SUPPLEMENTARY MATERIALS

Energy Status of Stygophilous Amphipod *Synurella ambulans* as a Promising Biomarker of Environmental Stress in the Hyporheic Zone

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Ecohydrological characteristics of the hyporheic zone at selected sampling sites

The physicochemical properties and chemical composition of groundwater and surface water, including temperature, dissolved oxygen, organic carbon, and nutrient concentrations, can vary significantly. Depending on the difference between the hydraulic heads of the surface water and the adjacent groundwater, two different scenarios can occur in the HZ - "gaining river" or "upwelling" and "losing river" or "downwelling" [19].
In our study, lower dissolved oxygen concentrations (and oxygen saturation) were found in the interstitial water of the HZ compared to the surface water of the river (Table S1). This difference was particularly evident at 2-DOWN sampling site, where dissolved oxygen concentrations were approximately 50% lower than in the river water. Comparing the sampling sites, there was no difference in oxygen concentration in the surface water, but higher values were found in the interstitial water from the HZ at 1-UP site (Table S1).

Table S1. Concentrations of dissolved oxygen (mg L^{-1}) and oxygen saturation level (%) in the river water (river) and in the interstitial water from the hyporheic zone (HZ) measured in four seasons at two sampling sites at the Sava River.

	1-UP - river		**1-UP - HZ**		**2-DOWN - river**		**2-DOWN - HZ**	
	mg L^{-1}	**%**	**mg L^{-1}**	**%**	**mg L^{-1}**	**%**	**mg L^{-1}**	**%**
Winter 2018	10.7	94	8.7	78	10.7	89	6.0	42
Spring 2019	11.0	106	8.0	79	11.5	105	5.9	57
Summer 2019	7.2	85	6.1	65	8.6	98	4.4	44
Autumn 2019	10.8	107	8.9	88	11.0	109	7.4	74

Generally, hyporheic water temperatures are cooler in summer and warmer in winter compared to water in the channel [19]. This trend was also observed in our study, especially at the sampling site 2-DOWN, where the temperature in the HZ was 13.1 °C in winter, while the water temperature in the river was 7.5 °C (Table S2). Thus, it was 5.6 °C warmer in the HZ than in the surface water in winter, while it was 5.5 °C cooler in the HZ than in the surface water in summer (Table S2).

Table S2. Temperatures (° C) of the river water (river) and the interstitial water from the hyporheic zone (HZ) measured in four seasons at two sampling sites at the Sava River.

	1-UP - river	**1-UP - HZ**	**2-DOWN - river**	**2-DOWN - HZ**
Winter 2018	9.0	10.4	7.5	13.1
Spring 2019	12.3	12.1	13.2	12.7
Summer 2019	23.3	23.3	22.0	16.5
Autumn 2019	14.5	14.5	14.9	16.1
Δ Summer – Winter	14.3	12.9	14.6	3.5

Based on the water temperature and dissolved oxygen concentrations, the interstitial water from the HZ at sampling site 1-UP was more influenced by the river, so "downwelling" could occur at this site. At the sampling site 2-DOWN, the HZ was more influenced by groundwater, which was colder and lower in oxygen, so "upwelling" could occur.

Only at site 2-DOWN were higher concentrations of dissolved macroelements (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}) determined in the interstitial water from the HZ than in the river water (Figure S1). This could be due to the greater influence of groundwater, which generally contains higher concentrations of macroelements than surface water [19]. This, in turn, confirms our finding that "upwelling" has occurred in the HZ at sampling site 2-DOWN.

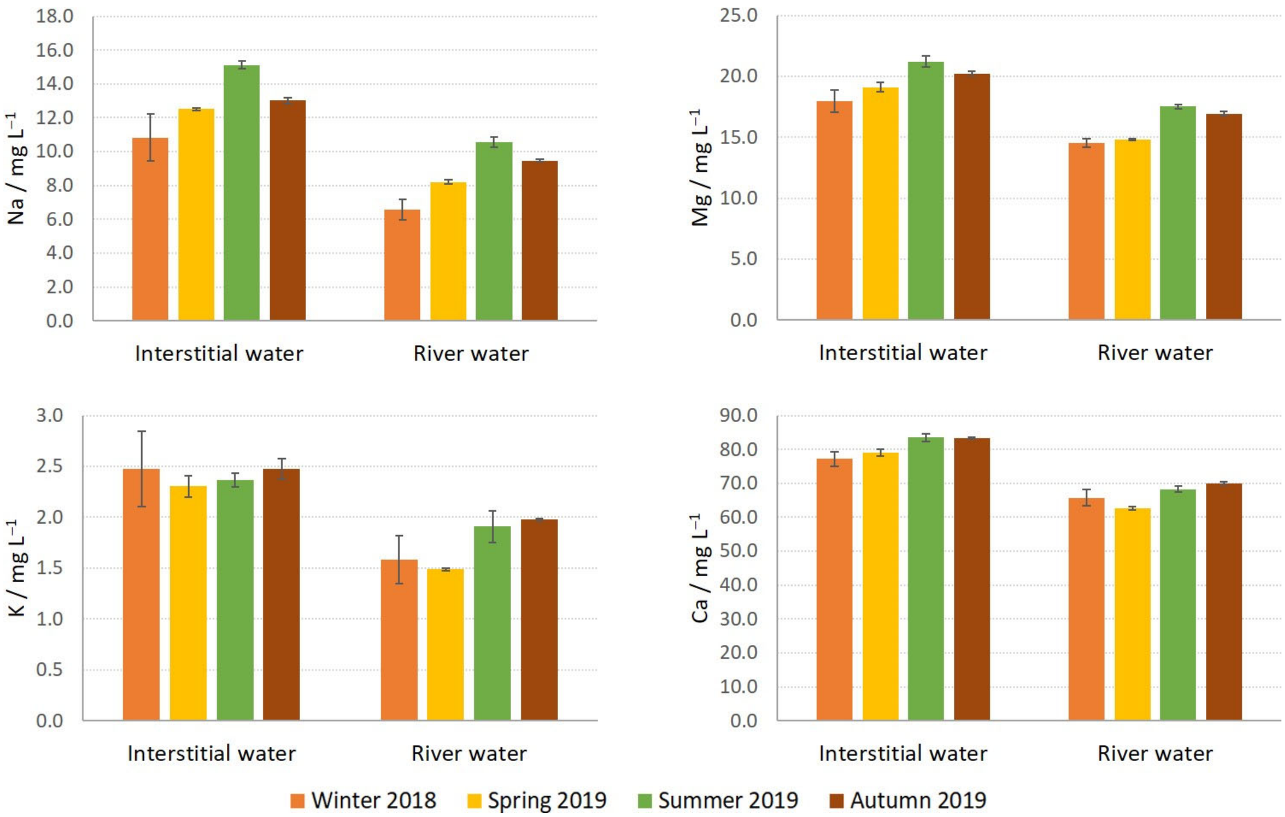

Figure S1. Concentrations of macroelements (dissolved form) in the interstitial water from the HZ and in the river water (mean value and standard deviations, N = 3) determined at the sampling site 2-DOWN in four sampling campaigns.

Concentrations of dissolved organic carbon (DOC) were higher in surface water than in interstitial water of the HZ at both sampling sites (Table S3a). Concentrations of DOC in interstitial water of the HZ were higher at site 1-UP than at site 2-DOWN in all seasons, with the highest values measured at site 1-UP in spring and summer (1.367 mg L^{-1} in both seasons) and at site 2-DOWN in spring (0.825 mg L^{-1}) and autumn (0.904 mg L^{-1}) (Table S3a).

Table S3a. Dissolved organic carbon concentrations (mg L^{-1}) in the river water (river) and the interstitial water from the hyporheic zone (HZ) measured in four seasons at two sampling sites at the Sava River.

	1-UP - river	1-UP - HZ	2-DOWN - river	2-DOWN - HZ
Winter 2018	1.512	1.364	1.027	0.623
Spring 2019	1.404	1.367	1.441	0.825
Summer 2019	1.462	1.367	1.252	0.700
Autumn 2019	0.967	0.938	1.426	0.904

Total organic carbon (TOC) in sediment from the HZ was higher at site 1-UP than at 2-DOWN, with a maximum value of 2.97% in summer (Table S3b). Previous studies near the Medsave village (site 1-UP) showed maximum TOC concentrations in river sediments of 0.97% [24], which were much lower than in our study. The elevated TOC concentrations in sediments from the HZ in the Medsave area could be related to the intensive use of agricultural fertilizers. All forms of organic carbon present in interstitial water and sediment are very important for determining contaminant mobility, its potential bioavailability, and toxicity to biota inhabiting the HZ.

Table S3b. Total organic carbon (%) in fine fraction (< 63 μmm) of sediment from the hyporheic zone measured in three seasons at two sampling sites at the Sava River.

	1-UP	2-DOWN
Spring 2019	2.7	1.8
Summer 2019	3.0	1.9
Autumn 2019	2.8	1.7

In general, higher concentrations of phosphates and nitrates were found in the interstitial water of the HZ compared to the surface water of the river (Figure S2) at both sampling sites. In the interstitial water at site 2-DOWN phosphate concentrations were higher than threshold values established for good water quality status for river type HR_R-5b [32] in all seasons except winter. In spring, phosphate concentration (0.236 mg L^{-1}) exceeded the threshold value for good groundwater quality status [32]. Concentrations of phosphates and nitrates in the interstitial water from the HZ in the studied area may be considered moderately increased, probably due to agricultural activities and wastewater treatment discharge [31].

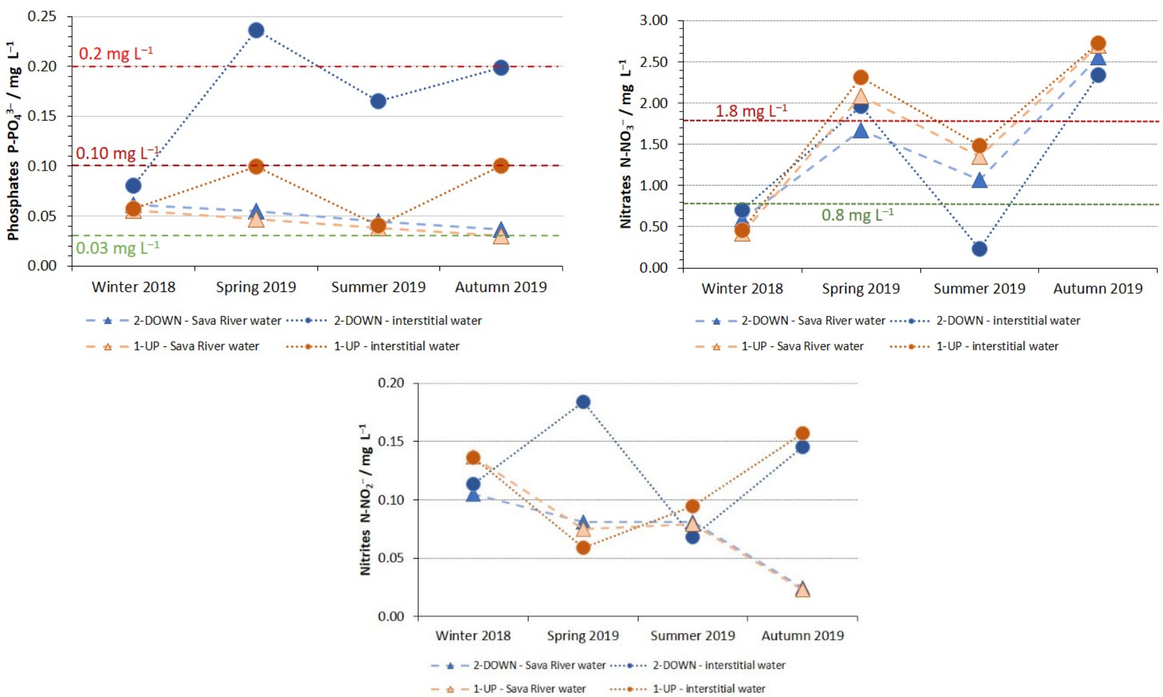

Figure S2. Concentrations of phosphates, nitrates and nitrites in the interstitial water from the HZ and in the surface water from the Sava River determined at both sampling sites in four sampling campaigns. In the graphs, threshold values are indicated for phosphates and nitrates: i) good water quality (dark red dash line), ii) very good water quality (green dash line) established for surface water of the river type HR_R-5b and iii) good groundwater quality status (red dash dot line is shown only for phosphates; threshold values in groundwaters for nitrates 50 mg L^{-1} [32] and nitrites 0.5 mg L^{-1} [32] are not shown in graphs).

Concentrations of nutrients and organic carbon were influenced not only by the release of treated wastewater, but also by the hydrological conditions (river water level and discharge, Figure S3) as the consequence of the amount of atmospheric precipitation and the extent of snowmelt occurring in the spring. Similarly, seasonal variations in dissolved O_2 and temperature related to discharge (Figure S3) were observed; e.g. in summer, low dissolved oxygen concentrations in the HZ (Table S1) coincided with low discharge (Figure S3) and elevated water temperature in the Sava River (Table S2).

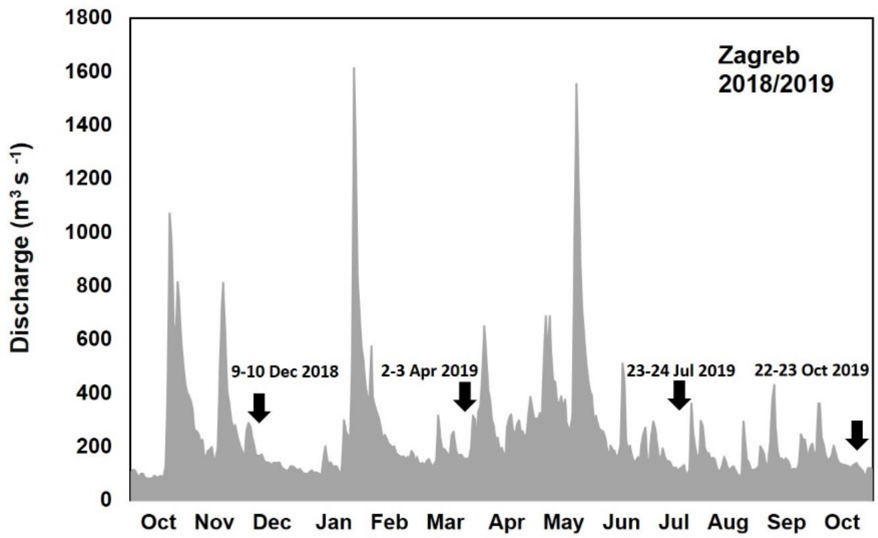

Figure S3. Daily discharge (m^3 s^{-1}) in the Sava River, measured at the Zagreb gauging station (45°47'11.2"N, 15°57'20.2"E, near the sampling site 2-DOWN) during four sampling periods (period from 1st October 2018 to 31st October 2019 is shown). Data are provided from Meteorological and Hydrological Service of Croatia (DHMZ). Arrows point to the seasonal sampling campaigns.

References

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Table S4. Wet weight and number of pooled samples of *Synurella ambulans* (mean ± S.D) analysed per sampling site in each season.

	1-UP		2-DOWN	
	g	***N***	**g**	***N***
Winter 2018	0.031 ± 0.006	10	0.012 ± 0.010	4
Spring 2019	0.025 ± 0.003	6	0.026 ± 0.004	6
Summer 2019	0.030 ± 0.010	6	0.021 ± 0.006	6
Autumn 2019	0.034 ± 0.007	6	0.032 ± 0.006	6