Studies of Some Morphological and Biochemical Parameters Concerning the Health Condition of Some Fish Species from Prut River, Romania

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The aim of this study was to assess the biometric and biochemical indices collected from seven fish species belonging on the two families, namely: Cyprinidae (Squalius cephalus, Alburnus alburnus, Carassius gibelio) and Percidae (Perca fluviatilis) from the Prut River. Body measurements and weighting were used to determine the increase in length of the fishes and to evaluate the general physiological condition. Also, the oxidative stress status in white muscle was assessed using superoxide-dismutase, glutathione-peroxidase, catalase and peroxidase specific activities along with malondialdehyde level. Biochemical parameters register variations one species to another and the values of the main morphological indices indicate a good maintenance state for all fish species taken into study (e.g. profile index oscillates between 2.34±0.124 cm at Carassius gibelio and 3.948±0.089 cm at Alburnus alburnus; Fulton coefficient between 0.998±0.035 % at Alburnus alburnus and 2.118±0.098 % at Perca fluviatilis; fleshy index between 18.44±0.958 % at Alburnus alburnus and 29.464±4.464 % at Perca fluviatilis). Taken together, our results showed that the Prut River aquatic environment offers good conditions for maintenance and health of studied fish population.

Keywords: Biochemical parameters, oxidative stress fish, malondialdehyde level, bodily indices, muscle.

River's ecosystems are extremely vulnerable to alterations in the environment due either to natural changes or to anthropic impact, the human activity influencing the biodiversity reduction of freshwater ecosystems, drastically [1].

Fishes may be influenced by specific changes or pressures in both the natural environment and in the intensive growth system in aquaculture conditions.

The response to stress factors such as climate change, temperature [2, 3], ultraviolet radiations [4], various pollutants [5, 6] anthropic impact [7], transport, experimental manipulation [8, 9], over-density [10], pesticides, heavy metals etc. [11-17] it is considered an adaptive mechanism that allows to fishes' organism to perceive and to react to these factors in order to maintain the state of homeostasis [18].

The fishes' response to these factors involves all levels of organization: from the cell to the individual organism and even to the population structure. In these conditions in which the fishes' responses to a certain change are to maintain the homeostasis essence, it is not surprising that fishes respond in a generalized manner to all these levels of organization. Furthermore, fishes are used as a model for the biomonitoring of the aquatic environment and as sentinel agents for pollutant agents [19-21].

As a response to the action of stress factors in body's fishes take part a series of physiological and biochemical changes to compensate the effect of stress factors.

It has been reported that oxidative stress may be induced in different aquatic organisms by various compounds and accelerating the generation of highly reactive oxygen species (ROS), including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and singlet oxygen species (O_2). Moreover, these ROS can oxidize proteins, lipids, and nucleic acids, often leading to damages in different cellular targets or even cell death [22]. The antioxidant system in aquatic animals comprises both-low molecular mass and high molecular mass antioxidants [23]. Low molecular mass antioxidants described to date include water-soluble compounds such as reduced glutathione, ascorbic acid, and lipid-soluble ones such as carotenoids (including β-carotene), retinol, α-tocopherol. They usually operate as free radical scavengers. However, other mechanisms can be implicated here. For example, glutathione may serve as a cofactor for antioxidant enzymes such as glutathione-dependent peroxidases, or glutathione-S-transferases, a second phase detoxification enzyme [24]. High molecular mass antioxidant group consists of specific or non-specific proteins. A specific group includes antioxidant enzymes such superoxide dismutase (E.C. 1.15.1.1), catalase (E.C. 1.11.1.6), Se-dependent glutathione peroxidase (E.C. 1.11.1.9) and associated ones providing needed cofactors-glutathione reductase (E.C. 1.6.4.2), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) [25, 26]. Non-specific high molecular mass antioxidants are represented by proteins that prevent ROS-induced damage by binding to transition of metal ions (mainly iron and copper) such as metallothioneins and ferritin [20].

The aim of this study is to assess biometric and biochemical biomarkers of seven fish species captured in the Prut River. Lipid peroxidation in white muscle along with the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) were measured. The biochemical evaluation was complemented with morphological indices analysis such as profile, Kiselev and fleshy index, Fulton coefficient.

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Experimental part

Site description, sample collection and preparation

Prut River springs from the southeast slope of the peak Goverla, at 15 km south-southwest of Vorokhta from Carpathians Forests Mountain massif (Ukraine) and has a length of 953 km, flowing first to east and then on southeast direction up to flowing into the Danube near Reni, at east to the Galati City. In Romania's territory the river has a length of 742 km, a hydrographic basin of 10990 km² and an annual average flow of 110 m³ /sec (before flowing to the Danube). On a stretch of 39.4 km marks the Romanian-Ukrainian border and on a section of 681.3 km (from which 73.9 km are made of the Stânca-Costesti Lake) marks the border between Romania and Moldova.

Perca fluviatilis, *Squalius cephalus*, *Alburnus alburnus*, and *Carassius gibelio* (40 specimens, namely each 10 individuals per species) were collected using electric fishing from the Prut River (Stanca-Costesti zone) in September 2014. All collected specimens were males of one summer-old, and consequently were measured and weighed for studying morphological characters. The fishes were brought to the laboratory and then the tissue samples (white muscle) were isolated. Tissue samples were frozen at -80°C until further processing. Sigma-Aldrich chemicals (Germany) were used for the analyses.

Assessment of the morphological parameters

By calculation of the profile index (PI) it can be determined whether the external shape of fish livestock corresponds to the desired character searching by the pisciculturist, highlighting the body size of the fish and allows the employment of individuals of a population in a certain type of profile. The profile index can be calculated using the following formula:

$$PI = ls/H,$$

where: ls- standard length of body (cm); H- maximum height of body (cm).

The morphometric measurements (body standard length, head length, peduncle caudal length) were taken as a projection along the longitudinal axis.

Fulton coefficient (K) reflects the so-called *general condition* of fish or physiological condition (patho-physiological). As much as the values of fattening coefficient are higher, the fish is better developed [27, 28].

$$K = gx100/ls^3;$$

where: g- body weight (g); ls- standard length of body (cm), The fish weight was measured with an analytical balance $KERN_{EWB}$.

The setting of Kiselev index (K) is used to determine quickly the quality of fish, without the need for weights and other measurements. As much as the values of Kiselev index are lower, the fish meets better condition for selection [29]. For calculation can be used the following formula:

$$K = ls/Ci.$$

where: ls- standard length of body (cm); Ci - body circumference.

The fleshy index (Ic) expresses the proportion of head or caudal peduncle from the standard length of the body. As much as the values of Fleshy index are lower, the fish has a higher fleshy [30]. For its calculation, the following formula was used:

$$Ic = lcx100/ls;\ Ic = lcx100/ls.$$

where: lc = length of the head (cm); ls = standard length of body (cm); lp = length of caudal peduncle, in cm.

Biochemical parameter assay

Tissue samples (white muscles) were weighted and homogenized (1:10) with Potter Homogenizer coupled with Cole-Parmer Servodyne Mixer in ice-cold buffer (*p*H = 7.4) (0.08 M Tris-HCl, 250 nM sucrose, 5 mM $MgCl_2$ and 1 mM EDTA for SOD extraction, 0.154 M KCl for GPX extraction and 0.175 M KCl for MDA). Homogenates were centrifuged at 960 x *g* for 15 min and the supernatant was used for assays of SOD, GPX, CAT activities and malondialdehyde (MDA) levels.

Determination of SOD activity

The activity of SOD (E.C. 1.15.1.1) was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). Each 3 mL reaction mixture contained 0.067 M KH_2PO_4 (*p*H 7.8), 1.5 mM NBT, 0.12 mM riboflavin, 0.08 M EDTA and 0.1 mL of supernatant. The monitoring of the increase in absorbance at 560 nm is followed by the production of blue formazan. One unit of SOD is defined as the quantity required to inhibit the rate of NBT reduction by 50% [31]. The enzyme activity is expressed as units/mg protein.

Determination of GPX activity

GPX (E.C. 1.11.1.9) activity was analyzed by a spectrophotometric assay. A reaction mixture consisting of 1.3 mL of 0.25 M phosphate buffer (*p*H 7.0) containing 0.1 mL of 0.25 mM EDTA, 0.1 mL of 0.4 M NaN_3, 0.3 mL of 50 mM glutathione (GSH), and 0.2 mL of supernatant was pre-incubated at 37°C for 5 min. Then 0.1 mL of 50mM H_2O_2 were added and incubated at 37°C for further 5 min. The excess amount of GSH was quantified by the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method [31]. One unit of GPX is defined as the amount of enzyme required to oxidize 1 nmol GSH/min. The enzyme activity is expressed as units/mg protein.

Determination of muscular CAT activity

The CAT (E.C. 1.11.1.6) is an antioxidant enzyme involved in detoxifying hydrogen peroxide, its activity being determined through the *Sinha method* (1972), quoted by *Artenie et al.* [31]. The method is based on colorimetric determination (at λ = 570 nm) of chromic acetate obtained through reduction of potassium dichromate in acid medium by the hydrogen peroxide remained after enzyme inactivation. 0.1 mL of supernatant was taken and added to 0.4 mL potassium phosphate buffer, 0.5 mL substrate solution of hydrogen peroxide and 2 mL of potassium dichromate solution - acetic acid. One unit of catalase represents the amount of enzyme which breakdowns one micromole of hydrogen peroxide in one minute at a temperature of 20°C and *p*H of 7.0. The enzyme activity is expressed as units/mg protein.

Determination of MDA level

The MDA which is an indicator of lipid peroxidation, was spectrophotometrically measured by using the thiobarbituric acid assay [32]. 0.2 mL of supernatant was added and briefly mixed with 1 mL of 50% trichloroacetic acid in 0.1 M HCl and 1 mL of 26 mM thiobarbituric acid. After vortex mixing, samples were maintained at 95°C for 20 min. Afterwards, samples were centrifuged at 960 x g for 10 min and supernatants were read at 532 nm. A calibration curve was constructed using MDA as standard and the results were expressed as nmol/mg protein.

Estimation of protein concentration

The total soluble protein content expressed as mg/g was determined using Bradford method which is based on the observation that in the acid environment the Coomassie Brilliant Blue G - 250 reactive forms with proteins a complex with maximum absorbance at 595 nm. Thus, more than

0.1 mL of the supernatant were added 0.4 mL of distilled water and 1.5 mL of Bradford reagent [33]. To calculate the amount of proteins in the sample analyzed we used to build a standard curve of known concentrations (between 1 and 25 micrograms of protein in a volume of 0.5 mL) of bovine serum albumin.

Statistical analysis

For each species in part, tissue samples were taken from five individuals, the biochemical investigations being made in triplicate for each exemplar. Then, we realized the descriptive statistics calculation with standard statistical packages (Microsoft Excel). The results were expressed as means ± standard error.

Results and discussions

The study of morphological characters of fish species from the Prut River by means of biometry consisted in determining the variability of characters in groups of individuals, by direct measurement, weighting and statistical processing of obtained data. Using biometry we can assess the general physiological condition of the fish population. On the base of the somatic measurements could be calculated a series of corporal indexes which offers information regarding fish maintenance state and corporal shape of body [34, 35].

In the present study we assessed to the biometric and also to the biochemical indices collected from 4 fish species in the river Prut. The species identified were: *Perca fluviatilis*, *Squalius cephalus*, *Alburnus alburnus*, and *Carassius gibelio.*

For the fish biometry study to give the best results, the following conditions must be followed: the number of specimens to be large enough so that the errors to be more lower, the state of fish to be more fresh as possible, to take into account the physiological state in which the fish were in the moment of fishing, and during the researches do not measure than the dimensions which varies with the physiological state.

So, in terms of biometry data obtained by calculating the main indexes such as profile index, fattening coefficient, quality index (Kiselev index) and fleshy index, our results showed a corresponding increase, correlated with good maintenance and health of the Prut River fish population.

The analysis of the profile index (fig. 1) shows that the highest values were observed in *Alburnus alburnus* (3.948±0.089 cm), followed by *Squalius cephalus* (3.364±0.114 cm), which corresponds to the fact that these fish species have high lengths and height, back straight as compared to the crop species. In return, the lowest values of this index were registered by *Carassius gibelio* (2.345±0.124 cm), which signify a body more slender comparative to the fishes who registered the maximum values.

The estimation of the fattening coefficient (fig. 2) shows that the highest values were observed in *Perca fluviatilis* (2.118±0.098%) and *Carassius gibelio* (2.111±0.329%) which corresponds to the fact that these the fish species have a good physiological condition and favorable conditions for development.

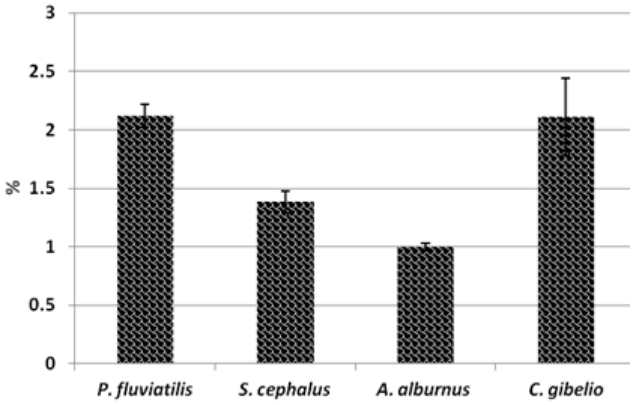

Fig. 2. Fattening coefficient (Fulton coefficient) of the fish population collected from the Prut River. Values are mean ± mean standard error, n=20 specimens/group

Wolnicki et al. (2001) [2] pointed out that no data are available in the literature regarding the relationship between the Fulton's coefficient, which describes fish condition, and the value of fish as stocking material. However, it should be assumed that higher values of this coefficient are advantageous, just as, undoubtedly, larger individual size is also preferable.

On the other hand, recently data [36] underline that Fulton coefficient is generally used as an indicator of the nutritional status of the fish. Being a biological parameter, it is expected that this index to have physiological limits. These limits would be determined by biological constraints related to the implication of the nutritional status of the fish. Below a certain value of this index, fish would have died of starvation and cannot function physiologically. On the other hand, going beyond an upper value for a particular species would be anatomically impossible [37].

The experimental results refering to Kiselev index (fig. 3) indicate that the lowest values were observed in *Perca fluviatilis* (1.317±0.102 cm) and *Carassius gibelio* (1.216±0.039 cm) which corresponds to the fact that these fish species show very good conditions for selection. In the opposite pole lies the *Alburnus alburnus* species, with a value of Kiselev index of 1.88±0.101 cm.

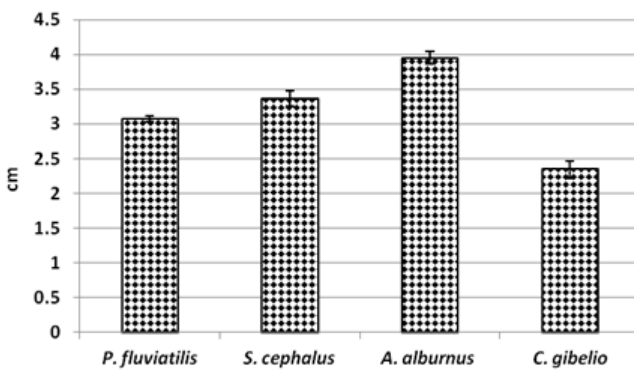

Fig. 1. Profile index (height) of the fish population collected from the Prut River. Values are mean ± mean standard error, n=20 specimens/group

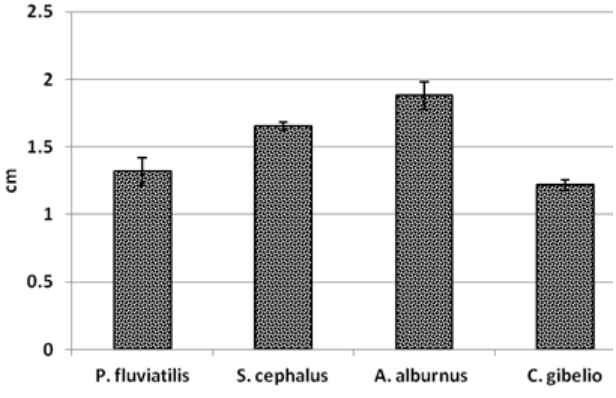

Fig. 3. Kiselev index (quality index) of the fish population collected from the Prut River. Values are mean ± mean standard error, n=20 specimens/group

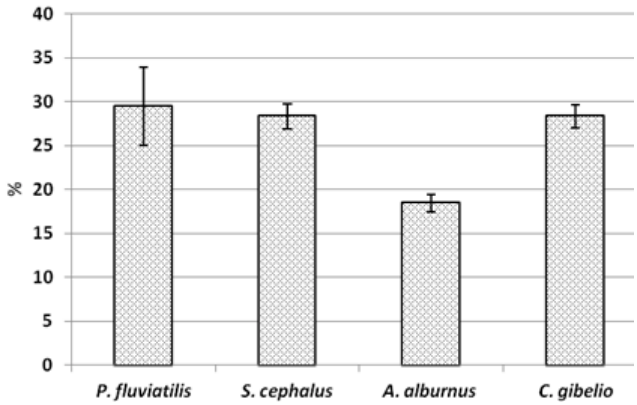

Fig. 4. Fleshy index of the fish population collected from the Prut River. Values are mean ± mean standard error, n=20 specimens/ group

In what concerns the fleshy index (fig. 4), our results denote that the lowest values were observed at *Alburnus alburnus* (18.441±0.958%), which corresponds to the fact that this species of fish has a large fleshy. *Perca fluviatilis* registers the maximum value of fleshy index (34.101±0.767%), followed by *Squalius cephalus* and *Carassius gibelio*, each of them with approximately 28.3%.

Unlike terrestrial animals, fishes deal with a variable living environment, with temporal and spatial changes and, in consequence, with a variation of the available oxygen, in these aquatic species, the body's response can be influenced by the action of various external factors like temperature, light, water quality, salinity or stress [38].

In the present study we were interested to know if the biometry evaluated by specific indexes is related to oxidative stress status of fish population form the Prut River. For this purpose, we analyzed the oxidative stress status of the selected fish species by using antioxidant enzymes activity like SOD, GPX, CAT, POX and a specific product of lipid peroxidation as MDA.

The literature data highlight, furthermore, the importance of antioxidant enzymes like indicators of waters pollution level [39, 40]. In the same time, fishes have been proposed as indicators for monitoring land-based pollution because they may concentrate indicative pollutants in their tissue, directly from water through respiration and also through their diet [41, 42].

Other data [43], generalized that the stress response involves some changes at the cellular level, which also can include an increase in the production of specific proteins, namely the so-called stress protein family, Iwama et al. (1998) [44] specifying their physiological and defensive role after the exposure of fish to various environmental factors. Data from the literature suggests, moreover, increased levels of these proteins in the presence of bacterial germs [45], industrial effluent and polycyclic aromatic hydrocarbons [46] or pesticides [47].

It must be taken into account that there are a lot of intrinsic factors to the fish itself, such as age, feeding behavior and phylogenetic position, as well as environmental factors such as the type of diet supplied, dissolved oxygen, daily or seasonal changes in temperature, pathologies, or parasites, can either fortify or weaken antioxidant defenses [48, 49].

Under most physiological states, ROS production is closely matched by antioxidant responses. Natural or anthropic changes in the environment introduce oxidative stress that results in disequilibrium in the cell by increasing the production of ROS [18]. The enzymatic antioxidants, such as superoxide-dismutase, glutathione-peroxidase, catalase and peroxidase, form an important part of the antioxidant response [50].

The SOD enzyme is the first antioxidant enzyme involved in antioxidant defense, being an oxido-reductase which catalyzes the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide which afterwards is detoxified by CAT [51, 34]. The literature on the field suggests, furthermore, that in aerobic organisms SODs constitute a group of metalloenzymes who play a crucial antioxidant role in the primary de-fense mechanism against the toxic effect of oxygen [26].

In term of its activity, the lowest values were observed in *Alburnus alburnus* (3.656 USOD/mg protein ± 0.363) and *Perca fluviatilis* (3.87 USOD/mg protein ± 0.507) which corresponds to the fact that these fish species showed a reduced oxidative stress given to the good physiological conditions in aquatic environment (fig. 5).

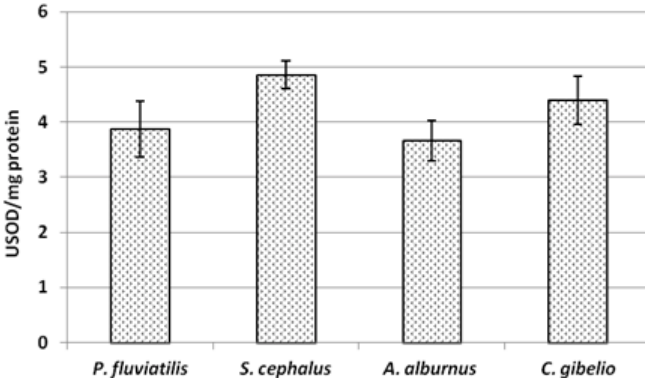

Fig. 5. SOD specific activity in the white muscle homogenates of the fish population collected from the Prut River. Values are mean ± mean standard error, n = 5 specimens/group

The statistical analysis of experimental results show that there wasn't a pronounced interindividual variability, the confidence interval limits being threshold relatively close, in *Squalius cephalus* and *Carassius gibelio*, for example, the SOD activity being between 4.534 - 5.168, respectively 3.851 - 4.945 USOD/ mg protein.

Therefore, a simultaneous activity induction of SOD and CAT is usually an expected response. However, this relation is not always observed [52] and it is known to be species dependent [5].

The enzyme CAT is widely distributed in biological tissues and is involved in the decomposition of hydrogen peroxide into oxygen and water. It is one of the most prominent enzymes involved in the defense against oxidative stress in both vertebrates and invertebrates [7, 53].

Considering the fact that, as some authors have shown [54, 55], in the case of fish, catalase is an *enzyme adaptation*, we appeal to the determination of this enzyme activity too, in samples of muscle tissue.

Thus, in *Alburnus alburnus* species was registered the minimum activity threshold value 25.442 UCAT/mg protein ± 3.241), followed by *Squalius cephalus* (33.699 UCAT/ mg protein ± 1.768) and *Perca fluviatilis* (38.269 UCAT/ mg protein ± 2.364). The maximum value was highlighted in *Carassius gibelio* (43.171 UCAT/mg protein ± 1.039), which suggests that oxidative stress in this species reached the highest levels (fig. 6).

In commenting the results it should be taken into account the environment variability, various studies showing that, in fish, temperature, salinity, weather, and feeding habitats may cause changes in peroxisome enzyme activity which, moreover, varies greatly depending on the species [56, 57], catalase being un well-known peroxisomal enzyme marker [58, 59].

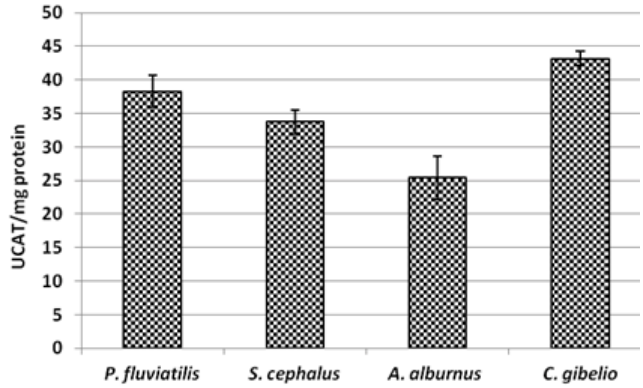

Fig. 6. CAT specific activity in the white muscle homogenates of the fish population collected from the Prut River. Values are mean ± mean standard error, n=5 specimens/group

The GPX, a component of antioxidant system, plays a crucial role in maintaining cell homeostasis, being one of the most useful biomarkers of contaminant-mediated oxidative stress in a variety of aquatic organisms, their induction reflecting a specific response to pollutants [60, 61]. The induction of antioxidants can provide sensitive early warning signals of incipient oxidative stress [62].

In the case of this enzyme (fig. 7), the lowest values were observed in *Carassius gibelio* (0.757 UGPX/mg protein ± 0.123) and *Perca fluviatilis* (0.928 UGPX/mg protein ± 0.095) which corresponds to the fact that these fish species show a reduced oxidative stress, probably, due to good physiological conditions in aquatic environment.

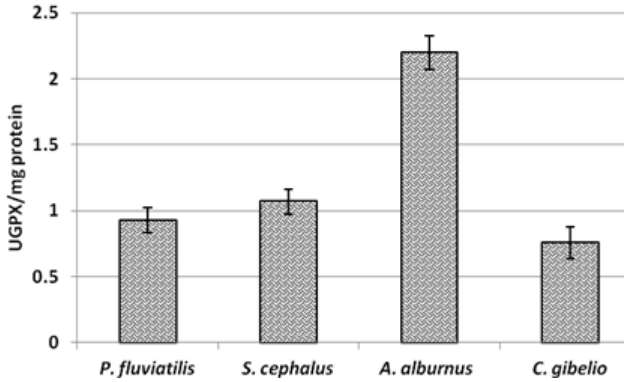

Fig. 7. GPX specific activity in the white muscle homogenates of the fish population collected from the Prut River. Values are mean ± mean standard error, n=5 specimens/group

The activity of these antioxidant enzymes are in parallel with the MDA level (fig. 8). Some ROS can initiate lipid peroxidation, a self-propagating process in which a peroxide radical is formed, the reaction of ROS with lipids

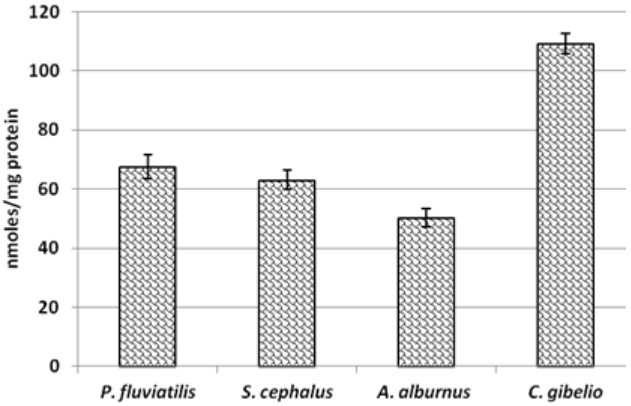

Fig. 8. MDA level in the white muscle homogenates of the fish population collected from the Prut River. Values are mean ± mean standard error, n = 5 specimens/group

being considered one of the most prevalent mechanisms of cell damage [25]. It is well-known that then fish are exposed to oxidative stress con-ditions, polyunsaturated fatty acid peroxidation can occur, MDA - an indica-tor of lipid peroxidation, being an oxidation product of those acids, influencing the fluidity and integrity of cell membranes [63, 64].

Lipid peroxidation is a manifesta-tion of oxidative damage induced by heavy metals [63, 65] and has a predictive importance as biomarkers of ecosystem pollution [60]. Moreover, both antioxidant enzymes and non-enzymatic antioxidants have been intensely employed in aquatic monitoring studies [52, 66].

The analysis of experimental results in what concerns the level of MDA in biological samples studied revealed that in all selected fish species this parameter registered low values (50.15 nmoles/mg protein ± 3.105 in *Alburnus alburnus*, 63.07 nmoles/mg protein ± 3.198 in *Squalius cephalus* and 67.567 nmoles/mg protein ± 4.219 in *Perca fluviatilis*), with except of *Carassius gibelio* (109.252 nmoles/mg protein ± 3.523), the MDA lowest values corresponding to the fact that these fish species show a reduced oxidative stress.

Conclusions

In conclusion, biometric markers and antioxidant enzymes activity are useful tools to evaluate the health status of the fish population from the Prut River. Therefore, these biomarkers can be used in the next studies for monitoring the health of fish stocks from Prut River.

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