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# Ecological status assessment using periphytic diatom communites - case study Krka River

## Проценка на еколошкиот статус со помош на перифитонски дијатомејски заедници - студија на река Крка

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### Abstract

In the light of ever-growing issue of climate changes, a special focus has been directed towards freshwater periphytic algal communities. Diatoms, a commonly dominant group of protists in periphytic assemblages, are frequently used for ecological water-quality assessments of rivers. Diatom-based indices for ecological status assessment are based on a weighted-averaging model using relative abundances and the environmental optima of taxa. The aim of this study was to assess the ecological status of the Krka River based on taxonomic analyses of its periphytic communities. Sampling of periphytic and environmental variables was carried out in September 2017 and September 2018 on nine stations along the Krka River course, with 3 microhabitats at each station. Taxonomic analysis of samples in 2017 revealed a total of 120 diatom species, whilst in 2018 a total of 226 diatom species was noted. Ecological status of the Krka River, evaluated using $TDI_{HR}$ (Croatian Trophic Diatom Index) and the corresponding ecological quality ratio (EQR), was classified as high in most of the watercourse of the Krka River.

**Key words**: diatoms, periphyton, ecological status, Krka River, trophic index

Слатководните перифитонски алгални заедници добиваат посебен фокус во рамките на зголемениот интерес за климатските промени. Дијатомеите (силикатни алги) се доминантна група од протистите во перифитонските заедници и често се користат за проценка на еколошкиот квалитет на водите во реките. Индексите за проценка на еколошкиот статус кои ги вклучуваат дијатомеите се базирани на моделирања на коригирани просечни вредности од релативната абудантност и еколошкиот оптимум на таксоните. Цел на оваа студија е да

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се процени еколошкиот статус на реката Крка врз база на таксономски анализи на перифитонските заедници. Собирањето на податоци за перифитонот и за еколошките фактори беше спроведено во септември 2017 и септември 2018 година во девет локалитети по течението на реката Крка при што во секој локалитет беа анализирани по три микрохабитати. Таксономската анализа во 2017 година покажа присуство на 120 дијатомејски таксони, додека во 2018 година беа регистрирани 226 дијатомејски таксони. Еколошкиот статус на реката Крка беше проценет користејќи го TDIHR (Croatian Trophic Diatom Index = Хрватски трофички дијатомејски индекс), а еколошкиот однос на квалитет (EQR - ecological quality ratio) беше висок во поголемиот дел од течението на реката Крка.

**Клучни зборови**: дијатомеи, еколошки статус, река Крка, трофички индекс

## Introduction

In the light of ever-growing issue of climate changes, a special focus has been directed towards freshwater periphytic algal communities. These communities refer to assemblages of benthic photoautotrophic algae and prokaryotes associated with submerged substrata (Poulíčková et al., 2008). The ecological importance of periphyton arises from a multitude of ecosystem functions they provide, including accretion and biostabilisation of sediments (Droppo et al., 2007), regulation of nutrient cycling (Dodds, 2003), as well as playing the fundamental role in primary production and trophic interactions (Dodds et al., 1999; Giller & Malmqvist, 1999). As being commonly dominant in periphytic assemblages (Stevenson et al., 1996; Smol & Stoermer, 2010), diatoms are frequently used for routine monitoring and ecological status assessmentof rivers. They inhabit various microhabitats within a lotic ecosystem, thus enabling targeted habitat sampling and point source assessment allowinghigh spatial resolution, as well as multi-habitat, composite characterization (Wehr et al., 2015). They are easily sampled and processed by the well established, standardized laboratory procedures (Kelly et al., 1998). Most common diatom species can beeasily discernible to species level based solely on morphological features. Furthermore, diatoms reproduce quickly and respond rapidly to environmental changes, making them highly sensitive indicators of organic pollution, degradation and eutrophication (Feio et al., 2007). Diatoms are used in water-quality assessments in all EU member states, where each country has established methods for assessing ecological status on the basis of phytobenthos in running waters.

Diatom-based indices for ecological status assessment are calculated based on a weighted-averaging model using relative abundances of all taxa in a sample from a site and the environmental optima or autecological values for the taxa. The weighted-average optima and tolerances of many diatom taxa have been developed with respect to geographic region and to environmental variables (Smol & Stoermer, 2010). Strength of such autecological indices is evident in their broad geographic performance and applicability (Stevenson et al., 2008).

The research on diatom based ecological status assessments of European rivers has grown extensively in the last two decades (Kelly, 2012; Poikane et al., 2016), much owing to the WFD and the consequent intercalibration exercises (Poikane et al., 2014) carried out among Member States within similar bio-geophysical types in order to harmonise the upper two class boundaries of all national assessment methods (Birk et al., 2013). Although most assessment methodologies prefer sampling diatoms from submerged naturally occurring moveable hard surfaces like large pebbles, cobbles and boulders, periphytic assemblages on tufa are considered proxies in rivers where karst bedrock is the most representative substratum.

The Croatian national method for ecological status assessment of rivers considers benthic diatoms as proxies for phytobenthos. It is compliant with normative definitions of WFD used by other MS and takes into account both taxonomic composition and species' relative abundance of benthic diatom assemblages. The aim of this study is to assess the ecological status of the Krka River based on taxonomic analyses of its periphytic communities.

## Materials and methods

### Study area

Krka is a 73 km long karstic river situated in the Dinaric Western Balkan ecoregion (ER5; sensu Illies, 1978) in Croatia, pertaining to national type HR-R_12: medium and large upland rivers (Narodne novine, 2019). According to the intercalibration river typology (Schöll et al., 2012) it belongs into the IC type R-M2: Mediterranean rivers with catchment between 100-1000 $km^2$, mixed geology (except non-siliceous) and high seasonality. The Krka River springs at the base of Dinara Mountain near the city of Knin, after which it flows downstream through a series of valleys (poljes) and canyon formations until reaching the Adriatic sea near the town of Šibenik. Along the course of the Krka River there are 7 prominent tufa deposits forming barrages and cascades which cause the water to change flow and speed with alternating lotic and lentic microhabitats, thus governing the river dynamic. Some parts of the Krka River have been protected since 1948 due to specific geomorphological, hydrological and landscape values. In 1985 a status of National Park was given to the Krka River and its drainage area (Narodne Novine, 1985, 1988, 1997).

### Sampling procedure

Sampling was carried out during the low water period for two consecutive years: from 21st to 23rd September 2017 and from 10th to 11th September 2018 along the Krka River course (Table 1). According to „multi habitat sampling" at each station 3 microhabitats (3 subsamples) on a 10 m long transect in the main river current were sampled (AQEM Consortium, 2002; HRN EN 13946:2014). Periphytic diatom samples were scrubbed from at least five tufa/stone substrates on each microhabitat, placed into 50 ml volume plastic vials and preserved in a 4% final concentration formaldehyde solution (samples from 2017) or in a 70% final concentration ethanol (samples from 2018). For the physico-chemical analysis of water *in situ* measurements of water temperature, pH, conductivity, oxygen concentration and saturation were done with a Hach HQ40D portable multimeter (HACH, USA). Samples for chemical analysis of water were collected simultaneously with biological samples and stored at -20 °C until laboratory processing. Chemical analysis included quantification of total nitrogen (TN), nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), phosphates ($P\text{-}PO_4^{3-}$), total inorganic carbon (TIC), dissolved inorganic carbon (DIC), total organic carbon (TOC) and dissolved organic carbon (DOC) according to stadardized methodology (American Public Health Association et al., 2017).

For the preparation of diatom slides, samples were cleaned by removing all organic material using acid digestion with the addition of 2 ml saturated $KMnO_4$ and 4 ml HCl(Taylor et al., 2007)and afterwards mounted in Naphrax (Brunel Microscopes, UK). On each slide at least 400 valves were counted using random transects under the Olympus BX51 (Olympus, Japan) and Nikon E–80i light microscopes (Nikon Corporation, Japan). Identification of diatoms was performed using relevant taxonomic literature (Krammer & Lange-Bertalot, 1986, 1991a, 1991b; Round et al., 1990; Krammer, 1997a, 1997b; Krammer, 2000; Lange-Bertalot, 2001; Hofmann et al., 2013; Lange-Bertalot et al., 2017).

### Statistical analyses and ecological status assessment

Computer program PRIMER v7 for Windows (Primer-E Ltd., U.K.) was utilised for calculation of diversity indices: total species number (*S*), Margalef Index of species richness (*d*), Pielou's Evenness Index (*J'*), Shannon-Wiener Diversity Index (*H'*), Simpson Index of dominance ($1\text{-}\lambda$), as well as all statistical analyses and pertaining figures. Statistical analyses included the following: Principal Component Analysis (PCA) based on the Euclidean distance was used to check for statistical significance of the environmental variables and to single out the most important ones, and Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis similarity distance was used to elucidate the relationship between diatom samples and environmental parameters.

Ecological status was evaluated using $TDI_{HR}$ (Croatian Trophic Diatom Index), a diatom metric modified from Rott's Trophic Index (Rott et al., 1999). Trophic indicator values and weights of all identified diatom species were defined according to the extended Operational list of diatom taxa for rivers included in the "Methodology for sampling, laboratory analyses and determination of ecological quality ratios for biological quality elements" (Narodne novine, 2019). Taxa list of diatoms with assigned indicator values and weights and with corresponding relative abundances is used for calculation of $TDI_{HR}$ by using the modified Zelinka-Marwan equation (1961):

**Table 1.** List of sampling stations on the Krka River, with pertaining tufa microhabitats, from which diatom samples were taken in 2017 and 2018.

| STATION | CODE | DATE | MICROHABITAT | GEOGRAPHIC COORDINATES (DMS) |
|---|---|---|---|---|
| Krka spring | P1 | 22.09.2017. | P1-1<br>P1-2<br>P1-3 | N 44° 2' 30.869''<br>E 16° 14' 6.478'' |
| Krka spring - bridge | P1a | 22.09.2017. | P1-4<br>P1-5<br>P1-6 | N 44° 2' 28.45''<br>E 16° 14' 1.334'' |
| Krka Marasovine | P2 | 23.09.2017. | P2-1<br>P2-2<br>P2-3 | N 44° 0' 37.375''<br>E 16° 5' 37.975'' |
| Bilušića buk waterfall | P3 | 10.09.2018. | P3-1<br>P3-2<br>P3-3 | N 44° 0' 47.286''<br>E 16° 4' 6.171'' |
| Brljan waterfall | P4 | 10.09.2018. | P4-1<br>P4-2<br>P4-3 | N 44° 0' 44.942''<br>E 16° 2' 5.549'' |
| Manojlovića buk waterfall | P5 | 10.09.2018. | P5-1<br>P5-2<br>P5-3 | N 44° 0' 55.663''<br>E 16° 1' 31.753'' |
| Rošnjak waterfall | P6 | 11.09.2018. | P6-1<br>P6-2<br>P6-3 | N 44° 0' 26.302''<br>E 16° 1' 41.718'' |
| Miljacka waterfall | P7 | 11.09.2018. | P7-1<br>P7-2<br>P7-3 | N 44° 0' 12.769''<br>E 16° 1' 11.503'' |
| Roški slap waterfall - cascades | P8 | 22.09.2017. | P8-1<br>P8-2<br>P8-3 | N 43° 54' 29.48''<br>E 15° 58' 38.114'' |
| Roški slap waterfall | P8a | 22.09.2017. | P8-4<br>P8-5<br>P8-6 | N 43° 54' 8.421''<br>E 15° 58' 32.307'' |
| Skradinski buk waterfall | P9 | 21.09.2017. | P9-1<br>P9-2<br>P9-3 | N 43° 48' 17.239''<br>E 15° 57' 49.475'' |
| Skradinski buk waterfall - experimental reach | P9a | 21.09.2017. | P9-4<br>P9-5<br>P9-6 | N 43° 48' 22.882''<br>E 15° 57' 55.314'' |

$$TDI_{HR} = \frac{\sum_{i=1}^{n} Ai \times IVi \times IWi}{\sum_{i=1}^{n} Ai \times IWi}$$

Where:
Ai = Total number of cells/valves of a species in the sample, representing the number of a certain species on 400 counted diatoms.
IVi = Indicator value (tolerance) of a species
IWi = Indicator weight (sensitivity) of a species

The ecological status was assessed on the basis of ecological quality ratio (EQR) values of $TDI_{HR}$. EQR was calculated using the formula described in the "Methodology for sampling, laboratory analyses and determination of ecological quality ratios for biological quality elements" (Narodne novine, 2019):

$$EQR\_TDI_{HR} = \frac{Index\ value - Poorest\ value}{Reference\ value - Poorest\ value}$$

## Results

The environmental variables of water measured at the sampling stations are shown in Table 2. The water temperature showed a downstream increase from the minimum values (10.3 °C) at Krka spring to the maximum (20.6 °C) at Skradinski buk. Similarly, the lowest pH (7.75) was recorded at Krka spring, whilst the highest (8.58) at Skradinski buk. The highest concentration of dissolved oxygen was measured at Bilušića buk (11.16 mg $L^{-1}$) and the lowest at Skradinski buk (8.19 mg $L^{-1}$). The lowest oxygen saturation of water was recorded at Krka spring (94.5%), whilst the highest at Bilušića buk (108.8%). The electrical conductivity of water ranged from a minimum of 391 μs $cm^{-1}$ at the Krka spring to a maximum of 690 μs $cm^{-1}$ at the upper course near Marasovine. Nitrite concentrations in water were below detection level at all investigated stations (<0.001 mg $L^{-1}$), as well as ammonium concentrations (<0.01 mg $L^{-1}$), except at Roški slap (0.02 mg $L^{-1}$). Nitrate concentrations varied from the lowest concentrations (<0.1 mg $L^{-1}$) measured at sites in the upper and middle section of the Krka River, while the highest concentrations were recorded at two downstream stations, Roški slap and Skradinski buk (6.6 and 6.2 mg $L^{-1}$, respectively). Correspondingly, the highest concentrations of total nitrogen were also measured at Roški slap (7.1 mg $L^{-1}$) and Skradinski buk (6.4 mg $L^{-1}$), while the lowest at Krka spring, Krka Marasovine and Roški slap - cascades (<0.1 mg $L^{-1}$). The highest concentrations of orthophosphates were recorded at Krka spring and Roški slap (0.31 and 0.27 mg $L^{-1}$, respectively), whereas at other sites it was considerably lower. Silicon dioxide ranged from 0.8 mg $L^{-1}$ at Krka spring to 6.9 mg $L^{-1}$ at Brljan. Slightly higher concentrations of total and dissolved organic carbon were recorded only at Skradinski Buk (TOC = 2.17 mg $L^{-1}$, DOC = 1.10 mg $L^{-1}$). The highest concentrations of total and dissolved inorganic nitrogen were recorded at Manojlovića buk (TIC = 12.79 mg $L^{-1}$, DIC = 11.30 mg $L^{-1}$). Due to probe shutdown at Krka Marasovine oxygen concentration, oxygen saturation and water temperature could not be measured. Skradinski buk and Roški slap stations were differentiated from other sampling stations according to higher concentrations of nitrogen compounds. Bilušića buk was singled out on the basis of dissolved oxygen concentration and saturation, while Manojlovića buk differred by total and dissolved inorganic carbon.

The PCA performed for the 12 environmental parameters (Table 3) singled out 4 most important ones thus indicating eigenvalues for the first two axes of 4.46 and 2.99, respectively, explaining 57.2 % of total variance on the first two PCA axes. The most important parameter for the PCA axis 1 were $N\text{-}NO_3^-$ and pH (intra-set correlations: -0.426 and -0.415, respectively). Regarding axis 2, $P\text{-}PO_4^{3-}$ and temperature (intra-set correlations: -0.513 and 0.371, respectively) were the variables that weighted most for ordination. PCA arranged samples (Figure 1) into three groups: first group consisted of samples from stations Krka spring, Krka Marasovine, Bilušića buk, Brljan, Manojlovića buk, Rošnjak and Miljacka (P1 to P7), the second group included only samples from Roški slap waterfall (P8), and the third one comprised all samples from Skradinski buk waterfall (P9 and P9a).

Taxonomic analysis of samples collected in 2017 at the sampling stations Krka spring (P1-1, P1-2, P1-3), Krka spring – bridge (P1-4, P1-5, P1-6), Krka Marasovine (P2-1, P2-2, P2-3),Roški slap – cascades (P8-1, P8-2, P8-3), Roški slap (P8-4, P8-5, P8-6), Skradinski buk (P9-1, P9-2, P9-3) and Skradinski buk – experimental reach (P9-4, P9-5, P9-6) has revealed a total of 120 diatom species. Diversity indices for the sampling stations on the Krka River sampled in 2017 are shown in Table 4. The lowest number of diatom species (*S*) was recorded at the station Krka spring (14), while the highest numberwas recorded at the station Skradinski buk (40). Margalef Index of species richness (*d*) ranged from 2.27 (at Krka spring)

**Table 2.** Environmental variables of water measured at the sampling stations on the Krka River during the sampling period.

| Code | pH | Cond. (µS cm$^{-1}$) | $O_2$ (mg L$^{-1}$) | $O_2$ (%) | T (°C) | TN (mg L$^{-1}$) | N-$NO_3^-$ (mg L$^{-1}$) | N-$NO_2^-$ (mg L$^{-1}$) | N-$NH_4^+$ (mg L$^{-1}$) | P-$PO_4^{3-}$ (mg L$^{-1}$) | $SiO_2$ (mg L$^{-1}$) | TIC (mg L$^{-1}$) | DIC (mg L$^{-1}$) | TOC (mg L$^{-1}$) | DOC (mg L$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P1** | 7.75 | 391 | 10.26 | 94.5 | 10.3 | 0.05 | 0.05 | 0.0005 | 0.005 | 0.310 | 0.9 | 10.77 | 10.53 | 0.61 | 0.26 |
| **P1a** | 7.76 | 405 | 10.40 | 95.4 | 10.4 | 0.05 | 0.05 | 0.0005 | 0.005 | 0.310 | 0.8 | 10.78 | 10.64 | 1.44 | 0.23 |
| **P2** | 7.88 | 690 | - | - | - | 0.05 | 0.05 | 0.0005 | 0.005 | 0.005 | 1.7 | 10.46 | 10.20 | 0.96 | 0.46 |
| **P3** | 7.92 | 528 | 11.16 | 108.8 | 13.6 | 1.00 | 0.20 | 0.0005 | 0.005 | 0.05 | 3.9 | 10.81 | 10.11 | 0.36 | 0.29 |
| **P4** | 8.06 | 680 | 9.80 | 103.5 | 17.5 | 0.50 | 0.05 | 0.0005 | 0.005 | 0.005 | 6.9 | 11.48 | 11.25 | 1.56 | 0.59 |
| **P5** | 8.13 | 668 | 9.31 | 99.5 | 17.9 | 0.50 | 0.05 | 0.0005 | 0.005 | 0.030 | 3.4 | 12.79 | 11.30 | 0.78 | 0.45 |
| **P6** | 7.94 | 649 | 8.46 | 89.8 | 18.3 | 0.50 | 0.05 | 0.0005 | 0.005 | 0.005 | 2.5 | 11.94 | 11.21 | 0.84 | 0.68 |
| **P7** | 8.06 | 640 | 9.54 | 102.6 | 18.9 | 1.00 | 0.10 | 0.0005 | 0.005 | 0.020 | 3.3 | 12.13 | 10.90 | 0.63 | 0.60 |
| **P8** | 7.96 | 653 | 9.50 | 95.2 | 15.4 | 0.05 | 0.05 | 0.0005 | 0.005 | 0.005 | 2.4 | 11.06 | 10.73 | 0.72 | 0.44 |
| **P8a** | 8.35 | 648 | 9.75 | 97.2 | 15.4 | 7.10 | 6.60 | 0.0005 | 0.020 | 0.270 | 2.0 | 10.79 | 10.45 | 0.61 | 0.45 |
| **P9** | 8.53 | 523 | 8.19 | 98.1 | 20.2 | 2.00 | 1.80 | 0.0005 | 0.005 | 0.005 | 1.2 | 9.78 | 8.88 | 2.17 | 1.10 |
| **P9a** | 8.58 | 505 | 9.16 | 101.5 | 20.6 | 6.40 | 6.20 | 0.0005 | 0.005 | 0.005 | 0.8 | 10.55 | 10.15 | 1.37 | 1.09 |

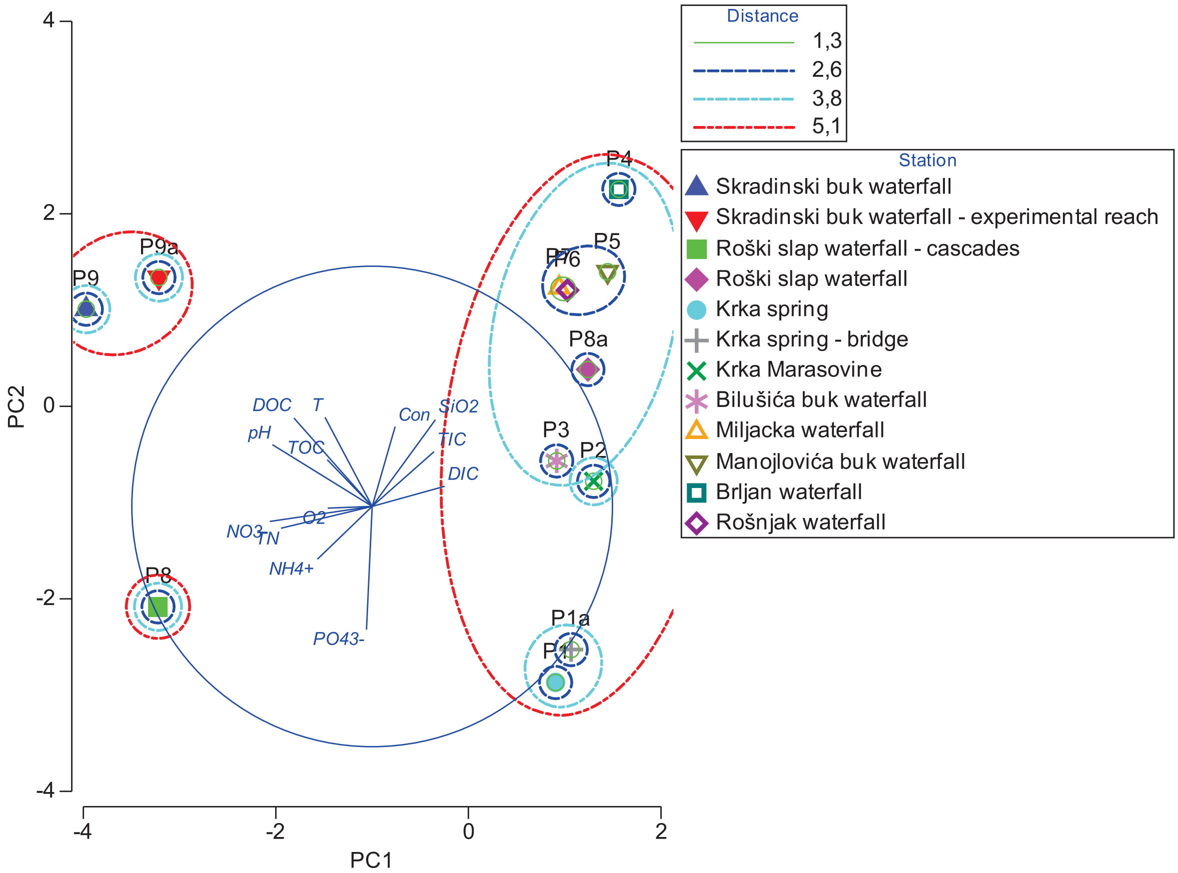


**Figure 1.** Principal Component Analysis (PCA) ordination diagram performed on the environmental parameters for all sampling stations in the Krka River during the investigated period.

**Table 3.** Relative variance explained and factor coordinates of the variables for the first two principal components (PC1 and PC2) of the Principal Component Analysis (PCA).

| Variable | PC1 | PC2 |
|---|---|---|
| **% Variation** | 34.3 | 23.0 |
| **Cumulative % variation** | 34.3 | 57.2 |
| **Eigenvalues** | 4.46 | 2.99 |
| **pH** | -0.415 | 0.257 |
| **Con** | 0.095 | 0.332 |
| **$O_2$** | -0.185 | -0.007 |
| **T** | -0.196 | 0.371 |
| **$N\text{-}NO_3^-$** | -0.426 | -0.063 |
| **$N\text{-}NH_4^+$** | -0.228 | -0.220 |
| **$P\text{-}PO_4^{3-}$** | -0.024 | -0.513 |
| **$SiO_2$** | 0.264 | 0.361 |
| **TN** | -0.379 | -0.091 |
| **TIC** | 0.258 | 0.228 |
| **DIC** | 0.302 | 0.084 |
| **TOC** | -0.187 | 0.195 |
| **DOC** | -0.326 | 0.368 |

to 6.26 (at Skradinski buk). Pielou's Evenness Index (*J'*) ranged between 0.36 - 0.90, noted at stations Krka spring – bridge and Skradinski buk – experimental reach, respectively. The lowest Shannon-Wiener Diversity Index (*H'*) recorded at Krka spring – bridge was 1.01, whilst the highest recorded at Skradinski buk – experimental reach was 2.92. The Simpson Index of dominance (*1-λ*) ranged from 0.37 at Krka spring – bridge to 0.94 at at Skradinski buk – experimental reach.

In the samples from 2018 taken at stations Bilušića buk (P3-1, P3-2, P3-3), Brljan waterfall (P4-1, P4-2, P4-3), Manojlovića buk waterfall (P5-1, P5-2, P5-3), Rošnjak waterfall (P6-1, P6-2, P6-3) and Miljacka waterfall (P7-1, P7-2, P7-3) a total of 226 diatom species were identified. Diversity indices for the sampling stations on the Krka River sampled in 2018 are shown in Table 5. The lowest number of diatom species (*S*) was recorded at the station Bilušića buk (26), while the highest number was recorded at the station Manojlovića buk (72). Margalef Index of species richness (*d*) ranged from 4.72 (at Bilušića buk) to 11.85 (at Manojlovića buk). Pielou's Evenness Index (*J'*) ranged between 0.55 - 0.90, noted at stations Bilušića buk and Miljacka, respectively. The lowest Shannon-Wiener Diversity Index (*H'*) recorded at Bilušića buk was 1.80, whilst the highest recorded at Miljacka was 3.52. The Simpson Index of dominance (*1-λ*) ranged from 0.64 at Bilušića buk to 0.95 at Manojlovića buk.

According to NMDS analysis based on Bray-Curtis similarity distance of the taxonomic composition of diatom community in 2017 (Figure 2), the two uppermost sampling stations at the Krka spring area differred significantly (stress 0.13) from the samples at Marasovine, Roški slap and Skradinski buk. The NMDS analysis of the taxonomic composition of diatom community in 2018 (Figure 3) indicated clustering of samples from Manojlovac, Brljan and Rošnjak waterfalls with 2 of the Miljacka samples, whilst the Bilušića buk samples comprised a separate group (stress 0.1). One sample from Miljacka was singled out from both groups.

For phytobenthos, in which diatom community was analysed, the morphological approach yielded results from which the

**Table 4.** Diversity indices calculated at the sampling stations on the Krka River sampled in 2017. Abbreviations as follows: total species number (*S*), Margalef Index of species richness (*d*), Pielou's Evenness Index (*J'*), Shannon-Wiener Diversity Index (*H'*), Simpson Index of dominance (*1-λ*).

| Sample | *S* | *d* | *J'* | *H'* | *1- λ* |
|---|---|---|---|---|---|
| **P1-1** | 20 | 3.20 | 0.76 | 2.28 | 0.86 |
| **P1-2** | 14 | 2.27 | 0.73 | 1.92 | 0.80 |
| **P1-3** | 27 | 4.31 | 0.78 | 2.57 | 0.89 |
| **P1-4** | 16 | 2.76 | 0.36 | 1.01 | 0.37 |
| **P1-5** | 19 | 3.49 | 0.80 | 2.37 | 0.86 |
| **P1-6** | 18 | 3.63 | 0.75 | 2.16 | 0.82 |
| **P2-1** | 28 | 4.60 | 0.83 | 2.78 | 0.92 |
| **P2-2** | 24 | 4.00 | 0.79 | 2.51 | 0.87 |
| **P2-3** | 19 | 3.95 | 0.79 | 2.31 | 0.83 |
| **P8-1** | 28 | 5.16 | 0.73 | 2.43 | 0.85 |
| **P8-2** | 29 | 4.51 | 0.42 | 1.42 | 0.51 |
| **P8-3** | 27 | 4.35 | 0.52 | 1.70 | 0.57 |
| **P8-4** | 29 | 5.10 | 0.75 | 2.53 | 0.85 |
| **P8-5** | 29 | 5.77 | 0.81 | 2.73 | 0.91 |
| **P8-6** | 29 | 5.09 | 0.49 | 1.66 | 0.58 |
| **P9-1** | 40 | 6.26 | 0.75 | 2.78 | 0.90 |
| **P9-2** | 32 | 5.69 | 0.81 | 2.81 | 0.92 |
| **P9-3** | 20 | 4.35 | 0.88 | 2.62 | 0.92 |
| **P9-4** | 23 | 4.45 | 0.51 | 1.59 | 0.56 |
| **P9-5** | 26 | 5.72 | 0.90 | 2.92 | 0.94 |
| **P9-6** | 30 | 5.67 | 0.68 | 2.31 | 0.74 |

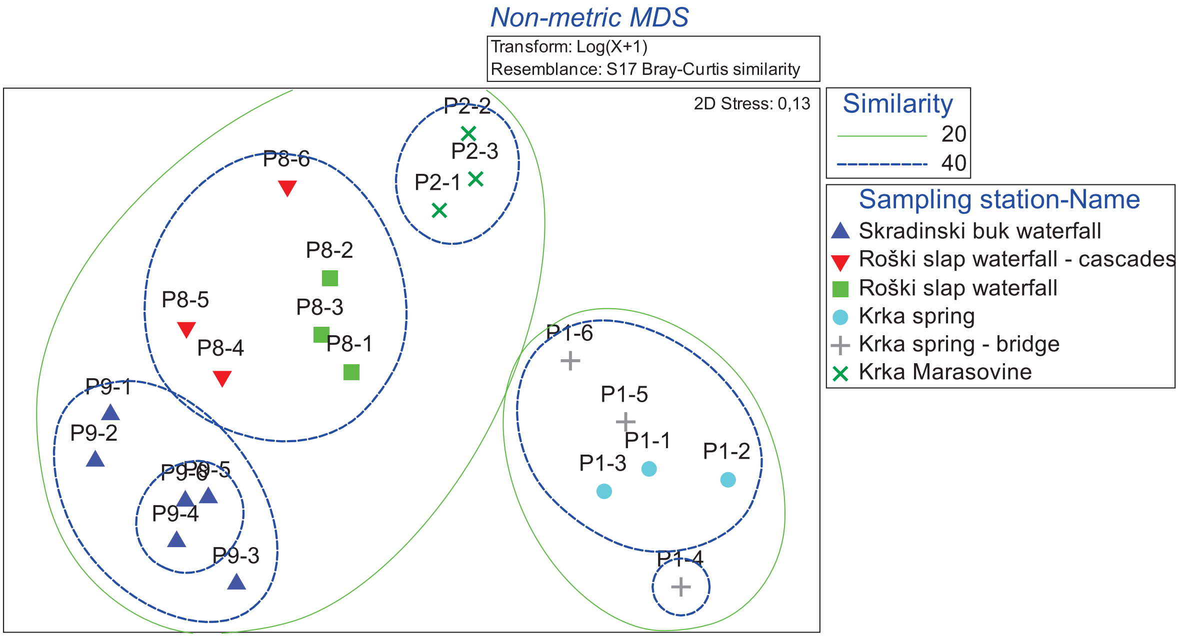


**Figure 2.** Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis similarity distance in taxonomic composition of diatom community according to sampling stations from 2017.

**Table 5.** Diversity indices calculated at the sampling stations on the Krka River sampled in 2018. Abbreviations as follows: total species number (*S*), Margalef Index of species richness (*d*), Pielou's Evenness Index (*J'*), Shannon-Wiener Diversity Index (*H'*), Simpson Index of dominance (*1-λ*).

| Sample | *S* | *d* | *J'* | *H'* | *1- λ* |
|---|---|---|---|---|---|
| **P3-1** | 41 | 6.68 | 0.68 | 2.53 | 0.81 |
| **P3-2** | 26 | 4.72 | 0.55 | 1.80 | 0.64 |
| **P3-3** | 43 | 7.01 | 0.73 | 2.73 | 0.86 |
| **P4-1** | 71 | 11.68 | 0.74 | 3.15 | 0.91 |
| **P4-2** | 63 | 10.35 | 0.79 | 3.25 | 0.93 |
| **P4-3** | 54 | 9.89 | 0.82 | 3.28 | 0.94 |
| **P5-1** | 69 | 11.35 | 0.82 | 3.47 | 0.95 |
| **P5-2** | 72 | 11.85 | 0.82 | 3.51 | 0.95 |
| **P5-3** | 66 | 10.85 | 0.79 | 3.30 | 0.93 |
| **P6-1** | 61 | 10.01 | 0.80 | 3.28 | 0.92 |
| **P6-2** | 46 | 7.51 | 0.73 | 2.78 | 0.89 |
| **P6-3** | 58 | 9.51 | 0.77 | 3.11 | 0.91 |
| **P7-1** | 34 | 6.23 | 0.62 | 2.18 | 0.73 |
| **P7-2** | 50 | 9.25 | 0.90 | 3.52 | 0.96 |
| **P7-3** | 63 | 10.35 | 0.84 | 3.46 | 0.96 |

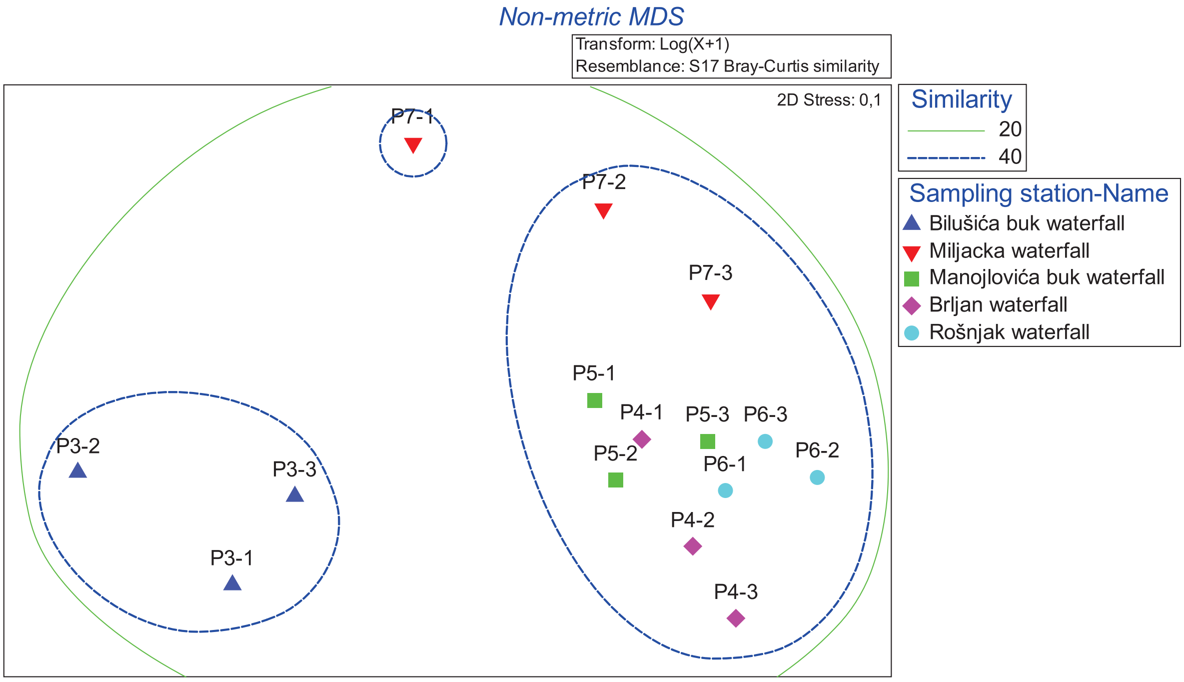


**Figure 3.** Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis similarity distance in taxonomic composition of diatom community according to sampling stations from 2018.

**Table 6.** Values of $TDI_{HR}$ index on the investigated stations and pertaining microhabitats on the Krka River in 2017 and 2018.

| CODE | P1-1 | P1-2 | P1-3 | P1-4 | P1-5 | P1-6 | P2-1 | P2-2 | P2-3 | P3-1 | P3-2 | P3-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $TDI_{HR}$ | 2.71 | 1.97 | 2.16 | 3.56 | 2.29 | 2.36 | 2.71 | 2.83 | 2.49 | 1.97 | 2.91 | 1.92 |
| **CODE** | **P4-1** | **P4-2** | **P4-3** | **P5-1** | **P5-2** | **P5-3** | **P6-1** | **P6-2** | **P6-3** | **P7-1** | **P7-2** | **P7-3** |
| $TDI_{HR}$ | 2.32 | 2.11 | 3.03 | 2.11 | 1.97 | 2.82 | 2.13 | 2.18 | 2.27 | 1.84 | 1.94 | 1.91 |
| **CODE** | **P8-1** | **P8-2** | **P8-3** | **P8-4** | **P8-5** | **P8-6** | **P9-1** | **P9-2** | **P9-3** | **P9-4** | **P9-5** | **P9-6** |
| $TDI_{HR}$ | 2.4 | 2.24 | 2.43 | 2.28 | 2.44 | 2.49 | 1.88 | 1.76 | 2.23 | 2.22 | 2.55 | 2.44 |

ecological status of the Krka River was assessed by calculating the Croatian Trophic Diatom Index ($TDI_{HR}$). $TDI_{HR}$ values and the corresponding ecological status of all sampled stations with pertaining microhabitats are shown in Tables 6 and 7, respectively. The ecological status of the Krka River, in most of its course, is classified as high.

## Discussion

The karst tufa barriers arising along the course of Krka River represent one of its most unique and recognizable natural attributes. The tufa-forming process is strongly dependent on the balance and interplay between distinct physical properties of water, specific chemical conditions and biological primary component.

The studied environmental parameters mostly demonstrated a well-documented trend present in karst riverine systems of the Dinaric area. The downstream increase in

**Table 7.** Ecological status, expressed as ecological quality ratio (EQR), of the investigated stations and pertaining microhabitats on the Krka River in 2017 and 2018.

| CODE | P1-1 | P1-2 | P1-3 | P1-4 | P1-5 | P1-6 | P2-1 | P2-2 | P2-3 | P3-1 | P3-2 | P3-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EQR | 0.76 | 1.03 | 0.96 | 0.45 | 0.91 | 0.88 | 0.76 | 0.71 | 0.84 | 1.03 | 0.68 | 1.04 |
| | GOOD | HIGH | HIGH | MODE-RATE | HIGH | HIGH | GOOD | GOOD | GOOD | HIGH | GOOD | HIGH |
| **CODE** | **P4-1** | **P4-2** | **P4-3** | **P5-1** | **P5-2** | **P5-3** | **P6-1** | **P6-2** | **P6-3** | **P7-1** | **P7-2** | **P7-3** |
| EQR | 0.9 | 0.97 | 0.64 | 0.97 | 1.03 | 0.72 | 0.97 | 0.95 | 0.92 | 1.07 | 1.04 | 1.05 |
| | HIGH | HIGH | GOOD | HIGH | HIGH | GOOD | HIGH | HIGH | HIGH | HIGH | HIGH | HIGH |
| **CODE** | **P8-1** | **P8-2** | **P8-3** | **P8-4** | **P8-5** | **P8-6** | **P9-1** | **P9-2** | **P9-3** | **P9-4** | **P9-5** | **P9-6** |
| EQR | 0.87 | 0.93 | 0.86 | 0.91 | 0.86 | 0.84 | 1.06 | 1.1 | 0.93 | 0.93 | 0.82 | 0.86 |
| | HIGH | HIGH | HIGH | HIGH | HIGH | GOOD | HIGH | HIGH | HIGH | HIGH | GOOD | HIGH |

water temperature, along with an increase in pH values from the uppermost station (Krka spring) to the lowermost station (Skradinski buk) were in accordance with the spatial variability presented by previous studies (Cukrov et al., 2008; Filipović Marijić et al., 2018). As the groundwater surfaces the river begins warming up along its watercourse, with further insolation intake in the lacustrine parts after each tufa barrier, until reaching the Adriatic sea. Rivers on limestone and dolomite waterbed are characterized by having a relatively high pH values of water, as a consequence of dissolution of the substrate. Moreover, calcareous hardwater rivers show typical pH increase in the downstream direction due to $CO_2$ degassing, causing a significant decrease in calcium carbonate solubility with pronounced precipitation (Golubić, 1973; Cukrov et al., 2008; Rugel et al., 2016). The dissolved oxygen (DO) is a particularly important parameter for the assessment of water quality (American Public Health Association et al., 2017). The oxygen content was near the saturation level at all sampling stations, with higher values at the Krka spring and the upper tufa barriers (Bilušića buk and Brljan) owing to higher solubility in the lower water temperatures, narrowstreambed and faster waterflow. As was the case with pH, the observed spatial fluctuations of conductivity also exhibited a general downstream increase, thus indicating natural calcite precipitation along the watercourse. The overall nutrient concentrations were rather low, thus reflecting the good water quality of the system. The majority of nitrogen in rivers usually comes from direct terrestrial runoff compared to the atmospheric sources. Since it can be rapidly oxygenized, the concentration of $NO_2^-$ is usually very low (Wetzel, 2001), as was previously exhibited in the Krka River system (Sertić Perić et al., 2018).The extremely low values (<0.001 mg $L^{-1}$) of $NH_4^+$ ions, whose main natural source is microbiological decomposition of nitrogenous compounds (EPA, 2007), indicated very low content of organic matter. As opposed, $NO_3^-$ and TN levels have shown a clear trend of increasing concentrations in the downstream direction. Very low values of $NO_3^-$and TN recorded in the upper stations were in congruence with previous studies (Sertić Perić et al., 2018), whilst higher values on the last two tufa barriers (Roški slap and Skradinski buk) suggested possible bacterial denitrification processes (Bandurski, 1965). Low concentration of soluble reactive phosphorus at all stations, except for Krka spring and Roški slap, signalled processes of enhanced formation of calcium carbonate with coprecipitation of available phosphate along with carbonates (Otsuki & Wetzel, 1972) and removing it from the water.Inorganic carbon is an important indicator of the biological productivity and buffer capacity of water ecosystems (Polesello et al., 2006). A major part of total inorganic carbon (TIC) is dissolved inorganic carbon (DIC), an indicator of primary productivity, namely providing bioavailable C source for photosynthesis (Jarvie et al., 2017). The concentrations of TIC and DIC demonstrated narrow-range fluctuations, hence demonstrating balance between photosynthesis and respiration by biota and carbon dioxide transfers from the water column to the atmosphere. Since being resistant to microbial degradation, organic carbon is used as a proxy for detecting humic substances and partly degraded biotic materials, as well as allochtonous organic pollutants (EPA, 2007; Lee et al., 2016). Both DOC, mainly defined as the input of allochthonous materials, and TOC, as the measure of total organic matter in the system, were very low and did not vary significantly along the stations, suggesting very low presence of organic substances in sediments. Low concentration of dissolved organic matter is a prerequisite for tufa deposition process (Srdoč et al., 1985) and coincides with a higher values of dissolved oxygen concentrations (EPA, 2007), thus indicating good water quality of the Krka River.

The forming of tufa involves a precipitation of calcium carbonate from water (Pentecost, 2005) with a simultaneous interaction of biotic elements. A crucial biological component involved in this dynamic process are algae, namely diatoms, with a role of primary production and mucus excretion for plastering calcite microcrystals, as well as embedding themselves into barriers (Emeis et al., 1987; Chafetz et al., 1994). Besides having a crucial part in biogenic development of tufa barriers, diatoms are excellent proxies for the water quality evaluation. The ability to reflect even the slightest environmental changes in real-time, fast growth and short generation time and their ubiquity justifies the use of diatoms as a biological quality element forecological assessments of waterbodies. Therefore, composition and relative abundance of diatoms at the investigated sampling stations clearly reflects their ecological status. The total diatom species number steadily increased from the uppermost stations on the Krka River to the maximum at middle reaches (Manojlovića buk waterfall), and was followed by a downstream decline. The presented dynamics is accordant with the disposition of local microhabitats, which regulary follows the pattern from being relatively uniform at the river spring to more heterogenous and diverse environments crafted by the river through the terrain (Vannote et al., 1980; Minshall et al., 1985; Molloy, 1992; Markert et al., 2003; Barren, 2015; Rusanov & Khromov, 2016). This was further corroborated by all diversity indices, specifically Margalef Index describing the species richness and Pielou's Evenness Index describing how evenly distributed abundance is among the taxa, both clearly displaying the aforementioned trend in qualitative species composition. The complementary results were also given by the Shannon-Wiener Diversity Index (*H'*), which shows sensitivity with regards to having more unique species in samples, and the Simpson Index, which measures the strength of dominance based on the abundance of the most common species. Moreover, high discrepancy in species number detected over the two years can be explained by spatial differences in sampling. Namely, sampling in 2017 was conducted at the uppermost and lowermost stations, whilst the samples from 2018 included the middle section of the Krka River. The uppermost part of the Krka (Krka spring and Marasovine) represents typical Mediterranean karst spring zone, which is very variable to the hydrological cycle, rainfall regime and climate change, and thus usually exhibits lower number of taxa and high values of abundance of several dominant taxa (Kamberović et al., 2016; Lai et al., 2019). The lowermost stations on the Krka River (Roški slap, Skradinski buk) are exposed to increased tourism-related anthropogenic pressure, as well as historical (e.g. old watermills and hydroplants) and recent hydromorphological alterations (e.g. walking trail constructed over the river), which inevitably cause habitat degradation and impoverishment of the biota (Fehér et al., 2012). Adversely, middle parts of the Krka River are mostly inaccessible to general public, and therefore more preserved, offering more heterogenous, natural, mosaic-like structures or patches of different aquatic microhabitats (e.g. mosses and large woody debris) which can host a larger number of species (Wondzell & Bisson, 2003).

The official Croatian methodology for the ecological status assessment is compliant with the propositions of the WFD and has recently been successfully intercalibrated. For that purpose, Croatian trophic diatom index ($TDI_{HR}$) was developed in order to integrate all of its distinct watershed features. The ranges of $TDI_{HR}$ were set to reflect the specificities and the differences in the diatom communites with regards to the ecological preferences of individual diatom species (Rott et al., 1999). On the basis of these settings, the ecological quality ratios (EQRs) can be expressed as a standard for ecological status assesment (European Commision, 2000). Besides Krka Marasovine, the upper sampling stations on the Krka River were characterized with low values of $TDI_{HR}$ and high ecological status. Lower recorded values of $TDI_{HR}$ on one microhabitat (P1-4, Krka spring - bridge), with 'only' moderate ecological status could be interpreted by the uniformity of the selected stone microhabitat and the stochasticity in the choosing process with no prior selection. Moreover, the good ecological status of station Krka Marasovine is most likely a result of the anthropogenic impact from the nearby town of Knin, documented for this particular section of the river (Cukrov et al., 2008; Filipović Marijić et al., 2018; Sertić Perić et al., 2018). Further downstream the waters of Krka River show signs of significant self-purification through several small lakes formed by tufa barriers (Cukrov et al., 2008), especially the Bilušića buk, Brljan and Manojlovića buk waterfalls, thus reflecting the consistently high EQRs ecological status. Due to such intense purification processes, the water at all downstream stations of Rošnjak, Miljacka, Roški slap and Skradinski buk showed high ecological status. Nevertheless, the sentient karst aquatic systems, such as Krka River, are highly sensitive to the anthropogenic influence and require constant monitoring and implementation of strict protection regimes.

## Further goals

Further plans in monitoring of the Krka River include introducing metabarcoding as a novel method of detecting species and predicting biodiversity from short nucleotide sequences of a DNA molecule, the so-called 'barcode', where the combination of DNA taxonomy and high-throughput sequencing technologies merge. The advantages of this approach are the speed of sample processing and obtaining data on the overall biodiversity of the studied systems, which include data on small species, species that are difficult to preserve in samples, and cryptic species that are morphologically difficult or almost impossible to identify or distinguish.

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