascual et al. (2003) in gilthead seabream, S. aurata, exposed to food deprivation and Sayeed et al. (2003) in freshwater fish, Channa punctatus Bloch, exposed to pesticides. The explanation of why catalase activity decreased after exposure remains to be determined but it could be provoked by the flux of superoxide radicals, (O_2^-) induced by the pollutants (Ahmad et al., 2000).

In general terms, when compared with other tissues, such as liver or kidney, it is observed that gills antioxidant enzyme potential is very poor as was suggested previously by different studies (Pandey et al., 2003; Sayeed et al., 2003).

The present work has also revealed that the gills of Sparus aurata were affected after acute exposure to a sublethal concentration of malathion, but later than antioxidant enzyme activities. In this line, histopathological features from exposed gills were observed as early as at 48 h exposure, but the most severe damage occurred at 96 h exposure. In general terms these alterations were time-dependent and similar to those observed previously by Cengiz and Unlu (2003) and Fanta et al. (2003). Further, Dutta et al. (1996) reported that the gills of H. fossilis were highly affected by a sublethal dose of malathion as early as at 24 h exposure. It may be explained since they used a higher concentration to assess sublethal effects.

The evidence presented here, together with other data from the literature, unequivocally established oxidative-stress-inducing effects of malathion in gilthead seabream *Sparus aurata*. It is also concluded that antioxidants employed (SOD, CAT and GPX) changed significantly after 24 h exposure, a long time before histopathological alterations of gills became evident. Consequently, these antioxidant enzymes may be highly

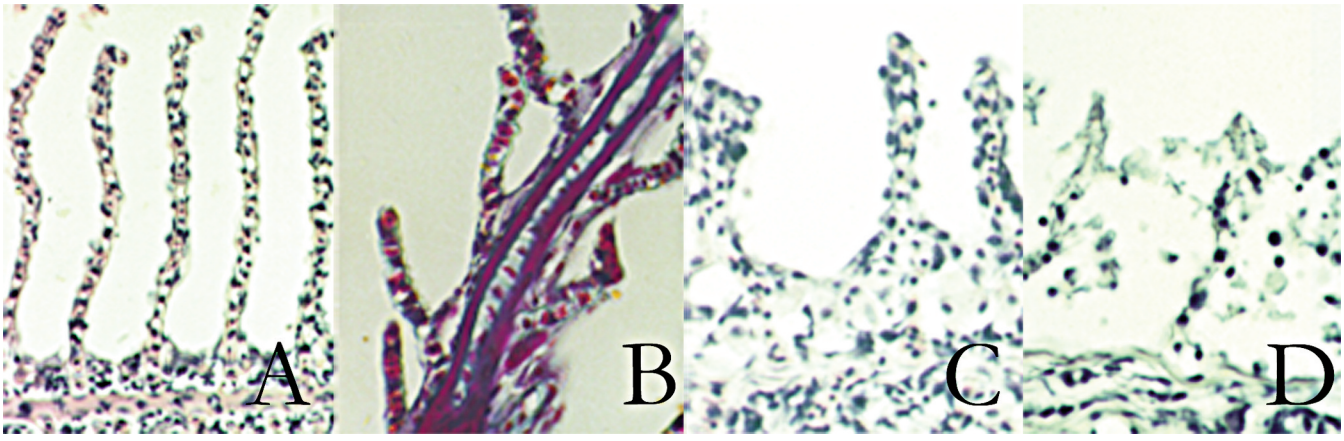

Fig. 1. Gilthead seabream gills from control and exposed specimens to a sublethal concentration of malathion (0.4 mg/l), stained with Harris´ hematoxylin and acetic eosin and Harris' hematoxylin-VOF (Gutierrez, 1967). **A.** control individuals. **B.** At 48 h exposure, the tendency of some secondary lamellae to fuse was observed. **C.** After 72 h exposure, histopathological assessment revealed the hyperplasia of the respiratory epithelium as well as the shortening of lamellae. **D.** At 96 h, the detachment of the epithelial layer at the primary lamellae as well as some areas with shrinkage of the lamellar cells were evident. x 400

recommended as useful early-warning bioindicators of environmental pollution by malathion in the areas where it is proposed to be used in pest control activities.

Acknowledgements. This research has received financial support from a National Grant (Nº Exp.: 01/0250). Instituto de Salud Carlos III. Ministerio de Sanidad y Consumo. España.

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Accepted April 22, 2005