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Antioxidant enzymes in the liver of *Chelidonichthys obscurus* from the Montenegrin coastline

Research Article

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Abstract: The activities of antioxidant defence enzymes - total, manganese and copper zinc containing superoxide dismutase (Tot SOD, Mn SOD, CuZn SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and biotransformation phase II enzyme glutathione-S-transferase (GST) - in the liver of longfin gurnard (*Chelidonichthys obscurus*) from the Montenegrin coastline (Adriatic sea) were investigated. The specimens were collected in winter (February) and late spring (May) at two localities: Platamuni (PL, potentially unpolluted) and the Estuary of the River Bojana (EB, potentially polluted). The obtained results show that the activities of Mn SOD, CAT, GSH-Px and GST in winter were significantly lower at EB than at PL. In spring, the activities of CAT and GST were decreased, while GR activity was increased at EB in comparison to PL. The activities of Mn SOD and GST at PL were decreased and GSH-Px, GR and GST activities at EB were increased in spring compared to winter. Our work represents the first study of liver antioxidant enzymes of longfin gurnard from the Montenegrin coastline and reveals that locality, as a variable, has a greater influence on antioxidant enzymes and biotransformation phase II enzyme GST activities compared to season.

Keywords: *Antioxidant enzymes • Reactive oxygen species • Pollution • Season • Longfin gurnard*

1. Introduction

Some reactive oxygen species (ROS) (superoxide anion radicals, hydrogen peroxide and hydroxyl radicals) are produced as side products of an aerobic metabolism. They can also be formed intracellularly under the influence of various xenobiotics. ROS can arise as by-products in some metabolic processes or in some signal pathways [1]. They are very reactive molecules and thus very dangerous for normal cellular function [2]. During the evolution of the aerobic metabolism, cells developed various mechanisms in order to defend themselves from ROS. One of these mechanisms includes antioxidant defence enzymes, such as: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR). Phase II biotransformation components glutathione-S-transferase (GST) and reduced/oxidized glutathione system (GSH/GSSG) are also included in the defence against ROS [3]. ROS generation and antioxidant defences may be influenced by many environmental factors. Consequently, many abiotic and biotic influences should be taken into account when interpreting antioxidant defence biomarkers [4]. Marine ecosystems possess many specificities and many marine organisms have fine cellular control between production of ROS and antioxidant defence mechanisms [5]. The activity of antioxidant defence enzymes can be used as potential biomarkers for various environmental influences and aquatic contamination because these factors can directly or indirectly change the balance between the pro-oxidants and antioxidants [6]. Antioxidant defence enzymes are also related to changes in environmental factors such as temperature, salinity, food availability and dissolved oxygen levels, as well as to intrinsic biological factors such as gonadal development or the reproductive cycle [7]. Since changes at the organism level lead to changes at the population and community

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levels, antioxidant defence enzymes can be used as early warning signals of environmental disturbance [8].

Fishes play a number of roles in the food chain, bioaccumulate toxic substances and respond to low concentration of xenobiotics, and thus are often use as bioindicators [9]. Fishes are thermo- and oxygen-conformer organisms, meaning that ROS levels are dependent on these physical variables. Studies on oxidative stress biomarkers in poikilotherms show strong correlation between environmental influences and their metabolic needs [3,10]. Lower metabolic rate implies lower capacity of antioxidant defence. The main problem in fishes is to determine the role of individual components of antioxidant defence system in the achieving and in maintaining of homeostasis. Fishes are suitable model organisms, because they are on the top of the food chain and also very sensitive in response to stress conditions [11]. Fish liver plays a major role in various processes, such as the uptake, biotransformation and excretion of pollutants. The teleost liver has been shown to be very sensitive to pollutant exposure [9]. As many toxic compounds tend to accumulate in this organ, the magnitude of liver exposure to contaminants is greater than that of the environment or of other organs, resulting in various biochemical and histo-cytopathological changes [12].

Longfin gurnard *Chelidonichthys obscurus* (Walbaum 1792) was chosen for investigation because it is a territorial fish of commercial interest in fisheries and aquaculture. Longfin gurnard is a perciform fish which occurs in the benthic zone and inhabits sand, muddy sand or gravel bottoms. Longfin gurnard feeds mainly with fish and various invertebrates, such as crustaceans and mollusks. It has three rays on the pectoral fin which mainly help them in locating food on the soft bottom [13]. During their life-span, longfin gurnard is always close to the sediments when they find food and protection from predators. Therefore, it was expected to be exposed to contaminants associated with the sediment. Maturation of gonads in female gurnards in the Mediterranean takes place from October to May. In January and February more than 80% of females were sexually mature, while from June to September no mature females were observed [14].

The general aim of our study was to establish differences in antioxidant enzyme activities in the liver of longfin gurnard *C. obscurus* between two investigated localities in the Adriatic Sea: Platamuni (PL, northwestern part of the Montenegrin coastline) and the Estuary of the River Bojana (EB, southeastern part of the Montenegrin coastline), as well as between two different seasons: winter and spring. We determine the activities of total superoxide dismutase (Tot SOD), manganese containing superoxide dismutase (Mn SOD), copper zinc containing superoxide dismutase (CuZn SOD), (EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), as well as the activity of biotransformation phase II enzyme glutathione-S-transferase (GST, EC 2.5.1.18).

2. Experimental Procedures

2.1 Study area and sampling

Fish samples were caught by trawling in winter (February) and late spring (May) at two localities: Platamuni (PL) and the Estuary of the River Bojana (EB) in the Adriatic Sea (Figure 1). The localities were chosen based on our earlier investigations [15,16] in order to compare the activity of antioxidant defence enzymes in the liver of longfin gurnard between the northwestern part of the Montenegrin coastline (open sea, 5 km from the coast) potentially with lower levels of pollution (PL) and the southeastern part of the Montenegrin coastline (1 km from the coast) with higher levels of urban and agricultural pollution (EB), as well as between periods of low metabolic activity in winter and basal metabolic activity in spring [17]. Investigated areas have similar climate conditions, with the lowest mean water temperature in February and the highest in August. The bottoms of the biotopes are covered with a thick layer of fine terrigenous sludge containing particles of detritus. From each investigated location, 10 specimens of male longfin gurnard were collected in winter and 10 in spring (in total, 40 individuals).

2.2 Measurements of environmental parameters

Environmental parameters (depth, salinity, temperature, oxygen concentration and oxygen saturation) were

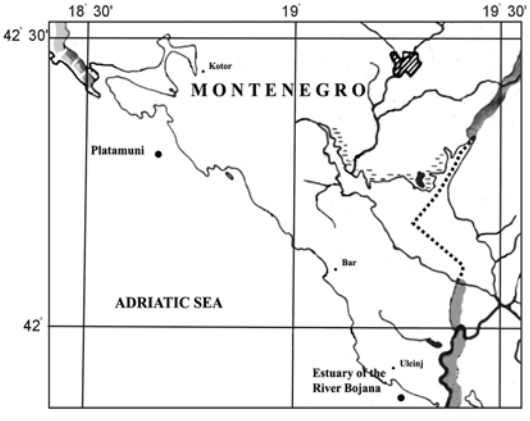

Figure 1. The geographical position of investigated localities: Platamuni (PL) and Estuary of the River Bojana (EB) in the Adriatic Sea.

measured with a WTW (Wissenschaftlich-Technische Werkstatten, Weilheim, Germany) multilab system (spot measurements at the time of sampling).

2.3 Tissue preparation

After collection, samples of longfin gurnard were immediately transferred to seawater tanks. Specimens of the same size class weighing 100-120 g were selected to ensure uniformity of samples. Fish were killed on board with a sharp blow to the head and dissected within 3 minutes on ice. In each group, the liver was rapidly dissected, washed in ice-cold 0.6% NaCl and frozen in liquid nitrogen (-196°C) before storage at -80°C. The liver tissue was ground and homogenized in 5 volumes of 25 mmol/L sucrose containing 10 mmol/L Tris-HCl, pH 7.5, at 1500 rpm [18] using a Janke & Kunkel (Staufen, Germany) IKA-Werk Ultra-Turrax homogenizer at 4°C. The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes [19] and sonicates were then centrifuged at 100,000×*g* for 90 min at 4°C. The resulting supernatants were used for further biochemical analyses. All chemicals used in this study were obtained from Sigma (Germany).

2.4 Antioxidant enzyme activities

Protein concentration in the supernatants was determined according to the method of Lowry *et al*. [20] using bovine serum albumin as a standard and expressed in mg/g wet mass. The activity of antioxidant defence enzymes was measured simultaneously in triplicate for each sample using a Shimadzu UV-160 spectrophotometer and a temperature-controlled cuvette holder. Total SOD activity was measured by the epinephrine method [21]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C and was expressed as specific activity (U/mg protein). For the determination of Mn SOD activity, the assay was performed after pre-incubation with 8 mmol/L KCN. CuZn SOD activity was calculated as a difference between total SOD and Mn SOD activities.

The activity of CAT was evaluated by the rate of hydrogen peroxide (H_2O_2) decomposition [22]. The method is based on H_2O_2 degradation by the action of CAT contained in the examined samples. In this procedure 30 mM H_2O_2 was used as substrate. CAT activity was expressed as μmol H_2O_2/min/mg protein.

GSH-Px activity was assayed following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide [23]. This reaction proceeds by the action of GSH-Px contained in the samples in the presence of *t*-butyl hydroperoxide (3 mM) as substrate in 0.5 M phosphate buffer, pH 7.0, at 37°C. The activity of GSH-Px was expressed as nmol NADPH/min/mg protein.

The activity of GR was measured as described by Glatzle *et al*. [24]. The method is based on the capability of GR to catalyze the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as substrate in the phosphate buffer (pH 7.4). GR activity was expressed as nmol NADPH/min/mg protein.

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined by the method of Habig *et al*. [25]. The method is based on the reaction of CDNB with the -SH group of GSH catalyzed by GST contained in the samples. The reaction proceeded in the presence of 1 mM GSH in phosphate buffer (pH 6.5) at 37°C. GST activity was expressed as nmol GSH/min/mg protein.

The experiments were approved by the Animal Care Committee of the Institute for Biological Research "Siniša Stanković" (Belgrade, Serbia) in conformity with the recommendations provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

2.5. Statistical analysis

The data are expressed as means ± S.E. (Standard Error). The non-parametric Mann-Whitney U-test was used to seek significant differences between means. A minimum significance level of $P<0.05$ was accepted. Principal component analysis (PCA) was employed to detect variables that significantly contributed to differences in the activity of the investigated antioxidant enzymes between the examined localities and seasons. *Post-hoc* pairwise comparisons were performed using the Spearman rank order correlation between the activities of antioxidant enzymes and localities and seasons to determine which values differed significantly. Analytical protocols described by Darlington *et al*. [26] and Dinneen and Blakesley [27] were followed.

3. Results and Discussion

Geographical coordinates of the investigated locations are presented in Table 1 and the data on physico-

		Longitude	Latitude
Winter	PL	42°19'56''	18°35'05''
	EB	41°52'60''	19°14'87''
Spring	PL	42°16'56''	18°41'66''
	EB	41°52'60''	19°14'87''

Table 1. Geographical coordinates of investigated locations (Platamuni-PL and Estuary of the River Bojana-EB) in the Southern Adriatic Sea in winter and spring.

		Depth (m)	Salinity (‰)	Temp. (ºC)	O_2 conc. (mg/L)	O_2 sat. (%)	NO_2^- (mg/L)	NO_3^- (mg/L)
Winter	PL	80	33.07	12.8	8.1	91	0.724	2.880
	EB	30	37.70	11.6	8.4	95	0.804	3.330
Spring	PL	80	38.00	17.2	7.6	98	0.175	1.223
	EB	28	37.20	17.9	7.4	97	0.233	2.466

Table 2. Physico-chemical parameters of the seawater (depth, salinity, temperature, O_2 concentration and O_2 saturation) and concentrations of nitrites (NO_2^-, mg/L of water) and nitrates (NO_3^-, mg/L of water) at the examined locations (Platamuni-PL and Estuary of the River Bojana-EB) in the Southern Adriatic Sea in winter and spring.

chemical characteristics of seawater are presented in Table 2. The obtained results show that seawater temperature was markedly higher in spring than in winter at both investigated localities. Consequently, concentration of dissolved oxygen was higher in winter, because oxygen solubility increases in cold water. Also, the water salinity was lower in winter in comparison to spring at all investigated localities.

The antioxidant and phase II biomarkers are closely related to changes in physico-chemical characteristics of seawater [7]. For example, salinity of the seawater fluctuates widely during the year. This makes it a factor that can affect the biochemical and physiological processes in living organisms. It is observed that changes in the seawater salinity induce various adaptations at all levels of organisation, from biochemical to behavioral [28]. It is well known that in aquatic ecosystems temperature and dissolved oxygen are likely to influence oxidative processes even more than xenobiotics do. The solubility of oxygen increases at lower temperatures and there is a direct relationship between ROS production and the partial pressure or concentration of oxygen [29], which contributes to the enhanced antioxidant enzyme activities. Aquatic hypoxia triggers a complex set of physiological and biochemical alterations in fish, including decreased metabolic rate, increased ventilation rate and increased anaerobic respiration [30]. It is known that temperature rise induces higher metabolic rate, oxygen consumption, ROS formation and oxidative stress, and theoretically, low temperature could reduce the metabolic activities and hence, lower enzymatic activities in general.

The Montenegrin coastline of the Adriatic Sea is very short, but with high demographic activity, and receives different levels of anthropogenic pressure (industrial, urban and agricultural activities). The present study is part of a larger investigation and our previous results at the same localities [15] show differences in concentrations of some pollutants. Namely, polychlorinated biphenyls (PCBs) were detected only in sediments at EB in spring. Concentrations of PCB-28 and PCB-101 were 147 ng/g and 457 ng/g, respectively. Concentrations of all other classes of PCBs were less than 10 ng/g. Of all investigated polycyclic aromatic hydrocarbons (PAHs), concentration of phenantrene was higher in the water at PL (373 ng/L) and anthracene and benzo(A) pyrene at EB in spring (200 ng/L and 2089 ng/L, respectively). Concentrations of all other classes of PAHs were less than 50 ng/L. Concentrations of nitrites and nitrates were higher in the water of both localities in winter, as well as at EB than at PL in both seasons (Table 2). These results indicate that EB received a higher content of various organic pollutants (PCB-28, PCB-101, anthracene, benzo(A)pyrene, higher nitrites and nitrates) than PL (only phenantrene), which can explain some of the obtained results for antioxidant defence enzyme activities. However, because seasonal factors can change the activity of investigated enzymes, it is very difficult to foresee connection between toxic compounds from seawater and antioxidant defence enzymes in our study.

In marine fish, antioxidant status is in direct correlation to organism activity level and oxygen consumption [10]. Fish live in an environment where there are both daily and seasonal variations in water temperature and oxygen concentration [31,32]. Influence of some stressors depends on their toxicity and from the ability of the organisms to protect themselves. Therefore, in natural populations we often detect either increase or decrease of antioxidant enzymes and it is difficult to draw conclusions on the basis of individual parameters. Often reduced level of some antioxidant component is related with elevated oxidative stress, but this effect is complicated with other forms of defences [33]. The use of individual antioxidant defence enzymes in biomonitoring studies is additionaly complicated with seasonal variations in concentration of some pollutants [17].

Seasonal patterns of antioxidant defence enzyme activities were also obtained in other marine organisms from the Adriatic Sea, such as thinlip gray mullet *Liza*

ramada [17], red mullet *Mullus barbatus* [34,35] and mussel *Mytilus galloprovincialis* [36].

SOD is an antioxidant enzyme which catalyses the destruction of the superoxide anion radical generating hydrogen peroxide, which is subsequently degraded to water and oxygen by CAT (7). In our work, the activity of Mn SOD (Figure 2) was markedly lower at EB than at PL in winter ($P<0.05$), as well as at PL in spring in comparison to winter ($P<0.05$). No changes in the activities of Tot SOD and CuZn SOD were observed. This is in accordance with our previous investigations on thinlip gray mullet *L. ramada* [17] and red mullet *M. barbatus* [34]. Many other laboratory and field studies show fluctuations of SOD activity in various aquatic organisms. Ferreira *et al*. [37] demonstrated increased SOD activity in the liver of mullet *Mugil cephalus* and flounder *Platichthys flesus*, which may be related to increased pro-oxidant concentration and higher metabolic level.

The obtained results show that CAT activity (Figure 3) was significantly lower at EB ($P<0.05$) than at PL in both winter and spring. GSH-Px activity (Figure 3) was also lower at EB, but only in winter. We also detected increased GSH-Px activity at EB in spring compared to winter ($P<0.05$).

SOD, CAT and GSH-Px are critically important antioxidant enzymes in the detoxification of ROS to nonreactive molecules in fish liver. However, response to pollution varies among different species, enzymes and single or mixed contaminants, and greater variability is found in field studies [38]. According to Van der Oost *et al*. [3], the activity of antioxidant enzymes can be higher, unchanged or lower in polluted compared to cleaner environments. In our study, the activities of Mn SOD, CAT, GSH-Px and GST in the liver were significantly lower at EB in comparison to reference locality PL. Investigations of other authors also show lower SOD and CAT activities in polluted sites, such as in *M. cephalus* in India [39] and *Dentex labrax* in Portugal [40]. Contrary to these findings, some studies demonstrated increased activities of these enzymes in polluted environments [41]. In general, antioxidant enzymes can be induced by enhanced production of ROS as a protection mechanism, or inhibited in case of deficiency of the antioxidant system [42].

Low environmental temperatures lead to increased polyunsaturation of mitochondrial membranes in fish, and consequently to elevated mitochondrial respiration. As a result there is an increase of ROS production, increase of proton leak and lipid peroxidation of membranes. The mitochondria exhibit seasonality of the maximum rates of protein-specific substrate oxidation at any given temperature. The increase in lipid mobilization leads to increased formation of organic hydroperoxides which causes increased activity of GSH-Px. Increased activity of GSH-Px depletes GSH storage and increases concentration of their oxidized form (GSSG). Consequently, in order to maintain a sufficient level of reduced equivalents and normal homeostasis, results in increased activity of GR. In many field studies in fish exposed to organic pollutants (PAHs, PCBs) induction of GR activity were observed [3,43].

The activities of GR and biotransformation phase II enzyme GST are presented in Figure 4. GR activity

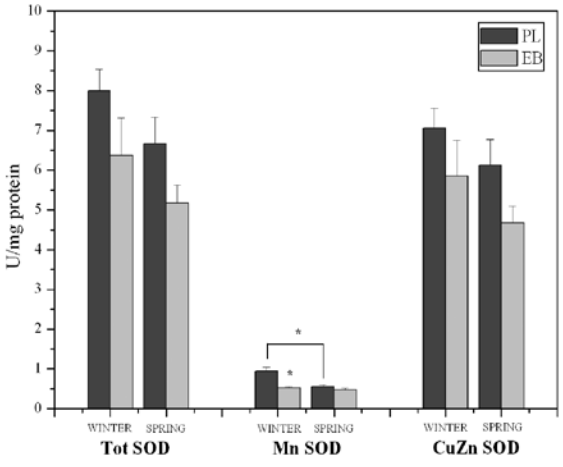

Figure 2. Tot SOD, CuZn SOD and Mn SOD activities (U/mg protein) in the liver of longfin gurnard (*C. obscurus*) from Platamuni (PL) and Estuary of the River Bojana (EB) in winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. $P<0.05$ represents a minimal significant level.

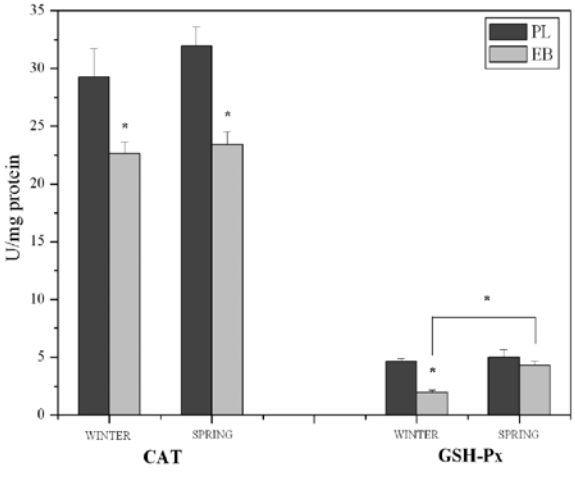

Figure 3. CAT and GSH-Px activities (U/mg protein) in the liver of longfin gurnard (*C. obscurus*) from Platamuni (PL) and Estuary of the River Bojana (EB) in winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. $P<0.05$ represents a minimal significant level.

was higher at EB than at PL in spring, as well as at EB in spring in comparison to winter (P<0.05). The most pronounced changes in enzyme activity were obtained for GST, with markedly lower activity of this enzyme at EB than at PL in both seasons (P<0.05). We also obtained significantly decreased activity of GST at PL in spring and EB in winter (P<0.05). GST, part of the biotransformation phase II enzyme system, has been used as a biomarker of organic industrial effluents [44]. In addition, GST has been used as a biomarker of exposure to anthropogenic organics [45]. Many enzymes, such as biotransformation phase II enzyme GST, have reduced activities at lower environmental temperature [46], but some enzymes increase their activities in winter (etoxycoumarin and etoxyresorufin O-dealkylases in red mullet) [47]. Some researchers showed that biotransformation phase II enzyme GST is influenced by the levels of organic substrates, and both enhancement and inhibition of these enzymatic activities have been reported in field studies [33]. GST metabolizes xenobiotics, which reduces cellular damage resulting from ROS [48]. From the presented results, the influence of season on the activities of glutathione (GSH)-dependent enzymes is evident. Namely, increased activities of GSH-Px and GST at EB in spring compared to winter indicate greater utilization of GSH and formation of its oxidized form (GSSG). Consequentely, higher activity of GR is necessary to provide sufficient reduction of GSSG to GSH for further undisturbed function of GSH-Px and GST.

In Figure 5, the principal component analysis (PCA) of all investigated antioxidant defence enzymes is presented. The PCA referred to relative contribution of every antioxidant enzyme in the liver and shows that Factor 1 and Factor 2 explain over 65% of the total variance in the data matrix. Factor 1 explains 47.35% of the total variance and is mainly characterized by negative loading of the variables Tot SOD and CuZn SOD and by positive loading of the variables Mn SOD, CAT, GSH-Px and GST. Factor 2 explains 17.90% of the total variance and is mainly characterised by positive loading of the variable GR.

Figure 6 represents summary results of PCA for both investigated sampling localities in winter and spring and shows that Factor 1 and Factor 2 explain over 92% of the total variance. Regarding the position of sites, Factor 1 (72.91%) discriminates between EB-winter/EB-spring and PL-winter/PL-spring. Factor 2 (19.65%) discriminates between EB-winter/PL-winter and EB-spring/PL-spring, showing clear separation of investigated localities (Factor 1), as well as investigated seasons (Factor 2). In order to determine which factor (locality or season) has a dominant influence on the

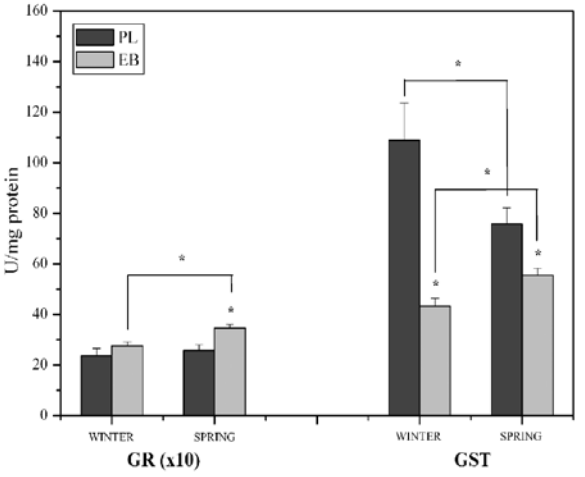

Figure 4. GR and biotransformation phase II enzyme GST activities (U/mg protein) in the liver of longfin gurnard (*C. obscurus*) from Platamuni (PL) and Estuary of the River Bojana (EB) in winter and spring. The data are expressed as means ± S.E. The non-parametric Mann-Whitney U-test was used to seek significant differences between means. P<0.05 represents a minimal significant level.

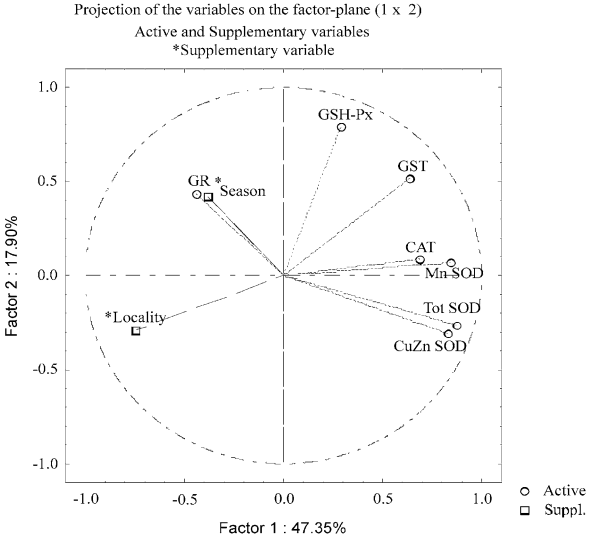

Figure 5. Principal component analysis (PCA) based on correlations; projection of all investigated antioxidant defense enzyme activities on the factor plane.

antioxidant enzyme activities, we performed pair-wise comparisons using Spearman rank order correlations (Table 3). We obtained a significant positive correlation between localities and GR, and negative correlations between localities and all of the investigated antioxidant defence enzymes (P<0.05). The effect of season expressed a significant negative correlation with Mn SOD activity, as well as a positive correlation with GR activity (P<0.05).

From the presented results it can be concluded that at the locality with a higher impact of pollutants (EB) we obtained depletion of antioxidant defence enzyme activities (except GR) in the liver of longfin gurnard *C. obscurus* compared to the reference site (PL). Differences between the two localities were more pronounced in winter, indicating higher susceptibility of hepatic antioxidant enzymes to pollutants in winter. Seasonal effects were also greater at the polluted site (EB), especially on activities of GSH-dependent enzymes (GSH-Px, GR and GST). We confirmed these results using principal components analysis based on correlations and Spearman rank order correlations. The obtained results show that locality as a variable has greater influence on antioxidant enzyme activities than season does. Observed changes represent physiological adaptation to differences in environmental factors in the Adriatic Sea and also in the presence of various anthropogenic influences.

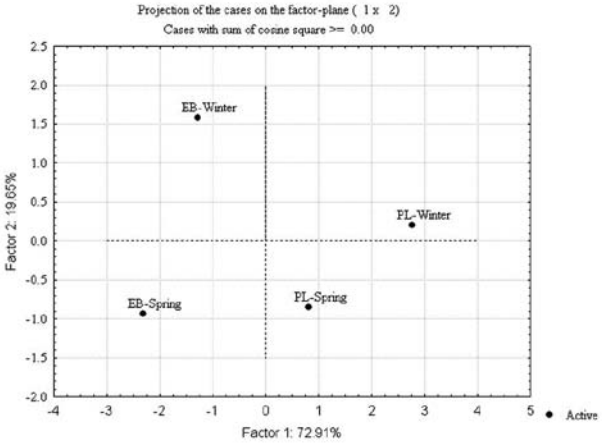

Figure 6. Principal component analysis (PCA) of each site (Platamuni-PL and Estuary of the River Bojana-EB) and season on the factor plane.

Our work is the first study on liver antioxidant enzymes of *C. obscurus* on the Montenegrin coastline and represents a contribution to the general knowledge of longfin gurnard biology.

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	Spearman R	P-level
Season & Tot SOD	-0.353851	0.064701
Season & Mn SOD	*-0.500569**	*0.006669**
Season & CuZn SOD	-0.300732	0.119942
Season & CAT	0.088451	0.654459
Season & GSH-Px	0.265352	0.172346
Season & GR	*0.402505**	*0.033711**
Season & GST	-0.088463	0.654414
Locality & Tot SOD	*-0.495391**	*0.007351**
Locality & Mn SOD	*-0.615744**	*0.000487**
Locality & CuZn SOD	*-0.468788**	*0.011859**
Locality & CAT	*-0.619154**	*0.000443**
Locality & GSH-Px	*-0.521858**	*0.004396**
Locality & GR	*0.504237**	*0.006218**
Locality & GST	*-0.831549**	*0.000000**

Table 3. Spearman rank order correlations in the liver of longfin gurnard (*C. obscurus*) between seasons and localities. Marked correlations are significant at $P<0.05$.

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