CAN EPHEMEROPTERA, PLECOPTERA, TRICHOPTERA (EPT) ASSEMBLAGES REFLECT NITROGEN AND PHOSPHORUS LOAD IN A RIVERINE ECOSYSTEM?

Lidija Kladarić[1*], Renata Ćuk[1], Ivor Dukić[2], Aleksandar Popijač[3] & Andrea Marinović Ruždjak[1]

[1]Hrvatske vode, Central Water Management Laboratory, Ulica Grada Vukovara 220, HR-10000 Zagreb, Croatia

[2]student, Faculty of Science in Split, Ruđera Boškovića 33, HR-21000 Split, Croatia

[3]Kopački rit Nature Park Public Institution, Mali Sakadaš 1, 31327 Kopačevo, Bilje, Croatia

Kladarić, L., Ćuk, R., Dukić, I., Popijač, A. & Marinović Ruždjak, A.: Can Ephemeroptera, Plecoptera, Trichoptera (EPT) assemblage reflect nitrogen and phosphorus load in the riverine ecosystem? Nat. Croat., Vol. 30, No. 1, 217–230, Zagreb, 2021.

The aim of this paper is to make a comparative analysis of benthic macroinvertebrate compositions in streams and rivers in Croatia with relation to different physical-chemical factors, especially nutrients. Samples were collected according to the AQEM method. At all the sites, 20 higher taxonomic categories were recorded of which Turbellaria, Gastropoda, Bivalvia, Oligochaeta, Hirudinea, Crustacea, Ephemeroptera, Odonata, Plecoptera, Heteroptera, Trichoptera, Coleoptera and Diptera were included in the present study. Water temperature mostly affected the composition of benthic macroinvertebrates to which it was inversely proportional. Nutrient enrichment, i.e., higher concentrations of ammonium, nitrates, nitrites, total nitrogen, orthophosphates and total phosphorus mostly affected Ephemeroptera, Plecoptera, Trichoptera and Diptera, by decreasing their diversity.

Keywords: nutrient enrichment, water temperature, watercourse, macrozoobenthos, Croatia

Kladarić, L., Ćuk, R., Dukić, I., Popijač, A. & Marinović Ruždjak, A.: Može li zajednica Ephemeroptera, Plecoptera, Trichoptera (EPT) odraziti opterećenje dušikom i fosforom u riječnom ekosustavu? Nat. Croat., Vol. 30, No. 1, 217–230, Zagreb, 2021.

Cilj ovog rada bio je izvršiti usporednu analizu sastava bentoskih makrobeskralježnjaka u potocima i rijekama u Hrvatskoj u odnosu na različite fizikalno-kemijske čimbenike, posebno hranjive tvari. Uzorci su prikupljeni prema AQEM metodi. Na svim nalazištima zabilježeno je 20 viših taksonomskih kategorija od kojih su u ovo istraživanje uključeni: Turbellaria, Gastropoda, Bivalvia, Oligochaeta, Hirudinea, Crustacea, Ephemeroptera, Odonata, Plecoptera, Heteroptera, Trichoptera, Coleoptera i Diptera. Temperatura vode je najviše utjecala na sastav makrozoobentosa. Što je temperatura vode viša, raznolikost svojti makrozoobentosa je niža. Obogaćivanje hranjivim tvarima, odnosno više koncentracije amonijaka, nitrata, nitrita, ukupnog dušika, ortofosfata i ukupnog fosfora, najviše je utjecalo na Ephemeroptera, Plecoptera, Trichoptera i Diptera, smanjujući njihovu raznolikost.

Ključne riječi: hranjive tvari, temperatura vode, tekućice, vodeni beskralježnjaci, Hrvatska

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[*] Author for correspondence (lidija.kladaric@voda.hr)

INTRODUCTION

Freshwaters are among the most threatened ecosystems globally (Vörösmarty *et al.*, 2010).

A wide range of human-generated stressors that degrade water quality and hydro-morphology affect life in European rivers. One of the main stressors on river quality is nutrient enrichment, which is the reason for 34.4% deviance in ecological status, hydro-morphological degradation causes 39.4% deviance (morphology: 23.2% and hydrology: 16.2%), whereas the rest, 26.2% of deterioration, is a result of toxic substances (Lemm *et al.*, 2020). The eutrophication and organic pollution of freshwater ecosystems is considered a pan-European problem "of major concern" (Dahl & Johnson, 2004; EEA, 2018).

Agriculture contributes most to nitrogen and phosphorus pollution (Fierro *et al.*, 2017). Thanks to the EU Nitrates Directive and national measures, nitrogen pollution from agriculture has been reduced in some regions over the last 15–20 years. The reduced pressure is reflected in lower river nitrate concentrations. However, the apparent stabilisation of river nitrate concentrations in recent years may call for further measures to be taken (EEA, 2021).

The average concentration of phosphates in European rivers decreased twice over the period 1992–2012. The decrease in river phosphate concentration can be related to the measures introduced by national and European legislation, in particular the Urban Waste Water Treatment Directive, which involves the removal of nutrients. Also, the change to phosphate-free detergents has contributed to the lower phosphorus concentrations (EEA, 2021).

Aquatic macrophyte and algae quantities are good indicators of nutrient enrichment (Conley *et al.*, 2009), as they require several macronutrients for growth (especially nitrogen and phosphorus), and freshwater macroinvertebrates provide good evidence of response to their fluctuations (Rader & Richardson, 1992; Gafner & Robinson, 2007; Ouyang *et al.*, 2018; Everall *et al.*, 2019;).

Anthropogenic pressure, especially nutrient enrichment, has many negative impacts on freshwater organisms (Allan, 2004; Moore & Palmer, 2005; Riseng *et al.*, 2011; Cui *et al.*, 2017). It has been noticed that nutrient enrichment influences aquatic invertebrates by decreasing their richness (Paul & Meyer, 2001), particularly eliminating the sensitive taxa such as Ephemeroptera, Plecoptera and Trichoptera (EPT) (Lenat, 1988).

Although there are increasing numbers of studies on the effects of nutrient enrichment on biota (Alcaraz *et al.*, 1999; Berenzen *et al.*, 2001; Lawrence & Gresens, 2004; Matthaei *et al.*, 2010; Prater *et al.*, 2015), comparative studies among different macroinvertebrate taxa are scarce.

The object of this study was to present the response of EPT assemblages (i.e., EPT species richness) to nitrate, nitrite, ammonium and orthophosphate concentrations within 71 Croatian streams and rivers. The questions addressed in the study were: (1) which physical-chemical parameters have a significant effect on the EPT assemblage and (2) whether nutrient enrichment has the strongest impact on the EPT assemblage.

MATERIAL AND METHODS

Study Area

The Republic of Croatia lies at the intersection of two ecoregions: Dinaric Western Balkan (ER5) and Hungarian Lowlands (ER11) according to Illies (1978). Considering the geographical location of Croatia and its position within Central Europe and the Mediterranean, as well as climatic and geological conditions, is the country can be divided into the Pannonian and karst parts. Sava, Drava and Danube rivers flow through the lowland Pannonian part. Aquifers of the lowland rivers consist of thick layers of deposited gravel. The karst area is determined by special karst hydro-morphological features formed due to the solubility of the carbonate rocks that make up the terrain. Karst covers approximately half of the total area of the country (Šafarek & Šolić, 2011).

Sampling and laboratory work

Biological data were obtained as a part of the monitoring and assessment programme conducted by the Central Water Management Laboratory of Hrvatske vode. The samples of benthic macroinvertebrates were taken over a period of 3 years (2015–2017).

The sampling was conducted in the period of low and stable water levels, mostly in the warm period of the year (spring and summer). In total, 114 samples from 106 sampling stations in 71 Croatian streams and rivers were collected and analysed (Fig. 1).
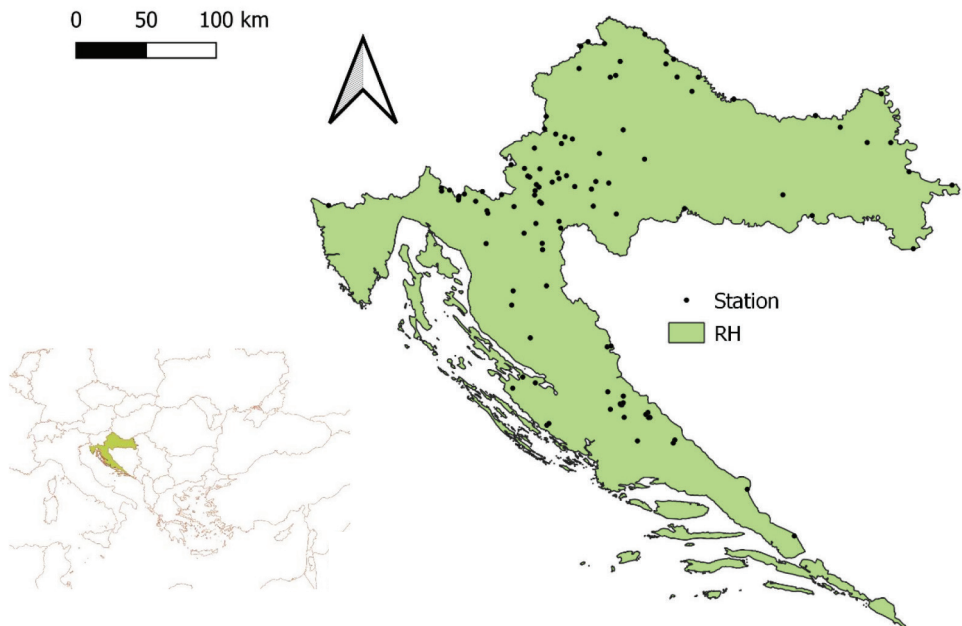

Fig. 1. Map of Croatia with sampling stations (106 sampling stations in 71 Croatian streams and rivers)

Samples were collected with a hand net (25 × 25 cm aperture and mesh size 500 µm) according to the AQEM sampling procedure (AQEM, 2002) in a littoral zone of rivers where 20 quantitative replicative samples were collected (0.0625 m²) from all microhabitats represented by more than 5%. Endangered or protected species were identified in the field and immediately released back into the stream. Macroinvertebrates and associated organic material were immediately placed in plastic bottles and preserved in 80% ethanol.

Isolation and determination of benthic macroinvertebrates were conducted in the laboratory with the use of a binocular stereomicroscope (Olympus SZX9 and SZX10). The organisms were determined to the lowest possible taxonomic level, usually to species or genus level (with the exception of chironomids and oligochaetes) and separated into the higher taxonomic categories. The result was a list of taxa comprising the number of individuals found in each sample (Official Gazette, 2020; Hrvatske vode, 2015). At the end, the abundance of the macroinvertebrates was calculated as number of individuals per m².

In order to perform statistical analysis, higher taxonomic categories represented with a single taxon were excluded from further analyses (Coelenterata, Nematoda, Polychaeta, Araneae, Megaloptera, Lepidoptera and Hydrachnidia).

Standard determination keys were used (Kerovec & Čičin-Šain, 1986; Campaioli *et al.*, 1994; Schwab, 1995; Nilsson, 1996; Waringer & Graf, 1997; Grabow, 2000; Bauernfeind & Humpesch, 2001; Zwick, 2004; Engelhardt *et al.*, 2008). All specimens have been deposited in the macrozoobenthos collection in the Central Water Management Laboratory of Hrvatske vode.

Physical and chemical parameters

Sampling, preservation and chemical analyses of the water samples were performed according to standard methods for the examination of surface waters (ISO Standards, APHA 1998). Water samples were collected in appropriate containers, kept in the dark at 4°C and analysed within 24h. Selected water quality parameters included water temperature (WT), pH, electrical conductivity (EC), total suspended solids (TSS), m-alkalinity, total water hardness (dH), and concentrations of ammonium, nitrite, nitrate, total nitrogen (TN), orthophosphate (OP) and total phosphorus (TP) (Tab. 1).

WT was measured on-site while pH and EC were measured either on-site (portable pH and conductivity meters, HANNA instruments) or in the laboratory (Mettler Toledo SevenMulti dual pH and conductivity meter). Water samples for the analysis of ammonium, nitrite, nitrate and OP concentrations were field filtered through 0.45 µm cellulose nitrate membrane filter.

Inorganic nitrogen was obtained as the sum of the nitrite, nitrate, and ammonium concentrations while organic nitrogen was calculated by subtracting inorganic nitrogen from the obtained results of TN. TN measurements were performed on a Shimadzu TOC-V/TNM-1.

The ammonium molybdate spectrophotometric method (on Perkin Elmer Lambda 25) was applied for the determination of OP. The TP was determined using acid digestion followed by the molybdate method. Since 2016, TP analyses have been performed on an Agilent 8900 ICP-MS.

Tab. 1. Descriptive statistics of physical-chemical parameters and nutrients

Variable		n	Mean	Min	Max
Physical-chemical parameters	WT (°C)	114	12.34	7.48	24.42
	pH	114	7.99	7.23	8.46
	EC (µS/cm)	114	509.46	78.64	6199.17
	TSS (mg/L)	114	9.18	0.76	53.42
	Alkalinity (mgCaCO3/L)	114	193.89	30.64	387.23
	dH (mgCaCO3/L)	114	244.85	31.53	1368.60
Nutrients	Ammonium (mgN/L)	114	0.11	0.00	1.78
	Nitrite (mgN/L)	114	0.01	0.00	0.08
	Nitrate (mgN/L)	109	0.82	0.05	3.43
	TN (mgN/L)	114	1.91	0.28	60.06
	OP (mgP/L)	114	0.02	0.00	0.34
	TP (mgP/L)	87	0.08	0.00	0.53

Data analysis

The number of taxa of higher taxonomic categories found in samples was calculated with the software ASTERICS version 1.0. (AQEM/STAR Ecological River Classification System) for each sampling site. This metric was selected in order to assess the effect of nutrients (ammonium, nitrite, nitrate, TN, OP and TP) on macrozoobenthos diversity.

Box-plots were used to compare the number of taxa among sampling stations, streams and rivers. A correlation analysis was performed to examine the relationship between the number of taxa and physical-chemical parameters, including nutrients. Spearman correlation coefficients were calculated for this purpose. The significance of the Spearman rank-order correlation coefficients was assessed using a two-sided test. Levels of significance were set at $p<0.01$ and $p<0.05$. Finally, principal component analysis was used to reduce the dimensionality of the data and to summarise the variation in the number of taxa and relate it to the environmental variables.

Statistical analysis of the data was carried out using IBM SPSS Statistics Version 24 (IBM Corp., Armonk, NY, USA) and Statistica 12 (StatSoft, Inc., Tulsa, OK, USA). All ordinations were provided by the program CANOCO 5.0 (TER BRAAK & SMILAUER, 2012).

A principal component analysis (PCA) was conducted on log (x+1) transformed environmental data (except pH). A detrended correspondence analysis (DCA) was performed to determine the gradient length. The choice of PCA was justified, since response data had a gradient 1.8 SD units long. According to the Canoco Advisor expert system, the response variables (number of taxa within the investigated macrozoobenthos higher taxonomic categories) were not transformed. However, given the fact that different taxa from various watercourses and ecoregions were analysed, centralization and standardization were performed.

RESULTS

The box-plot diagram suggests that some higher taxonomic categories, like Diptera and Trichoptera are represented by more taxa than other taxonomic categories. There are no extreme variables, but outliers are present (Fig. 2).

Tab. 2 shows the Spearman rank-order correlation coefficients between the number of taxa within individual higher taxonomic categories, and physical-chemical parameters including nutrients. Some taxonomic categories are more strongly correlated with the environmental variables than others. The results imply that higher nutrient concentrations result in lower Ephemeroptera, Plecoptera, Trichoptera and Diptera numbers. Thereby, the highest correlation was obtained with the nitrites and OP (Tab. 2). Trichoptera and Diptera are negatively correlated with TP (–0.511 and 0.407, respectively). Some higher correlations were found for ammonium and Plecoptera and Trichoptera (–0.449 and–0.470, respectively) and between EPT and TN (–0.403,–0.447 and–0.468, respectively).

Plecoptera richness was significantly negatively correlated to WT (–0.491). EPT taxa best react to the increase of EC, by reduction of number of species. The results presented in Table 2 also indicate that EPT and Diptera negatively correlated to TSS.

Inorganic nutrients (ammonium, nitrite, nitrate, TN, OP, TP) were positively correlated with richness of some taxonomic categories, e.g., Bivalvia, Gastropoda, Hirudinea, Odonata and Crustacea.
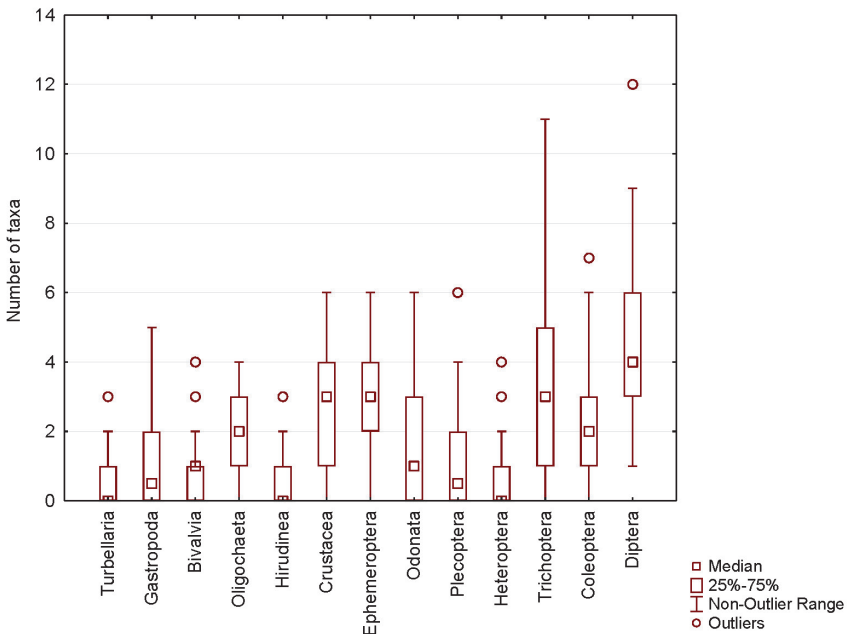

Fig. 2. Box plots of the number of taxa (species, genus) in certain higher taxonomic categories found at 106 sampling stations in 71 streams and rivers

Tab. 2 Spearman's correlation coefficients between the number of taxa in certain higher taxonomic categories and physical-chemical parameters including nutrients

Order	Nutrients						Physical-chemical parameters					
	TP(mg P/L)	OP (mg P/L)	TN (mg N/L)	Nitrate (mg N/L)	Nitrite (mg N/L)	Ammonium (mg N/L)	dH (mg CaCO₃/L)	Alkalinity (mg CaCO3/L)	TSS(mg/L)	EC (µS/cm)	pH	WT(°C)
Turbellaria	–0.163	–0.175	–0.242**	–0.208*	–0.319**	–0.281**	0.078	0.024	–0.235*	0.022	0.075	–0.193*
Gastropoda	0.204	0.173	0.249**	0.266**	0.081	0.201*	–0.155	–0.122	0.120	–0.132	0.134	**0.324****
Bivalvia	0.179	0.232*	0.100	0.154	0.065	0.098	–0.181	–0.201*	0.208*	–0.111	0.166	0.270**
Oligochaeta	0.120	–0.016	0.082	0.044	0.028	0.200*	0.035	0.077	0.024	0.043	0.022	0.099
Hirudinea	0.238*	0.232*	0.141	0.018	0.168	0.241**	0.073	0.066	0.070	0.038	–0.203*	0.023
Crustacea	–0.065	0.056	0.108	0.090	0.092	0.191*	0.046	0.037	–0.025	0.089	–0.155	**0.362****
Ephemeroptera	**–0.306****	**–0.415****	**–0.403****	–0.289**	**–0.568****	**–0.354****	–0.188*	–0.146	**–0.353****	–0.291**	0.122	**–0.324****
Odonata	0.127	0.177	0.211*	0.170	0.230*	**0.373****	0.184*	0.214*	0.121	0.231*	–0.041	**0.551****
Plecoptera	**–0.385****	**–0.490****	**–0.447****	–0.361**	**–0.476****	**–0.449****	–0.186*	–0.119	**–0.443****	–0.288**	0.091	**–0.491****
Heteroptera	0.183	0.132	0.195*	0.245*	0.151	0.100	–0.039	–0.044	0.197*	0.027	0.063	0.365**
Trichoptera	**–0.511****	**–0.547****	**–0.468****	–0.401**	**–0.545****	**–0.470****	–0.134	–0.134	**–0.468****	–0.269**	0.045	**–0.463****
Coleoptera	–0.226*	–0.233*	–0.133	–0.144	–0.096	0.016	0.204*	0.217*	–0.239*	0.109	–0.249**	–0.092
Diptera	**–0.407****	**–0.412****	**–0.396****	–0.389**	**–0.362****	–0.280**	0.098	0.096	**–0.342****	–0.039	–0.139	**–0.370****

** **Correlation** is significant at the 0.01 level

 * **Correlation** is significant at the 0.05 level

Principal components analysis

The first four PCA axes explain 62.55% of the variance in the data on the number of taxa, with the first component explaining 24.72% and the second component 18.31% of the total variance (Tab. 3).

According to PCA, Plecoptera was negatively correlated to WT. Ephemeroptera, Trichoptera and Diptera are negative with TSS, TP, ammonium and nitrate. pH is at least correlated with EPT and Diptera.

The results of the unconstrained ordination (PCA) are presented by a biplot (Fig. 3). The diagram shows the first two PCA axes. In addition to the response variables (number of taxa of higher taxonomic categories (Fig. 2), physical-chemical parameters and nutrients are also presented in the biplot. The first principal component is mostly negatively correlated with environmental variables. The orders like Ephemeroptera, Plecoptera, Trichoptera and Diptera tend to have larger abundance in colder waters with reduced concentrations of TSS, TP, nitrate, nitrite, ammonium and OP.

Tab. 3. Loadings, eigenvalues, and cumulative explained variation of the first four extracted components

Higher taxonomic category	Component			
	1	2	3	4
Turbellaria	0.0164	0.1439	0.7360	–0.3455
Gastropoda	–0.5674	0.4166	–0.1236	–0.3465
Bivalvia	–0.4757	–0.0656	–0.2306	–0.6191
Oligochaeta	–0.3710	0.4946	–0.0841	–0.2127
Hirudinea	–0.4050	0.3661	0.5714	–0.0888
Crustacea	–0.5452	0.2179	–0.1565	–0.0232
Ephemeroptera	0.4362	0.5078	–0.3825	–0.2400
Odonata	–0.4978	0.4032	–0.3109	0.4044
Plecoptera	0.8290	0.2278	–0.1619	–0.1245
Heteroptera	–0.4709	0.4388	–0.0787	0.1778
Trichoptera	0.6841	0.5210	–0.1479	–0.2465
Coleoptera	0.0160	0.7081	0.1662	0.4043
Diptera	0.5009	0.5642	0.2612	0.0842
Eigenvalues	0.2472	0.1831	0.1047	0.0905
Explained variation (cumulative)	24.72	43.03	53.50	62.55

DISCUSSION

According to PCA, the increase of WT is most reflected in the number of taxa of Plecoptera, compared to the other higher taxonomic categories. This is in line with many previous studies (e.g., Meštrov *et al.*, 1978; Živić *et al.*, 2006; Chinnayakanahalli *et al.*, 2011; Krepski *et al.*, 2014; Strbad *et al.*, 2015). Increased temperature generally reduces species richness. Many researchers, who studied water bodies with higher temperature (e.g., post-cooling water from power plant) have found negative effect of temperature to qualitative structure of benthic invertebrate (Ciemiński & Zdanowski, 2009).

A large proportion of our sampling sites (ca. 50%) were situated in the Dinaric karst system, and karst rivers are vulnerable to nutrients, particularly nitrates, nitrites and phosphates (Peterson *et al.*, 2002), due to rapid surface infiltration, anthropogenic activity, and the flow of water through sinkholes and groundwater-influenced river networks (Caetano Bicalho *et al.*, 2012).

This investigation is in line with numerous previous investigations (Shokri *et al.*, 2014; Zhang *et al.*, 2018; Kelly-Quinn *et al.*, 2020; Rawat *et al.*, 2020; Leitner *et al.*, 2021) showing that samples from the least anthropogenic disturbed sites (i.e., nitrogen and phosphorus enriched watercourses) are characterized by the presence of pollution sensitive EPT species, indicating that these sites are more pristine. Other organisms, in turn, represented by Gastropoda, Bivalvia, Oligochaeta are known to be present in highly enriched environments (Armendáriz *et al.*, 2012; Wang *et al.*, 2012; Chase *et al.*, 2017) as rather insensitive to nitrogen and phosphorus overload, and can stand in moderately and high enriched waters, which is also seen in results (Tab. 2).

As indicated by Spearman correlations (Tab. 2) and PCA analysis (Fig. 3), it seems that nitrogen in water is equally as important as phosphorus in regulating macroin-
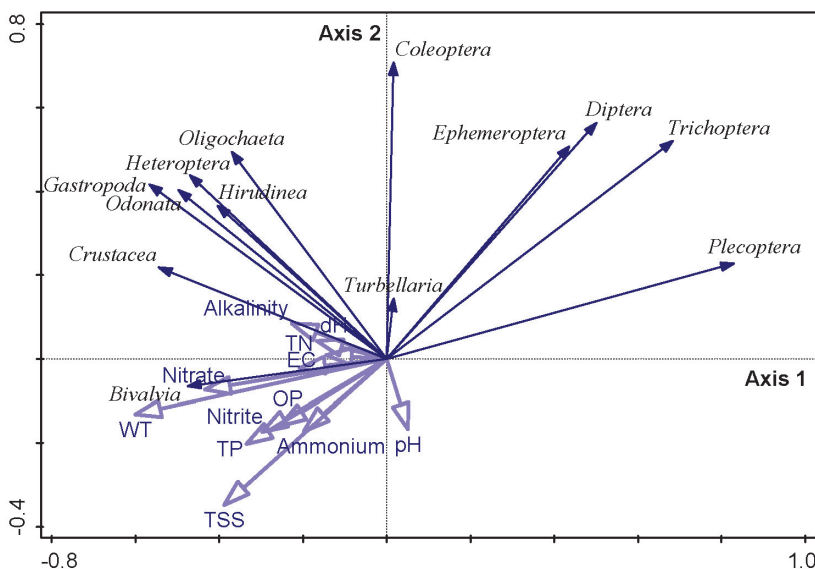
Fig. 3. Ordination of a PCA based on measurements (n =114) of 12 physical and chemical variables at each site during the study. Codes of the variables are given in material and methods. The first two axes of the PCA explain 43.08% of the total variation (PCA-1: 24.72%, PCA-2: 18.31%)

vertebrate distribution. The importance of nitrogen as a variable is not surprising as the agriculture in our area uses lot of (1,200,000 t per year) mineral fertilizers (Gugić *et al.*, 2014), which causes an amount of nitrogen leaching to the waterbodies. Intensification of agriculture can lead to soil condition alteration and increase runoff of minerals, which can increase nitrogen amounts in waters (Stefanidis *et al.*, 2016). The study of Lassaletta *et al.*(2009) concluded that agricultural cover explained 82% of the variation in nitrite concentration in streams in Ebro River Basin.

National plans to build numerous waste water treatment plants (WWTP) (Official Gazette, 2020) seems very promising as it will improve water quality by removing nitrogen, phosphorus and their compounds. EEA (2018) reported TP discharges decreased by circa 70% from 1983 to 2014 in agricultural Germany, mainly due to improved WWTP.

The study revealed many significant correlations between the physical-chemical variables (including nutrients) and macrozoobenthos, but these correlations were relatively weak. Some positive correlation of inorganic nutrients to macrozoobenthos can be explained by functional feeding groups, and their relationship to periphyton which is affected by nutrients (Makarewicz*et al.*, 2000; Garg *et al.*, 2009). The collector-gatherer functional feeding group show positive response to nutrient enrichment (Lawrence & Gresens, 2004). Some previous studies (e.g. Lawrence & Gresens, 2004) found interesting results in contrast to ours, reporting that Chironomidae (Diptera), especially grazing taxa, showed a strong response to nutrient manipulation. Chironomidae larvae appeared to follow the patterns of increased primary production at phosphorus-enriched sites. In addition, they have a rapid population growth and high densities in nutrient-enriched streams. In impacted aquatic ecosystems, chirono-

mid assemblages play a key role, particularly in the food web, due to their high densities in impacted ecosystems and rapid population growth (Benke, 1998; Maasri *et al.*, 2008).

Benthic invertebrates, especially EPT, respond well to phosphorus enrichment. Development of the DJ multimetric index (Dahl & Johnson, 2004), showed that EPT was highly correlated to total phosphorus concentration, whether it was the number of EPT taxa, representing richness measures or proportion of EPT taxa, representing composition measures. Perhaps other factors also influence the composition of benthic invertebrates, but they have not been considered in the present study, for example: structure and the composition of the bottom sediments (Kerakova & Varadinova, 2020), flow velocity (Habdija *et al.*, 2004), oxygen content (Jacob *et al.*, 1984; Galic *et al.*, 2019), heavy metal load (Hirst *et al.*, 2002; Stoyanova *et al.*, 2012; Kladarić *et al.*, 2020), hydro-morphological alterations (Urbanič, 2014; Villeneuve *et al.*, 2018; Urbanič *et al.*, 2020; Zelnik & Muc, 2020).

Further investigations are needed in order to see how nutrient enrichment affects the benthic macroinvertebrate assemblage in the presence of other stressors such as hydromorphological alteration and/or the presence of toxic substances (EEA, 2018; Birk 2019; Poikane *et al.*, 2019) to have a better understanding of river status deterioration. It is obvious that individual stressors affect the ecological status of freshwater waterbodies (i.e., water quality) more than a single stressor (Grizzetti *et al.*, 2017; Reid *et al.*, 2019; Lemm *et al.*, 2020).

CONCLUSIONS

Among the studied parameters, WT is the best predictor for diversity of freshwater macroinvertebrates.

EPT and Diptera are the most sensitive to nutrient enrichment among all freshwater invertebrates, according to their number of taxa in the sample. A strong negative Spearman correlation coefficient between physical-chemical parameters including nutrient enrichment and EPT implies this interdependence. This confirms the EPT taxa as useful indicators in monitoring stream health, especially nutrient enrichment.

Overall, nutrient levels are the likely cause of variations in diversity and distribution of macroinvertebrates in Croatian streams and rivers. However, future comprehensive analyses are needed to detect the detailed patterns of this interdependence.

Received May 20, 2021

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