Article

Epilithic Diatom Community Shows a Higher Vulnerability of the River Sava to Pollution during the Winter

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Abstract: The aim of the research was to investigate the influence of environmental factors on the structure of epilithic diatom communities in the Sava River from the source to the state border 220 km downstream. The river had numerous human influences along its course, such as municipal and industrial wastewater, agriculture, hydroelectric power plants, etc. The main objective of the research was to find out the influence of human pressure on the structure of the epilithic diatom community under winter and summer conditions. Winter and summer samples were taken at nine sites. At each sampling site, a set of abiotic factors was measured and another set of environmental parameters was evaluated. The analyses showed that nitrogen and phosphorus concentrations increased downstream. We identified 118 different species of diatoms. The most common taxa were *Achnanthes minutissimum* and *A. pyrenaicum*. Planktonic species *Cyclotella meneghiniana* was only found in the samples of the lower part of the Sava, which is unusual for the epilithic community. The composition of the epilithic diatom community was significantly influenced by conductivity and water temperature, pH and distance from the source. The similarity between diatom communities closer to the source of the river was higher than between communities from the lower part of the Sava River. The values of the trophic and saprobic indices increased downstream and showed that the pollution of the river with nutrients and organic matter increases with the distance from the source. Both indices were significantly higher in winter and showed a more impaired river ecosystem during the winter.

Keywords: periphyton; diatoms; the Sava River; Slovenia; saprobic index; pollution of the rivers

1. Introduction

Periphyton is the basis for the self-purification of the aquatic ecosystem, which enables the absorption of various substances from the water and thus represents a fundamental component in the food chain [1,2]. Periphyton algae are the main primary producers in most watercourses [3] and provide high-quality food for various groups of vertebrates and invertebrates [4]. Periphytic communities are very sensitive to the deterioration as well as to amelioration of water quality, which affects algae growth [5]. Deterioration of water quality has negative effects on river basins, food webs the diversity of communities, and causes the uncontrolled growth of species, which can become a threat to public health [6]. Periphytic diatoms are important mediators of anthropogenic influences, since the diatoms are the most abundant group in the periphyton, especially in spring and autumn [7]. Their sorption capacity and large surface area represent a great potential for the removal of dissolved pollutants from the water. Moreover, they are builders of the biofilm, as many species are firmly attached to the substrate and have an anchoring function. The epilithic diatom communities are more stable than

the epiphytic and epipelic ones due to the stability of their substrate [8,9]. Diatoms are adapted to poor light conditions and turbulent water flow due to the synthesis of silica cell-wall [10]. Due to the combination of relatively narrow ecological tolerances of many species and morphological variability, diatom analysis is a useful tool for assessing ecological status [11], and they are also indicators used to assess natural and human-related characteristics of aquatic ecosystems [12]. Their diversity and different responses to environmental factors have contributed to the fact that many river quality indices are based on diatom data only [13,14].

Watercourses are strongly influenced by their catchment areas and react to physical and ecological processes in the landscape [15]. Vitousek and Reniers [16] and Vallet et al. [17] claim that watercourses allow us to assess the biogeochemical status of the catchment area because they can integrate the response of the catchment. Parr and Mason [18] found that 70% of total nitrogen and 86% of phosphorus in freshwaters are anthropogenic. The uptake of phosphorus and nitrogen accelerates the periphyton growth, changing the structure of the community and the function of the river ecosystem [4]. Nutrient concentrations have a significant influence on the growth of periphyton and the extinction of certain taxa [19]. Dissolved organic matter can serve as food for many diatoms and indirectly as a source of nutrients [20]. At moderately elevated levels of dissolved organic matter, photosynthesis rates are increased due to elevated nutrient concentrations [21]. The source of organic matter depends on the land use in the catchment: tree leaves, flushing from the catchment area and transport of organic matter downstream [22]. Increased amounts of suspended organic matter reduce the penetration of solar radiation into the depths [23] and thus affect primary producers [24].

Phytobentos and periphytic diatoms, respectively, are included in the assessment of the ecological status of water bodies in Water Framework Directive [25], where they are used to assess nutrient and organic pollution [14]. The assessment of water quality can be complicated by changes over time, which are of critical importance, but the factors that influence the temporal occurrence of species are poorly understood. However, the variability of the epilithic diatom community in a torrential river is explained by water temperature, conductivity and altitude [7], while light and temperature indicate seasonality [3]. In addition to environmental factors, spatial factors can also contribute significantly to algae distribution, as well as the aforementioned temporal factors, which we know much less about [6]. Alternative methods for water quality assessment use the functional or morphological characteristics of diatoms instead of taxonomic information [6,14]. Taxa are classified into ecological groups based on different adaptation strategies [6,26]. The classification of benthic diatoms according to sensitivity or tolerance of nutrient concentration is also used for evaluation of water quality.

The Sava River is exposed to many human influences [27], such as municipal and industrial sewage, flushing of farmland, large dams. These influences are reflected to a certain extent in the composition and diversity of the periphyton diatom community. So far, no studies dealing with the epilithic diatom community have been performed on the Sava river, the exceptions are its tributaries in Slovenia (e.g., [7]) or in other countries within the catchment area (e.g., [28]). The aims of this study were to determine how the structure of the epilithic diatom community changes seasonally and longitudinally and which environmental factors have the greatest influence on its diversity and species composition.

We hypothesized that: (a) the differences in species composition of the epilithic diatom community between the sites will be smaller in winter than in summer; (b) the influence of inflowing nutrients and dissolved organic matter on the epilithic diatom community will increase downstream due to the inflow of insufficiently treated wastewater and nutrients from agricultural areas; (c) human pressure in the form of nutrient and organic matter load of Sava River will be higher in winter than in summer, due to the lower activity of primary producers.

2. Materials and Methods

2.1. Study Area

The Sava River is the longest Slovenian river and measures 220 km from the source of Sava Dolinka in Zelenci (Slovenia) to the border with Croatia. The total length of the river from its source to its mouth into the Danube is 946 km and it is its largest right tributary. The Sava flows through three ecoregions: Alpine, Dinaric and Pannonian. It has its source at an altitude of 834 m and leaves Slovenia at the state border at an altitude of 134 m, so the difference in altitude of 700 m is a relatively steep natural gradient. The Sava flows through Croatia and Serbia, where it joins the Danube River. There are various sources of pollution; in addition to the washing away of agricultural land, point sources also have a significant impact. Many tributaries flow into the river, some of which are contaminated with nutrients, organic matter or other pollutants.

The construction of dams and reservoirs for hydropower plants creates completely new living conditions for biota [29]. Below the dams, significant morphological changes occur in the river bed, water regime and sediments [30]. Dams stop the transport of sediments and their accumulation downstream, while erosion processes continue and lead to a deepening of the river bed.

2.2. Sampling Sites

We selected 9 sampling sites (Figure 1) which were sampled twice—in the beginning of March and in the middle of July 2015. We sampled periphyton before the beginning of the growing season and during the summer when the typical summer periphytic communities are already established. At each sampling site, we checked the water level, estimated the proportions of different substrate types, the land use of the catchment area, the shading of the river bed and other factors that can significantly influence the conditions in the river.

Figure 1. Map of locations of all sampling sites within the territory of Slovenia. The positions of dams and accumulations on the river Sava are also presented. The sampling sites: ZE - Zelenci, DO—Dovje, OT—Otoče, ST—Struževo, SE—Šentjakob, KR—Kresnice, PO—Podkraj, KK—Krško, JE—Jesenice na Dolenjskem.

The sampling sites Zelenci (ZE), Dovje (DO), Otoče (OT), Struževo (ST) and Šentjakob (SE) are located within the alpine ecoregion or upstream, in the middle part we have the sampling sites Kresnice (KR) and Podkraj (PO), which are part of Dinaric Sava, while the sites Krško (KK) and Jesenice na

Dolenjskem (JE) are located in the Pannonian ecoregion. The Sava riverbed is cut into a lithologically heterogeneous base consisting of four petrographic types (according to [31]): triad limestones and dolomites (Zelenci, Dovje, Podkraj); carbonaceous marls and sandstones (Kresnice); conglomerate or older gravel (Otoče, Struževo); pleistocene gravel and other deposits (Šentjakob, Krško, Jesenice) [31].

2.3. Measurements of Abiotic Parameters

The water current velocities were measured with a current velocity sensor (Hydro-Bios, Kiel, Germany) at 60% depth. Water temperature, dissolved oxygen concentration and saturation, conductivity, total dissolved solids (TDS) and pH were measured with a multimeter (Eutech Instruments PCD 650, Singapore). For other analyzes the water was stored and analyzed in the laboratory. HACH Lange cuvette tests determined the concentrations of certain nutrients in the water. The contents of nitrate (LCK 339), ammonium (LCK 304), orthophosphate (LCK 349) and total nitrogen (LCK 138) were analyzed. The final values of the individual parameters in the sample were determined with a spectrophotometer (HACH Lange DR 3900, Dusseldorf, Germany). The total suspended solids content (TSS) and the dry mass were determined by filtration and drying of the samples at 105 °C.

2.4. Periphyton Sampling and Laboratory Analyses

Periphyton samples were collected quantitatively with a stone scraping method using a toothbrush and scalpel. At each sampling site, a section up to 10 m long was investigated, in which 5–7 stones (6–20 cm in size) were collected [32]. We chose stones whose upper surface was fairly parallel to the surface or which were equally exposed to the flow of water and sunlight. Using a 2 × 3 cm (6 cm²) rectangle, we scraped the periphyton with brush and scalpel into a plastic container and fixed the samples with 37% formaldehyde to achieve a ratio of 1:9.

Each sample was homogenized with a magnetic stirrer and 5 mL of the suspension was poured into a glass centrifuge tube. The samples were centrifuged at 4000 rpm for 10 min. The supernatant was removed and 2 mL of 65% HNO₃ was added. The sample was heated over the flame until the resulting vapor turned white. The sample was centrifuged again. The supernatant was poured and 2 mL of distilled water was added. Four permanent slides of each sample were prepared using Naphrax[®] mountant (Brunel Microscopes Ltd, Chippenham, UK).

The slides were examined with the microscope (Olympus CX41 microscope, Tokyo, Japan) at a magnification of 1000×. From each sample, 400 frustules were counted and determined. For the determination of diatom taxa and their nomenclature we followed Hofmann et al. [12] and Krammer and Lange-Bertalot [33–36].

2.5. Data Analysis

The trophic index (TI) was calculated according to Rott et al. [37]. The saprobic index (SI) was calculated on the basis of the saprobic value, the frequency and the indicator value of the taxon, on the basis of which the river at each sampling section is classified in the appropriate quality class [38].

The influence of specific factors on the composition of the diatom community was tested by redundancy analysis (RDA). The linear gradients in the matrix of taxa were revealed beforehand with detrended correspondence analysis (DCA), where the eigenvalue for the first axis was 0.38, while the gradient length was 2.49 standard deviations. We used forward selection, where 499 permutations were performed in every round to rank the relative importance of explanatory variables and to avoid co-linearity. Statistically significant factors were used for creation of the RDA diagram. These analyses were performed with the program package CANOCO 4.5 [39].

Correlations between SI, TI, ecological types of diatoms and explanatory variables were tested with a Spearman's rho correlation coefficient. Correlations were calculated with PAST [40]. Differences in characteristics between the seasons were checked with a *t*-test (MS Excell).

Diatom taxa were classified into ecological groups or “guilds” according to Passy [6] and Rimet and Bouchez [26]. Grouping species according to ecological guilds can strengthen relations with certain environmental stressors or factors, when compared to species data [26].

3. Results

3.1. Influence of Environmental Parametres on the Composition of the Diatom Community

The detrended correspondence analysis (DCA) shows that the differences in species composition of the diatom community are greater in winter than in summer (Figure 2), when the stability and similarity of the assemblages along the course is greater. The samples from the KK sampling site are conspicuous in both seasons and were most different from the others. The associations in the samples ZE.w and ZE.s are quite similar, as are the expected positions on the left edge of the group of samples (Figure 2).

Figure 2. Ordination diagram of detrended correspondence analysis (DCA). The symbols for winter samples are blue, labeled “Site_w” and the symbols for summer samples are yellow, labeled “Site_s”.

Using RDA analysis, we calculated that factors that statistically significantly explain the taxonomic composition of the epilithic diatom community are conductivity (19% of the total explained variance), water temperature (12%), distance from source (11%) and pH (8%). The assemblages at the sampling sites ZE and DO are relatively similar compared to the other samples (Figure 3). With the distance from the source, the differences between the winter and summer samples from the same sites increase, as do the differences between the samples. The distribution pattern becomes less clear downstream and varies greatly depending on the season, which is influenced by the greater temperature differences in the lower part compared to the upstream part (see Table A1).

Figure 3. Ordination diagram of the samples and significant parameters ($p < 0.05$) according to redundancy analysis (RDA). Blue symbols represent winter samples (e.g., 1.ZE.w etc.), and yellow symbols represent summer samples (e.g., 1.ZE.s).

3.2. Water Quality along the Course and between the Seasons

SI values at different locations vary between winter and summer samples (Figures 4 and 5). The smallest differences are found in the sampling site ZE and KR, but especially at OT, PO and JE. The highest value of SI was 3.19 in winter and 2.78 in summer, but in both cases in the site KK. The lowest value of SI (1.35) was calculated for the ZE.w sample.

Figure 4. Saprobic index values for all winter samples (Sampling sites are defined in Figure 1).

Figure 5. Saprobic index values for all summer samples (Sampling sites are defined in Figure 1).

In general, the values of SI increase downstream and indicate a lower level of organic matter pollution in the upper part of Sava River, while they increase in the middle part and reach the highest values in the KK sampling site, where the value of SI represents a very high level of organic matter pollution. According to the values of SI, the ZE and OT sampling sites can be classified in the 1st quality class or in the oligosaprobic level. The sampling sites DO and ST are oligosaprobic in winter, while in summer they are classified in the β -mesosaprobic level, i.e., 1st–2nd quality class. The samples OT.w, KR.w, KR.s and PO.s indicate a moderate pollution of the watercourse and belong to the β -mesosaprobic level, JE.w to α -mesosaprobic, while in summer the condition improved by one quality class. The PO.w and KK.s samples are also in the α -mesosaprobic level, while the water from the KK.w sample is heavily polluted (α -meso to the polysaprobic level).

TI values in winter samples range from 1.46 to 2.11 (Figure 6), which characterizes oligo-mesotrophic to meso-eutrophic ecosystem. In the summer samples the differences were smaller (1.5–1.94) and all values belong to the same trophic class. The highest trophic index was calculated in the sample KK.w (meso-eutrophic), and the lowest in the sample ZE.w (oligo-mesotrophic).

Figure 6. Trophic index values for all winter samples (Sampling sites are defined in Figure 1).

In most cases, the values of TI are higher in winter than in the summer samples (Figures 6 and 7). Both the winter and summer values increase downstream, as indicated by the high determinant coefficient ($R^2 = 0.80$) for the winter samples, which means that nutrient inputs to the Sava increase downstream despite increasing discharge. The trend line based on the diagram of the summer samples is flatter (Figure 7) and R^2 is only 0.20, indicating that the nutrient load downstream is more or less the same.

Figure 7. Trophic index for all summer samples (Sampling sites are defined in Figure 1).

In the alpine part, the river is oligo-mesotrophic and in the lower part (from KR downstream), while its status changes to mesotrophic with increasing nutrient input (the effects of the outflows of wastewater treatment-plants Ljubljana and Domžale-Kamnik). According to the results of winter samples, the river is meso-eutrophic in the lower part.

The values of SI and TI are positively correlated with distance from the source, channel width and in-stream parameters such as conductivity, total dissolved solids (TDS) and all measured forms of nitrogen (TN, NH₄-N, NO₃-N). The values of SI are also positively correlated with the periphyton dry mass and phosphate concentration, but negatively with current velocity (Table 1).

Table 1. Spearman correlation coefficients (ρ) between quality indices, ecological groups of diatoms and environmental parameters. Statistically significant ($p < 0.05$) correlations are shown only. TI—trophic index, SI—saprobic index, LP—low profile species, HP—high profile species, M—motile species, PL—planktonic species.

	Quality Index		Ecological Groups			
	TI	SI	LP	HP	M	PL
Altitude	−0.660	−0.784			−0.552	
Distance from the source	0.660	0.784			0.552	
Channel width	0.776	0.743	−0.554		0.659	
Periphyton dry mass		0.550				
Current velocity		−0.546				
T water				−0.593		
Conductivity	0.717	0.792			0.676	
PO ₄ -P		0.533			0.497	
TDS	0.771	0.839	−0.473		0.721	
TN	0.530	0.548			0.604	
NH ₄ -N	0.520	0.551			0.481	
NO ₃ -N	0.589	0.638			0.611	

Beside the water temperature, a set of seven measured parameters differed significantly between winter and summer samplings, as well as three indices determined on the base of epilithic diatom communities (Table 2).

Table 2. Average values of winter and summer samples for the parameters and indices that were statistically significant according to *t*-test.

Parameter:	Winter Samples Average	<i>p</i> -Value	Summer Samples Average
Water temperature (°C)	7.1	<0.001	17.5
Conductivity (µS/cm)	348	<0.001	321
TDS (mg/L)	341	<0.001	316
TN (mg/L)	1.54	0.017	0.98
NH ₄ -N (mg/L)	0.39	0.048	0.03
NO ₃ -N (mg/L)	1.06	0.005	0.74
[O ₂] (mg/L)	12.9	<0.001	10.3
O ₂ saturation (%)	99	0.050	112
SI	2.22	0.043	1.93
Species number	36.1	0.003	26.6
Shannon diversity index	3.70	0.012	2.94

3.3. The Structure of the Diatom Community

Table 3 lists taxa with a frequency of at least 10% in the diatom assemblage. The two most dominant taxa in the samples were *Achnanthes minutissimum* and *A. pyrenaicum*, which co-dominated 11 out of 18 samples: ZE.w, ZE.s, DO.w, DO.s, OT.s, ŠE.w, ŠE.s, KR.w, KR.s, PO.s and JE.s. (Table 3). These taxa (*A. minutissimum* and *A. pyrenaicum*) account for up to 84% in the epilithic diatom community.

Table 3. Dominance of taxa in winter (w) and summer (s) samples. The proportions of the two most abundant taxa are in shaded cells. Other species are considered co-dominant because their proportion is >10%. For full names of the sampling sites and their locations along the river Sava see Figure 1.

Taxa	ZE		DO		OT		ST		ŠE		KR		PO		KK		JE	
	w	s	w	s	w	s	w	s	w	s	w	s	w	s	w	s	w	s
<i>Achnanthes minutissimum</i>	41	51	30	48	23	41	22		38	30	26	16		27			16	36
<i>Achnanthes pyrenaicum</i>	20	21	25	35	13	38	12		37	29	14	17		24				42
<i>Encyonema minutum</i>					15		10						17					
<i>Gomphonema tergestinum</i>							15	32										
<i>Navicula lanceolata</i>													17		27			
<i>Nitzschia fonticola</i>							15				13							
<i>Cyclotella meneghiniana</i>																42		
<i>Amphora pediculus</i>																	20	
<i>Surirella minuta</i>															11			
<i>Navicula reichardtiana</i>																7		
<i>Cocconeis placentula</i>													17					
<i>Diatoma ehrenbergii</i>							13											
<i>Mayamaea atomus</i>											10							
<i>Nitzschia gracilis</i>							10											

In the samples from ST.s, PO.w, KK.w and KK.s none of these taxa was dominant. In the KK.s summer samples *Cyclotella meneghiniana*, the planktonic species, is the most dominant, and *Navicula reichardtiana* is also common. In the winter sample KK.w the dominant species are *Surirella minuta* and *Navicula lanceolata*. *N. lanceolata* also appears in PO.w among the dominant species, with *Encyonema minutum* among the more common ones. It is still present in the sample OT.w. Both in the sample ST.w and in the sample ST.s *Gomphonema tergestinum* is dominant.

ST.w contained five co-dominant species with uniform representation: *A. minutissimum*, *A. pyrenaicum*, *Encyonema minutum*, *Gomphonema tergestinum* and *Nitzschia gracilis*. Four species dominated in KR.s (*A. minutissimum*, *A. pyrenaicum*, *Nitzschia fonticola* and *Mayamaea atomus*) and three dominate in OT.w, ST.s and PO.s (see Table 3). Elsewhere, only two species reach values above 10% and all others are present in smaller proportions.

Ecological Groups of Diatoms

Low- and high-profile diatoms are present in all samples (Figure 8). Motile species occur in all samples except OT.s. Planktonic species occur in ZE.s, DO.w, ŠE.s, KR.s, PO.s, KK.s and JE.s. They occur mainly in summer samples, even in KK.s, the dominant ecotype. Most other samples are dominated by low-profile species, except for the sampling site ST where both samples are dominated by high-profile species and PO.w and KK.w where the motile diatoms predominate. The density of mobile and planktonic species increases downstream, but the proportion of high-profile species decreases.

Figure 8. Proportion of different ecological types of diatoms (ecotypes) in a single sample during winter and summer sampling on the Sava River. Light green: LP—low profile species, dark green: HP—high profile species, orange: M—motile species, blue: PL—planktonic species. For full names of the sampling sites and their locations along the river Sava see Figure 1.

The proportion of low-profile species is negatively correlated with channel width and TDS (Table 1). High-profile species are negatively correlated with water temperature, while proportion and motile species correlate positively with source distance, channel width, conductivity, TDS, water temperature and nutrients like phosphate, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ (Table 1).

4. Discussion

4.1. Influence of Environmental Parametres on the Composition of Epilithic Diatom Community

Conductivity, water temperature, distance from the source and pH had a statistically significant influence on the species composition of the epilithic diatom communities (Figure 3). These results are largely consistent with the results reported by Toman et al. [7]. Cantonati [41] also finds that the species composition of the diatom community is most strongly influenced by pH and conductivity. Soininen [11] reports that conductivity, trophic status, as well as spatial factors have a significant influence on the composition of diatom communities in rivers. Common to all mentioned studies are conductivity and spatial dimension as factors that significantly influence the epilithic diatom community.

In addition to ecological and spatial factors, the composition of diatom communities is also influenced by temporal variables about which we do not know much [6]. In our study, water temperature is a matter of time, since we sampled in winter and summer, and of space, since in summer the water temperature increases significantly with distance from the source, which is a general pattern [42]. This gives the water temperature a complex character. Temperature differences cause differences in the growth rate, biomass and taxonomic composition of algae [43] and differences in ecosystem processes such as organic matter degradation and nutrient uptake [2].

Distance from the source is a spatial factor, but as concentrations of organic matter and nutrients increase downstream, this factor may also include factors arising from human pressure, which is reflected in conductivity.

Although the differences in water temperature were almost five times greater in summer than in winter, we found that there are greater differences between diatom assemblages in winter than in summer (Figure 3). They were also more heterogeneous (with higher Shannon diversity index) in winter than in summer (Table 2). This indicates the strong influence of human activities, which most probably caused these differences.

4.2. Water Quality along the Course and between the Seasons

The dry mass of the periphyton and SI values of the samples increased downstream, indicating an increase in the concentration of dissolved organic matter (DOM). The values of SI were higher in the lower part of Sava River in both seasons studied. Cole and Caraco [44] report that although headwaters through mid-order rivers export large amounts of organic matter to the lower reaches, large rivers may be able to mineralize a large amount of carbon entering from terrestrial ecosystems. Despite this fact, the values of SI were increasing along the Sava River, reflecting the increasing pressure in the form of inputs of degradable organic matter.

A statistically significant negative correlation between SI and the current velocity indicated that the saprobity increased with decreasing current velocity, since lower velocity means also lower metabolic rate in the periphyton and was another reason for the lower self-purification capacity of the river, as the values from SI showed. Therefore, the possible construction of additional dams and reservoirs would negatively influence the self-purification ability of the Sava river.

The river Sava changed from oligosaprobic (1st quality class) in the headwater section to β -mesosaprobic (2nd class) in the middle section and deteriorated in the lower part of the river to α -mesosaprobic or even polysaprobic (3rd to 4th quality class) waterbody during winter according to the values in Sladeček [45]. There was a substantial increase of SI between sites PO and KK (Figures 4 and 5), which reached the maximal values in the latter, although that the distance between them was the biggest (Figure 1). The most possible reason is the regulated part of the river (with four large dams and reservoirs), which has lower ability for self-purification [46]. By contrast, the values of SI and TI dropped in all cases between the sites KK and JE, but there are no impoundments.

TI and SI correlated significantly with nitrogen, conductivity and distance from the source. The Sava River changes along its course from the oligo-mesotrophic environment to the meso-eutrophic environment. In the lower part of the river, the values of TI were higher, as more nutrients from the catchment area entered the watercourse.

The values at the KR site were very high because it is located a few kilometers downstream of two large wastewater treatment-plants with the total capacity of 560,000 population equivalents. Elias-Kowalska and Wojtal [47] also report that increased nutrient concentrations are the result of inflowing treated wastewater containing more phosphate and nitrate. Nitrate and ammonium concentrations increased downstream in both seasons, but the increase in the lower part was more pronounced in summer, probably due to their leaching from agricultural land. However, Exner-Kittridge et al. [48] report that the timing of fertilizer application was not found to be significant in relation to the seasonality of nitrogen concentrations and surface water pollution. Bailey and Ahmadi [49] report that upstream concentrations of NO_3 have less influence on these concentrations in the lower part during the summer and more in the winter, due to higher water temperatures in the summer.

Differences between Winter and Summer

Seasonal variation in water temperature has a strong influence on respiration rates and thus on the utilization of organic matter [2,50], standing stock of which is inversely related to temperature. In the present study, the values of SI were significantly higher in winter than in summer ($p = 0.043$) (Table 2). The rates of microbial activity are strongly temperature dependent and the breakdown of organic matter is slower at lower temperature [2]. Higher water temperature and nutrient concentrations lead to favorable conditions for microbial activity [51], which leads to the higher decomposition rate of

DOM in the summer and the higher uptake of the released nutrients by bacteria and primary producers. Consequently, the reduced self-purification capacity of the Sava River made it more vulnerable to pollution in winter. Chenier et al. [51] report that the microbial community within the biofilm varies greatly over the year and consequently the response to stress varies with the season. The important role of bacteria in the breakdown of organic matter and their ability to utilize labile DOM from the water is essential. The uptake of human-introduced organic matter by decomposers is often associated with insufficient $[O_2]$ and mortality of aquatic organisms [49].

TI values in winter samples (Figure 6) were higher in most sites than in summer (Figure 7). The concentrations of TN, NH_4-N and NO_3-N , were statistically significantly higher in winter, indicating a higher nutrient load of the river in winter as a consequence of a lower nutrient uptake by vegetation on land and in the riparian zone [16]. As a result, more nutrients are washed into the river. During the summer, most of the discharge is provided by deep aquifers containing low nitrate concentrations, while in winter about half of the discharge contributes to shallow aquifers, containing about 20-times higher nitrogen concentrations [48]. However, seasonal temperatures and vegetation growth rates are not the primary factors for differences in nitrogen concentrations.

During winter sampling, there were smaller temperature changes downstream than in summer. During the winter, significantly more oxygen was dissolved in the water than in summer (Table 2), due to its better solubility at low temperatures [2], but saturation showed the opposite, which was the consequence of higher algae production. Water temperature influences both respiration and the growth and primary production of algae and therefore has a stronger influence on $[O_2]$ in summer [49] than in winter. Biggs et al. [52] claim that the production of periphytic algae increases with temperature of the water and the maximum ratio between photosynthesis and respiration could be 3-times higher in summer than in winter.

Cunha de Menezes et al. [53] find a different response during the warmer period, when high primary productivity enables the reaeration of the stream. The majority of the sites have high values of dissolved O_2 due to high productivity of the algae and not by the assimilation capacity of organic matter.

The lower conductivity in summer was influenced by increased primary production and nutrient uptake, while in winter the average conductivity values were higher (+27 $\mu S/cm$).

4.3. The Structure of the Diatom Community

4.3.1. The Dominance of Taxa

The two most dominant taxa in the samples were *Achnanthes minutissimum* and *A. pyrenaicum* (Table 3), which accounted for up to 84% in the epilithic diatom community. Eliaz-Kowalska and Wojtal [47] consider the dominance of *Achnanthes minutissimum* and *Encyonopsis microcephala*, as indicators of anthropopressure, especially with regard to high nutrient contents.

In the KR.s sample, in addition to *A. minutissimum* and *A. pyrenaicum*, the co-dominant species *Mayamea atomus*, which is one of the most saprobity-tolerant species [54,55] was also found. The KK.s sample was dominated by the planktonic species *Cyclotella meneghiniana* (Table 3), which is very resistant to organic pollution [56]. Its high proportion (42%) was influenced by high nutrient contents and the reservoirs from which this species came from drift.

In winter samples (OT.w, ST.w, PO.w), *Encyonema minutum*, which is often found in anthropogenically little disturbed sites [55], was also dominant. The ST samples in both seasons were dominated by *Gomphonema tergestinum*, which is characteristic for fast flowing waters [55]. The average current velocity was the highest in this site.

At low temperatures and solar radiation, the species of the genus *Navicula* become dominant [43]. In our case, the species *Navicula lanceolata*, which is characteristic of polytrophic and α -mesosaprobic habitats [55], was actually the dominant species in winter samples PO and KK occurring with more than 10% in the community (Table 3).

Nitzschia dissipata, characteristic of meso-eutrophic habitats [55], was present in all winter samples (except in ZE), when TI values were higher. *N. palea* is a species common in α -meso to polysaprobic water [57,58] and it occurred mainly in the lower part of our sites (summer and winter).

4.3.2. Ecological Groups

The water current velocity influences the thickness of the periphyton and the taxonomic composition of the diatom community [52]. When velocities exceed 10–15 cm/s, the hydrodynamic shear stress begins to affect the structure of the periphyton and the taxonomic composition [59,60]. In the upstream samples, smaller and low-profile species dominated, while in the downstream samples, motile and high-profile species occurred with higher proportions (Figure 8), which is consistent with the longitudinal decrease of current velocities [61]. In our study, no significant correlation was calculated between the ecological types and velocity (Table 1). The lower current velocities mean lower frequency of disturbance, and a lower disturbance frequency results in more high-profile species [62] and motile species. The proportion of motile taxa positively correlated with distance from the source and channel width, as well as with contents of phosphorus, nitrogen and TDS (Table 1). Motile taxa reached the highest proportion in the site KK (Figure 8), which is downstream the chain of four accumulations (Figure 1). Discontinuous release of water from these accumulations and transport of higher contents of suspended solids could explain the high proportion of motile taxa (see [63,64]). On the other hand, low-profile taxa negatively correlated with channel width and TDS. Motile species are more efficient at absorbing substances from the water because they migrate to the upper layers of the periphyton where they have better access to nutrients and light, and because of their faster metabolism [26]. Planktonic species found in the lower Sava River, mainly in summer samples, have drifted out of the reservoirs.

5. Conclusions

The composition of the diatom community was statistically significantly influenced by conductivity, water temperature, distance from the source and pH.

In both seasons, the human pressure on the Sava River increased downstream. With the distance from the source, we recorded the increase of nitrate, ammonium, total nitrogen, conductivity and the values of TI and SI. Consequently, the amount of periphyton dry mass also increased downstream. The Sava River changed along its course from the oligo-mesotrophic to the meso-eutrophic ecosystem. With regard to saprobity, we evaluated the headwater section as oligosaprobic (1st quality class), which changes to β -mesosaprobic (2nd class) in the middle part of the river and deteriorated to an α -mesosaprobic or even polysaprobic (3rd to 4th class) water body in the lower part of the river in winter.

Higher TI values in sites KR than in PO indicate a higher eutrophic potential in the middle part of the Sava than in the lower Sava River, which could indicate a worse situation in the potential/planned reservoirs in the middle part of the Sava River. Additional reservoirs would negatively influence the self-purification ability of the river.

We can conclude that the self-purification ability of the river Sava is significantly lower in winter, which makes the river more vulnerable to human pressures in colder periods, especially in terms of organic pollution. We also strongly advise against the construction of large dams in the middle part of the Sava to prevent the impairment of water quality and loss of numerous species.

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Appendix A

Table A1. Physical and chemical characteristics of water at sampling sites. All concentrations are in mg per liter.

Sample	Max. Velocity (m/s)	T Water (°C)	pH	Conductivity (µS/cm)	[O ₂]	Sat. with O ₂ (%)	TDS	PO ₄ -P	TN	NH ₄ -N	NO ₃ -N
ZE.w	0.76	4.8	7.2	262	10.4	83	258	0.03	1.11	0.03	0.86
ZE.s	1.20	8.1	7.1	254	8.0	79	250	0.00	0.79	0.02	0.31
DO.w	1.70	7.2	7.8	278	12.0	98	273	0.23	0.59	0.03	0.42
DO.s	1.74	10.2	7.5	263	9.9	95	256	0.00	0.35	0.02	0.44
OT.w	1.33	6.5	8.0	310	14.3	111	304	0.00	1.12	0.03	0.72
OT.s	1.39	16.5	8.0	280	10.9	117	276	0.14	0.86	0.03	0.71
ST.w	1.38	6.9	8.0	327	14.7	114	321	0.76	0.92	0.02	0.72
ST.s	1.53	18.3	8.0	300	10.0	110	297	0.01	0.84	0.03	0.55
ŠE.w	0.93	7.9	7.5	376	13.1	100	356	0.01	1.46	0.04	1.26
ŠE.s	0.37	17.2	7.6	340	10.1	104	329	0.00	1.09	0.04	0.81
KR.w	0.87	7.2	7.5	392	13.0	98	385	0.14	3.13	0.08	1.59
KR.s	0.83	18.5	7.8	363	10.7	116	357	0.03	1.44	0.06	1.15
PO.w	0.94	7.2	7.5	392	13.0	96	385	0.01	1.41	0.08	1.16
PO.s	0.85	20.1	7.9	370	11.0	123	365	0.06	1.38	0.05	1.00
KK.w	0.15	8.1	7.6	391	13.0	96	385	0.53	1.76	0.05	1.41
KK.s	0.04	22.3	8.1	359	11.7	133	354	0.01	0.72	0.02	0.73
JE.w	0.06	8.5	7.5	406	13.0	99	398	0.20	2.35	0.04	1.43
JE.s	0.59	26.3	8.1	363	10.7	132	357	0.23	1.38	0.04	0.99

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