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Article

Aquatic Insects (Ephemeroptera, Plecoptera and Trichoptera) Metric as Important Tool in Water Quality Assessment in Hilly and Mountain Streams

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Abstract: The aim of the study was to test the significance of the EPT index in the water quality assessment of three types of water bodies in hilly and mountainous region of Serbia. The aquatic macroinvertebrate community was dominated by the group of insects, of which 95 taxa represent the EPT group. We compared the obtained values of biological indices used for the assessment of water quality according to the national legislation with the overall status assessment represented by the ecological quality classes (EQC). The results of the Spearman correlation test showed a negative correlation of EQC with the EPT index, BMWP score, H', total number of taxa and number of sensitive taxa, while a positive correlation was observed for the values of SI and Tubificinae %. The values of EQC and biological indices were subjected to principal component analysis (PCA). The results showed that the parameters that contributed most to the differences were the EPT index, the BMWP score and the number of sensitive taxa. The results indicate that the EPT index is an excellent indicator of changes in water quality and an important tool for the ecological categorization of water bodies in mountain regions.

Keywords: aquatic insects; EPT group; diversity; biological indices; water quality assessment; hilly and mountain streams; Serbia

1. Introduction

The benthic macroinvertebrate fauna is an effective tool for documenting changes in overall ecological status, and also one of the most prominent biological quality elements (BQEs) used for the ecological assessment of rivers under the European Water Framework Directive (WFD) [1]. Aims of WFD are to prevent further deterioration and to protect and improve the status of aquatic ecosystems, with the explicit goal of achieving at least "good ecological status" for all surface waters by 2027 [2].

Macroinvertebrate offer numerous advantages for biomonitoring. Sampling is relatively simple and has minimal adverse effects on the resident biota [3–5]. Macroinvertebrate taxa generally occur in characteristic and limited habitats within their geographic range and are usually most abundant near their respective ecological optimum [6,7]. The benthic macroinvertebrates are good indicators of local conditions and are particularly suitable for assessing site-specific impacts, as many of them have restricted migration patterns or a sessile lifestyle [8]. Larval stages will respond quickly to stress. Many of these groups are relatively easy to identify to the lower taxonomic levels such as genus and species [9,10].

Many aquatic insects are intolerant to various types of pressures, therefore along with increased pollution, lower diversity can be expected. Barbour et al. [10] compared a large number of macroinvertebrate indices and found that simple species counts, especially sensitive taxa, were most effective in determining water body impairment.

Some of the macroinvertebrate based indices of river health have been proven to be particularly useful and effective, such as EPT (Ephemeroptera + Plecoptera + Trichoptera) index. This index was named after three orders of aquatic insects common in the benthic macroinvertebrate community: Ephemeroptera (mayflies), Plecoptera (stoneflies) and Trichoptera (caddisflies). It represents the sum of the taxa richness of these three orders [11].

Publications from 1950s stated that species of these three orders are generally intolerant of pollution [12]. The EPT larvae are easy to sort and identify and are often used as an indicator of water quality. The EPT index has been recommended as possibly the most efficient of the macroinvertebrate indices, especially in lotic ecosystems where they are the dominant component of the representative community [13,14].

A complex river network covers most of the territory of Serbia and belongs to the catchment area of Black, Adriatic and Aegean Sea. The Serbian territory belongs to south-east Europe, covering the central part of the Balkan Peninsula and the southern part of the Pannonian Plain. It can be clearly divided into two regions – the Pannonian plain and the hilly and mountainous region south of the Danube and Sava rivers [15]. The distribution of aquatic organisms in this area is therefore a complex issue. The diversity of benthic macroinvertebrates in this area is significant and it is considered the main diversity hotspot of aquatic insects in Europe, especially the EPT groups [16]. Paunović et al. [17] used the distribution of macroinvertebrates to delineate the boundaries of ecoregions on the territory of Serbia with regard to the original concept of Illies [15], which was accepted by the WFD. According to these authors [17], the hilly and mountainous region of the country belongs to the ecoregion 5 (Dinaric Western Balkan) and ecoregion 7 (Eastern Balkan).

The aims of the study were: to assess the significance of the EPT index in the evaluation of the water quality assessment of three types of water bodies in the mountainous regions of Serbia; to test the relationship between the obtained values of biological indices used for the assessment of water quality of these types of water bodies according to the national legislation and the overall ecological status assessment represented by the ecological quality classes (EQC); to test whether the EPT index is sufficiently meaningful and self-sufficient for the assessment of water quality of hilly and mountainous streams.

2. Materials and Methods

Study area

The collection of benthic macroinvertebrate samples in spring and fall 2019 was conducted to supplement the data for the revision of the Water Management Plan for the territory of the Republic of Serbia. It included 119 watercourses that previously haven't been part of the routine water quality monitoring led by the Environmental Protection Agency of the Republic of Serbia. The analyzed dataset in this paper included the group of hilly and mountainous small to medium sized streams with predominantly hard bottom substrate, classified according to the Serbian typology of watercourses [18]. The focus was on 44 sites located on three selected stream types (Figure 1). The selected types of watercourses were: type 3 - small and medium streams, altitude up to 500 m a.s.l., dominance of larger substrate (mesolithal, macrolithal, megalithal) (24 sites); type 4 - small and medium streams, altitude above 500 m a.s.l. and dominance of larger substrate (mesolithal, macrolithal, megalithal) (6 sites) and type 6 - small watercourses outside the area of the Pannonian Plain that do not fall under types 3 and 4 and are not covered by the regulation on the establishment of surface and groundwater bodies (combination of different types of substrate) (14 sites) [19] (Table 1). Most of the investigated sites have microhabitat substrate characterized by boulders and cobbles and fast velocity of flow. The size of the microhabitat substrates were defined according to Hering et al. [20].

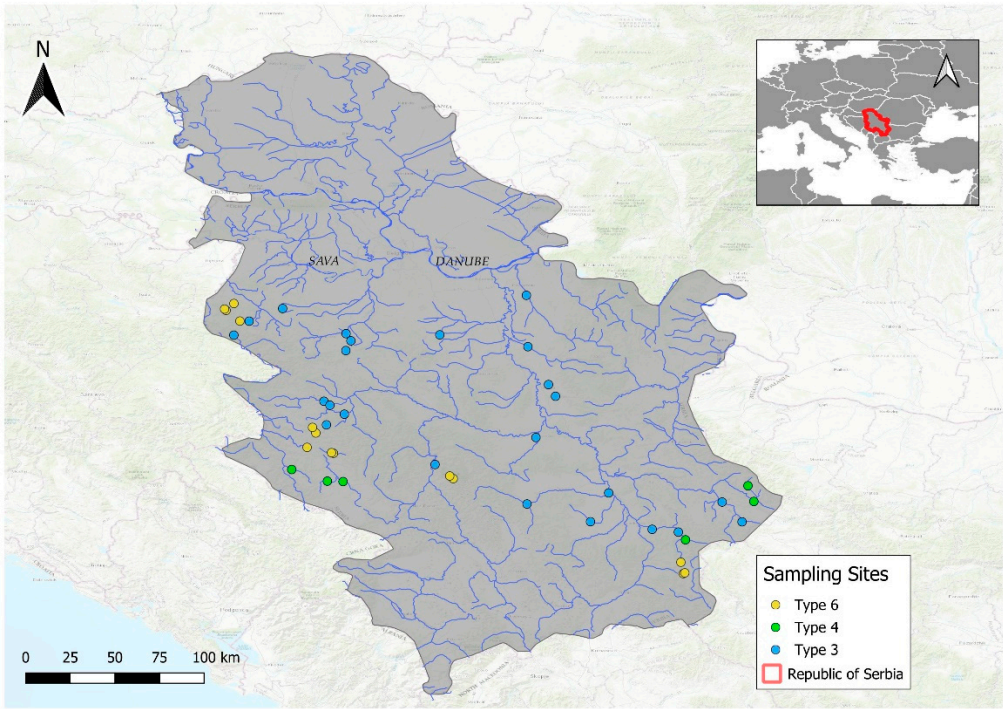


Figure 1. Map of sampling localities.

Table 1. Sampling localities by watercourse type with GPS coordinates.

No.	Type of watercourse	Localities	N	E	Altitude (m)
1	Type 3	Kamenica	44.27879	20.69594	203
2		Grza	43.86491	21.47602	210
3		Ravanica	43.94375	21.42883	176
4		Likodra	44.37108	19.41078	390
5		Krupinska reka	44.27692	19.30736	313
6		Veliki Rzav 1	43.67234	19.93187	498
7		Veliki Rzav 2	43.74391	20.05248	480
8		Nišava 1	43.01863	22.73374	413
9		Nišava 2	43.15188	22.59950	381
10		Pusta reka	43.01950	21.71199	246
11		Toplica 1	43.21316	21.83449	272
12		Toplica 2	43.13895	21.28398	425
13		Vlasina 1	42.96810	22.12787	329
14		Vlasina 2	42.94951	22.30421	410
15		Rasina	43.58722	21.34405	152
16		Mlava	44.54519	21.27974	93
17		Jošanica	43.40491	20.66400	463
18		Resava	44.19887	21.28998	148
19		Ribnica 1	44.28707	20.06403	163
20		Ribnica 2	44.23879	20.09669	260
21		Ribnica 3	44.17276	20.06269	375
22		Tamnava 2	44.45617	19.63632	236
23		Đetinja 1	43.83131	19.91463	642
24		Đetinja 2	43.80448	19.95518	458
25	Type 4	Mileševka	43.37128	19.69763	726
26		Uvac	43.29290	19.93810	1134
27		Vapa	43.29000	20.04355	1023
28		Dojkinačka reka 1	43.26172	22.77364	1223

29		Dojkinačka reka 2	43.15595	22.81343	1009
30		Gradska reka	42.89735	22.35245	846
31	Type 6	Tisovica	43.48065	19.97928	1167
32		Trudovačka reka	43.48476	19.96600	1167
33		Čađavica	44.37178	19.34831	491
34		Korenita	44.48985	19.30783	261
35		Štira 1	44.44366	19.25502	526
36		Štira 2	44.45272	19.24496	391
37		Pritoka Uvca	43.52039	19.80133	948
38		Ljubišnica	43.61730	19.86022	1018
39		Katušnica	43.65501	19.83772	896
40		Cvetkova reka	42.74631	22.32128	1380
41		Jarčev potok	42.66968	22.33745	1258
42		Simonova reka	42.67491	22.34782	1258
43		Samokovska reka 1	43.30976	20.78675	1506
44		Samokovska reka 2	43.32760	20.76281	1506

Macroinvertebrate sampling and processing

Samples were collected using the kick and sweep sampling method from all microhabitat types according to European standards [21] with an FBA hand net (25x25 cm, mesh size 500 μm). According to Tubić et al. [22] the kick and sweep sampling method is more effective compared to quantitative Surber net sampling in terms of general taxa richness and taxa richness within the main components of the benthic communities in the water body type of small to medium sized streams with predominantly coarse bottom substrate.

The biological material was pooled and transferred to sample containers (250 ml) and preserved with 70% ethanol. The benthic macroinvertebrates were identified based on their morphological characteristics using stereomicroscope ZEISS Stemi 2000C (x50) and Nikon SMZ 800N (x75), ZEISS Axio Lab. A1 (x630) at the lowest possible taxonomic level using appropriate identification keys [23–26].

Biological metrics

The following biological metrics were used for the analysis and comparison: EPT index, total number of taxa per sample (No. of taxa), Saprobic Index (SI) [27], Biological Monitoring Working Party (BMWP score) [28], Diversity Index/Shannon-Wiener Index (H') [29], number of sensitive taxa (No. of sensitive taxa), percentage participation of subfamily Tubificinae (Oligochaeta) (Tubificinae %). Average Score Per Taxon (ASPT) is a slightly modified version of BMWP score. It is calculated by dividing the BMWP values by the sum of the relative abundances of the present families. As it is based on the values of the BMWP score, it was not taken into account.

In accordance with national legislation and the established class boundaries for the metrics, the relevant parameters were used to assess ecological status of each river type based on macroinvertebrate metrics [19]. This is represented with the ecological quality classes (EQC). The ecological status assessed as high corresponds to class I, good corresponds to class II, moderate corresponds to class III, poor corresponds to class IV and bad corresponds to class V. The EQC obtained for selected types of watercourses were taken into account in order to compare them with the values of the biological indices used for the assessment of water quality according to the national legislation.

Data analyses

All metrics calculations, based on macroinvertebrate taxa lists, were performed using ASTERICS 4.04 software package [30]. This software is commonly used in similar studies [22,31] as a tool for assessing ecological quality in European streams with benthic macroinvertebrates.

The obtained metric values were tested for normality by the Kolmogorov-Smirnov test. Since the variables lacked normality of distribution, nonparametric tests were applied. Spearman's nonparametric correlation test ($p < 0.05$) was used to assess the relationship between the EQC and EPT index, No. of taxa, SI, BMWP score, H' and No. of sensitive taxa and Tubificinae % for three types of watercourse. Relationship between EQC and biological indices was analyzed using principal component analysis (PCA). PCA was done for all ecological indices with respect to EQC aiming to understand the ordination. IBM SPSS Statistics for Windows Software (Version 22.0; IBM Corp, Armonk, NY, USA) was used for the data processing.

3. Results

The ecological characteristics of the community recorded at the 44 studied sites correspond to the communities typical of hilly and mountainous watercourses. Insects were the most dominant group in the community. Mayflies, stoneflies and caddisflies were recorded at all sampling sites as an important component of the community. Together they represented 28 families and 95 taxa (8/30 mayflies, 6/18 stoneflies, 14/47 caddisflies families/taxa). Species *Taeniopteryx nebulosa* (Linnaeus, 1758), *Baetis (Baetis) pavidus* Grandi, 1949 and *Epeorus (Ironopsis) yougoslavicus* (Samal, 1935) classified as strictly protected according national legislation were recorded) [32]. The number of recorded representatives of the EPT group and their presence or absence in the three types of analyzed water bodies is shown in the Table 2 below.

Table 2. List of recorded EPT taxa in three types of watercourses [18].

No.	Order	Family	Taxon	Type 3	Type 4	Type 6
1	Ephemeroptera	Baetidae	<i>Baetis (Acentrella) sp.</i>	x		x
2			<i>Baetis (Baetis) alpinus</i> (Pictet, 1843)	x	x	x
3			<i>Baetis (Baetis) fuscatus</i> (Linnaeus, 1761)	x	x	x
4			<i>Baetis (Nigrobaetis) muticus</i> (Linnaeus, 1758)	x	x	x
5			<i>Baetis (Baetis) lutheri</i> Müller-Liebenau, 1967	x		x
6			<i>Baetis (Baetis) meridionalis</i> Ikononov, 1954	x		
7			<i>Baetis (Rhodobaetis) rhodani</i> (Pictet, 1843)	x	x	x
8			<i>Baetis (Baetis) pavidus</i> Grandi, 1949*		x	x
9			<i>Baetis (Baetis) scambus</i> Eaton, 1870			x
10			<i>Baetis (Baetis) vernus</i> Curtis, 1834	x		
11			<i>Baetis sp.</i>	x	x	x
12			<i>Cloeon (Cloeon) dipterum</i> (Linnaeus, 1761)	x	x	
13		Ephemeridae	<i>Ephemera (Ephemera) danica</i> Müller, 1764	x	x	x
14		Heptageniidae	<i>Ecdyonurus (Helvetoraeticus) subalpinus</i> Klapalek, 1907.		x	
15			<i>Ecdyonurus (Ecdyonurus) aurantiacus</i> (Burmeister, 1839)		x	
16			<i>Ecdyonurus (Helvetoraeticus) helveticus</i> Eaton, 1883.		x	
17			<i>Epeorus (Epeorus) sylvicola</i> (A.E Pictet, 1865)	x	x	x
18			<i>Epeorus (Ironopsis) yougoslavicus</i> (Samal, 1935)*	x	x	x
19			<i>Ecdyonurus (Ecdyonurus) venosus</i> (Fabricius, 1775)	x	x	x
20			<i>Ecdyonurus sp.</i>			

21		<i>Heptagenia</i> (<i>Heptagenia</i>) <i>sulphurea</i> (Müller, 1776)	x	x	x
22		<i>Rhithrogena</i> gr. <i>semicolorata</i> (Curtis, 1834)	x	x	x
23	Caenidae	<i>Caenis macrura</i> Stephens, 1835	x		
24		<i>Caenis luctuosa</i> (Burmeister, 1839)	x	x	x
25	Potamanthidae	<i>Potamanthus luteus</i> (Linnaeus, 1767)	x		
26	Oligoneuriidae	<i>Oligoneuriella rhenana</i> (Imhoff, 1852)	x	x	x
27	Leptophlebiidae	<i>Habrophlebia fusca</i> (Curtis, 1834)		x	x
28		<i>Paraleptophlebia submarginata</i> (Stephens, 1835)	x	x	x
29	Ephemerellidae	<i>Ephemerella ignita</i> (Poda, 1761)	x	x	x
30		<i>Torleya major</i> (Klapálek, 1905)	x	x	x
31	Plecoptera	Chloroperlidae			
32		<i>Chloroperla</i> sp.			x
33		<i>Siphonoperla torrentium</i> (Pictet, 1841)			x
34	Leuctridae	<i>Leuctra</i> gr. <i>hippopus</i> Kempny, 1899	x	x	x
35		<i>Leuctra fusca</i> (Linnaeus, 1758)		x	
36	Nemouridae	<i>Amphinemura sulcicollis</i> (Stephens, 1836)		x	x
37		<i>Nemoura</i> sp.	x	x	x
38		<i>Protonemura montana</i> Kimmins, 1941		x	x
39		<i>Protonemura praecox</i> (Morton, 1894)		x	
40		<i>Protonemura</i> sp.			x
41	Perlidae	<i>Perla marginata</i> (Panzer, 1799)	x	x	x
42		<i>Dinocras megacephala</i> (Klapálek, 1907)	x	x	x
43		<i>Dinocras</i> sp.		x	
44	Perlodidae	<i>Isoperla gramatica</i> (Poda, 1761)	x	x	x
45		<i>Isoperla obscura</i> (Zetterstedt, 1840)			x
46		<i>Isogenus nubecula</i> Newman, 1833			x
47		<i>Perlodes microcephalus</i> (Pictet, 1833)			x
48	Taeniopterygidae	<i>Taeniopteryx nebulosa</i> (Linnaeus, 1758)*	x	x	x
49		<i>Rhabdiopteryx acuminata</i> Klapálek, 1905.		x	x
50	Trichoptera	Rhyacophilidae			
51		<i>Rhyacophila pubescens</i> Pictet 1834			x
52		<i>Rhyacophila fasciata</i> Hagen, 1859	x		x
53		<i>Rhyacophila dorsalis</i> (Curtis, 1834)	x		x
54		<i>Rhyacophila tristis</i> Pictet, 1834	x	x	x
55		<i>Rhyacophila torrentium</i> Pictet, 1834			x
56		<i>Rhyacophila oblitterata</i> McLachlan, 1863	x		x
57		<i>Rhyacophila vulgaris</i> Pictet, 1834.		x	
58		<i>Rhyacophila</i> sp.	x		x
59	Glossosomatidae	<i>Glossosoma</i> sp.		x	x
60	Hydroptilidae	<i>Hydroptila occulta</i> (Eaton, 1873)	x		
61	Philopotamidae	<i>Philopotamus montanus</i> (Donovan, 1813)		x	
62	Hydropsychidae	<i>Hydropsyche incognita</i> Pitsch, 1993	x		
63		<i>Hydropsyche instabilis</i> (Curtis, 1834)	x		
64		<i>Hydropsyche fulvipes</i> (Curtis, 1834)	x		x
65		<i>Hydropsyche pellucidula</i> (Curtis, 1834)	x	x	x
66		<i>Hydropsyche tabacari</i> Botosaneanu, 1960	x		
67		<i>Hydropsyche</i> sp.	x	x	x

66		<i>Cheumatopsyche lepida</i> (Pictet, 1834)	x	x	
67	Polycentropodi dae	<i>Plectrocnemia conspersa</i> (Curtis, 1834)	x		x
68		<i>Polycentropus flavomaculatus</i> (Pictet, 1834)		x	
69		<i>Cyrnus trimaculatus</i> (Curtis, 1834)		x	x
70	Psychomyidae	<i>Psychomyia pusilla</i> (Fabricius, 1781)	x	x	
71		<i>Tinodes</i> sp.			x
72	Brachycentridae	<i>Brachycentrus montanus</i> Klapalek, 1892.	x	x	x
73		<i>Brachycentrus subnubilus</i> Curtis, 1834	x		
74		<i>Micrasema morosum</i> (McLachlan, 1868)			x
75		<i>Micrasema setiferum</i> (Pictet, 1834)		x	
76	Limnephilidae	<i>Ecclisopteryx madida</i> (McLachlan, 1867)		x	x
77		<i>Halesus digitatus</i> (Schrank, 1781)			x
78		<i>Micropterna lateralis</i> (Stephens, 1837)			x
79		<i>Potamophylax cingulatus</i> (Stephens, 1837)	x	x	x
80		<i>Limnephilus auricula</i> Curtis, 1834	x	x	
81		<i>Limnephilus sparsus</i> Curtis, 1834			x
82		<i>Melampophylax melampus</i> (McLachlan, 1876)		x	x
83		<i>Anabolia furcata</i> Brauer, 1857.	x		
84		<i>Allogamus uncatatus</i> (Brauer, 1857)			x
85		<i>Chaetopteryx villosa</i> (Fabricius, 1798)			x
86		<i>Glyphotaelius pellucidus</i> (Retzius, 1783)		x	
87	Goeridae	<i>Goera pilosa</i> (Fabricius, 1775)		x	x
88		<i>Silo pallipes</i> (Fabricius, 1781)		x	
89		<i>Silo nigricornis</i> (Pictet, 1834)		x	
90	Lepidostomatidae	<i>Lepidostoma hirtum</i> (Fabricius, 1775)	x		
91	Leptoceridae	<i>Athripsodes aterrimus</i> Stephens, 1836	x		
92		<i>Athripsodes cinereus</i> (Curtis, 1834)	x		
93		<i>Adicella</i> sp.		x	
94	Sericostomatidae	<i>Sericostoma personatum</i> (Kirby & Spence, 1826)	x	x	x
95	Odontoceridae	<i>Odontocerum albicorne</i> (Scopoli, 1763)		x	x

x- taxa recorded at certain type of watercourses; *strictly protected species according to Official Gazette [32].

In type 3 watercourses 194 taxa of benthic macroinvertebrates were identified. Insects were the principal components of the macroinvertebrate communities with 142 taxa. The EPT groups (53 taxa) were one of the main components of macroinvertebrate communities. A significant number of recorded taxa belongs to orders Ephemeroptera (23.8% of the total community, 23 taxa), Plecoptera (1.03%, 6 taxa) and Trichoptera (7.21%, 24 taxa) (Figure 2).

In type 4 watercourses a total of 136 taxa were identified in the macroinvertebrate community at the analyzed sites. In terms of taxa richness and number of individuals, insects were the dominant group in the community (78.16%, 121 taxa). Of the identified insects, almost half of the community (57 identified taxa) belonged to the target group (EPT) organisms in terms of taxa richness and number of individuals with 89.64%/22 taxa, 7.21%/12 taxa and 3.14%/19 taxa, respectively (Figure 2).

Of the 14 sites analyzed that belonged to a type 6 watercourse, 159 benthic macroinvertebrate taxa were identified. In terms of the number of taxa, the most diverse group was the Insecta (132 taxa). The EPT groups represented an important component of the community at the sites surveyed and were represented by a total of 63 taxa. Regarding the percentage of these three taxonomic groups, Ephemeroptera were the most represented with 22.12% of the total macroinvertebrate community

recorded, while Plecoptera and Trichoptera were represented with 7.61% and 5.4% of the total number of taxa recorded, respectively. Ephemeroptera with 21 taxa and Plecoptera with 15 taxa recorded, had a high diversity in the total community. Trichoptera (27 taxa) had the highest number of taxa in the total community (Figure 2).

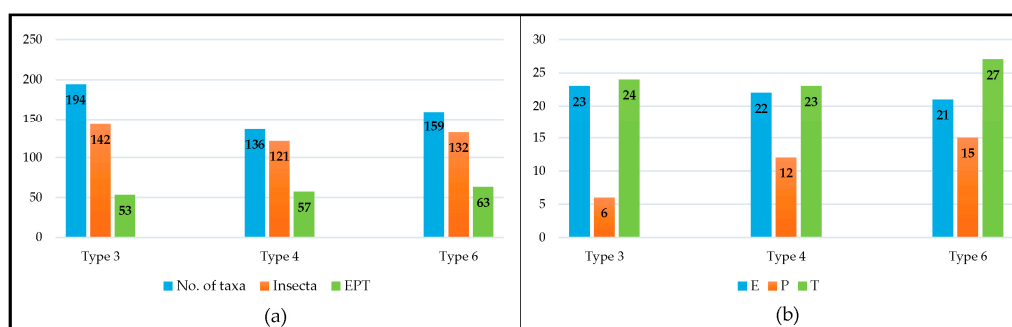


Figure 2. (a) Total number of taxa, number of Insecta taxa, and number of EPT taxa at three types of watercourses; (b) Number of taxa of EPT orders, Ephemeroptera (E), Plecoptera (P), Trichoptera (T) at three types of watercourses.

Differences in the distribution of target groups of insects in analyzed types of watercourses were evident. The structure of the Ephemeroptera community in respect to three different types of watercourses is shown in Figure 3. The family Baetidae was present with the largest number of species (12) and the species *Baetis rhodani* (Pictet, 1843) was the representative of the Ephemeroptera group with a significant abundance in all three types of studied water bodies. The species *Ecdyonurus (Helvetoraeticus) subalpinus* Klapalek, 1907 was only found in type 4, but it was the most abundant species of the Ephemeroptera group, along with *Baetis (Baetis) alpinus* (Pictet, 1843).

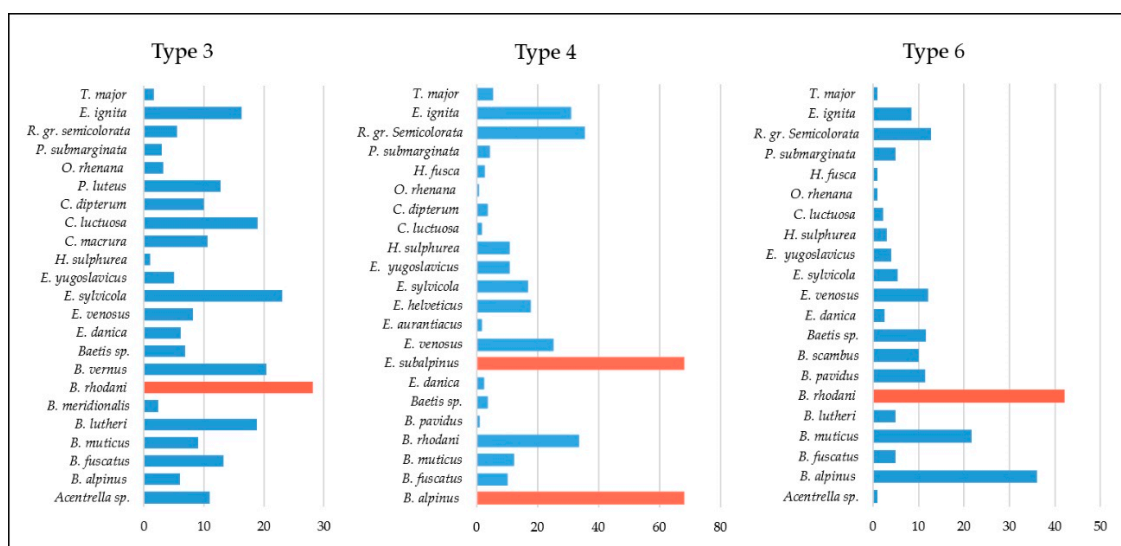


Figure 3. Average abundance of Ephemeroptera taxa at three types of watercourses (individuals/m²).

The species *Leuctra gr. hippopus* Kempny, 1899 was the representative of the Plecoptera group, and was recorded with a significant abundance in all three types of studied water bodies, with type 3 showing the highest values. In water types 4 and 6, species *Protonemura montana* Kimmins, 1941 and *Siphonoperla torrentium* (Pictet, 1841), which favor watercourses at higher altitudes, with domination of larger substrate, were the most numerous among the Plecoptera. (Figure 4).

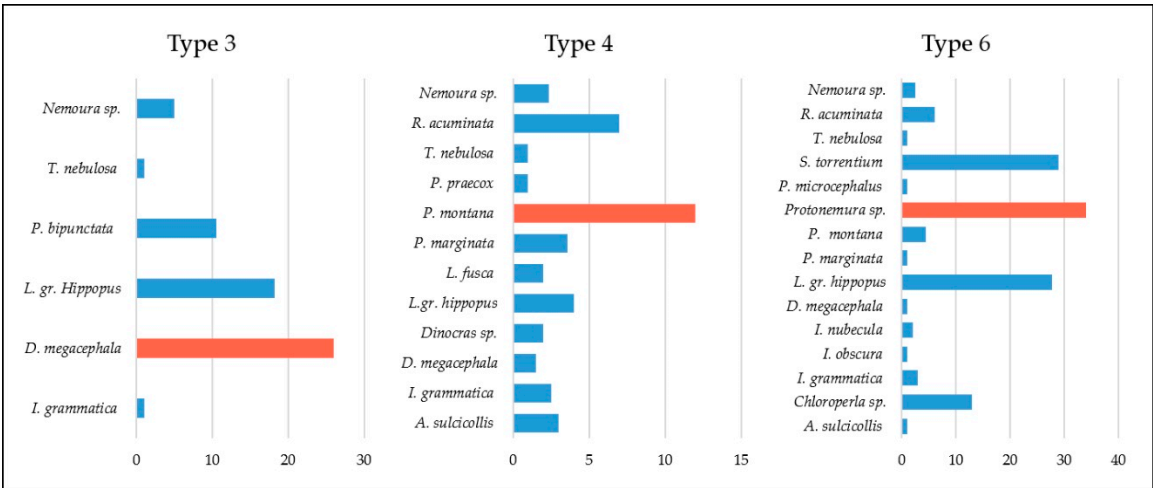


Figure 4. Average abundance of Plecoptera taxa at three types of watercourses (individu-als/m2).

With regard to the participation of the Trichoptera group at the investigated localities, the dominant family was the Limnephilidae with 11 species recorded. Depending on the type of water body, different species were found. *Psychomyia pusilla* (Fabricius, 1781) in type 3, *Micrasema setiferum* (Pictet, 1834) in type 4 and *Chaetopteryx villosa* (Fabricius, 1798) in type 6 were the most abundant species (Figure 5).

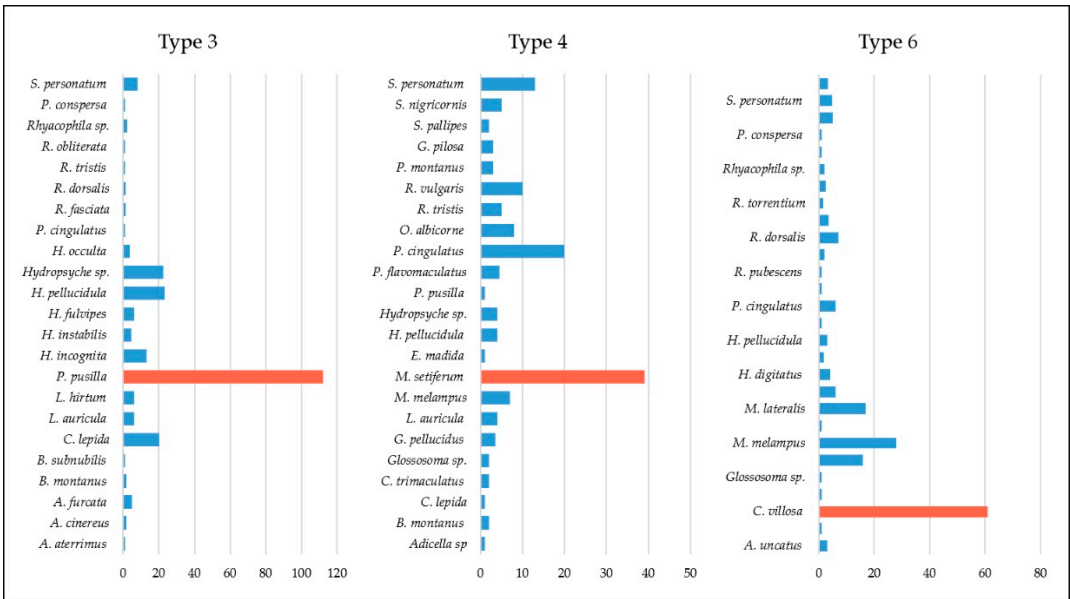


Figure 5. Average abundance of Trichoptera taxa at three types of watercourses (individuals/m²).

Indicative status assessment was carried out according to the procedure based on the class boundaries and in accordance with current legislation [19]. Analyzed localities were classified in the ecological quality classes from I (high ecological status) to V (bad ecological status).

In the type 3 watercourses, most sites have a poor or bad ecological status, mainly due to the low values of EPT index. Good ecological status was achieved on 5 sites, while moderate ecological status was recorded on 6 sites (Table 3).

Table 3. Values of biological indices and assessed ecological quality class for each type 3 locality according to the national legislative of the Republic of Serbia [21].

Metrics/Localities	1	2	3	4	5	6	7	8	9	10	11	12
No. of taxa	28	35	26	12	17	19	40	16	29	17	11	6
	I	I	I	III	II	II	I	II	I	II	III	IV

SI	2.389	1.769	1.99	2.196	2.589	1.919	1.759	2.433	1.818	2.256	1.947	2.179
	III	II	II	II	III	II	II	III	II	III	II	II
BMWP Score	93	88	76	49	30	75	136	41	113	42	50	20
	I	II	II	IV	IV	II	I	IV	I	IV	III	V
ASPT	5.812	5.867	6.333	6.125	4.286	6.25	6.182	4.1	6.278	5.25	5.556	5
	II	II	II	II	III	II	II	III	II	II	II	II
H'	2.211	1.819	1.791	1.441	2.369	1.829	2.002	1.046	1.059	2.428	2.159	0.703
	I	II	II	III	I	II	II	IV	IV	I	II	IV
EPT index	10	11	10	3	2	9	17	1	12	4	5	2
	III	III	III	V	V	III	I	V	II	IV	IV	V
No. of Families	19	20	15	11	11	13	32	13	21	9	9	4
	I	I	I	II	II	I	I	I	I	III	III	IV
Tubificinae %	2.78	0.00	0.34	2.13	0.00	0.00	0.14	1.65	0.00	4.27	0.00	0.00
	*	*	*	*	*	*	*	*	*	*	*	*
EQC	III	III	III	IV	V	III	II	V	IV	IV	IV	V

Metrics/Localities	13	14	15	16	17	18	19	20	21	22	23	24
No. of taxa	15	16	51	19	32	30	28	19	26	5	9	20
	II	II	I	II	I	I	I	II	I	IV	IV	I
SI	1.949	1.97	2.198	2.078	1.096	1.939	1.98	2.213	1.956	2.98	2.705	2.493
	II	II	II	II	I	II	II	III	II	IV	III	III
BMWP Score	32	36	128	33	121	138	87	82	122	21	11	31
	IV	IV	I	IV	I	I	II	II	I	V	V	IV
ASPT	4	6	5.333	4.714	6.722	6.9	6.214	6.308	7.625	5.25	2.75	3.875
	III	II	II	III	II	II	II	II	I	II	V	IV
H'	2.46	2.473	3.103	2.328	2.627	2.943	2.506	1.74	2.42	1.427	1.665	2.634
	I	I	I	I	I	I	I	II	I	III	II	I
EPT index	3	3	11	6	20	13	14	8	15	2	1	3
	V	V	III	IV	I	II	II	III	II	V	V	V
No. of Families	9	7	29	13	22	22	19	17	21	5	6	11
	III	III	I	I	I	I	I	I	I	III	III	II
Tubificinae %	2.13	0.00	1.47	5.11	0.00	0.00	3.35	0.41	0.00	44.44	23.91	5.88
	*	*	*	/	*	*	*	*	*	/	/	/
EQC	V	V	III	IV	II	II	II	III	II	V	V	V

* Good status; / Good was achieved

In type 4 watercourses, good ecological status was achieved at three analyzed sites. One site was characterized by poor ecological status, while moderate ecological status was recorded at the other two sites (Table 4).

Table 4. Values of biological indices and assessed ecological quality class for each type 4 locality according to the national legislative of the Republic of Serbia [21].

Metrics/Localities	1	2	3	4	5	6
No. of Taxa	39	22	45	44	26	26
	I	I	I	I	I	I
SI	1.409	1.701	2.177	1.413	1.396	2.019
	I	II	III	I	I	II
BMWP Score	151	106	130	184	118	70
	I	I	I	I	I	II
ASPT	7.55	7.067	6.842	6.815	6.556	7.778
	I	I	II	II	II	I

H'	2.127	1.402	2.715	2.697	1.861	2.585
	II	III	I	I	II	I
No. of sensitive taxa	13	8	13	13	6	3
	I	I	I	I	I	III
EPT index	23	17	19	27	15	9
	I	II	I	I	II	IV
Tubificinae %	0.00	0.00	2.59	0.00	0.00	0.00
	*	*	*	*	*	*
EQC	II	III	III	II	II	IV

* Good status; / Good status was achieved

Based on the values obtained, it can be concluded that, overall, good ecological status was achieved in the studied type 6 watercourses, based on most of the parameters analyzed. A moderate ecological status was found at four localities (Table 5).

Table 5. Values of biological indices and assessed ecological quality class for each type 6 locality according to the national legislative of the Republic of Serbia [21].

Metrics/Localities	1	2	3	4	5	6	7	8	9	10	11	12	13	14
No. of Taxa	34	26	19	28	27	23	26	38	48	20	7	4	16	32
	I	I	I	I	I	I	I	I	I	I	I	II	I	I
SI	1.391	1.784	2.084	1.898	1.717	2.05	1.686	1.444	1.657	1.257	1.967	1.686	2.116	1.828
	I	II	III	II	II	III	II	I	II	I	II	II	III	II
EPT index	16	13	8	12	12	7	11	23	21	9	5	2	4	13
	*	*	*	*	*	*	*	*	*	*	*	*	*	*
No. of sensitive taxa	11	5	3	7	9	2	8	8	10	6	3	1	2	4
	*	*	*	*	*	*	*	*	*	*	*	/	*	*
Tubificidnae %	0.00	0.00	0.00	0.00	0.00	0.17	2.27	0.00	0.00	0.00	0.00	0.00	4.57	0.00
	*	*	*	*	*	*	*	*	*	*	*	*	*	*
EQC	II	II	III	II	II	III	II	II	II	II	II	III	III	II

* Good status; / Good status was achieved

Results of Spearman correlation test ($p<0.05$), showed correlation of EQC with all biological indices (Table 6). It showed negative correlation with BMWP score, H', No. of taxa, EPT index and No. of sensitive taxa, while positive correlation was observed for the values of SI and Tubificinae %. Results showed strong negative correlation of EQC with EPT index, BMWP score and No. of sensitive taxa, while other obtained correlations are of medium strength.

Table 6. Statistically significant values of Spearman correlation coefficient among values of EQC and SI, BMWP score, H', No. of taxa, Tubificinae %, EPT index, and No. of sensitive taxa in analyzed watercourses.

Metrics	EQC
SI	0.6818
BMWP score	-0.7356
H'	-0.5239
No. of taxa	-0.6879
Tubificinae %	0.4379
EPT Index	-0.8165
No. sensitive taxa	-0.7625

Two principal components were extracted from the biplot and accounted for 77.14% of the total variation in the dataset (Figure 6). The first principle component (PC1) accounted for 60.51% of the variability, with EPT index, BMWP score and No. of sensitive taxa as the parameters that contributed most to the separation (Figure 6, Table 7). The second PC (PC2) explained 16.63% of the total variance, with SI, No. of taxa and Tubificinae % as the parameters that contributed most to the separation (Figure 6, Table 7).

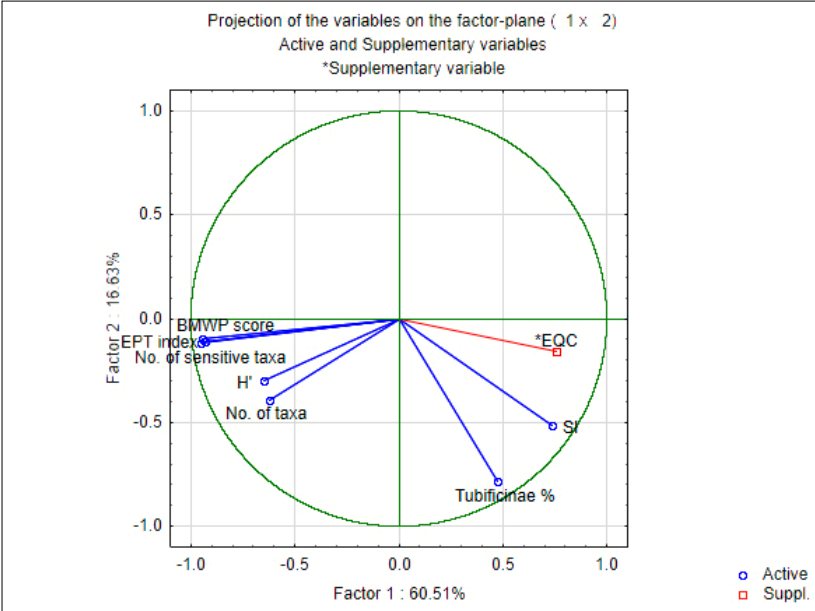


Figure 6. Principal Component Analysis (PCA) – projection of the relative contribution of the biological indices used in the assessment of ecological status and obtained EQC.

Table 7. Loadings of the variables on the principal components (PC). The parameters that contributed most to the separation are marked in bold.

Metrics	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
SI	0.12873	0.22732	0.00423	0.11183	0.49766	0.01228	0.01795
BMWP score	0.21024	0.00821	0.00007	0.06461	0.12799	0.14680	0.44208
H'	0.09873	0.07702	0.53114	0.19859	0.09095	0.00101	0.00257
No. of taxa	0.09158	0.13495	0.46399	0.23881	0.06641	0.00108	0.00317
Tubificinae %	0.05256	0.52954	0.00047	0.29940	0.10380	0.00847	0.00575
EPT Index	0.21460	0.01177	0.00003	0.07411	0.00810	0.16843	0.52297
No. of sensitive taxa	0.20356	0.01118	0.00008	0.01264	0.10510	0.66194	0.00551

4. Discussion

The ecological characteristics of the recorded community at the analyzed sites correspond to the communities usually found in the hilly and mountainous rivers [18,19,33]. The EPT larvae is generally prevalent in the upper reaches of rivers and the assessment of water quality based on these three insect orders is sufficiently accurate [34,35]. According to Pastuchova [36], the composition and distribution of the three orders is determined by their physiological tolerance to a wide range of environmental variables.

The largest number of taxa was found in the Trichoptera group in all three types of analyzed water bodies. Individual taxa within the Trichoptera group, which are numerous, are typical representatives of a particular water body type in which they occur in large numbers. Trichoptera are a very important component of aquatic ecosystems. Many Trichoptera species are sensitive to pollution, so their presence and relative abundance are used for biological assessment and monitoring of water quality [37].

They are followed by the Ephemeroptera group, which has slightly fewer taxa at the analyzed sites, but more abundant occurrence. A similar structure of the Ephemeroptera community can be observed in the 4 and 6 watercourse types. It's one of the most abundant groups of aquatic macroinvertebrates in all types of freshwater habitats, but its higher species diversity is characteristic of lotic habitats, especially the upper reaches of fast-flowing streams and rivers [38,39].

It can be seen that the Plecoptera community is more diverse and abundant in sites with a higher altitude. It's significant component of running water ecosystems. The most members from this order are known to be intolerant to variation in their environmental conditions [40,41]. The stonefly sensitivity to variation in abiotic factors may lead to the extinction of taxa. In this regard, the Plecoptera is one of the most endangered groups of aquatic insects [42].

Species *Taeniopteryx nebulosa* (Linnaeus, 1758), *Baetis* (*Baetis*) *pavidus* Grandi, 1949 and *Epeorus* (*Ironopsis*) *yugoslavicus* (Samal, 1935) classified as strictly protected according national legislation were important from conservational point of view [32]. These stenovalent species are considered endangered in the lotic habitats of Serbia due to the small number of populations and the relatively distant and isolated biotopes, especially with regard to the parameters of temperature, oxygen and water velocity.

The biological metrics analyzed in our research (No. of taxa, SI, BMWP score, H' index, No. of sensitive taxa, Tubificinae %) are known to be some of the most important indicators of water quality [31,43,44]. All these indices are a measure of the occurrence of taxa that are considered bioindicators of water quality. They vary considerably with the specific type of stressor, which means that they describe changing environmental conditions very well [45].

Shannon-Wiener - H' index measuring the diversity of species in a community, which takes into account both abundance and evenness, is used to characterize species diversity. Values may vary directly with water quality and low diversity may indicate an unstable community [29]. Results showed medium strength correlation H' index with EQC (Table 6).

On the other hand, results showed strong negative correlation of EQC with BMWP score and EPT index. The BMWP is the sum of individual values of all families present in the sample, multiplied by their relative abundance, which are actually indicators of the sensitivity of the taxa to organic pollution. This study confirms the importance of taking into account the family level in ecological assessments and biomonitoring programs development. Indices based on the genus and species taxonomic levels, such as the EPT index among others, are needed to improve the understanding of responses on the family level and the detection of specific pollution [46]. As the EPT group is included in the calculation of the BMWP score, since they have a low tolerance to pollution, many families from these three insect groups are assigned high scores. Therefore, it was expected that the values of the BMWP score would not deviate much from the values of the EPT index [47].

Furthermore, the results showed strong negative correlation of EQC with number of sensitive taxa. The number of sensitive taxa is based on the concept of the presence or absence of indicator taxa at the sampling site. Most indicator taxa belong to the EPT group as they tend to be very sensitive to different forms of pollution [8]. The list was included as part of the Fauna Aquatica Austriaca in 2004 [48]. Taxa that are considered sensitive have a narrow range of environmental requirements (e.g. stenotopic, stenoeceous) and react intolerantly to environmental disturbances. The inventory contains taxa with a wide range of sensitivity to physical, chemical and hydromorphological degradation.

The PCA also confirms previous results, given that biological indices that contributed the most to the separation on the PC1 axis were EPT index, BMWP score and No. of sensitive taxa (Figure 6, Table 7).

On the other hand, the values of the metrics SI and the percentage participation of the subfamily Tubificinae (Oligochaeta) in the macroinvertebrate community, whose higher values reflect a higher degree of pollution, were accompanied by a decrease in the values of the EPT index at most sampling sites. The values of the SI and Tubificinae indices showed statistically positive correlation with the values of the EQC (Table 6). The results of PCA analysis are in accordance with the previous

mentioned, given that the parameters that contributed the most to the separation on the PC2 axis were No. of taxa and Tubificinae % (Figure 6, Table 7).

The Saprobic Index (SI) is one of the most traditional biological metrics commonly used to assess water quality. It focuses on the tolerance of species to organic pollution and is measured by a combination of the biological oxidation demand of a water sample and the presence of certain indicator organisms in the habitat. High values in the SI indicate a high level of organic pollution and a moderate to poor ecological status [27].

The oligochaete group comprises of a large number of species that cover a broad spectrum of pollution sensitivity. Oligochaetes are usually the most dominant taxa in fine/sandy freshwater sediments. Analyzing the percentage of Tubificinae gives an indication of sediment contamination. The presence of the subfamily Tubificinae (Oligochaeta) indicates poor water quality of these sites, but also the presence of a habitat suitable for these organisms (silt, clay mud and sand) [43].

Pollution by organic matter and nutrients, as well as hydromorphological degradation, as the main factors affecting aquatic ecosystems, in Serbia are most pronounced in the lowland regions. Population density, agricultural activities and industry in the country are mainly located in the lowlands [49,50]. As the benthic community changes under the various pressure, the abundance of populations of sensitive taxa decreases and the abundance of tolerant species increases [51]. Percentage participation of subfamily Tubificinae and values of and SI describe a change of environmental conditions in this type of watercourses very well [43,52].

However, sites where higher values of BMWP score and parameters based on the number of taxa (EPT index and H') were expected, indicated poor to bad condition as well, especially at altitudes under 500 m (Table 3 and 5). Therefore, the overall status of these sites has been categorized as poor or bad, taking into account the poorer value of the individual metrics used to assess the overall water quality class [2]. On most investigated sampling sites the value of the EPT index determined the overall EQC. Results confirmed strong negative correlation of EQC with EPT index.

The macroinvertebrate fauna of aquatic ecosystems has changed as a result of various pressures such as organic and chemical pollution, land use and hydromorphological alterations, as well as biological invasions. There is a growing need for cost and time efficient methods that can provide rapid results and assess a wide range of water quality statuses [53,54]. This has led to the use of rapid bioassay protocols [9] and the selection of appropriate biological indices that effectively and adequately reflect the state of the aquatic ecosystem.

The EPT metrics are also included in multimeric indices that have broader applications for assessing the condition of streams, such as the Benthic Macroinvertebrate Index of Biotic Integrity (B-IBI) [55] and the Invertebrate Community Index (ICI) [56]. Richness metrics including that of EPT have a direct connection to biodiversity studies conducted in a wide variety of flowing waters, which is why this index is suitable for use for water assessment outside the country of Serbia as well.

This study has shown that water quality assessments based on the EPT index are more reliable in less polluted watercourses, especially in pristine environments. The EPT index is a relatively accurate and effective tool compared to other indices we have used to detect water disturbance and classify water quality.

5. Conclusions

The representatives of the orders Ephemeroptera, Plecoptera and Trichoptera were recorded at all sampling sites, making more than half of the macroinvertebrate community at investigated hilly and mountainous ecosystems in Serbian waters. The results confirmed the sensitivity of the EPT index, represented by the sum of the taxa richness of these three orders, to changes of the macroinvertebrate communities in these types of water ecosystems. Higher values of the EPT index are found in water bodies with no or low pollution, while lower values of this index indicate an increase in pollution. In addition, these aquatic insects respond to a wide range of potential pollutants and respond to both short-term and long-term conditions that affect water quality.

This study showed strong negative correlation between EQC and EPT index, BMWP score and number of sensitive taxa. Also, results of PCA analysis are in accordance with the obtained results.

As the EPT group is included in the calculation of these two metrics, results showed that the values of the BMWP score and number of sensitive taxa would not deviate much from the values of the EPT index. It can be concluded that the EPT index values effectively and adequately confirm changes in water quality in hilly and mountainous small to medium sized streams with predominantly hard bottom substrates. This index reflects the state of the aquatic ecosystem and provides accurate overall picture of water quality. It proved to be self-sufficient and reliable for water quality assessment in these types of water bodies. In addition, it proved to be an important tool for the prioritization of measures and the revision of the Water Management Plan for the territory of the Republic of Serbia. Furthermore, this index can also serve as a useful tool for early detection of pollution.

The EPT index should not be used to assess watercourses known to have low EPT taxa richness, such as lowland rivers at altitudes under 500 m, especially large rivers with fine substrate (silt, clay mud and sand) or slow flowing/stagnant water bodies (artificial channels and reservoirs - heavily modified water bodies) where the pollution tolerant groups are more significant. Moreover, this index is not suitable for the assessment of urban watercourses which are under higher anthropogenic pressure.

Having the above in mind, the overall status assessment is a complex matter. There is still a need for more intensive studies and further testing of the effectiveness of various indices used to assess water quality of different types of watercourses.

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