I.J.S.N., VOL. 2(2) 2011: 279-286 ISSN 2229 – 6441

INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

www.scienceandnature.org

EFFECT OF CHRONIC POLLUTION ON HEPATIC ANTIOXIDANT SYSTEM OF BLACK SEA FISH SPECIES

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ABSTRACT
Hepatic antioxidant enzyme activities of four Black Sea teleost fish species inhabited Sevastopol Bay (Black Sea, Ukraine) at the period of 1991-1995 and 2001-2006 were studied. The antioxidant enzymes included superoxide dismutase (SOD), catalase (CAT), peroxidase (PER) and glutathione reductase (GR). Significant interspecies differences were evidenced which were much more higher in 1999s as compared to 2000s. Hepatic antioxidant enzyme system of examined fish species was modified during the period of 10 years and it was adopted to the changes of ecological status of Sevastopol Bay. In spite of pollution decrease in 2000s as compared with 1990s the hepatic biomarkers were not restored to the level of 1990s and were elevated at another functional level resulted the chronic contamination in 2000s. The biomarker responses were generally higher in benthic *S. porcus* than those in pelagic *T. mediterraneus.* The response of suprabenthic and suprabenthic-pelagic fish species was intermediate between benthic and pelagic forms. The obtained results demonstrated the main trends of hepatic antioxidant enzyme system in two sampling periods and can be applied for development monitoring management and for perspectives of conservation ecology and biodiversity.

KEY WORDS: antioxidant enzymes, Black Sea fish, liver, chronic pollution, ecological variations

INTRODUCTION

Long-term and large-scale monitoring studies indicate the changes of anthropogenic impact on the water ecosystems, which can be chronically stressed by multiple environmental factors. These stressors may vary both spatially and temporally and combined with synergetic and cumulative effects and caused the responses in biota (Adams, 2001). Usually the investigators demonstrate the elevated levels of the man-made pollution and its negative effects on marine organisms in all levels of their biological organization (Adams *et al*., 1996; Balk *et al*., 1996). Such a situation was described for Black Sea (Rudneva and Petzold-Bradlry, 2001). Sewage and chemical pollution from industrial, agricultural, transport and domestic sector are the main contribution of the Black Sea. Sewage is an important source of organic substances, heavy metals, persistent organic pollutants, detergents and pathogenic bacteria. The coastal areas are the main recipients of discharges and combine effects of the various kinds of contaminants resulted dramatic ecological consequences such as eutrophication, biodiversity loss, elimination of some species, worsening of their health and decline the population size.

In polluted areas the exposure of aquatic organisms to xenobiotics results to interaction between these compounds and biological systems which may give elevation to biochemical and physiological damage or/and adaptive mechanisms via the induction of defense immune and antioxidant systems (Goksoyr *et al*., 1996). Biochemical and physiological parameters are used as biomarkers for contaminants and could be applied for evaluation of environmental stress and its after-effects. Biomarkers exposure to environmental stressors vary widely depending on the type of anthropogenic activity involved (Adams, 2001). However, a clear changes and general trends were not found since in most cases the response is depended on species biology, examined tissues and parameters and/or time and season (Hotard and Zou, 2008; Martinez-Alvarez *et al*., 2005).

Fish are very sensitive to anthropogenic pollution and some of them may be tested as biomonitors for the evaluation of the ecological status and risk assessment of marine environments. Fish biochemical parameters could be directly related to the area the fish were sampled (Balk *et al*., 1996). Previously we described the variation in blood antioxidant enzymatic system of some Black Sea elasmobranch and teleosts which reflect the adaptive strategy of fish and their ability to cope with the environment (Rudneva, 1997). Then we determined the age-related blood antioxidant enzyme fluctuations and demonstrated their associations with fish biology and ecology (Rudneva *et al*., 2010). At present study we selected hepatic antioxidant enzymes because liver is the organ with high metabolic activity where the main biomolecules are synthesis and xenobiotics are metabolized. Information of comparative studies of hepatic antioxidant enzymes of animals of different classes is very limited and to the best of our knowledge it needs to investigate of a large number of species (Rocha-e-Silva *et al*., 2004). Additionally hepatic biomarkers are applied in international monitoring programs and scientific communities for the evaluation of contamination effects on marine ecosystems (Goksoyr *et al*., 1996; Sole *et al*., 2009). Thus the further study of the biochemical mechanisms of fish resistance against environmental stress led the anthropogenic pollution is very important for the understanding the different ways of adaptations of aquatic organisms and for risk assessment.

Many environmental factors and chemical pollutants induce the generation of reactive oxygen species (ROS).

Special adopted enzymes defense organisms against oxidative stress and damage. In our study we used hepatic biomarkers superoxide dismutase (SOD), catalase (CAT), peroxidase (PER), and glutathione reductase (GR). SOD protects against oxidative damage by catalyzing the reaction of dismutation of the superoxide anion to H_2O_2, which removes by CAT to H_2O. PER reduces both hydrogen peroxide and hydroperoxides also. GR maintains the homeostasis of a ratio of GSH/GSSG under oxidative stress (Livingstone, 2001; Richardson *et al*., 2008). The aim of the present work was to compare the trends of hepatic antioxidant enzymes activity in four Black Sea teleost fish species inhabited Sevastopol Bay (Black Sea, Ukraine) at the period of 1991-1995 and 2001-2006.

MATERIAL AND METHODS

Description of sample site

Fish were performed at Sevastopol Bay (Black Sea, Ukraine) at the period of 1991-1995 and 2001-2006. The is influenced by high anthropogenic impact including domestic, industrial and agricultural sewage which input in the marine area (Fig. 1). The effluents contain broad spectrum of chemicals such as biogens, heavy metals, chlorinated hydrocarbons, synthetic detergents, oil, etc. More then 30 treatment points act at the coastal area of Sevastopol Bay and the total volume of their discharges is estimated approximately 10-15 000 m^3 per day (Pavlova *et al*., 1999). Additionally pollution of the marine area associated with maritime transport and shipyard activities.

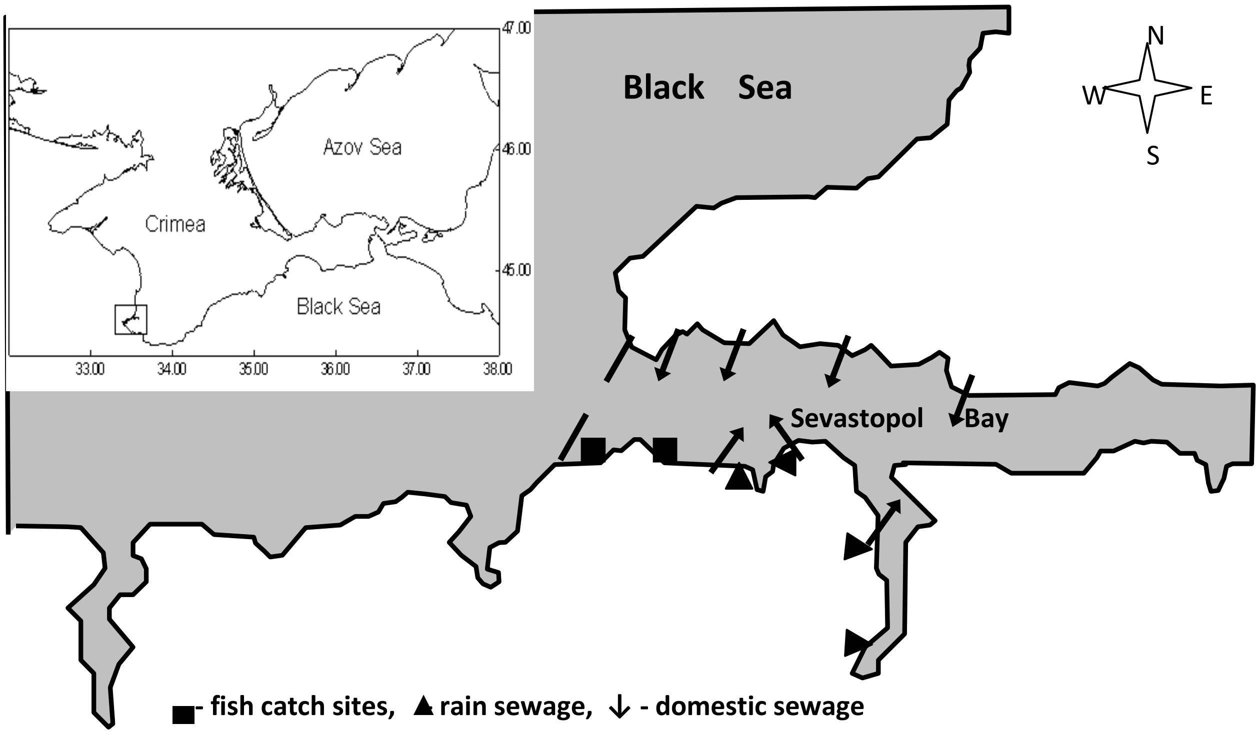

Fig.1. Sampling sites of fish specimens in Sevastopol Bay (44°36'N - 33°32'E, Sevastopol, Black Sea, Ukraine)

Sample collection and preparation

Four highly distributed Black Sea fish species were investigated: horse mackerel *Trachurus mediterraneus* (Steindachner), high body pickerel *Spicara flexuosa* (Rafinesque), red mullet *Mullus barbatus* (Essipov), scorpion fish *Scorpaena porcus* (L.). The species were classed in four groups: pelagic (*T. mediterraneus)*, suprabenthic/pelagic (*S. flexuosa),* suprabenthic (*M. barbatus)* and benthic (*S. porcus).*

The fish were captured by net with a mesh size of 5 mm in summer season in Sevastopol Bay in two periods of 1991-1995 and 2001-2006. The animals were immediately placed in the aerated tank and anesthesy.

Fish were individually measured, weighed and immediately processed for biochemical analysis. Liver was excised, had been washed three timed in cool 0.85% solution of NaCl and immediately homogenized in it and centrifuged at 8 000 g during 30 min at cool temperature. The supernatants were used for further biomarkers assay immediately after preparation.

Enzymatic activities assays

Antioxidant activities in fish liver were determined according to the methods which we described previously with small modifications (Rudneva, 1997).

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed on the basis of inhibition of the reduction of nitroblue tetrasolium (NBT) with NADH mediated by phenazine methosulfate (PMS) under basic conditions (Nishikimi *et al*., 1972). All measurements were performed in 0.017 M sodium pyrophosphate buffer pH 8.3 at 20 °C. The reaction mixtures contained 5 μM NBT, 78 μM NADH, 3.1 μM PMS and 0.1 ml sample; the final volume was 1.5 ml. The reaction was carried out in a spectrophotometer Specol-211 (Germany) at 560 nm.

Catalase (CAT, EC 1.11.1.6) was measured by the method involving the reaction of hydroperoxide reduction. Sample (0.5 ml) was added to 7.5 ml distilled water, containing 1 ml 1% solution of H_2O_2.The mixture was incubated for 30 min at room temperature and then the reaction of hydroperoxide reduction was stopped by adding of 10%

H_2SO_4. The titration of 1 N solution of $KMnO_4$ was carried out for determination of the content of H_2O_2 reduction by CAT.

Peroxidase (PER, EC 1.11.1.7) activity was detected by spectrophotometric method using benzidine reagent (Litvin, 1981). The reaction mixture contained 1 ml acetate buffer pH 5.4, 0.4 mL 0.09% benzidine, 0.2 ml 0.03% H_2O_2, and 0.2 ml sample. The reaction followed in a spectrophotometer for 1 min at 20 °C and at 600 nm.

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed spectrophotometrically using a method modified after Goldberg and Sparner (1987). The reaction mixture contained 0.1 ml mM NADPH, 0.5 mL 7.5 mM oxidized glutathione, 0.2 ml mM EDTA, and 2 ml 0.05 M phosphate buffer pH 8.0. After incubation for 10 min, the extinction of the mixture was determined at 340 nm.

Protein concentration in liver extracts was determined by the method of Lowry *et al*. (1951).

Statistical analysis

Statistical differences were processed using Student's t-test which was applied for pair-wise testing for fish hepatic antioxidant enzymes activity between two examined periods. All numerical data are given as means ± SEM (Halafyan, 2008). The significance level was 0.05.

RESULTS

SOD activity

SOD activities in the liver of examined fish species is shown in Fig. 2. Enzymatic activity was significantly higher ($P<0.05$) in fish collected at 2000s as compared with the period of 1990s. There are some interspecies differences of SOD activity in two periods also. At 1990s the lowest value was observed in *T. mediterraneus* while the highest was demonstrated in *S. flexuosa*. In 2000s the opposite tendency was indicated: the highest enzymatic activity was shown in *T. mediterraneus* liver and the lowest in *M. barbatus*.

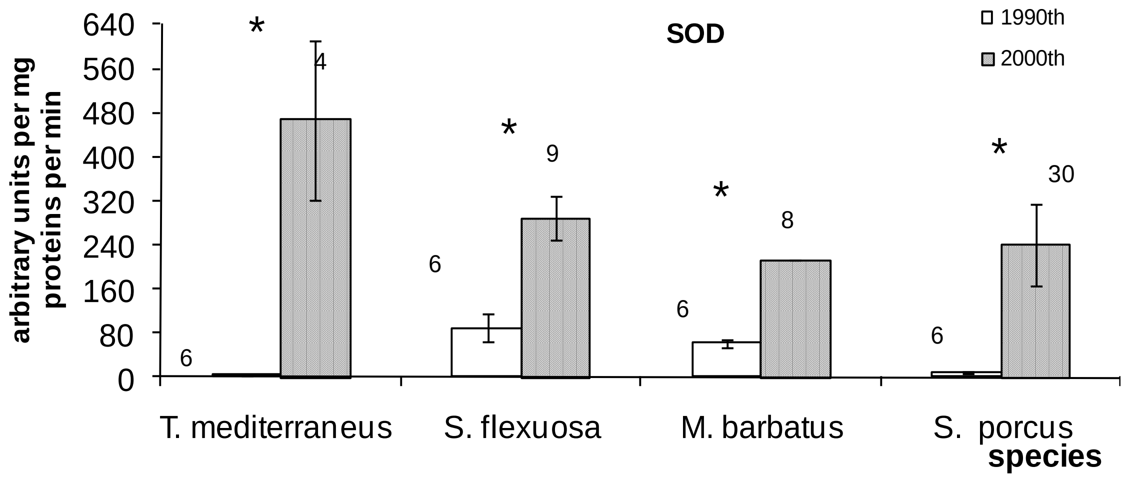

Fig 2. SOD activities in the liver of teleost fish species (arbitrary units per mg proteins per min, mean ± SEM) caught in Sevastopol Bay (Black Sea, Ukraine). Significant differences between examined periods according to Student t-test are indicated by an asterisk (*) at P≤ 0.05.

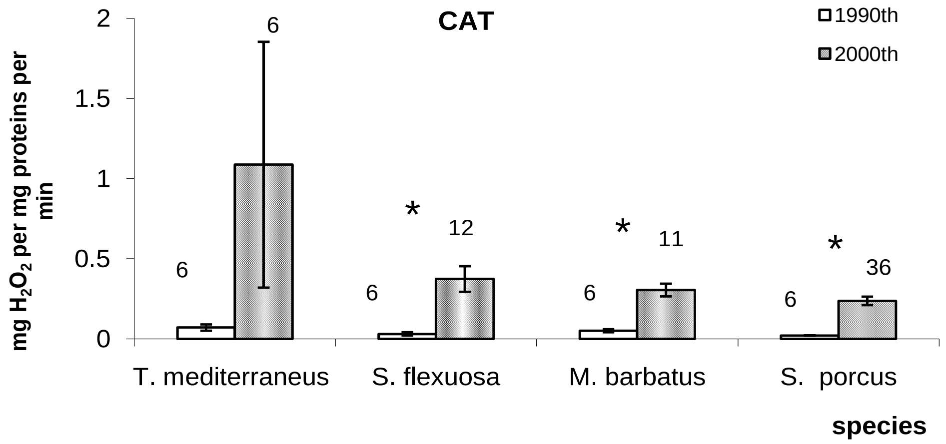

Fig 3. CAT activities in the liver of teleost fish species (mg H_2O_2 per mg proteins per min, mean ± SEM) caught in Sevastopol Bay (Black Sea, Ukraine). Significant differences between examined periods according to Student t-test are indicated by an asterisk (*) at P ≤ 0.05.

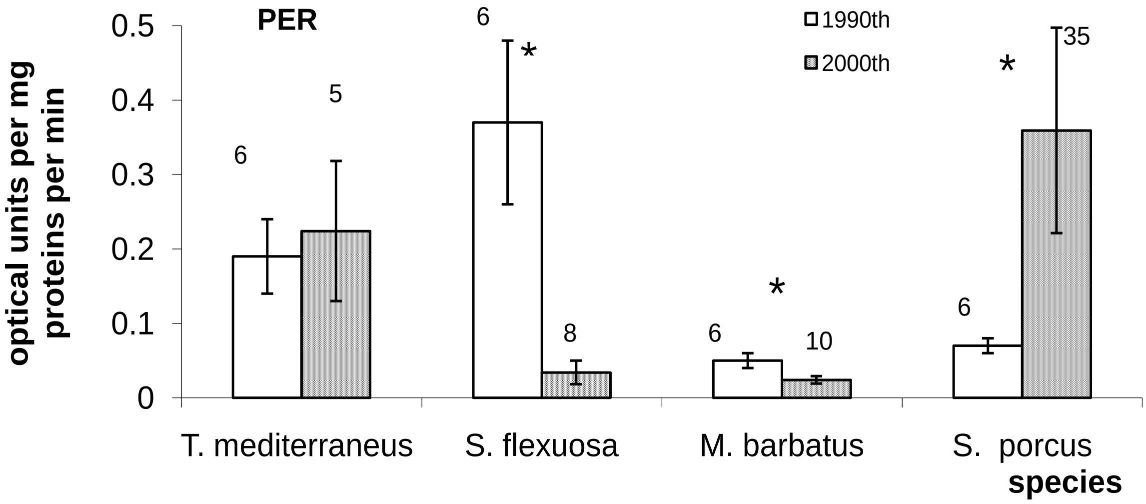

Fig 4. PER activities in the liver of teleost fish species (optical units per mg proteins per min, mean ± SEM) caught in Sevastopol Bay (Black Sea, Ukraine). Significant differences between examined periods according to Student t-test are indicated by an asterisk (*) at P ≤ 0.05.

CAT activity

Changes in hepatic CAT activity is presented in Fig. 3. The enzyme activity was increased significantly (P<0.05) in the liver of all examined fish species caught in 2000s with the exception of *T. mediterraneus* in which the changes were not significant. The highest value of CAT activity in 1990s was shown in the liver of *T. mediterraneus* and the lowest in *S. porcus*. Such a tendency was kept in 2000s also.

PER activity

No significant period-related changes in PER activity was observed in *T. mediterraneus* (Fig. 4). The enzymatic activity in the liver of *S. flexuosa* and *M. barbatus* was significant lower (P<0.05) in the period of 2000s as compared to 1990s, while in *S. porcus* it elevated (P<0.05). The highest values of hepatic PER activity in 1990s were detected in *S. flexuosa* and the lowest in *M. barbatus*. In 2000s the highest activity was indicated in *S. porcus* and the lowest in *M. barbatus*.

GR activity In

M. barbatus and in *S. porcus* hepatic GR activity was significantly (P<0.05) increased in 2000s as compared to 1990s (Fig. 5). In *T. mediterraneus* and *S. flexuosa* it elevated also but the values were not significant. In 1990s the highest hepatic GR activity was detected in *S. flexuosa*, and the lowest in *S. porcus*. In 2000s the highest value was indicated in *T. mediterraneus* and the lowest in *S. porcus*

The obtained results demonstrated that the environmental pollutants are changing the hepatic level of prooxidants in two examined periods and leading to differentiated enzyme response in 1990s and 2000s.

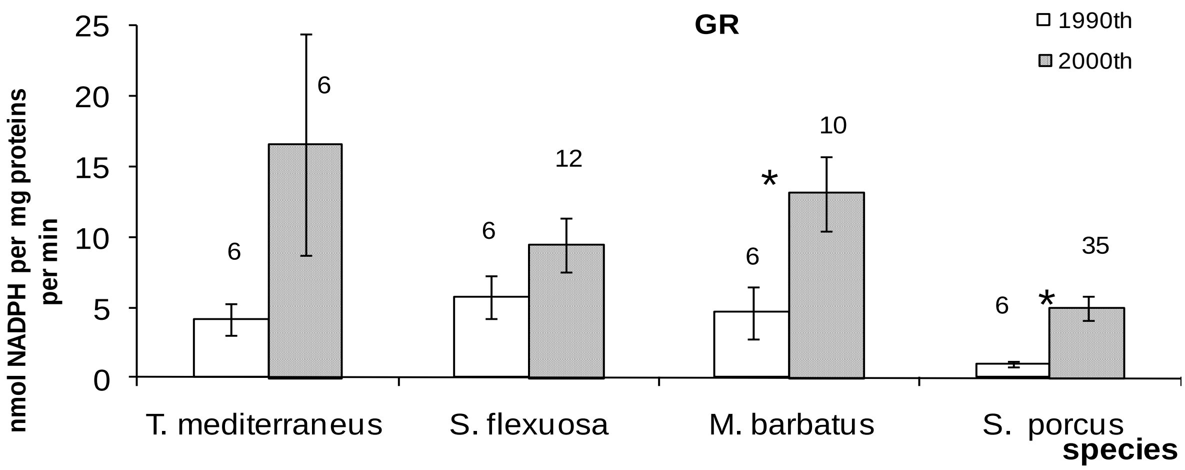

Fig 5. GR activities in the liver of teleost fish species (optical units per mg proteins per min, mean ± SEM) caught in Sevastopol Bay (Black Sea, Ukraine). Significant differences between examined periods according to Student t-test are indicated by an asterisk (*) at P ≤ 0.05.

DISCUSSION

Ecological status of Sevastopol Bay in the periods of 1991-1995 and 2001-2006

Sevastopol Bay is highly polluted enclosed marine area which total water turnover is very low. Marine transport traffic and sewage effluents contribute to the release of the waste in it. There are 30 outlets through which 15 000 cubic meters of liquid waste water enter its area per day. Annually 19 t of petroleum, 96 t of detergents, 9 t of heavy metals and 14 000 t of particular organic matter are dumped in Sevastopol Bay (Gordina *et al*., 1999). The highest polluted levels were indicated at the period of the end of 1980s to the beginning of 1990s.

Since the end of 1990s and at the present time the content of pollutants was decreased caused the economic crisis but not the ecosystem protective actions and special remedies. For example the phosphates concentrations were decreased to the level of 1946-1955, such a tendency was indicated for heavy metals and pesticides contents in the marine water (Pavlova *et al*., 1999). The highest concentration of PCB was detected in 1991 (0.33 μg per l) then its level was declined to 0.036 μg per in 1999 and to 0.010 μ g per l in 2000 (Jerko *et al*., 2001). Thus the changes of anthropogenic impact upon Sevastopol Bay has been caused the response in biota and changed its biological status including examined fish species.

Interspecies differences of fish hepatic antioxidant enzyme activities in two periods

Responses of organisms to pollutants can be modified by the environmental factors including physicochemical, biological and ecological. The biotic and abiotic factors as well as pollution impact act through direct and indirect metabolic pathways on biochemical and physiological functions of the organisms and modify them (Adams *et al*., 1996). Our results demonstrate that hepatic enzymatic activities depended on fish ecological status which agrees with the data of other investigators (Filho *et al*., 2001; Sole *et al*., 2009).

In 1990s the highest SOD activity was indicated in *S. flexuosa* and *M. barbatus* while in other fish species the values were significantly lower (Table 1). In 2000s no significant differences between SOD activity in the liver of examined fish species were shown. In both periods CAT activity was similar in all studied fish species.

The lowest PER activity was indicated in the liver of *S. porcus* and *M. barbatus* and the highest in *S. flexuosa* in 1990s. In 2000s we obtained the opposite tendency: the highest activity was demonstrated in *S. porcus* and in *T. mediterraneus* liver while the enzymatic activity in *S. flexuosa* and *M. barbatus* was 10-folds lower. Hepatic GR activity was the lowest in *S. porcus* in both examined periods.

Obtained results have been shown that in the period of 1990s the interspecies differences between hepatic antioxidant enzymes are much clear as compared to 2000s (9 positions opposite 3 respectively). It could be suggested that in spite of the peculiarities of fish biology and ecology the common trend to uniform response of antioxidant system was presented.

TABLE - 1. Interspecies differences between fish hepatic antioxidant enzyme activities in two examined periods

Fish specie	1990s				2000s			
	SOD	CAT	PER	GR	SOD	CAT	PER	GR
S. porcus→ *M. barbatus*	$P \leq 0.01$	n/s	n/s	n/s	n/s	n/s	n/s	n/s
S. porcus → *S. flexuosa*	$P \leq 0.01$	n/s	$P \leq 0.01$	$P \leq 0.01$	n/s	n/s	$P \leq 0.01$	$P \leq 0.01$
S. porcus → *T. mediterraneus*	$P \leq 0.01$	n/s	$P \leq 0.01$	$P \leq 0.01$	n/s	n/s	n/s	n/s
M. barbatus → *S. flexuosa*	n/s	n/s	$P \leq 0.01$	n/s	n/s	n/s	n/s	n/s
M. barbatus → *T.mediterraneus*	$P \leq 0.01$	n/s	$P \leq 0.01$	n/s	n/s	n/s	$P \leq 0.01$	n/s
S. flexuosa→ *T. mediterraneus*	$P \leq 0.01$	n/s	n/s	n/s	n/s	n/s	n/s	n/s

n/s – no significant variations

Changes of fish hepatic antioxidant enzyme activities in two periods

Liver was found to the stronger into the face of oxidative stress than the other tissues (Di Giulio *et al*., 1989). The significant increase of antioxidant enzyme activities in 2000s compared with 1990s may indicate an elevated exposure to and/or uptake of heavy metals and/or other potential xenobiotics induced antioxidant system in fish liver. The similar results were obtained for EROD activity in the liver of female perch collected in the polluted area of the Baltic Sea (Balk *et al*., 1996). In the liver tissues of fish *Silurus glanis* caught in petroleum-contaminated river SOD and GSH-Px activities were found to be increased, but no differences were observed in the CAT value (Avci *et al*., 2005). Flounders from the polluted site had the higher levels of hepatic CAT activity than those from the non-polluted site in summer and winter, but not in spring (Amado *et al*., 2006). The higher activities of the hepatic antioxidant enzyme CAT, GPx and GR in shorten sculpin *Myoxocephalus scorpius* caught in contaminated coastal area of Iceland indicated exposure of fish to prooxidant compounds (Stephensen *et al*., 2000).

In Table 2 the total antioxidant enzymatic activities in fish liver was shown. We see that in 2000s these parameters are much more greater than in 1990s in all examined fish species. At the same time the obtained tendency of increase of hepatic antioxidant enzyme activities was more clear in *T. mediterraneus* and *S. porcus* than in *S. flexuosa* and *M. barbatus.* Thus we could proposed that hepatic antioxidant enzyme system of *T. mediterraneus* and *S. porcus* is more sensitive to chronically stressed environmental factors than in *S. flexuosa* and *M. barbatus.* We could mark also that the ratio of 1990s/2000s values was approximately similar in *S. flexuosa* and *M. barbatus* and significantly lower than in *T. mediterraneus* and *S. porcus* (Table 2).

The variations of hepatic antioxidant enzyme response on environmental pollution were described by other investigations (Amado *et al*., 2006; Martinez-Gomez *et al*., 2006; Stephensen *et al*., 2000). At the same time the authors demonstrated that the fish biomarkers response depends on many biotic and abiotic factors including their specificity of biology and ecology (Martinez-Alvarez *et al*., 2005; Sole *et al*., 2009).

Thus we could proposed that the hepatic antioxidant enzyme system of examined fish species was modified during the period of 1990s-2000s and it was adopted to the changed ecological status of Sevastopol Bay. In spite of pollution decrease in 2000s as compared with 1990s the hepatic biomarkers were not restored to the level of 1990s and were elevated at another functional level resulted the chronic contamination in 1990s. At the other side we try to mark the specific trends taking into account the ecological status of investigated fish species.

TABLE 2. The total antioxidant enzymatic activity* in liver of fish caught in 1990s and 2000s in Sevastopol Bay (arbitrary units per mg protein per min)

Fish species	1990s	2000s	Ratio 1990s/2000s
T. mediterraneus	7.4	484.9	1:66.5
S. flexuosa	95.2	292.0	1:3.1
M. barbatus	67.1	225.7	1:3.6
S. porcus	10.2	247.2	1:24.3

*Total enzymatic activities was estimated as sum of each specific antioxidant enzyme activity (SOD+CAT+PER+GR)

Period-related hepatic antioxidant enzyme activities and fish ecological status

The present study supports the interspecies differences in hepatic antioxidant enzyme system between examined species, which agrees with the results of other researchers, who reported about the high variability of the enzymatic activities in fish liver and its dependence on animal ecological status (Martinez-Alvarez *et al*., 2005; Rosha-e-Silva *et al*., 2004; Sole *et al*., 2009; Zelinski and Portner, 2000). Interspecies differences and spatial variations in biomarkers were observed in two demersal fish *Lepidorhombus boscii* and *Callionymus lyra* from the two locations in the northern Iberian shelf after Prestige oil spill (Martinez-Gomez *et al*., 2006). In this case GR and CAT activities were significantly elevated in *L. boscii* in the most oil impacted area. The unambiguous biomarker responses observed in *L. boscii* and *C. lyra* in two locations seem to be more associated to the chronic pollution on this part of the continental shelf rather than to the oil spill consequences. We could shown the three types of fish hepatic antioxidant enzymes trends during the examined period (Table 3).

TABLE – 3. Period-related changes of hepatic antioxidant enzyme activities.

Fish	SOD	CAT	PER	GR
T. mediterraneus	I	III	III	III
S. flexuosa	I	I	II	III
M. barbatus	I	I	II	I
S. porcus	I	I	I	I

Type of the response: I – increase in 2000s as compared to 1990s,
II – decrease in 2000s as compared to 1990s,
III – no significant changes.

In all examined fish species the trend of SOD activity was uniform and it was significantly increased in 2000s. CAT activity in benthic *S. porcus*, suprabenthic *M. barbatus* and suprabenthic/pelagic *S. flexuosa* showed the similar tendency. In pelagic *T. mediterraneus* we could mark the growth of enzymatic activity, but it was not significant. The response of PER activity was not uniform: it significantly dropped in suprabenthic *M. barbatus* and suprabenthic-pelagic *S. flexuosa*, benthic *S. porcus* demonstrated the opposite tendency, while in pelagic *T. mediterraneus* it was not changed. GR activity increased in benthic *S. porcus* and suprabenthic *M. barbatus* and no significant period dependence was shown in pelagic and suprabenthic-pelagic fish.

Our study demonstrated that biomarker responses were generally higher in benthic form *S. porcus* than those in pelagic *T. mediterraneus.* The response of suprabenthic and suprabenthic-pelagic fish species was intermediate between benthic and pelagic forms. We could propose that benthic forms live in more contaminant environment because many pollutants accumulate in bottom sediments and low water layers. The other reason is associated with fish trophic level, feeding behavior and nutrition factors which also may affected antioxidant enzyme activities (Hamed *et al*., 2003; Martinez-Alvarez *et al*., 2005; Sole *et al*., 2009). Benthic invertebrates (mollusks, crustacean and worms) and fish which are the preferable prey for this fish ecological class might accumulate xenobiotics from the bottom sediments and transfer them via trophic nets to fish with the effect of concentration. Thus, chronic impact of stressors induces high level of antioxidant enzyme activities during fish life and leads the lack of its resources during 10 years.

At the same time the SOD response in pelagic *T. mediterraneus* was the greatest as compared with the other examined fish species in 2000s. We could suggest that in this case high SOD activities corresponds to enhanced metabolic rate because this fish is fast swimming and it inhabits the upper water layers in which the concentration of O_2 is very high. High oxygen concentrations together with xenobiotics level in upper water layers could led the synergetic effect for antioxidant system in *T. mediterraneus,* which is corresponded the highest ratio of the total antioxidant enzyme activities in 1990s/2000s

(Table 2). At the same time the variations of hepatic antioxidant enzyme activities in *T. mediterraneus* is higher as compared with the species belonging to other ecological groups and differences between the values of 1990s and 2000s are not significant with the exception of SOD. It could be proposed that the fish inhabited the high variable environment showed great fluctuations of biochemical parameters which was demonstrated by other investigators (Ferrante *et al*., 2008; Sole *et al*., 2009).

CONCLUSIONS

Biochemical biomarkers variables seem to be useful indicators of the health status of fish in monitoring studies. The obtained results demonstrated the increase of antioxidant enzyme defense system in the liver of all examined fish species in 2000s as compared to 1990s. Three types of response were shown which depended on fish ecological status.

In benthic form *S. porcus* the response was uniform and characterized the significant enhance of all examined hepatic enzyme activities. Pelagic *T. mediterraneus* demonstrated high variability of the biomarker values and only SOD activity was significant higher in 2000s. Suprabenthic *M. barbatus* and suprabenthic/pelagic *S. flexuosa* generally displayed the intermediate position between the pelagic and benthic forms. Thus in spite of the decrease of pollution level in Sevastopol Bay in 2000s as compared with 1990s we could postulated that hepatic antioxidant system in all examined fish species belonging to different ecological classes was not restored to the level of 1990s. This fact showed the long-term pollution after-effects in fish and their adaptation to another ecological conditions which were presented in Sevastopol Bay after chronic impact during ten years. At the same time benthic form *S. pocus* is more preferable as biomonitor then the others because its response was much more clear as compared with other species. The obtained results can be applied for development monitoring management and for perspectives of conservation ecology and biodiversity in impacted aquatic ecosystems.

ACKNOWLEDGEMENT

We would like to thank to the fishermen of the Institute of Biology of the Southern Seas for providing the examined fish species.

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