

## Influence of trout farm effluents on selected oxidative stress biomarkers in larvae of *Ecdyonurus venosus* (Ephemeroptera, Heptageniidae)

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**Abstract:** The aim of the present study was to establish the extent to which the outlet waters of trout farms affect the ecosystems of the Crnica and Skrapež rivers in Serbia. We monitored selected biomarkers of oxidative stress: superoxide dismutase (SOD), glutathione peroxidase (GPx) and total glutathione (GSH) in larvae of the species *Ecdyonurus venosus*, and simultaneously analyzed the changes in the physical and chemical parameters. The investigations were carried out in spring at four localities along the Skrapež and Crnica rivers: one upstream (the control localities), and three downstream from the fish farm outlets. On the Skrapež River, the fish farm was clearly visible and was markedly changed by the chemical parameters of the water, manifested as a decreased concentration of dissolved oxygen, increased concentrations of nitrates, nitrites, ammonium ions, total phosphorus and total organic carbon, and increased SOD and GPx activities and decreased GSH concentration in larvae from the first downstream locality as compared to the control locality. On the Crnica River, due to the high values of water flow (around 3 m<sup>3</sup>/s), effluents from the fish farm had no effect on the chemical parameters of the water or on the tested biomarkers.

**Keywords:** antioxidant enzymes; biomarkers; *Ecdyonurus venosus*; organic pollution; oxidative stress

### INTRODUCTION

Freshwater ecosystems are exposed to direct anthropogenic influence that negatively affects water quality and the ecological status of aquatic ecosystems [1]. The construction of dams and reservoirs modifies the flow of water, altering ecological characteristics [2]. Highland streams like the Skrapež and Crnica rivers are the least disturbed, primarily because of their inaccessibility [3]. In recent years, trout aquaculture has been observed to exert a strong influence on these sensitive aquatic ecosystems. With more intensive production in aquaculture, there has been an increase in the amount of fish-food and waste material, including organic matter, nutrients and suspended solid materials in the water, which directly affects the concentration of oxygen, eutrophication, and turbidity [4-6].

In Serbia, there has been a constant increase in the number of trout farms. In the 10-year period up to 2000,

their number has doubled and annual production has reached as much as 2000 tons of fish [7]. Trout farms in Serbia occupy an area of about 14 ha [8], but it can be expected that this area will increase even as much as from three- to five-fold [9]. How this will affect the environment depends on the size of the fish farm, the presence and types of water purification systems, the composition and structure of fish food, and on the characteristics of the stream itself, such as slope, capacity and the number of tributaries [10]. Aquatic ecosystems need to be protected from the negative influence of trout farms by legislation. This is a problem in developing countries where such regulation is inadequate or completely nonexistent [10]. To ensure effective legal regulation, it is necessary to conduct monitoring, which will give us an idea of the state of the aquatic ecosystems where fish farms are located.

Benthic macroinvertebrates are most often used in the monitoring of aquatic ecosystems, owing to their

limited mobility, high abundance and diversity, and their varying sensitivity to pollutants [11-13]. Larvae of Ephemeroptera are one of the most sensitive groups of macroinvertebrates [14]. They feed on detritus and are consequently very sensitive to changes in the substrate and to all toxic substances present. Moreover, the larvae are stenovalent in relation to water temperature, dissolved oxygen, pH, substrate type and river speed and size [15,16]. In aquatic organisms (including the larvae of Ephemeroptera), changes in the concentrations and activities of certain enzymatic antioxidants have been shown to be useful biomarkers of oxidative stress caused by pollutants [6,17-22]. These molecular biomarkers represent the most appropriate method of detecting the early influence of pollutants [18,23-26] and predicting their effects at the level of populations. They have wide application in the ecotoxicology of aquatic organisms exposed to various pollutants [27,28] and were therefore selected for the present research on the genus *Ecdyonurus*, which is one of the key bioindicators of water quality [29], and the species *E. venosus*, which is very sensitive to organic pollution that is especially pronounced downstream from trout farms.

In Serbia, among aquatic invertebrates, molecular biomarkers of oxidative stress have mainly been analyzed in Mollusca and Crustacea [20-22,30-33]. As far as the influence of outlet waters of trout farms is concerned, investigators have to date mainly concentrated their efforts on studying the structure of macrozoobenthic communities [3,10,34]. Investigations of the effects of trout farms on aquatic insects at the molecular level are still in their infancy [6]. In view of these facts and because the number of trout farms in Serbia is constantly increasing, the timely detection of negative influence of pollutants on aquatic ecosystems is especially important in order to prevent chronic negative effects of outlet waters from fish farms. Therefore, the aim of this study was to investigate the influence of effluents from two trout farms on selected components of antioxidative defense in the larvae of *E. venosus* as a model organism sensitive to pollution.

## MATERIALS AND METHODS

### Study area and sampling sites

Investigations into the influence of trout farms were carried out on the Crnica (CR) and Skrapež (SK)

rivers in April 2015 at four localities. One locality on both rivers (CR1, SK1) was upstream from the fish farm, while three localities (CR2, CR3, CR4; SK2, SK3, SK4) were downstream from it. Localities CR1 and SK1 represent the control localities above the fish farm. There is a trout farm with different production capacity on each of the rivers, a fact that makes them suitable for determination and comparison of the effects of organic pollution on the antioxidative status of *E. venosus* larvae. For nutrition, both trout farms used extruded fish food (Skreting Optiline he 3p gal) with a low phosphate content (0.9 %) and 42-44% protein.

The Crnica River is one of the most significant right-hand tributaries of the River Morava. It is 28.6 km long and has a drainage area of 338 km<sup>2</sup> [35]. The "Sisevac" Fish Farm is located at the very source of the Crnica, at the foot of the Kučaj Mountains. With a production area of 4200 m<sup>2</sup>, it has 43 concrete basins of different dimensions arranged in five batteries. The farm's production capacity is 70 tons of consumer rainbow trout. When the sampling was carried out in April 2005, the quantity of fish in the fish farm was 39.6 tons.

Locality CR1 was 250 m upstream from the fish farm, at latitude 43° 57' 19.6" N, longitude 21° 35' 24.4" E and elevation 348 m above sea level (a.s.l.). The second locality (CR2) was 20 m downstream from the outlet of water from the fish farm, at latitude 43° 57' 17.8" N, longitude 21° 34' 55.1" E, and elevation of 342 m a.s.l. At a distance of 400 m downstream from the second locality, the third (CR3) was at 43° 57' 16.7" N, 21° 34' 41.3" E and 340 m a.s.l. Locality CR4 was 900 m downstream from locality CR3, at 43° 57' 16.4" N, 21° 34' 07.1" E and 336 m a.s.l.

The Skrapež River is a left-hand tributary of the Đetinja (western Serbia). It is 47.7 km long and has a drainage area of 647.65 km<sup>2</sup> [35]. On the Skrapež, the "Kraj Vodenice" Trout Farm is located in the wider region of the village of Radanovci on the left-hand bank of the river, about 2 km downstream from the spring Taorska Vrela. The fish farm consists of four independent basins, supplied with water by a 220-m-long concrete canal. Water from the fish farm is emptied into the recipient through a common canal that passes over a pre-settler and settler. The farm's total production area is 588 m<sup>2</sup>, and its annual production

is 29 tons of trout. In April of 2015, the quantity of fish in the fish farm was 1 ton.

The control locality on the Skrapež River (SK1) was 180 m upstream from the fish farm at latitude 44° 04' 00.8" N, longitude 19° 50' 13.3" E, and elevation of 573 m a.s.l. Locality SK2 was 30 m downstream from the fish farm at the common channel, at 44° 03' 55.9" N, 19° 50' 21.2" E, and 566 m a.s.l. Locality SK3 was 300 m downstream from the second locality, at 44° 03' 47.8" N, 19° 50' 28.6" E and 552 m a.s.l. Locality SK4 was 300 m downstream from the third locality, at 44° 03' 40.9" N, 19° 50' 28.1" E and 550 m a.s.l.

### Sample collection

At the investigated localities, from 20 to 25 larvae of *Ecdyonurus venosus* in stage IV were collected by qualitative methods (using benthos sieves and tweezers) in April 2015. In the field, the larvae were put in a portable container with liquid nitrogen and transported to the laboratory, where the mass of each individual specimen was measured using a balance with a precision of 0.0001 g (AE163, Mettler-Toledo International). From each locality prior to freezing in liquid nitrogen, five larvae were separated and fixed with 96% alcohol for later identification. Identification was performed using a Zeiss Discovery V8 instrument and a Celestron CELE-822477 stereomicroscope, in addition to the key for identification [36].

### Analysis of physical and chemical parameters

The following basic physical and chemical parameters of the water were measured with the aid of a MULTI 340i/SET device (WTW, Germany) directly in the field: temperature (wt), pH, electroconductivity (ec) and dissolved oxygen concentration (DO) and saturation (DO%). Measurements at all localities were performed between 12:00 pm and 1:00 pm by immersing the corresponding electrodes 0.05 m below the water surface. Water flow rates (Q) were calculated from the cross-sectional area and longitudinal velocity data for every sampling site. The cross-sectional area was first determined by depth (d) and width (w) measurements and then divided into vertical sections where river velocity (v) was measured using a GEOPACKS Stream Flowmeter (Geopacks, UK). Total flow was

computed by summing the flow increments for all the vertical sections [3].

At every locality, water samples for laboratory analysis were taken with the aid of 500-mL polyethylene bottles, which were immersed 30 cm below the water surface against the current. In a laboratory of the Agency for Environmental Protection, the following chemical parameters were analyzed using methods prescribed by standards (SRPS ISO/IEC 17025:2006, ISO/IEC 17025:2005, <http://www.sepa.gov.rs>): concentrations of anions ( $\text{SO}_4^{2-}$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ ,  $\text{HCO}_3^-$ , and  $\text{Cl}^-$ ); concentrations of cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{NH}_4^+$ ,  $\text{Na}^+$ , and  $\text{K}^+$ ); the content of organic nitrogen ( $\text{N}_{\text{org}}$ ), total nitrogen ( $\text{N}_t$ ) and total phosphorus ( $\text{P}_t$ ); total water hardness (WH); alkalinity (al); total alkalinity (al); biological oxygen demand ( $\text{BOD}_5$ ); chemical oxygen demand (COD); and the content of total organic carbon (TOC).

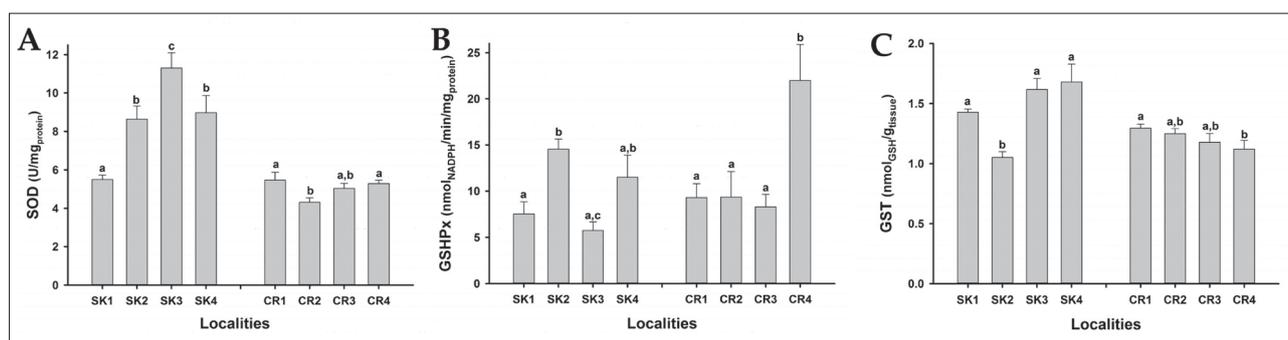
### Preparation of homogenates for biomarker analysis

Homogenates of whole larvae were used to determine components of antioxidative defense. Larvae were homogenized on ice in sucrose buffer (pH 7, 100 mg/2 mL) using an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) for 3 x 10 s at 2000 rpm followed by 3 x 15 s sonication steps using a 50-W sonifier (Bandelin Sonopuls HD2070, Berlin, Germany). After centrifugation at 105000 x g and 4°C (Beckman L7-55 ultracentrifuge, Beckman, Nyon, Switzerland), the supernatant was separated and kept at -24°C until use. The number of replications were as follows: n=5 at SK2, n=7 at SK1, n=8 at SK4 and CR2, n=9 at CR1 and CR4, n=10 at SK3 and n=11 at CR3.

### Determination of protein concentration and enzymatic activity

Protein concentration was determined by the Bradford method [37] using bovine serum albumin as the standard. The activity of SOD was determined by the method of Misra and Fridovich [38]; the activity of GPx was determined according to the method described by Tamura et al. [39]. In order to determine the concentration of total GSH, a portion of the sonicated homogenates was used to precipitate proteins with





**Fig. 2.** Influence of trout farms on selected components of antioxidative defense. Activities of SOD (A) and GPx (B), and the concentrations of total GSH (C) in *Ecdyonurus venosus* at the investigated localities along the Skrapež (SK1, SK2, SK3, and SK4) and Crnica (CR1, CR2, CR3, and CR4) rivers. Data are expressed as the mean±standard error. Bars marked with different letters (a, b or c) are statistically different, and those marked with the same letter are not.

immediately downstream from it (SK2) are on opposite sides of the F1 axis, which explains the greatest part of variability (51%), while the other two downstream localities (SK3 and SK4) are between them, indicating recovery from the influence of the fish farm. Therefore, values of abiotic parameters of the water located at the positive end of the F1 axis increased and those located at its negative end decreased at locality SK2 when compared to the control locality (Table 1). The most pronounced changes in abiotic parameters of the water at SK2 compared to SK1 were a decrease in DO and increases in  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{NH}_4^+$ ,  $\text{P}_t$  and TOC (Table 1, Fig. 1A).

In the case of the Crnica River, PCA showed no visible influence of the fish farm on the chemical parameters of the water (Fig. 1B), since the investigated localities are arranged along the F1 axis, which again explains the bulk of variability (60%) in a regular downstream series from CR1 at the positive end of the axis to CR3 and CR4 at its negative end.

One of the most pronounced differences in the abiotic parameters of the water between two rivers, which could account for the differences in the trout farms' influence observed by PCA, is in the water flow that is several times higher in the Crnica River as compared to the Skrapež River (Table 1).

### Influence of the trout farms on selected components of antioxidative defense

On the Skrapež, the influence of the trout farm on the activity of the tested molecular biomarkers in the

larvae of *E. venosus* mimicked the described effects on the chemistry of the water (Fig. 2). Thus, ANOVA showed that in the cases of all three molecular markers, statistically significant variation occurred between the investigated localities as follows:  $F=14.00$ ,  $P<0.001$  for activity of SOD;  $F=5.94$ ,  $P=0.003$  for activity of GPx; and  $F=7.28$ ,  $P=0.001$  for the concentration of total GSH in the larvae. Moreover, the Fisher LSD test used to compare pairs of localities indicated that in the cases of all three molecular markers a difference exists between SK1 and SK2. As regards SOD and GPx, there was an increase in activity at SK2 (Fig. 2A, B), whereas the concentration of GSH declined at SK2 (Fig. 2C), which clearly indicates that the observed statistically significant changes were the consequence of the action of the trout farm. In the case of SOD, the increase continued at SK3 ( $P=0.037$ ), while at SK4, a statistically significant decline was observed in relation to SK3 ( $P=0.026$ ) (Fig. 2A). Such changes in SOD activity are in strong positive correlation with changes in the total phosphorus concentration ( $R=0.971$ ,  $P=0.029$ ). The activity of GPx declined at SK3 ( $P<0.001$ ) and increased again at SK4 ( $P=0.009$ ) (Fig. 2B), which correlates with the changes in nitrite concentration ( $R=0.995$ ,  $P=0.005$ ) and total water hardness ( $R=0.973$ ,  $P=0.027$ ). The concentration of GSH at SK3 and SK4 returned to the values recorded at the control locality (Fig. 2C).

On the Crnica River, ANOVA (Fig. 2) revealed statistically significant variation between the investigated localities only in the case of GPx activity ( $F=5.00$ ,  $P=0.006$ ), while such variation was absent in the cases of SOD activity and GSH concentration. The activity

of GPx was practically identical at the first three localities, but increased markedly at locality CR4 (Fig. 2B), which cannot be a consequence of the action of the trout farm.

## DISCUSSION

The products of fish metabolism, remains of food, and dissolved nutrients from fish farms are the main pollutants released by them [41], and they represent one of the most frequent problems in pollution of the recipient [42]. Only about 30% of fish food is transformed into biomass of the object of aquaculture, while the remainder flows into the recipient and constitutes the most important parameter when considering the influence of a fish farm on an aquatic environment [43,44]. In view of this and the fact that certain amounts of phosphorus and nitrogen (which fish food contains) can cause eutrophication, in the present study, for comparison of the effects of trout farms with different production capacities, we selected the same type of fish food (see Materials and Methods). Enzymes of the antioxidative defense system (SOD and GPx), and total GSH were used as biomarkers.

In the present study, the influence of outlet waters from the fish farm on abiotic parameters of the water was recorded only in the case of the Skrapež River. The greatest increases in  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{NH}_4^+$ ,  $\text{P}_t$  and TOC concentrations were recorded directly below the fish farm at locality SK2, where its influence was the most noticeable.

SOD is an effective and sensitive biomarker and a reliable indicator of oxidative stress and the negative effects of various toxins [6], and it is considered to be the first line of defense against the harmful action of reactive oxygen species (ROS) [45,46]. GPx is an enzyme responsible for the reduction of  $\text{H}_2\text{O}_2$  and elimination of organic hydroperoxides [47]. It is of key significance to the detoxification of  $\text{H}_2\text{O}_2$  when the latter is present in low concentrations, whereas saturation of GPx by the substrate occurs at high concentrations of  $\text{H}_2\text{O}_2$  [33,48].

We analyzed the changes in the activities of both enzymes in *E. venosus* under the influence of waste waters from the trout farms. The results of this testing indicate that the activities of both SOD and GPx

were increased at locality SK2 on the Skrapež River, where the fish farm's influence was most pronounced, whereas an opposite trend in the activities of these two enzymes was in evidence at localities SK3 and SK4. At locality SK2, where the strongest influence of the fish farm was manifested, a rapid increase in the concentration of the superoxide radical probably occurred because of the powerful influence of the environmental stressor, which caused the activity of SOD to increase. Vranković et al. [20] also noticed a similar phenomenon, namely increased activity of SOD in the species *Holandriana holandrii*, indicating the presence of an increased concentration of the superoxide radical. However, in our study the increased activity of SOD probably was not effective enough to rapidly detoxify the increase in concentration of this radical. It can be concluded from this that the concentration of  $\text{H}_2\text{O}_2$  was not excessively high and that the increased activity of GPx was sufficient for its elimination at locality SK2. At locality SK3 the activity of SOD increased, which led to an increase in the concentration of  $\text{H}_2\text{O}_2$  and decreased GPx activity. Such a hypothesis is based on the results obtained by Łukaszewicz-Hussain and Moniuszko-Jakoniuk (2004) [48] when examining the changes in the activities of GPx, GR and CAT, and GSH and  $\text{H}_2\text{O}_2$  concentrations in the liver of rats after acute intoxication with an organophosphate insecticide. The authors noted a statistically significant negative correlation between  $\text{H}_2\text{O}_2$  concentration and GPx activity. Thus it is possible to explain the correlation of SOD and GPx activities at all localities on the Skrapež River investigated in the present study.

If we analyze the trend of changes in SOD and GPx activities from locality to locality, we can see that they are strongly correlated with the respective changes in concentrations of total phosphorus and nitrite along the Skrapež River. Mirčić et al. [6] investigated the influence of trout farms on the activities of the antioxidant enzymes SOD and catalase in the larvae of *Dinocras megacephala*. Contrary to our results, the results of their study indicated the greatest activity of SOD at the first locality downstream from the fish farm. However, in their study PCA analysis did not reveal any statistically significant correlation between SOD and the chemical parameters of the water [6]. Contrary to this, our results indicate that changes in SOD activity are in strong positive correlation with changes in the concentration of total phosphorus,

while changes in GPx activity correlate with changes in nitrite concentration. It can therefore be concluded that these chemical parameters of the water exert a strong influence on the activities of the investigated enzymes of *E. venosus* larvae in the Skrapež River. This is also supported by the results of Kelso et al. [49], who conducted nitrite toxicity tests on the genera *Ephemera* and *Hexagonia* and reported that both genera exhibited high sensitivity. They were also among the most sensitive to nitrite toxicity in comparison with other tested macroinvertebrates [50]. Additionally, values of  $P_t$  between 0.01 and 0.02 mg/L are considered to be critical levels [51] above which eutrophication is accelerated in surface waters, and they were exceeded at all three localities on the Skrapež River downstream from the trout farm, making it a good candidate for a prime stressor that caused changes in SOD activity.

The metabolism of reduced GSH is one of the main antioxidative defense mechanisms of living organisms [46]. For its realization, GPx utilizes low-molecular-weight thiols such as GSH [51], which can explain the decrease in its concentration in the Skrapež River at locality SK2, since the activity of GPx was significantly elevated. Despotović et al. [32] also noted that a decrease in GSH concentration was in positive correlation with the increase in GPx activity in snails of the species *Viviparus acerosus*.

In the case of the Crnica River, in contrast to the Skrapež River, the influence of the fish farm on the abiotic parameters of water and tested molecular biomarkers was lacking.

Comparing the results obtained on the two investigated rivers, it is clear that the characteristics of the watercourse have a very significant role in determining the influence of the fish farms on macrozoobenthic organisms. The absence of effects of the fish farm on abiotic parameters of the water and the investigated molecular markers in the Crnica River is a consequence of increased water flow. At the time of sample taking, the Crnica was characterized by very high values of water flow because of which the influence of the fish farm was reduced to below the limits of sensitivity of the analyzed biomarkers. Also, it is known that the influence of fish farms is inversely proportional to the water flow in streams [3]. In comparison with the Crnica, the water flow in the Skrapež River was significantly lower, and

influence of the fish farm on both chemical parameters and molecular markers was clearly discernible.

On the basis of the obtained results, it can be concluded that the trout farm on the Skrapež River exerted a mild but clear influence on both the water chemistry parameters in the receiving watercourse and the tested molecular biomarkers in the larvae of *E. venosus*, whereas such effects were lacking in the case of the trout farm on the Crnica River, probably because of the high water flow. Therefore, the changes in GPx, SOD and GSH in *E. venosus* proved to be reliable and sensitive biochemical biomarkers of environmental stress caused by the trout farm effluents, making this species an excellent bioindicator of freshwater pollution.

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