



Oxidative stress and metabolic adaptation in *Synurella ambulans*: Assessing pollution impact in the hyporheic zone[☆]

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ABSTRACT

Oxidative stress is a prevalent mechanism of physiological stress caused by exposure to pollutants or environmental hypoxia in aquatic organisms. The hyporheic zone (HZ), a transitional area between surface and groundwater with distinct ecological conditions, is highly susceptible to pollution and hypoxia, but the physiological responses of its aquatic inhabitants to these stressors remain poorly understood. The aim of this study was to investigate the effect of wastewater treatment plant (WWTP) effluent as a point source of contamination on the battery of biomarkers of antioxidant defence (catalase, CAT; glutathione reductase, GR), xenobiotic biotransformation (glutathione-S-transferase, GST), and aerobic/anaerobic energy metabolism (pyruvate kinase, PK; phosphoenolpyruvate carboxykinase, PEPCK; lactate dehydrogenase, LDH) in a stygophilous freshwater amphipod *Synurella ambulans* from the Sava River HZ. The samplings were conducted in relation to the Sava River's hydrologic regime after low river discharge (in September 2020) and after high river discharge (in April 2021) at one site (Medsave) upstream of the WWTP outflow and at two sites downstream (Podsused and Jarun). *S. ambulans* populations at sites downstream of the WWTP showed higher CAT, GR, GST and LDH activity than population at the upstream site. PK/PEPCK ratio was significantly lower in the *S. ambulans* populations at Podsused and Jarun sites compared to Medsave population after low river discharge, indicating lower aerobic capacity and greater reliance on anaerobic metabolic pathways. This study improves the understanding of the physiological responses of crustaceans exposed to chemical pollution and environmental stressors, thus contributing to the ecological assessment of groundwater connected ecosystems.

1. Introduction

In biological systems, reactive oxygen species (ROS) are produced naturally during aerobic metabolism by the partial reduction of oxygen

(O₂) to water (H₂O). ROS play important signalling roles in cells, but excessive ROS production can damage macromolecules including proteins, DNA and membrane lipids leading to oxidative stress (Halliwell and Gutteridge, 2015; Sies and Jones, 2020). Therefore, maintaining the

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balance between the metabolic ROS production and detoxification by cellular antioxidants plays a key role in metabolic regulation, signalling and cell survival (Sies and Jones, 2020). Although ROS are byproducts of normal aerobic metabolism, their efflux is usually increased by exposure to hypoxia or contaminants (Tafani et al., 2016; Tanabe et al., 2022). For example, the elevated production of ROS and the resulting oxidative stress in aquatic organisms can be triggered by a variety of contaminants, such as metals and organic contaminants (e.g., quinones, polycyclic aromatic compounds (PAHs), halogenated hydrocarbons, pesticides) (Livingstone, 2003; Lushchak, 2011). Furthermore, changes in environmental parameters such as temperature and oxygen levels affect the homeostasis of living organisms and can induce oxidative stress (Livingstone, 2003; Lushchak, 2011; van der Oost et al., 2003; Winston and Di Giulio, 1991). As a defence system against the ROS, organisms have enzymatic antioxidants such as the enzymes catalase (CAT), glutathione-S-transferase (GST), glutathione reductase (GR), and superoxide dismutase (SOD), as well as non-enzymatic low molecular weight antioxidants such as glutathione (GSH) and ascorbic acid (Frías-Espéricueta et al., 2022). Since antioxidant enzymes play an important role in the maintenance of redox homeostasis in tissues, changes in their activities are used as biomarkers in biomonitoring and in the assessment of the toxic effects of various contaminants (Valavanidis et al., 2006). Both induction and inhibition of antioxidant enzyme activities were observed as a consequence of exposure to metals, organic contaminants, warming and hypoxia, depending on the organism, contaminant concentration and exposure time (Frías-Espéricueta et al., 2022; Livingstone, 2003; Lushchak, 2011; Mesquita et al., 2023; Valarmathi and Azariah, 2003; Wang et al., 2012).

The hyporheic zone (HZ) is an active ecotone between surface water and groundwater that plays an important role in removing the pollutants from the stream water by adsorbing them on the sediment particles (Boulton et al., 1998; Lewandowski et al., 2019; Peralta-Maraver et al., 2018). Thus, the HZ provides a crucial ecosystem service by acting as a water-purifying bioreactor (Lewandowski et al., 2011) or 'river's liver' (Fischer et al., 2005). The adsorption may be followed by transformation or degradation of nutrients and pollutants that occur as a result of biofilm activities (Battin et al., 2016). Hyporheic communities of macro-invertebrates and meiofauna are very important for biofilm activities because their bioturbation and bioirrigation activities increase sediment permeability (Peralta-Maraver et al., 2018).

Hyporheic crustaceans such as a stygophilous crustacean *Synurella ambulans* are frequently exposed to hypoxic stress due to extremely rapid and unpredictable fluctuations of oxygen (O₂) concentrations in groundwater (Hervant and Malard, 2012). Although crustaceans generally act as O₂ regulators and are able to maintain a constant O₂ uptake when O₂ partial pressure drops, ventilation activities and aerobic metabolism cease when critically low oxygen levels are reached. Hyporheic crustaceans often have reduced metabolic rate, possibly due to the hypoxic conditions to which they are exposed (Havird et al., 2014; Hervant et al., 1995; Hervant and Mathieu, 1995). Such hypoxic or even anoxic conditions have directed the evolution of physiological and metabolic adaptation strategies in crustaceans, such as large amounts of stored glycogen and phosphagen arginine phosphate (Hervant, 1996). These adaptive strategies hinge on the regulation of key metabolic enzymes involved in aerobic and anaerobic metabolism, and ROS detoxification, which ensure the maintenance of energy and redox balance under fluctuating environmental conditions (Greenway and Storey, 2001; Li et al., 2023; Somero, 2004).

The enzymes pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), and lactate dehydrogenase (LDH) play an important role in orchestrating glucose oxidation for cellular energy metabolism – PK irreversibly converts phosphoenolpyruvate (PEP) into pyruvate and thus drives aerobic metabolism (Cameselle et al., 1980). In the absence of O₂, aerobic pyruvate oxidation is suppressed, and PEP is converted to oxaloacetate by PEPCK and subsequently channelled for anaerobic succinate production in the mitochondria (Li et al., 2019; Sussarellu

et al., 2012). Consequently, PEPCK and PK compete for a common substrate (PEP), with PK directing carbon flux towards aerobic oxidation and PEPCK towards anaerobic pathways (Bayne, 2017; Tielens et al., 1992). This competition positions PK and PEPCK as indicators of the transition between aerobic and anaerobic metabolic pathways, making the PK/PEPCK ratio a valuable index for assessing relative aerobic capacity, with a decreasing ratio indicating an increased reliance on anaerobic pathways for ATP production (Bayne, 2017; Falfushynska et al., 2016; Sussarellu et al., 2012). Furthermore, lactate dehydrogenase (LDH), a cytoplasmic enzyme, catalyzes the reversible reduction of pyruvate to lactate in glycolysis, making it a common biomarker for anaerobic metabolism (Havird et al., 2014; Jie et al., 2021).

Enzymatic biomarkers in crustaceans are influenced by seasonal and hydrological changes in abiotic factors (e.g., temperature, dissolved O₂ concentration, contaminants, food availability) (Jemec et al., 2017; Sroda and Cossu-Leguille, 2011; Strode et al., 2023; Vranković et al., 2018) or endogenous factors (e.g., reproductive cycle, age, and feeding habits) (Jemec et al., 2010; Livingstone, 2001). Hydrological fluctuations such as changes in water flow, salinity and seasonal shifts in discharge or freshwater supply can also significantly influence the activity and expression of these biomarkers (Paital and Chainy, 2013). Therefore, understanding both the seasonal and hydrological variation of biomarkers in organisms is crucial for adequate monitoring of anthropogenic impacts on the aquatic environment, as these factors can interact and influence organisms' responses to pollutants and other stressors.

In our previous research, we found that *S. ambulans* is a perspective bioindicator species of chronic metal contamination in the HZ of the Sava River and that the concentrations of metals, phosphates and nitrates in the interstitial water of the HZ can be considered moderately elevated, probably due to agricultural activities and wastewater treatment plant (WWTP) effluents (Redžović et al., 2023a). In addition, the adenylate energy charge (AEC) was shown to be a useful physiological biomarker of environmental stress in *S. ambulans*, with the strongest associations of AEC with Fe and Zn accumulated in amphipods (Redžović et al., 2023b). Therefore, the aim of this study was to further investigate the effects of WWTP effluents after low and high river discharge (September 2020 and April 2021, respectively) on the battery of enzymatic biomarkers related to the anaerobic capacity and antioxidant defence responses of an indigenous amphipod, *S. ambulans*, from the HZ of the Sava River, the longest tributary of the Danube. In this study we focused on the key marker enzymes involved in antioxidant defence (CAT and GR), biotransformation of xenobiotics (GST) and aerobic/anaerobic energy metabolism (PK, PEPCK and LDH) in *S. ambulans* collected from three study sites: one upstream of the WWTP outflow (Medsave) and two downstream sites (Podsused and Jarun). The selection of biomarkers in this study was guided by the need to capture a broad spectrum of physiological responses relevant to hyporheic amphipods, while simultaneously ensuring ecological relevance and methodological robustness. Given the complexity of environmental stressors in the HZ where organisms are exposed to fluctuating oxygen levels, variable organic loads, and mixtures of contaminants, our approach prioritized biomarkers that are sensitive to cumulative metabolic and oxidative stress. This strategy allows for the detection of sublethal effects under environmentally realistic (*in situ*) conditions. To our knowledge, this is the first *in situ* study reporting biochemical responses of the stygophilous amphipod *S. ambulans* inhabiting vulnerable HZ, exposed to anthropogenic contamination. Given the ecological importance of HZ for groundwater–surface water interactions, these results provide valuable insights into the ecological state of global freshwater ecosystems and the potential impact of wastewater discharges on vulnerable subterranean fauna.

2. Materials and methods

2.1. Study area

The study area (a section of the Zagreb aquifer system) is situated in the north-west of the Republic of Croatia (Fig. 1) and comprises the city of Zagreb and its surroundings. As the capital of Croatia and a centre of population and human activities, Zagreb plays a central role in shaping the ecological and hydrological dynamics of the region. This includes changes in groundwater recharge and discharge due to urban development, pollution from industrial or residential sources affecting water quality, alterations in land use affecting surface runoff and infiltration, and climate-related impacts on water availability (Parlov et al., 2012; Vlahović et al., 2009). Three sampling sites on gravel bars of the Sava River were selected at different distances from the point source of pollution, namely the effluent discharge from the WWTP of the town of Zaprešić. The first sampling site is located 3 km upstream of the WWTP outflow near the village of Medsave (45°50′04″N, 15°46′32″E), surrounded by agricultural land. The second sampling site is located 900 m downstream of the WWTP outflow near Podsused in the suburb of the city of Zagreb (45°49′38″N, 15°49′20″E), near the confluence of the Krapina River with the Sava River. The third sampling site is located about 13 km downstream of the WWTP outflow near the city district of Jarun (45°46′24″N, 15°55′56″E) in an urban area of the city of Zagreb. The WWTP in the town of Zaprešić receives treated wastewaters from the pharmaceutical industry, baker's yeast production and the wastewaters from the local municipality, which are then treated and discharged into the Sava River (Ferina, 2015). Only primary (mechanical) treatment is carried out in this WWTP (Česen et al., 2019). Although the effluent from the WWTP may be warmer than the surrounding river water, the thermal impact on the river is minimal, especially considering the high flow rates of the Sava River, which dilutes the effluent. The daily discharge volume of the Zaprešić WWTP (6000–8000 m³/day) is negligible compared to the river's flow of up to 1200 m³/s in winter (<https://hidro.dhz.hr/>). In addition, the WWTP outflow is located on the left bank of the Sava River, while the sampling site at Podsused is on the

right bank, which further reduces the thermal impact. This is further supported by Milaković et al. (2019), who found no significant temperature differences in the river upstream and downstream of the WWTP outflow, emphasising the limited thermal footprint of the wastewater discharge in winter. The physicochemical properties and organic carbon content of the interstitial water from the HZ at selected sampling sites are shown in Table 1, while a detailed methodological description and explanation of these properties is provided in the Supplementary material section.

2.2. Sampling dynamics

To account for differences in enzymatic activity related to hydrologic regime, we conducted two samplings: after low river discharge (15th and September 17, 2020) and after high river discharge (12th, 23rd, and April 30, 2021) (Fig. 2). These periods were chosen considering two distinct scenarios. Scenario 1: A prolonged low discharge could increase the concentrations of contaminants from the WWTP effluent in the surface water, consequently increasing their concentrations in the interstitial water in the HZ and affecting the antioxidant enzyme activities and aerobic capacity of the studied organism *S. ambulans*. Scenario 2: Increased river discharge could dilute the WWTP effluent, potentially lowering contaminant concentrations. However, increased rainfall could also enhance weathering and leaching processes, resulting in additional contaminant concentrations from diffuse pollution sources entering the river and thus increasing overall concentrations.

2.3. Amphipod sampling

Synurella ambulans was selected as a study species because it is a euryoecious species that usually inhabits both surface waters and groundwater connected habitats such as the HZ (Arbačiauskas, 2008; Boets et al., 2010; Gottstein Matočec et al., 2002; Karaman, 1974). The samples were collected from the shallow HZ in the gravel bank (average depth of 55 cm) using a Bou-Rouch piston pump (Bou and Rouch, 1967). A mobile steel pipe (Ø 50 mm, 110 cm long) with a perforated distal end



Fig. 1. Map of the study area on the Sava River (Croatia) with three sampling sites – Medsave, Podsused and Jarun. (Source: State Geodetic Administration of the Republic of Croatia).

Table 1

Physicochemical parameters in interstitial water of the Sava River (river type HR-R.5 b) and organic carbon concentrations from three sampling sites (Medsave, Podsused and Jarun) in two sampling campaigns (after low river discharge in September 2020 and after high river discharge in April 2021) and threshold values for surface water and groundwater according to Croatian Directive on Water Quality Standards (GRC, 2019). Indicated values (italicized) are those below good water quality status of surface water according to classification in Croatian Directive on Water Quality Standards (GRC, 2019).

Parameter	Unit	Medsave		Podsused		Jarun		Threshold values for river type HR-R.5 b	
		September 2020	April 2021	September 2020	April 2021	September 2020	April 2021	Very good	Good
								Surface water	Surface water
								Groundwater	Groundwater
Water temperature	°C	22.0	14.2	20.3	11.4	16.8	11.3		
DO	mg O ₂ L ⁻¹	8.10	7.93	7.50	8.80	5.30	5.40		
Saturation	% O ₂	95.3	79.3	82.0	82.5	55.3	51.0		
pH		8.15	7.96	7.89	8.07	7.42	7.54	7.40–8.50	7.00–7.40; 8.50–9.00
Conductivity	µS cm ⁻¹	410	427	440	421	631	572	N.E. ^a	N.E. 2500
Alkalinity	mg CaCO ₃ L ⁻¹	188	218	238	241	308	277		
Total water hardness	mg CaCO ₃ L ⁻¹	249	196	231	142	356	196		
P-PO ₄ ³⁻	mg L ⁻¹	0.029	0.024	0.031	0.037	0.075	0.013	0.03	0.10
N-NO ₂	mg L ⁻¹	<LOD ^b	0.058	<LOD	0.126	0.086	0.034	N.E.	0.20
N-NO ₃	mg L ⁻¹	1.69	1.59	2.14	1.35	0.88	0.93	0.80	N.E. 0.50
COD _{KMnO4}	mg O ₂ L ⁻¹	2.04	3.38	1.89	5.26	14.9	2.04	N.E.	1.80 50
DOC in water	mg L ⁻¹	1.43	0.96	1.37	1.44	0.87	0.76	2.50	5.50
POC in water	mg L ⁻¹	1.95	0.71	1.94	26.8	2.65	0.54	N.E.	N.E.
TOC in sediment	%	3.66	1.88	2.94	2.19	2.83	1.69		

^a N.E. – threshold values not established.

^b < LOD – below limit of detection (<0.001).

(thirty-five 5 mm diameter holes) was inserted into the gravel bar. For each sample, 50 L of the mixture of water and sediment were pumped out to obtain biological samples. This sampling procedure was repeated at a minimum of three points (along the gravel bar) per sampling site. During pumping, the mixture of water and sediment was sieved to collect invertebrates using a set of fine-mesh hand nets (100, 200 and 500 µm). The amphipods retained by the two largest nets (200 and 500 µm) were stored in polyvinyl chloride bottles filled with ambient water and transported to the laboratory in a portable refrigerator.

2.4. Amphipod identification

The specimens were identified under a stereomicroscope (Zeiss Stemi, 2000C, Jena, Germany) on the basis of the diagnostic features proposed by Karaman (1993) for *S. ambulans*. As microscopic sex determination in *S. ambulans* is extremely time-consuming, we did not determine the sex of each individual to avoid the risk of heating the specimens and thus jeopardising reliable enzyme measurements. Only ovigerous females were excluded from the analyses. The animals were sorted, counted, shock-frozen in liquid nitrogen and stored at −80 °C until enzyme analyses.

2.5. Homogenization of amphipod samples

The fresh mass of all individuals was measured prior to enzyme analysis. Amphipods were pooled due to their small size and mass (8–40 specimens per sample, wet mass of a sample: 0.026 ± 0.012 g; Table S1). The number of pooled samples per sampling site was six (n = 6).

The amphipod samples for measurement of PK, PEPCK, LDH were homogenised in seven volumes of ice-cold homogenization buffer containing 10 mM Tris-HCl (Merck, Germany, pH 7.0 at 4 °C) with 5 mM ethylenediaminetetraacetic acid, EDTA (Merck, Germany), 1 mM dithiothreitol, DTT (Sigma, USA) as a reducing agent and 0.1 mM phenylmethylsulfonyl fluoride, PMSF (Sigma, USA) as protease inhibitor.

The amphipod samples for the measurement of GR were homogenised in four volumes of ice-cold homogenization buffer containing 10 mM Tris-HCl (Merck, Germany, pH 8.2 at 4 °C) with 5 mM EDTA (Merck, Germany) and 0.1 mM PMSF (Sigma, USA). For CAT and GST measurements, the amphipod samples were homogenised in seven volumes of ice-cold homogenization buffer containing 100 mM Tris-HCl/base (Merck, Germany, pH 8.1 at 4 °C) with 1 mM DTT (Sigma, USA) and 0.5 mM PMSF (Sigma, USA). Homogenization of all samples was performed using Teflon pestle in ice-cooled Eppendorf tube (Glas-Col, USA) and the resulting homogenates were centrifuged in the Centric 260 R centrifuge (Domel, Železniki, Slovenia) for 8 min at 10,000×g and 4 °C. Supernatants were collected and stored at −80 °C until the assays were performed. Each sample was thawed only once, immediately before the assay.

2.6. Determination of CAT activity

The activity of CAT was measured according to the method of Claiborne (1985). In brief, sodium phosphate buffer (50 mM, pH 7.0) and hydrogen peroxide (30 %) were used to prepare 15.8 mM H₂O₂, which was added to 5-fold diluted samples; the reaction volume was 300 µL. The absorbance was measured every 10 s for 1 min at 340 nm at 25 °C. The specific enzyme activity was expressed as µmol of degraded H₂O₂ per min per g tissue wet mass (w.m.) or U g⁻¹ w.m. and calculated using a molar extinction coefficient of 0.0436 mM⁻¹ cm⁻¹.

2.7. Determination of GR activity

The activity of GR was measured according to the method of Mavis and Stellwagen (1968). Oxidation of 0.8 mM β-nicotinamide adenine dinucleotide phosphate (β-NADPH) by GR in the presence of 30 mM oxidized glutathione (GSSG), 1.0 % (w/v) bovine serum albumin (BSA) and 100 mM potassium phosphate buffer containing 3.4 mM EDTA (pH 7.6 at 25 °C) was monitored every 60 s for 30 min at 340 nm at 25 °C.

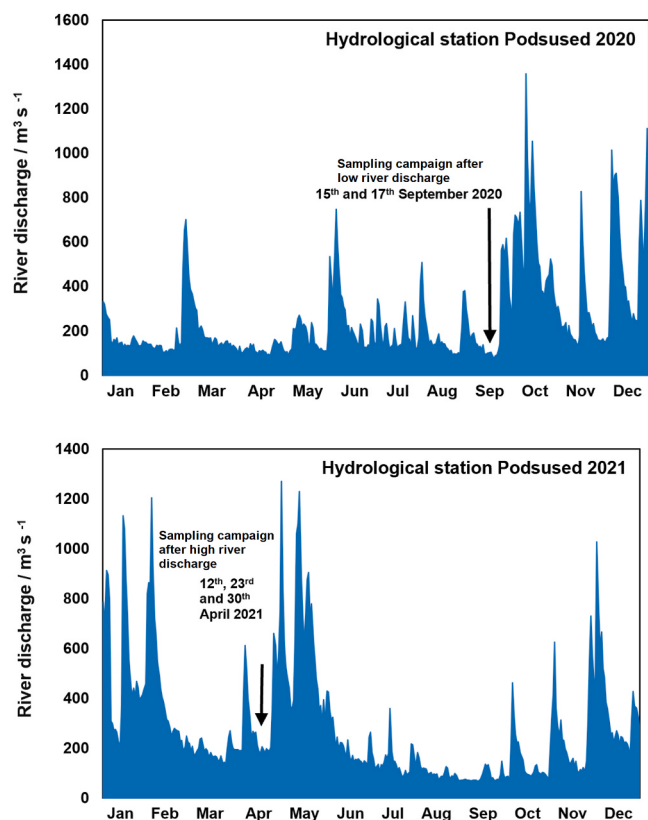


Fig. 2. Water discharge measurements at the hydrological station Podused on the Sava River (45°48'27"N, 15°50'19"E), along with the timing of sample collection in 2020 and 2021, are presented. Arrows on the hydrographs indicate the specific time periods during which samples were collected. Data provided from Meteorological and Hydrological Service of Croatia.

The activity of GR was calculated from the extinction coefficient of NADPH of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as μmol NADPH oxidized per min per g tissue wet mass (w.m.) or $\text{U g}_{\text{w.m.}}^{-1}$.

2.8. Determination of PK activity

The activity of PK was determined as described by [Simpfendorfer et al. \(1995\)](#). The assay mixture for PK was as follows: 83 mM Tris-HCl (pH 7.0), 250 mM KCl, 250 mM $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$, 100 mM adenosine diphosphate (ADP), 5.6 mM NADH, 550 U lactate dehydrogenase (LDH), and 50 mM phosphoenolpyruvate (PEP). Changes in NADH absorbance at 340 nm were monitored every 20 s for 7 min. The biochemical activity of PK was calculated from the extinction coefficient of NADH of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as μmol NADH oxidized per min per g of tissue wet mass (w.m.) or $\text{U g}_{\text{w.m.}}^{-1}$.

2.9. Determination of PEPCK activity

The activity of PEPCK was quantified as described by [Simpfendorfer et al. \(1995\)](#). Briefly, the assay consisted of 166.7 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES, pH 7.0), 11.5 mM $\text{MnCl}_2 \times 4 \text{ H}_2\text{O}$, 25 mM inosine 5'-diphosphate sodium salt (IDP), 499.4 mM KHCO_3 , 5.64 mM NADH, 1000 U malate dehydrogenase (MDH), and 250 mM PEP. Absorbance was measured at 340 nm every 20 s for 15 min. The activity of PEPCK was calculated from the extinction coefficient of NADH of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as μmol NADH oxidized per min per g of tissue wet mass (w.m.) or $\text{U g}_{\text{w.m.}}^{-1}$.

2.10. Determination of LDH activity

The activity of LDH was determined according to [Bergmeyer et al. \(1965\)](#) by monitoring the oxidation reaction with D-lactate as substrate. The assay consisted of glycine/hydrazine buffer (glycine, 0.5 M; hydrazine, 0.4 M, pH 9.0), 0.05 M β -nicotinamide adenine dinucleotide (NAD^+), and 0.5 M lactic acid potassium salt. Absorbance was measured at 340 nm every 20 s for 15 min. The enzyme activity was calculated from the extinction coefficient of NADH of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as μmol NADH oxidized per min per g of tissue wet mass (w.m.) or $\text{U g}_{\text{w.m.}}^{-1}$.

2.11. Determination of GST activity

The activity of GST was measured according to the method of [Habig et al. \(1974\)](#) with some modifications. In brief, the enzyme was assayed in 0.1 M sodium phosphate buffer (pH 6.5) with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as the electrophilic substrate and 1 mM GSH (reduced glutathione) as co-substrate. The reaction was started by the addition of CDNB. GST activity was measured at 340 nm every 15 s for 3 min. Initial rates of conjugate formation were determined, and the values were corrected for non-enzymatic conjugation (without addition of extract). The activity was expressed in μmol conjugated GSH per min per g of tissue wet mass (w.m.) or $\text{U g}_{\text{w.m.}}^{-1}$ and calculated using a molar extinction coefficient $\epsilon_{340} = 9.60 \text{ mM}^{-1} \text{ cm}^{-1}$.

All biochemical measurements described above were performed spectrophotometrically using the microplate spectrophotometer/fluorometer Infinite M200 (Tecan, Männedorf, Switzerland). The microplate was incubated for 5 min at 25 °C in the microplate reader before starting the enzymatic reaction. Before each measurement, the microplate was shaken for 5 s. All samples were measured in triplicates in microplates. For all enzyme activities, the assay conditions were chosen so that the concentrations of the substrates were not limited and the reactions were linear over the assay time.

2.12. Statistical analyses

Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, USA). Variability of enzyme activities in *S. ambulans* ($n = 6$, number of replicates per sampling site in each sampling campaign) between low and high river discharge were tested using the Mann-Whitney *U* test, as the assumptions of normality and homogeneity of variance were not always met. The significance of the variability of enzyme activities between three sampling sites was tested using the Kruskal-Wallis one-way analysis of variance on ranks, separately for each sampling campaign. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Biomarkers of antioxidative capacity – CAT and GR

The CAT activities in *S. ambulans* did not show significant differences between the low and high river discharge at any of the sampling sites (Fig. 3a). Across both discharge regimes, the average CAT activities in amphipod samples were $1665.0 \pm 174.0 \text{ U g}_{\text{w.m.}}^{-1}$ from the Medsave site, $2066.4 \pm 38.4 \text{ U g}_{\text{w.m.}}^{-1}$ from the Podused site, and $2247.9 \pm 158.6 \text{ U g}_{\text{w.m.}}^{-1}$ from the Jarun site. In general, *S. ambulans* populations at the sites downstream of the WWTP outflow had higher CAT activity than the population at the upstream site, with a significant difference between the upstream site Medsave and downstream site Jarun after high river discharge (Fig. 3a).

The GR activities in *S. ambulans* showed no significant differences between the low and high river discharge (Fig. 3b). Similar as observed for CAT activity, *S. ambulans* populations at sites downstream of the WWTP outflow had higher GR activity than population at upstream site. However, a significant difference between the upstream site Medsave

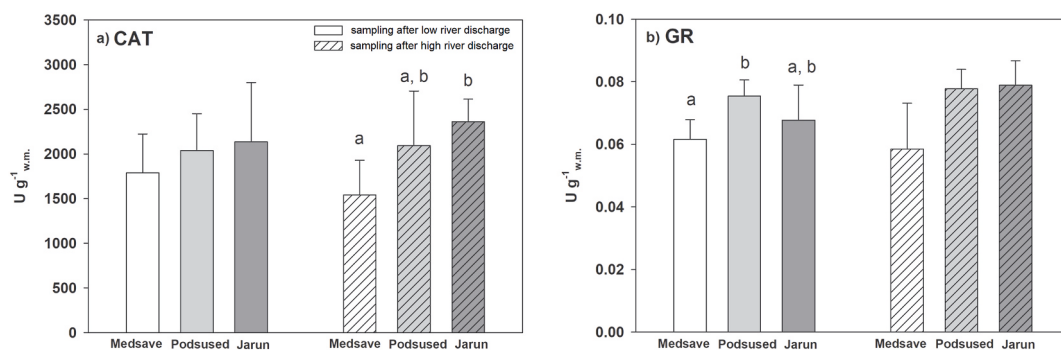


Fig. 3. Antioxidant enzyme activities (bars represent mean values and whiskers standard deviations; U g^{-1} on wet mass basis; $n = 6$) in *S. ambulans* from the HZ of the Sava River at three sampling sites (upstream of the WWTP outflow: Medsave; downstream of the outflow: Podsused and Jarun) and two hydrologic regimes (after low river discharge and after high river discharge) (a) catalase, CAT; (b) glutathione reductase, GR. Statistically significant differences (Mann-Whitney U test, $p < 0.05$) between three sampling sites within the same sampling campaign are assigned with different letters (Kruskal-Wallis one-way ANOVA on ranks, $p < 0.05$).

($0.06 \pm 0.01 \text{ U g}^{-1} \text{ w.m.}$) and the downstream site Podsused ($0.08 \pm 0.01 \text{ U g}^{-1} \text{ w.m.}$), which is closest to the WWTP outflow, only occurred after low river discharge (Fig. 3b).

3.2. Biomarker of exposure to xenobiotics – GST

The GST activities in *S. ambulans* were generally higher at sites downstream of the WWTP outflow than at the upstream site. At the Podsused site, the samples of *S. ambulans* showed a significant seasonal difference, with enzyme activity being significantly higher after high river discharge than after low discharge (Fig. 4). Furthermore, a significant spatial difference was observed after low river discharge, with higher average activity at the Jarun site ($1.23 \pm 0.12 \text{ U g}^{-1} \text{ w.m.}$) compared to upstream Medsave ($1.01 \pm 0.07 \text{ U g}^{-1} \text{ w.m.}$) (Fig. 4).

3.3. Biomarkers of anaerobic capacity of the organism - PK, PEPCK and LDH

Significant seasonal differences in the PK activity in *S. ambulans* were observed at the Medsave and Jarun sites, with higher values obtained

after low river discharge (Medsave: $18.02 \pm 1.17 \text{ U g}^{-1} \text{ w.m.}$; Jarun: $19.13 \pm 0.92 \text{ U g}^{-1} \text{ w.m.}$) than after high discharge (Medsave: $15.80 \pm 1.48 \text{ U g}^{-1} \text{ w.m.}$; Jarun: $14.88 \pm 1.98 \text{ U g}^{-1} \text{ w.m.}$) (Fig. 5a). The PK activities at the Podsused site were almost the same in both low and high discharge regimes. The average values of PK activity showed no significant spatial differences (Fig. 5a).

PEPCK activity was significantly higher in *S. ambulans* at the Jarun site after low river discharge than after high discharge, while PEPCK activities at the Medsave and Podsused sites were similar across both low and high discharge regimes (Fig. 5b). Spatial differences were observed after low river discharge, with the PEPCK activities being higher at the downstream Podsused ($0.98 \pm 0.05 \text{ U g}^{-1} \text{ w.m.}$) and Jarun ($1.07 \pm 0.09 \text{ U g}^{-1} \text{ w.m.}$) sites than at the upstream Medsave site ($0.87 \pm 0.09 \text{ U g}^{-1} \text{ w.m.}$). After high river discharge, significantly higher levels of PEPCK activity were observed in *S. ambulans* at the Podsused site ($1.01 \pm 0.08 \text{ U g}^{-1} \text{ w.m.}$), which is closest to the WWTP outflow, compared to the Jarun site ($0.82 \pm 0.07 \text{ U g}^{-1} \text{ w.m.}$), which is further away from the WWTP outflow (Fig. 5b).

Seasonal differences in the PK/PEPCK ratio as an indicator of relative aerobic to anaerobic capacity were observed in *S. ambulans* only at the Medsave site, with significantly higher values after low river discharge (20.85 ± 1.32) compared to the period after high discharge (16.77 ± 1.22), while the values at the other sites were similar in both low and high discharge regimes (Fig. 5c). The PK/PEPCK ratio in *S. ambulans* after low river discharge was significantly lower at the downstream Podsused (17.50 ± 2.34) and Jarun (18.02 ± 0.97) sites than at the upstream site Medsave (20.85 ± 1.32). No spatial differences in the PK/PEPCK ratio in *S. ambulans* were observed after high discharge (Fig. 5c).

Regarding LDH activity, a significant seasonal difference was observed in *S. ambulans* at the Jarun site, with increased enzyme activity after low river discharge (Fig. 5d). The comparison between sampling sites showed that LDH activities in *S. ambulans* were generally higher at the downstream sites Podsused and Jarun than at the upstream site Medsave. The average LDH activity in *S. ambulans* after low river discharge was significantly higher at the downstream Jarun site ($1.33 \pm 0.13 \text{ U g}^{-1} \text{ w.m.}$) than at the upstream Medsave site ($0.82 \pm 0.30 \text{ U g}^{-1} \text{ w.m.}$). A significant spatial difference was also observed after high river discharge, with higher average activity in *S. ambulans* at the Podsused site ($1.34 \pm 0.19 \text{ U g}^{-1} \text{ w.m.}$) than at the Medsave site ($1.05 \pm 0.13 \text{ U g}^{-1} \text{ w.m.}$), as shown in Fig. 5d.

4. Discussion

In crustaceans exposed to metals and organic contaminants, the activities of enzymes CAT, GR, GST, PK, PEPCK, and LDH have shown variable responses in both field and laboratory studies, depending on the dose, type of contaminant, species, or route of exposure (e.g., Barata et al., 2005; Havird et al., 2014; Hervant, 1996; Jemec et al., 2017; Tatar

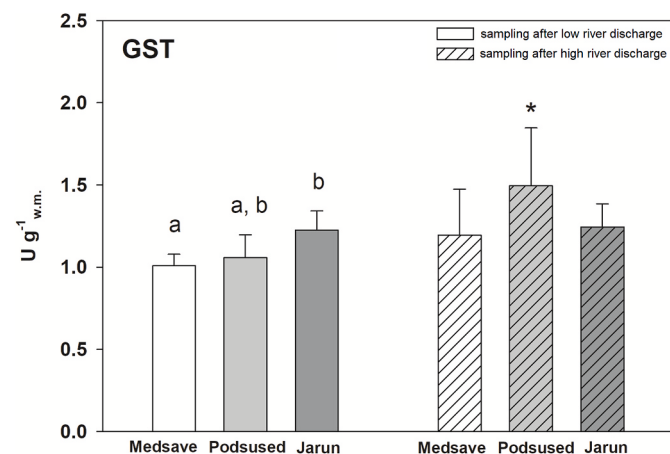


Fig. 4. Activity of enzymatic biomarker of exposure to xenobiotics – glutathione-S-transferase, GST (bars represent mean values and whiskers standard deviations; U g^{-1} on wet mass basis; $n = 6$) in *S. ambulans* from the HZ of the Sava River at three sampling sites (upstream of the WWTP outflow: Medsave; downstream of the outflow: Podsused and Jarun) and two hydrologic regimes (after low river discharge and after high river discharge). Statistically significant differences (Mann-Whitney U test, $p < 0.05$) between two hydrologic regimes at individual sampling sites are marked with asterisk (*) and differences between three sampling sites within the same sampling campaign are assigned with different letters (Kruskal-Wallis one way ANOVA on ranks, $p < 0.05$).

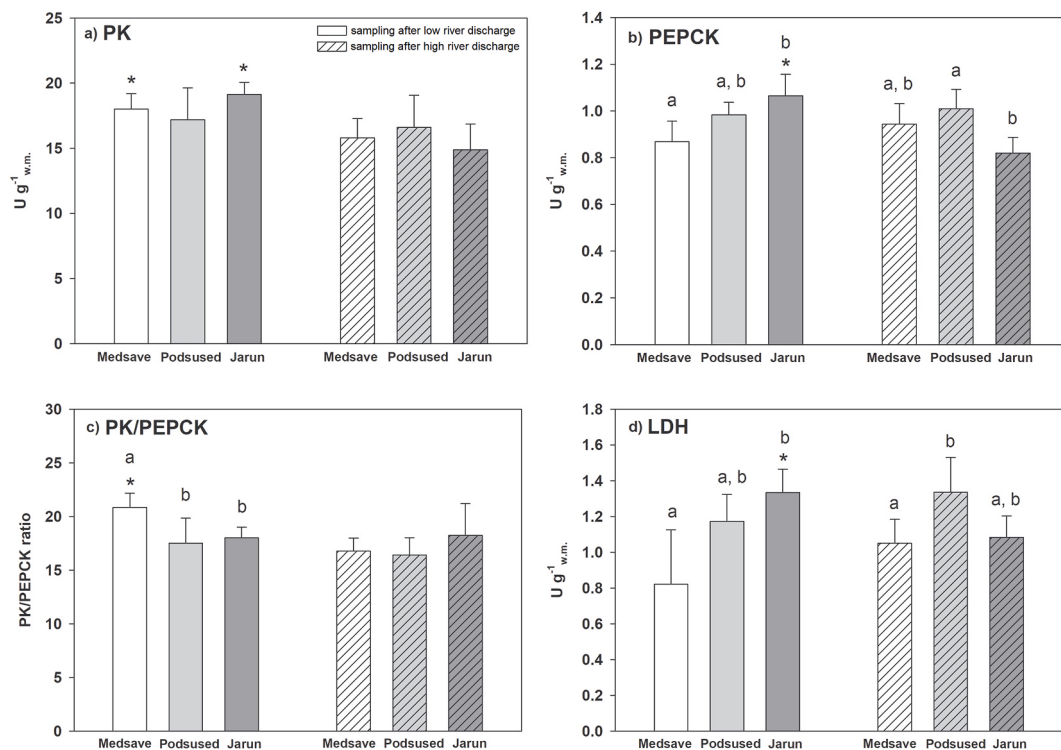


Fig. 5. Activities of enzymatic biomarkers of anaerobic capacity (bars represent mean values and whiskers standard deviations; $U\ g^{-1}$ on wet mass basis; $n = 6$) in *S. ambulans* from the HZ of the Sava River at three sampling sites (upstream of the WWTP outflow: Medsave; downstream of the outflow: Podsused and Jarun) and two hydrologic regimes (after low river discharge and after high river discharge) (a) pyruvate kinase, PK; (b) phosphoenolpyruvate carboxykinase, PEPCK; (c) PK/PEPCK ratio, and (d) lactate dehydrogenase, LDH. Statistically significant differences (Mann-Whitney U test, $p < 0.05$) between two hydrologic regimes at each sampling site are marked with asterisk (*) and differences between three sampling sites within the same sampling campaign are assigned with different letters (Kruskal-Wallis one way ANOVA on ranks, $p < 0.05$).

et al., 2018; Vranković et al., 2018). However, to our knowledge, there are no studies dealing with the effects of multiple contaminants *in situ* on the above-mentioned enzyme activities of the amphipods which inhabit HZ. The biomarker panel employed in this study was specifically designed to achieve an optimal balance between ecological relevance, methodological robustness and physiological scope.

4.1. Biomarkers of antioxidative capacity – CAT and GR

In a normal, healthy cell, there is a balance between pro-oxidant production and antioxidant defence. However, a significant increase in ROS production can overcome antioxidant defences, resulting in increased oxidative stress and biological damage. Many contaminants such as redox cycling compounds (quinones), PAHs, halogenated hydrocarbons (e.g., polychlorinated biphenyls, PCBs), and metals can act as pro-oxidants in aquatic invertebrates (Livingstone, 2003). For example, metals such as Cd, Ni, Pb, and Hg can cause oxidative stress by lowering glutathione levels or by metal-induced displacement of redox metal ions (Stojs and Bagchi, 1995).

The efficiency of the antioxidant defence system of *S. ambulans* was tested by analyzing the activity levels of two complementary enzymatic antioxidants CAT and GR. Our study showed that the CAT activity increased at the Jarun site (downstream of the WWTP outflow) compared to the upstream Medsave site in both discharge regimes, although statistically significant only after high discharge. Furthermore, higher GR activity observed in *S. ambulans* at two sites downstream of the WWTP outflow compared to upstream site indicated its active role in the glutathione-recycling pathways that promote ROS scavenging. Our previous research showed higher average concentrations of Al, Cr, Fe, Ni, and Pb accumulated in *S. ambulans* at the downstream Jarun site (Al 176.6 ± 46.6 ; Cr 0.85 ± 0.21 ; Fe 240.3 ± 64.3 ; Ni 0.59 ± 0.15 ; Pb 0.38

$\pm 0.09\ \mu g\ g_{w.m.}^{-1}$) compared to the upstream Medsave site (Al 118.0 ± 30.4 ; Cr 0.38 ± 0.09 ; Fe 155.4 ± 33.4 ; Ni 0.31 ± 0.05 ; Pb $0.19 \pm 0.04\ \mu g\ g_{w.m.}^{-1}$), suggesting a chronic metal contamination (Redžović et al., 2023a). The concentrations of Cr and Ni in the fine fraction of the sediment ($<63\ \mu m$ particles) from the HZ also tended to be higher at the downstream Jarun site (Cr 18.3 ; Ni $9.8\ mg\ kg^{-1}$) than at the upstream Medsave site (Cr 14.4 ; Ni $7.9\ mg\ kg^{-1}$) (Redžović et al., 2023a). In addition, the same previous study showed the difference in bio-accumulation factors (BAFs, which were calculated as the ratio of the metal concentration in total amphipod body to the metal concentration in the interstitial water from the HZ of the Sava River) between the studied populations of *S. ambulans* with higher BAF values in the population at the downstream Jarun site (Redžović et al., 2023a). The accumulation of Fe, Cr and Ni can be considered particularly important because these transition metals undergo redox cycling, catalysing the formation of ROS via Fenton and Haber-Weiss reactions (Atli and Canli, 2010). This can lead to an increase in intracellular H_2O_2 production and the production of hydroxyl radicals, placing the overall antioxidant defences of organisms under pressure. Therefore, the increased CAT activity in our study probably reflects enhanced detoxification of H_2O_2 , which prevents oxidative stress caused by hydroxyl radicals that might originate from metal pollution. At the same time, GR activity contributes to intracellular redox balance by regenerating reduced glutathione (GSH) from the oxidized form (GSSG), ensuring neutralisation of ROS and supporting conjugation reactions of xenobiotics, further catalysed by GST (Meister and Anderson, 1983). Overall, we observed a coordinated antioxidative defence against increased ROS production, which could be related to the higher concentrations of several elements in the environment and/or *S. ambulans*.

In contrast to our results, the amphipod *Gammarus dulensis* collected directly downstream of the trout farm showed reduced CAT activity

compared to a reference site (Vranković et al., 2018). GR activity also decreased at the site closest to the aquaculture discharge in autumn, probably due to inactivation of the enzyme caused by free radicals. GR activity varied spatially and seasonally, increasing in summer and winter at the affected site compared to the reference site (Vranković et al., 2018). The authors identified elevated concentrations of Cr, Ni and Cd, presumably from trout farming, as the main stressors affecting CAT and GR activity. The sediment concentrations of Cr and Ni at the contaminated sites downstream of the trout farm discharge (Cr 22.3; Ni 16.1 mg kg⁻¹) were higher than average concentrations in the sediments of HZ in our previous study (Cr 16.3; Ni 8.9 mg kg⁻¹) (Redžović et al., 2023a). Thus, it is possible that the lower concentrations of metals in our study area compared to the study by Vranković et al. (2018) were high enough to trigger some antioxidant responses, but did not reach the threshold that leads to inactivation of antioxidant enzyme. In another study, port activities that led to the accumulation of trace metals (especially Cd) in muddy sediments also affected the CAT and GR activity in the marine benthic amphipod *Monoporeia affinis*, with levels being higher at contaminated sites than at uncontaminated sites (Strode et al., 2023).

In addition to metals and organic contaminants, the sex of the organism, seasonal variations, and physicochemical variables of the water can also influence the biomarkers of oxidative stress in crustaceans. Water temperature is an important factor for ectothermic animals, as changes in water temperature can trigger the production of ROS (Lushchak, 2011). The seasonal variability of CAT and GR activities was studied in the benthic amphipod *M. affinis*, which showed higher values in summer than in autumn (Strode et al., 2023). In contrast, no significant seasonal differences in CAT and GR activities were observed in *S. ambulans* in the present study. The concentration of oxygen may be another factor influencing enzyme activity, as it exhibited a positive correlation with CAT and GR activities in amphipods (Strode et al., 2023). However, in our study, CAT and GR activities in *S. ambulans* showed the opposite pattern, as the amphipods from the Jarun site with the lowest dissolved O₂ concentrations and O₂ saturation (compared to Medsave and Podsused sites) after high discharge had the highest CAT and GR activities compared to Medsave site. This pattern indicates specific compensatory upregulation of antioxidant defences under hypoxic conditions.

As there are no data in the literature on the activities of antioxidant defence enzymes of *S. ambulans* or similar stygophilous species, we compared our results with two epigeal freshwater gammarid species. A comparison of CAT activity in *Gammarus roeseli* (80–290 μmol min⁻¹ mg⁻¹ of protein) and *S. ambulans* (26–36 μmol min⁻¹ mg⁻¹ of protein, unpublished data) showed that the values were higher in *G. roeseli* (Sroda and Cossu-Leguille, 2011) suggesting lower basal oxidative defence capacity in the investigated hyporheic species. When comparing GR activity in *G. dulensis* (1.30 × 10⁻³ to 5.10 × 10⁻³ μmol min⁻¹ mg⁻¹ protein, Vranković et al., 2018) with our results for *S. ambulans* (1.01 × 10⁻³ to 1.25 × 10⁻³ μmol min⁻¹ mg⁻¹ protein), similar activity levels were observed. However, *G. dulensis* exhibited a four times wider range of values, which is likely due to chronic exposure to higher contaminant concentrations from aquaculture effluent.

4.2. Biomarker of exposure to xenobiotics – GST

GST is a phase II detoxification enzyme that conjugates reduced glutathione (GSH) to electrophilic xenobiotics and secondary products of oxidative stress. Increased GST activity serves as a biomarker of oxidative stress defence and pollutant exposure, particularly induced by organic xenobiotics like PCBs and organochlorine pesticides (Cossu-Leguille and Vasseur, 2013; George, 1994). Our results indicated elevated GST activities in *S. ambulans* populations from Podsused and Jarun sites chronically exposed to the WWTP effluent, particularly after low river discharge, suggesting a detoxification response associated with increased accumulation of metals, particularly Al, Cr, Fe, Ni and Pb at

the downstream Jarun site, which was detected in our previous study (Redžović et al., 2023a) and already described in preceding section 4.1. This pattern of increased GST activity corresponds to the activation of enzymatic antioxidants in our study. As CAT and GR co-operate to maintain redox balance by removing ROS and recycling GSH, GST uses this regenerated GSH to conjugate xenobiotics and products of lipid peroxidation and oxidative stress. Thus, the observed overall induction of CAT, GR and GST reflects an integrated defence strategy involved in GSH metabolic pathways. Moreover, no inhibition of the enzymes was observed in our study, confirming that the level of pollution in the studied area was not so high as to lead to the breakdown of the antioxidant system, but nevertheless indicating the need for increased activity of antioxidants to cope with environmental stress.

Our results are consistent with literature studies showing that environmental stressors, including metals and other environmental factors, affect GST activity in various crustaceans. Higher GST levels have been observed in *G. dulensis* downstream of metal-contaminated aquaculture sites (Vranković et al., 2018), in the mudflat fiddler crab *Minuca rapax* (Decapoda) exposed to industrial and municipal wastewater (Capparelli et al., 2019), in the freshwater flea *Daphnia magna* (Branchiopoda) exposed to metals and organic pollutants (Barata et al., 2005), and in the amphipod *M. affinis* in metal-rich sediments (Strode et al., 2023).

Seasonal variability can also play a role on GST activity in crustaceans. Elevated GST activity in *M. affinis* (Strode et al., 2023) and *M. rapax* (Capparelli et al., 2019) in summer suggests temperature-related effects. However, in our study, no temperature-related changes in GST activity were observed between two river discharge regimes. Instead, the higher GST activity at Podsused after high river discharge could be related to the increased levels of phosphates, nitrites, and chemical oxygen demand COD_{KMnO4} (Table 1). This is consistent with the study on *G. dulensis*, where biological oxygen demand (BOD₅), total organic carbon (TOC), pH, NH₄⁺, NH₃, NO₂⁻, total hardness (TH), and total alkalinity (TA) had the strongest effects on GST activity (Vranković et al., 2018).

Life-history traits, particularly reproductive stages, further modulate GST activity (Jemec et al., 2017). The reproductive peak of *S. ambulans* in spring (Gottstein et al., 2023) could explain the fluctuations of GST activity related to the period after high discharge (April 2021), especially at Podsused site.

While this study focuses on capturing the ecological responses of *S. ambulans* to WWTP effluents *in situ*, we recognise that mechanistic experimental studies could provide valuable insights into the underlying biochemical processes. Future research exploring the roles of antioxidant enzymes (CAT, GR and GST) in glutathione metabolism, redox cycling of metals (e.g., Fe, Cr, Ni) and ROS production would provide a deeper mechanistic understanding and further enhance the scientific value of this work.

4.3. Biomarkers of anaerobic capacity of the organism – PK, PEPCK and LDH

Crustaceans living in HZ, as a groundwater connected ecosystem, have to cope with hypoxic conditions more or less frequently. To survive under such conditions, they have developed specific behavioural, biochemical, and physiological adaptations, such as the ability to decrease their metabolic rate during hypoxia by reducing activity and ventilation, and lowering glycolysis and respiration rates. High concentrations of stored glycogen and phospho-L-arginine in these organisms allow a longer duration of anaerobic metabolism and prolong their survival (Hervant et al., 1997, 1996; 1995; Hervant and Mathieu, 1995). In the present study, we attempted to value these adaptations indirectly by determining the activities of PK, PEPCK and LDH, as these enzymes play a crucial role in amphipod metabolism, especially in anaerobic and aerobic energy metabolism under changing environmental conditions.

In the HZ of a river, the oxygen dynamics and the associated

chemical parameters differ considerably between areas of downwelling where surface water infiltrates the sediment, and upwelling where groundwater or the hyporheic flow returns to the surface (Mueller et al., 2022). The eco-hydrologic characteristics of the HZ at three selected sampling sites, considering the physicochemical parameters in the interstitial water, especially water temperature, DO, oxygen saturation, pH, conductivity and DOC, were markedly different (Table 1). We can assume that Medsave and Podsused sites can be categorised as downwelling areas, which are more influenced by river water, while Jarun site can be categorised as upwelling area, which is more influenced by groundwater (Redžović et al., 2023b). However, more reliable claim could only be drawn if more detailed spatial and temporal multi-parameter monitoring of physicochemical parameters in the HZ is carried out. Based on the physicochemical parameters data from this study Jarun site was a more physiologically stressful environment for the native populations of *S. ambulans* regarding its sensitivity to oxygen availability (Supplementary material, section Physicochemical characteristics of the interstitial water from the hyporheic zone at selected sampling sites).

We compared our results on PK activity in *S. ambulans* inhabiting the HZ of the Sava River (average value at all sites and in both discharge regimes: $16.94 \text{ U g}^{-1} \text{ w.m.}$) with the literature data on hypogean (*Niphargus virei*, *N. rhenorhodanensis*, and *Stenasellus virei*; $7.50 \text{ U g}^{-1} \text{ w.m.}$) and epigean (*Gammarus fossarum* and *Asellus aquaticus*; $15.12 \text{ U g}^{-1} \text{ w.m.}$) peracarid crustaceans (Hervant, 1996) and found that it was more similar to the PK activity of epigean species. The same previous study showed that hypogean amphipods (*N. virei*, *N. rhenorhodanensis*) and isopods (*S. virei*) have about 8 times higher PEPCK activity ($0.95 \text{ U g}^{-1} \text{ w.m.}$) compared to epigean amphipods (*G. fossarum*) and isopods (*A. aquaticus*) ($0.12 \text{ U g}^{-1} \text{ w.m.}$) (Hervant, 1996). Our results showed that the average value of PEPCK activity in *S. ambulans* was similar to PEPCK activity in hypogean species. During anaerobic metabolism in the cytosol, PEP is carboxylated by PEPCK to oxaloacetate, which is reduced to malate, then transported into the mitochondria and reduced to succinate with concomitant ATP production (Tielens et al., 1992). Thus, the higher PEPCK activity indicates that hypogean organisms have a greater ability to function anaerobically.

The PK/PEPCK ratio as an index for assessing relative aerobic capacity differs between epigean and hypogean crustaceans, with epigean species showing a 17-fold higher ratio (131.48) than hypogean species (7.87) (Hervant, 1996). In our study, *S. ambulans* as a stygophilous species exhibited the PK/PEPCK ratio of 17.97, approximately twice that of hypogean species and seven times lower than epigean species. At downstream sites (Podsused, Jarun), the ratio decreased after low river discharge, indicating reduced aerobic capacity and greater reliance on anaerobic metabolism, consistent with lower dissolved O_2 levels at these sites (Table 1). The lower PK/PEPCK ratio at these sites may also reflect increased pollution, as $\text{COD}_{\text{KMnO}_4}$ and nitrate concentrations (Table 1) exceeded quality thresholds for surface waters of good quality (GRC, 2019).

LDH activity in *S. ambulans* as an index of anaerobic potential (Somero and Childress, 1980) followed a similar pattern to PEPCK, with higher activities observed after low river discharge at the Podsused and Jarun sites, both located downstream of the WWTP, compared to the upstream Medsave site. The reason for this similar response may be that both enzymes are involved in anaerobic metabolic pathways. Higher LDH activities at the Podsused and Jarun sites could possibly be due to lower dissolved O_2 concentrations compared to Medsave (Table 1) as already discussed for the PK/PEPCK ratio. This trend aligns with findings in the laboratory studies in freshwater and estuarine decapod crustaceans, in which LDH activity increased under hypoxia, indicating a shift to anaerobic metabolism (Bao et al., 2018; Jie et al., 2021). Similarly, in a field study hypogean shrimp exhibited higher LDH and lower malate dehydrogenase (MDH) activity than epigean species, reflecting greater anaerobic reliance (Bishop and Iliffe, 2012).

Other environmental factors such as metals and organic pollutants

have also been shown to influence the activities of PK, PEPCK and LDH. For example, exposure to Cu inhibited PK activity, as observed in the crabs *Neohelice granulata* and *Carcinus maenas*, where Cu likely displaced Mg^{2+} , resulting in inhibition of phosphoryl transport (Hansen et al., 1992; Lauer et al., 2012; Stryer, 1981). The pesticide glyphosate also affected PK activity, as seen in the juveniles of the crayfish *Cherax quadricarinatus*, where reduced muscle PK activity was detected, indicating metabolic depression (Avigliano et al., 2014). In our study, we did not observe a decrease in PK activity at sites with anthropogenic influence. However, we observed differences between discharge regimes with higher PK activities after low discharge.

Conversely, chronic exposure to PAHs, PCBs, Cu and tributyltin (TBT) can lead to an increase in PEPCK activity, as documented in the digestive gland of blue mussel *Mytilus* spp. Suggesting increased anaerobic metabolism and oxidative stress response (Lacroix et al., 2015). Similarly, in our study, *S. ambulans* showed significantly higher PEPCK activity at downstream sites, especially at Jarun, after the river discharge was reduced for three months (from June to August 2020), which may lead to higher concentrations of contaminants in the water. We could not link the higher PEPCK activity at Jarun after low river discharge to lower oxygen levels as a possible reason, as dissolved O_2 concentrations were virtually the same in both sampling campaigns at this sampling site (Table 1).

Similar to PEPCK, LDH is activated not only under hypoxic conditions but also when additional energy is needed for detoxification processes (Long et al., 2003). For example, decapod crustacean *Carcinus maenas* exposed to Zn and Hg in laboratory experiment showed increased LDH activity, suggesting enhanced anaerobic energy production (Elumalai et al., 2007). Similarly, Cu exposure in the estuarine crab *Sesarma quadratum* elevated LDH levels in muscles, gills, and hepatopancreas, likely due to Cu-induced gill damage impairing O_2 uptake (Valarmathi and Azariah, 2003). Based on our data on LDH activity in *S. ambulans*, it can be inferred that the activation of anaerobic metabolism observed in populations from sites downstream of the WWTP may result from the combined effects of low dissolved O_2 levels and the high energy demand for pollutant detoxification.

Temperature has also been shown to influence the function of PK in aquatic invertebrates. In *S. ambulans*, PK activity was significantly higher after low river discharge, when the interstitial water temperature was higher (16.8 and 22.0°C , Table 1) than after high river discharge (11.3 and 14.2°C , Table 1) at Jarun and Medsave sites, respectively (Fig. 5a). These results are consistent with findings in the oyster *Crassostrea gigas* (Le Moullac et al., 2007) and gastropod *Littorina littorea* (Greenway and Storey, 2001), where PK activity increased at higher temperatures. This higher PK activity observed after low river discharge may be related to an increased glycolytic capacity driven by the utilization of carbohydrates ingested during this period (Greenway and Storey, 2001). This is particularly evident in species that reproduce in the spring, such as *L. littorea* (Greenway and Storey, 2001) or in our study *S. ambulans* (Gottstein et al., 2023), which may store energy reserves during summer.

Temperature fluctuations can have significant effects on the PK/PEPCK ratio in aquatic invertebrates. In *S. ambulans*, the PK/PEPCK ratio at the Medsave site was significantly higher after low river discharge, when higher temperatures occurred, than after high river discharge, when lower temperatures were recorded. Similarly, in experiments with oysters (*C. gigas*), an increase in temperature led to an increased PK/PEPCK ratio and a lower anaerobic capacity of the organisms (Le Moullac et al., 2007). This indicates that organisms have a lower ability to survive anaerobiosis at high temperatures (Le Moullac et al., 2007). Global climate change, involving a global rise in temperature and a reduction in river discharge (Copernicus Climate Change Service, 2022), thus poses a major threat to *S. ambulans* populations and hyporheic communities in general, as they may be less able to survive anaerobiosis in HZ, especially in combination with contamination.

5. Conclusions

This study presents the first report on the biochemical responses of the stygophilous amphipod *S. ambulans*, which inhabits vulnerable HZ and is exposed to anthropogenic contamination. The activities of biomarkers of antioxidant defence of the organism (CAT and GR) and the biomarker of exposure to xenobiotics (GST) were generally higher at sites downstream of the discharge of WWTP effluent than at upstream site. The increase in CAT, GR and GST enzyme activities downstream of the WWTP outflow is most likely a response to environmental contamination and increased concentrations of toxic elements (Al, Cr, Fe, Ni and Pb) that have accumulated in *S. ambulans* and suggests an integrated defence mechanism involving GSH metabolic pathways. Changes in environmental parameters, especially increased values of P-PO_4^{3-} , N-NO_3^- , and $\text{COD}_{\text{KMNO}_4}$, could also affect GST activity in *S. ambulans* downstream of the WWTP effluent discharge. The biomarkers of anaerobic capacity (PK, PEPCK, LDH) in *S. ambulans* reflect the physiological adaptations to the lower oxygen concentrations that characterise the HZ of the Sava River. The increased LDH activity in the downstream populations could be due to the lower oxygen availability and the influence of WWTP effluents. Similarly, the observed decrease in the PK/PEPCK ratio in these populations indicates a lower aerobic capacity and a greater reliance on anaerobic metabolic pathways, possibly indicating an enhanced tolerance to hypoxic conditions. However, as higher PK/PEPCK ratios were associated with increased temperatures, increasing global temperatures due to climate change could severely threaten *S. ambulans* populations in the HZ, potentially exceeding their physiological limits.

This study contributes to a deeper understanding of the biological and physiological responses of amphipods exposed to chemical pollution and unfavourable environmental conditions that can cause oxidative stress, which is crucial for assessing the ecological status of groundwater-connected ecosystems. Given the ecological importance of HZ in mediating groundwater – surface water interactions, our results provide valuable perspectives on global freshwater ecosystem health and highlight the potential impact of wastewater effluents on vulnerable subterranean fauna.

CRediT authorship contribution statement

Zuzana Redžović: Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Conceptualization. **Marijana Erk:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sanja Gottstein:** Writing – review & editing, Supervision, Resources, Investigation, Conceptualization. **Inna M. Sokolova:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition. **Eugene P. Sokolov:** Methodology, Investigation. **Tatjana Mijošek Pavin:** Writing – review & editing, Investigation. **Sara Šariri:** Investigation. **Mirela Sertić Perić:** Writing – review & editing, Investigation. **Jelena Dautović:** Writing – review & editing, Investigation. **Željka Fiket:** Writing – review & editing, Investigation. **Vlatka Filipović Marijić:** Writing – review & editing, Resources, Funding acquisition. **Duška Ivanković:** Writing – review & editing, Methodology. **Mario Cindrić:** Writing – review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2025.127234>.

Data availability

Data will be made available on request.

References

- Arbačiauskas, K., 2008. *Synurella ambulans* (F. Müller, 1846), A new native amphipod species of Lithuanian waters. *Acta Zool. Lit.* 18, 66–68. <https://doi.org/10.2478/v10043-008-0006-z>.
- Atli, G., Canli, M., 2010. Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. *Ecotox Environ Safe* 73, 1884–1889. <https://doi.org/10.1016/j.ecoenv.2010.09.005>.
- Avigliano, L., Fassiano, A.V., Medesani, D.A., Ríos de Molina, M.C., Rodríguez, E.M., 2014. Effects of glyphosate on growth rate, metabolic rate and energy reserves of early juvenile crayfish, *Cherax quadricarinatus* M. Bull. Environ. Contam. Toxicol. 92, 631–635. <https://doi.org/10.1007/s00128-014-1240-7>.
- Bao, J., Li, X., Yu, H., Jiang, H., 2018. Respiratory metabolism responses of Chinese mitten crab, *Eriocheir sinensis* and Chinese grass shrimp, *Palaemonetes sinensis*, subjected to environmental hypoxia stress. *Front. Physiol.* 9. <https://doi.org/10.3389/fphys.2018.01559>.
- Barata, C., Lekumbarri, I., Vila-Escalé, M., Prat, N., Porte, C., 2005. Trace metal concentration, antioxidant enzyme activities and susceptibility to oxidative stress in the tricopter larvae *Hydropsyche exocellata* from the llobregat River basin (NE Spain). *Aquat. Toxicol.* 74, 3–19. <https://doi.org/10.1016/j.aquatox.2005.04.002>.
- Battin, T.J., Besemer, K., Bengtsson, M.M., Romani, A.M., Packmann, A.I., 2016. The ecology and biogeochemistry of stream biofilms. *Nat. Rev. Microbiol.* 14, 251–263. <https://doi.org/10.1038/nrmicro.2016.15>.
- Bayne, B.L., 2017. Metabolic expenditure. *Dev. Aquacult. Fish. Sci.* 41, 331–415. <https://doi.org/10.1016/B978-0-12-803472-9.00006-6>.
- Bergmeyer, H.-U., Bernt, E., Hess, B., 1965. Lactic dehydrogenase. In: Bergmeyer, H.-U. (Ed.), *Methods of Enzymatic Analysis*. Academic Press, pp. 736–743. <https://doi.org/10.1016/B978-0-12-395630-9.50134-1>.
- Bishop, R.E., Iliffe, T.M., 2012. Ecological physiology of the anchialine shrimp *barbouria cubensis*: a comparison of epigeal and hypogean populations. *Mar. Biodivers.* 42, 303–310. <https://doi.org/10.1007/s12526-012-0113-8>.
- Boets, P., Lock, K., Goethals, P.L.M., 2010. First record of *Synurella ambulans* (Müller 1846) (Amphipoda: Crangonitidae) in Belgium. *Belg. J. Zool.* 140, 244–245. <https://doi.org/10.26496/bjz.2010.176>.
- Bou, C., Rouch, R., 1967. Un nouveau champ de recherches sur la faune aquatique souterraine. *Comptes Rendus Acad Sci* 256, 369–370.
- Boulton, A.J., Findlay, S., Marmonier, P., Stanley, E.H., Valett, H.M., 1998. The functional significance of the hyporheic zone in streams and rivers. *Annu. Rev. Ecol. Systemat.* 29, 59–81. <https://doi.org/10.1146/annurev.ecolsys.29.1.59>.
- Cameselle, JoséC., Sánchez, JoséL., Carrión, A., 1980. The regulation of glycolysis in the hepatopancreas of the sea mussel *Mytilus edulis* L. *Comp Biochem Physiol B: Comp Biochem* 65, 95–102. [https://doi.org/10.1016/0305-0491\(80\)90116-9](https://doi.org/10.1016/0305-0491(80)90116-9).
- Capparelli, M.V., Gusso-Choueri, P.K., Abessa, D.M. de S., McNamara, J.C., 2019. Seasonal environmental parameters influence biochemical responses of the fiddler crab *Minuca rapax* to contamination in situ. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 216, 93–100. <https://doi.org/10.1016/j.cbpc.2018.11.012>.
- Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A. (Ed.), *Handbook Methods for Oxygen Radical Research*. CRC Press, Boca Raton, pp. 283–284.
- Copernicus Climate Change Service, 2022. River discharge. available at: <https://climate.copernicus.eu/esotc/2022/river-discharge>. (Accessed 31 July 2023).
- Cossu-Leguille, C., Vasseur, P., 2013. Aquatic biomarkers. In: *Encyclopedia of Aquatic Ecotoxicology*. Springer, Netherlands, Dordrecht, pp. 49–66. https://doi.org/10.1007/978-94-007-5704-2_6.
- Česen, M., Ahel, M., Terzić, S., Heath, D.J., Heath, E., 2019. The occurrence of contaminants of emerging concern in Slovenian and Croatian wastewaters and receiving Sava river. *Sci Tot Environ* 650, 2446–2453. <https://doi.org/10.1016/j.scitotenv.2018.09.238>.

- Elumalai, M., Antunes, C., Guilhermino, L., 2007. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NW Portugal) exposed to zinc and mercury. *Chemosphere* 66, 1249–1255. <https://doi.org/10.1016/j.chemosphere.2006.07.030>.
- Falfushynska, H.I., Phan, T., Sokolova, I.M., 2016. Long-term acclimation to different thermal regimes affects molecular responses to heat stress in a freshwater clam *Corbicula fluminea*. *Sci. Rep.* 6, 39476. <https://doi.org/10.1038/srep39476>.
- Ferina, S., 2015. Technical-Technological Solution of the Existing Plants of the Company PLIVA Hrvatska D.O.O. at Savski Marof - Annex to the Request for Determining Integrated Environmental Protection Conditions. PLIVA, Zagreb, Croatia.
- Fischer, H., Kloppe, F., Wilczek, S., Pusch, M.T., 2005. A river's liver – microbial processes within the hyporheic zone of a large lowland river. *Biogeochemistry* 76, 349–371. <https://doi.org/10.1007/s10533-005-6896-y>.
- Frías-Espicueta, M.G., Bautista-Covarrubias, J.C., Osuna-Martínez, C.C., Delgado-Alvarez, C., Bojórquez, C., Aguilar-Juárez, M., Roos-Muñoz, S., Osuna-López, I., Páez-Osuna, F., 2022. Metals and oxidative stress in aquatic decapod crustaceans: a review with special reference to shrimp and crabs. *Aquat. Toxicol.* 242, 106024. <https://doi.org/10.1016/j.aquatox.2021.106024>.
- George, S.G., 1994. Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. In: Malins, D.C., Ostrander, G.K. (Eds.), *Aquatic Toxicology: Molecular, Biochemical and Cellular Perspectives*. CRC Press, pp. 37–85.
- Gottstein, M., Matočec, S., Bakran-Petricoli, T., Bedek, J., Bukovec, D., Buzjak, S., Francićević, M., Jalžić, B., Kerovec, M., Kletečki, E., Kralj, J., Kružić, P., Kućinić, M., Kuhta, M., Matočec, N., Ozimec, R., Rada, T., Ternje, I., Tvrtković, N., 2002. An overview of the cave and interstitial biota of Croatia. *Nat. Croat.: Periodicum Musei Historiae Naturalis Croatiae* 11, 1–112.
- Gottstein, S., Redžović, Z., Erk, M., Sertić Perić, M., Dautović, J., Cindrić, M., 2023. Life history traits of the stygophilous amphipod *Synurella ambulans* in the hyporheic zone of the lower reaches of the upper Sava River (Croatia). *Water (Basel)* 15, 3188. <https://doi.org/10.3390/w15183188>.
- GRC (Government of the Republic of Croatia), 2019. Directive on Water Quality Standard. *Official Gazette No. 96. Narodne novine*, Zagreb.
- Greenway, S.C., Storey, K.B., 2001. Effects of seasonal change and prolonged anoxia on metabolic enzymes of *Littorina littorea*. *Can. J. Zool.* 79, 907–915. <https://doi.org/10.1139/cjz-79-5-907>.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. *J. Biol. Chem.* 249, 7130–7139. [https://doi.org/10.1016/S0021-9258\(19\)42083-8](https://doi.org/10.1016/S0021-9258(19)42083-8).
- Halliwell, Barry, Gutteridge, J.M.C., 2015. Oxygen: boon yet bane-introducing oxygen toxicity and reactive species. In: Halliwell, B., Gutteridge, J. (Eds.), *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford, pp. 1–29.
- Hansen, J.I., Mustafa, T., Depledge, M., 1992. Mechanisms of copper toxicity in the shore crab, *Carcinus maenas*. *Mar. Biol.* 114, 259–264. <https://doi.org/10.1007/BF00349528>.
- Havird, J.C., Vaught, R.C., Weeks, J.R., Fujita, Y., Hidaka, M., Santos, S.R., Henry, R.P., 2014. Taking their breath away: metabolic responses to low-oxygen levels in anacholine shrimps (Crustacea: Atyidae and Alpheidae). *Comp. Biochem. Physiol. Mol. Integr. Physiol.* 178, 109–120. <https://doi.org/10.1016/j.cbpa.2014.08.015>.
- Hervant, F., 1996. The activities of enzymes associated with the intermediary and energy metabolism in hypogean and epigean crustaceans. *Comptes Rendus Acad. Sci. Ser. III* (319), 1071–1077.
- Hervant, F., Malard, F., 2012. Responses to low oxygen. In: White, W.B., Culver, D.C. (Eds.), *Encyclopedia of Caves*. Elsevier, Oxford, UK, pp. 651–658. <https://doi.org/10.1016/B978-0-12-383832-2.00096-7>.
- Hervant, F., Mathieu, J., 1995. Ventilatory and locomotory activities in anoxia and subsequent recovery of epigean and hypogean crustaceans. *Comptes Rendus Acad. Sci. Ser. III* 318, 585–592.
- Hervant, F., Mathieu, J., Garin, D., Freminet, A., 1996. Behavioral, ventilatory, and metabolic responses of the hypogean amphipod *Niphargus virei* and the epigean isopod *Asellus aquaticus* to severe hypoxia and subsequent recovery. *Physiol. Zool.* 69, 1277–1300. <https://doi.org/10.1086/physzool.69.6.30164261>.
- Hervant, F., Mathieu, J., Garin, D., Freminet, A., 1995. Behavioral, ventilatory, and metabolic responses to severe hypoxia and subsequent recovery of the hypogean *Niphargus rhenorhodanensis* and the epigean *Gammarus fossarum* (Crustacea: Amphipoda). *Physiol. Zool.* 68, 223–244. <https://doi.org/10.1086/physzool.68.2.30166501>.
- Hervant, F., Mathieu, J., Messana, G., 1997. Locomotory, ventilatory and metabolic responses of the subterranean *Stenasellus virei* (Crustacea, Isopoda) to severe hypoxia and subsequent recovery. *Comptes Rendus Acad. Sci. Ser. III* (320), 139–148. [https://doi.org/10.1016/S0764-4469\(97\)85005-6](https://doi.org/10.1016/S0764-4469(97)85005-6).
- Jemec, A., Drobne, D., Tišler, T., Sepčić, K., 2010. Biochemical biomarkers in environmental studies - lessons learnt from enzymes catalase, glutathione S-transferase and cholinesterase in two crustacean species. *Environ. Sci. Pollut. Res.* 17, 571–581. <https://doi.org/10.1007/s11356-009-0112-x>.
- Jemec, A., Škufca, D., Prevorčnik, S., Fišer, Ž., Zidar, P., 2017. Comparative study of acetylcholinesterase and glutathione S-transferase activities of closely related cave and surface *Asellus aquaticus* (Isopoda: Crustacea). *PLoS One* 12, e0176746. <https://doi.org/10.1371/journal.pone.0176746>.
- Jie, Y.-K., Cheng, C.-H., Wang, L.-C., Ma, H.-L., Deng, Y.-Q., Liu, G.-X., Feng, J., Guo, Z.-X., Ye, L.-T., 2021. Hypoxia-induced oxidative stress and transcriptome changes in the mud crab (*Scylla paramamosain*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 245, 109039. <https://doi.org/10.1016/j.cbpc.2021.109039>.
- Karaman, G.S., 1993. Crustacea - Amphipoda (d'acqua dolce), vol. XXXI. *Fauna d'Italia*, Calderini, Bologna.
- Karaman, G.S., 1974. Contribution to the knowledge of the amphipoda. Genus *Synurella* Wrzes in Yugoslavia with remarks on its all World known species, their synonymy, bibliography and distribution (fam. Gammaridae). *Poljoprivreda i šumarstvo*, pp. 83–133 20.
- Lacroix, C., Richard, G., Segueineau, C., Guyomarch, J., Moraga, D., Auffret, M., 2015. Active and passive biomonitoring suggest metabolic adaptation in blue mussels (*Mytilus* spp.) chronically exposed to a moderate contamination in Brest harbor (France). *Aquat. Toxicol.* 162, 126–137. <https://doi.org/10.1016/j.aquatox.2015.03.008>.
- Lauer, M.M., de Oliveira, C.B., Yano, N.L.I., Bianchini, A., 2012. Copper effects on key metabolic enzymes and mitochondrial membrane potential in gills of the estuarine crab *Neohelice granulata* at different salinities. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 156, 140–147. <https://doi.org/10.1016/j.cbpc.2012.08.001>.
- Le Moullac, G., Quéau, I., Le Souchu, P., Pouvreau, S., Moal, J., René Le Coz, J., François Samain, J., 2007. Metabolic adjustments in the oyster *Crassostrea gigas* according to oxygen level and temperature. *Mar. Biol. Res.* 3, 357–366. <https://doi.org/10.1080/17451000701635128>.
- Lewandowski, J., Arnon, S., Banks, E., Batelaan, O., Betterle, A., Broecker, T., Coll, C., Drummond, J., Gaona Garcia, J., Galloway, J., Gomez-Velez, J., Grabowski, R., Herzog, S., Hinkelmann, R., Höhne, A., Hollender, J., Horn, M., Jaeger, A., Krause, S., Löchner Prats, A., Magliozzi, C., Meinikmann, K., Mojarrad, B., Mueller, B., Peralta-Maraver, I., Popp, A., Posselt, M., Putschew, A., Radke, M., Raza, M., Rimpl, J., Robertson, A., Rutere, C., Schaper, J., Schirmer, M., Schulz, H., Shanfield, M., Singh, T., Ward, A., Wolke, P., Wörman, A., Wu, L., 2019. Is the hyporheic zone relevant beyond the scientific community? *Water (Basel)* 11, 2230. <https://doi.org/10.3390/w11112230>.
- Lewandowski, J., Putschew, A., Schwesig, D., Neumann, C., Radke, M., 2011. Fate of organic micropollutants in the hyporheic zone of a eutrophic lowland stream: results of a preliminary field study. *Sci. Tot. Environ.* 409, 1824–1835. <https://doi.org/10.1016/j.scitotenv.2011.01.028>.
- Li, L., Shen, S., Bickler, P., Jacobson, M.P., Wu, L.F., Altschuler, S.J., 2023. Searching for molecular hypoxia sensors among oxygen-dependent enzymes. *eLife* 12. <https://doi.org/10.7554/eLife.87705>.
- Li, Q., Sun, S., Zhang, F., Wang, M., Li, M., 2019. Effects of hypoxia on survival, behavior, metabolism and cellular damage of Manila clam (*Ruditapes philippinarum*). *PLoS One* 14, e0215158. <https://doi.org/10.1371/journal.pone.0215158>.
- Livingstone, D.R., 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. *Rev. Med. Vet. (Toulouse)* 154, 427–430.
- Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull.* 42, 656–666. [https://doi.org/10.1016/S0025-326X\(01\)00060-1](https://doi.org/10.1016/S0025-326X(01)00060-1).
- Long, S.M., Ryder, K.J., Holdway, D.A., 2003. The use of respiratory enzymes as biomarkers of petroleum hydrocarbon exposure in *Mytilus edulis planulatus*. *Ecotoxicol. Environ. Saf.* 55, 261–270. [https://doi.org/10.1016/S0147-6513\(02\)00137-9](https://doi.org/10.1016/S0147-6513(02)00137-9).
- Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol.* 101, 13–30. <https://doi.org/10.1016/j.aquatox.2010.10.006>.
- Mavis, R.D., Stollwagen, E., 1968. Purification and subunit structure of glutathione reductase from bakers' yeast. *J. Biol. Chem.* 243, 809–814. [https://doi.org/10.1016/S0021-9258\(19\)81737-4](https://doi.org/10.1016/S0021-9258(19)81737-4).
- Meister, A., Anderson, M.E., 1983. Glutathione. *Annu. Rev. Biochem.* 52, 711–760. <https://doi.org/10.1146/annurev.bi.52.070183.003431>.
- Mesquita, A.F., Gonçalves, F.J.M., Gonçalves, A.M.M., 2023. Effects of inorganic and organic pollutants on the biomarkers' response of *Cerastoderma edule* under temperature scenarios. *Antioxidants* 12, 1756. <https://doi.org/10.3390/antiox12091756>.
- Milaković, M., Vestergaard, G., González-Plaza, J.J., Petrić, I., Šimatović, A., Senta, I., Kublik, S., Schlöter, M., Smalla, K., Udiković-Kolić, N., 2019. Pollution from azithromycin-manufacturing promotes macrolide-resistance gene propagation and induces spatial and seasonal bacterial community shifts in receiving river sediments. *Environ. Int.* 123, 501–511. <https://doi.org/10.1016/j.envint.2018.12.050>.
- Mueller, B.M., Schulz, H., Lewandowski, J., 2022. Hyporheic zone and processes. In: *Encyclopedia of Inland Waters*. Elsevier, pp. 301–311. <https://doi.org/10.1016/B978-0-12-819166-8.00103-1>.
- Paital, B., Chainy, G.B.N., 2013. Seasonal variability of antioxidant biomarkers in mud crabs (*Scylla serrata*). *Ecotox. Environ. Safe* 87, 33–41. <https://doi.org/10.1016/j.ecoenv.2012.10.006>.
- Parlov, J., Nakić, Z., Posavec, K., Bačani, A., 2012. Origin and dynamics of aquifer recharge in Zagreb area. *Water observation and information system for decision support*. In: *The Fifth International Scientific Conference on Water, Climate and Environment*. BALWOIS, Ohrid: Faculty of Civil Engineering, Skopje.
- Peralta-Maraver, I., Reiss, J., Robertson, A.L., 2018. Interplay of hydrology, community ecology and pollutant attenuation in the hyporheic zone. *Sci. Total Environ.* 610–611, 267–275. <https://doi.org/10.1016/j.scitotenv.2017.08.036>.
- Redžović, Z., Erk, M., Gottstein, S., Sertić Perić, M., Dautović, J., Fiket, Ž., Brkić, A.L., Cindrić, M., 2023a. Metal bioaccumulation in stygophilous amphipod *Synurella ambulans* in the hyporheic zone: the influence of environmental factors. *Sci. Total Environ.* 866, 161350. <https://doi.org/10.1016/j.scitotenv.2022.161350>.
- Redžović, Z., Erk, M., Gottstein, S., Cindrić, M., 2023b. Energy status of stygophilous amphipod *Synurella ambulans* as a promising biomarker of environmental stress in the hyporheic zone. *Water (Basel)* 15, 3083. <https://doi.org/10.3390/w15173083>.
- Sies, H., Jones, D.P., 2020. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nat. Rev. Mol. Cell Biol.* 21, 363–383. <https://doi.org/10.1038/s41580-020-0230-3>.
- Simpfendorfer, R.W., Vial, M.V., López, D.A., Verdala, M., González, M.L., 1995. Relationship between the aerobic and anaerobic metabolic capacities and the vertical distribution of three intertidal sessile invertebrates: *Jehlius cirratus* (Darwin) (Cirripedia), *Perumytilus purpuratus* (Lamarck) (Bivalvia) and *Mytilus chilensis* (Hupé)

- (Bivalvia). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 111, 615–623. [https://doi.org/10.1016/0305-0491\(95\)00026-5](https://doi.org/10.1016/0305-0491(95)00026-5).
- Somero, G.N., 2004. Adaptation of enzymes to temperature: searching for basic “strategies”. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 139, 321–333. <https://doi.org/10.1016/j.cbpc.2004.05.003>.
- Somero, G.N., Childress, J.J., 1980. A violation of the metabolism-size scaling paradigm: activities of glycolytic enzymes in muscle increase in larger-size fish. *Physiol. Zool.* 53, 322–337. <https://doi.org/10.1086/physzool.53.3.30155794>.
- Sroda, S., Cossu-Leguille, C., 2011. Seasonal variability of antioxidant biomarkers and energy reserves in the freshwater gammarid *Gammarus roeseli*. *Chemosphere* 83, 538–544. <https://doi.org/10.1016/j.chemosphere.2010.12.023>.
- Strode, E., Barda, I., Suhareva, N., Kolesova, N., Turja, R., Lehtonen, K.K., 2023. Influence of environmental variables on biochemical biomarkers in the amphipod *Monoporeia affinis* from the Gulf of Riga (Baltic sea). *Water (Basel)* 15, 248. <https://doi.org/10.3390/w15020248>.
- Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* 18, 321–336. [https://doi.org/10.1016/0891-5849\(94\)00159-H](https://doi.org/10.1016/0891-5849(94)00159-H).
- Stryer, L., 1981. *Biochemistry, second ed.* Freeman and Company.
- Sussarellu, R., Fabioux, C., Camacho Sanchez, M., Le Goïc, N., Lambert, C., Soudant, P., Moraga, D., 2012. Molecular and cellular response to short-term oxygen variations in the Pacific oyster *Crassostrea gigas*. *J. Exp. Mar. Biol. Ecol.* 412, 87–95. <https://doi.org/10.1016/j.jembe.2011.11.007>.
- Tafani, M., Sansone, L., Limana, F., Arcangeli, T., De Santis, E., Polese, M., Fini, M., Russo, M.A., 2016. The interplay of reactive oxygen species, hypoxia, inflammation, and sirtuins in cancer initiation and progression. *Oxid. Med. Cell. Longev.* 2016. <https://doi.org/10.1155/2016/3907147>.
- Tanabe, S., O'Brien, J., Tollefsen, K.E., Kim, Y., Chauhan, V., Yauk, C., Huliganga, E., Rudel, R.A., Kay, J.E., Helm, J.S., Beaton, D., Filipovska, J., Sovadinova, I., Garcia-Reyero, N., Mally, A., Poulsen, S.S., Delrue, N., Fritsche, E., Luettich, K., La Rocca, C., Yepiskoposyan, H., Klose, J., Danielsen, P.H., Esterhuizen, M., Jacobsen, N.R., Vogel, U., Gant, T.W., Choi, I., FitzGerald, R., 2022. Reactive oxygen species in the adverse outcome pathway framework: toward creation of harmonized consensus key events. *Front Toxicol* 4. <https://doi.org/10.3389/ftox.2022.887135>.
- Tatar, S., Cıkıkcıoglu Yildirim, N., Serdar, O., Yildirim, N., Ogedey, A., 2018. The using of *Gammarus pulex* as a biomonitor in ecological risk assessment of secondary effluent from municipal wastewater treatment plant in Tunceli, Turkey. *Hum. Ecol. Risk Assess.* 24, 819–829. <https://doi.org/10.1080/10807039.2017.1400374>.
- Tielsens, A.G.M., Horemans, A.M.C., Dunnewijk, R., van der Meer, P., van den Bergh, S.G., 1992. The facultative anaerobic energy metabolism of *Schistosoma mansoni* sporocysts. *Mol. Biochem. Parasitol.* 56, 49–57. [https://doi.org/10.1016/0166-6851\(92\)90153-B](https://doi.org/10.1016/0166-6851(92)90153-B).
- Valarmathi, S., Azariah, J., 2003. Effect of copper chloride on the enzyme activities of the crab *Sesarma quadratum* (Fabricius). *Turk. J. Zool.* 27, 253–256.
- Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullou, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.* 64, 178–189. <https://doi.org/10.1016/j.ecoenv.2005.03.013>.
- van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* 13, 57–149. [https://doi.org/10.1016/S1382-6689\(02\)00126-6](https://doi.org/10.1016/S1382-6689(02)00126-6).
- Vlahović, T., Bačani, A., Posavec, K., 2009. Hydrogeochemical stratification of the unconfined Samobor aquifer (Zagreb, Croatia). *Environ. Geol.* 57, 1707–1722. <https://doi.org/10.1007/s00254-008-1452-4>.
- Vranković, J., Živić, M., Radojević, A., Perić-Mataruga, V., Todorović, D., Marković, Z., Živić, I., 2018. Evaluation of oxidative stress biomarkers in the freshwater gammarid *Gammarus dulensis* exposed to trout farm outputs. *Ecotoxicol. Environ. Saf.* 163, 84–95. <https://doi.org/10.1016/J.ECOENV.2018.07.061>.
- Wang, Z., Yan, C., Yan, Y., Chi, Q., 2012. Integrated assessment of biomarker responses in caged shrimps (*Litopenaeus vannamei*) exposed to complex contaminants from the Maluan Bay of China. *Ecotoxicology* 21, 869–881. <https://doi.org/10.1007/s10646-011-0849-0>.
- Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* 19, 137–161. [https://doi.org/10.1016/0166-445X\(91\)90033-6](https://doi.org/10.1016/0166-445X(91)90033-6).