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# Are Intermittent Rivers in the Karst Mediterranean Region of the Balkans Suitable as Mayfly Habitats?

**Marina Vilenica [1,*], Fran Rebrina [2], Lea Ružanović [2], Mario Rumišek [2], Renata Matoničkin Kepčija [2] and Andreja Brigić [2,*]**

[1] Faculty of Teacher Education, University of Zagreb, Trg Matice Hrvatske 12, 44250 Petrinja, Croatia
[2] Division of Zoology, Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia
* Correspondence: marina.vilenica@ufzg.hr (M.V.); andreja.brigic@biol.pmf.hr (A.B.)

**Abstract:** Intermittent rivers, common in dry parts of the world, such as the Mediterranean region, are hydrologically harsh habitats characterized by periodical flow cessation. Ephemeroptera are aquatic insects common in both lotic and lentic waterbodies, where they show a high sensitivity to anthropogenic modifications of their habitat. Therefore, they are widely used as bio-indicators of the freshwater's health. However, mayfly assemblages and their ecological requirements in the karst Mediterranean intermittent habitats are still not sufficiently known. Thus, the work presented here includes an analysis of mayfly assemblages and their relationship with environmental variables in the lotic phase of four intermittent rivers in the karst Mediterranean region of Croatia. Considering that the studied intermittent rivers are hydrologically extreme environments, a total of 12 recorded mayfly species could be considered as rather high species richness. Nevertheless, species richness per river was quite low (between three and six), and was highly influenced by river morphology, physico-chemical water properties (especially conductivity, water velocity, and concentrations of dissolved oxygen), and anthropogenic pressures. Our results could contribute to the mayfly species protection in karst Mediterranean intermittent freshwater habitats in the Balkans, as well as to the development of conservation measures for those threatened habitats.

**Keywords:** Ephemeroptera; diversity; environmental variables; temporary habitats; lotic habitats



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## 1. Introduction

Intermittent rivers are lotic habitats characterized by a temporary flow of water, i.e., by the cessation of water flow and the occurrence of dry events during certain periods of the year. One or more river reaches of the river basin could be affected by drying events, i.e., it could occur in particular reaches of the river, such as the headwaters, or it could be more widespread, occurring throughout the entire network of the river [1–4]. In intermittent rivers, three distinct hydrological phases can be distinguished: the lotic phase (the flowing phase characterized by the continuous water flow), the lentic phase (the non-flowing phase characterized by the water flow cessation, which leads to the formation of connected or isolated pools), and the dry phase with no surface water [5]. Such an extreme hydrological regime (with different hydrological phases, including intense floods and droughts) has a strong influence on the physico-chemical water properties (i.e., discharge, water temperature, oxygen saturation, conductivity, concentration of nutrients, etc.) and significantly affects the biotic communities in an intermittent river and its catchment [6].

Intermittent rivers and streams are naturally widespread and common freshwater habitats in semi-arid regions of the world, such as the Mediterranean region, where they are an essential resource of water for many organisms [7–9]. However, due to the combined effects of climate change and increasing water demand [10,11], many perennial rivers and streams are increasingly changing their hydrological regime to an intermittent type, even in more humid climates [12–14]. This trend towards rivers' hydrological change into intermittency is expected to continue in the near future [15], which will certainly lead to permanent changes in communities of the riverine biota [7,8].

Intermittent freshwater habitats provide various ecosystem services, such as freshwater supply, flood control, and water regulation, and they have recreational value [16]. They also provide habitats for aquatic and terrestrial organisms [17]. Aquatic macroinvertebrate communities of intermittent rivers and streams are generally less diverse than those in perennial lotic habitats [7,8,18]. Such communities are typically dominated by generalists, that also occur in perennial streams, but could also contain some rare species specialized for intermittent habitats [19,20].

Mayflies are amphibious insects often present in high abundances in freshwater habitats [21], where they play an important role in secondary production and are a valuable food source for a wide range of aquatic, but also terrestrial predators [22]. Mayfly nymphs inhabit both running and standing freshwater habitats where their assemblages are shaped by various physical and chemical water parameters, such as the current velocity, water temperature, the concentration of dissolved oxygen in water, pH, microhabitat composition, and the availability of food resources [23–27]. Mayflies show a high sensitivity to anthropogenic modifications of their habitat and water pollution [28–30], which is why they are widely used as bio-indicators of the freshwater's health [22,31].

Although research interest in the hydrology and biology of intermittent freshwater habitats is increasing worldwide [6], the ecology of aquatic macroinvertebrates populating such habitats, including mayflies, is still poorly understood. Therefore, the main goals of the study presented here were: (i) to determine mayfly species richness, diversity, and abundance; (ii) to identify the most influential environmental variables that shape mayfly assemblages; (iii) to investigate relationships between individual mayfly species and environmental variables; and (iv) to analyze the influence of microhabitat type on mayfly assemblages and species in four intermittent rivers in the karst Mediterranean region of the Balkans.

## 2. Materials and Methods

### *2.1. Study Area*

The Croatian territory is situated in two limno-ecological regions: the Dinaric western Balkan ecoregion (ER5) and the Hungarian lowland ecoregion (ER11). Our study area was in the Dinaric Western Balkan ecoregion (ER5) of Croatia [32]. This region is characterized by a temperate humid climate with hot summers (Cfa, Köppen classification). The mean temperature of the warmest month is above 22 °C [33], the mean annual air temperature is 14 °C and the mean annual rainfall is 1000 mm [34]. This study included four Mediterranean intermittent karst rivers of the Adriatic Sea basin: the Krčić, Čikola, Miljašić Jaruga, and Guduča rivers (Figure 1). All rivers are characterized by several months of flow cessation during the summer [35–40]. In total, 12 study sites were sampled at those four rivers. Three sites were sampled per river, the first study site was closest to the river spring and the third study site was farthest from the river spring. A detailed description of each river is presented in [41,42].


**Figure 1.** Map showing the position of the study area in the karst Mediterranean region of the Balkans, with locations of the studied intermittent rivers in Croatia. Examples of the study sites at each river are shown in the photographs.

### 2.2. Environmental Variables

The study was conducted in April 2021, during the lotic phase of all four rivers (i.e., when all the rivers were flowing). At each study site, the following environmental variables were measured in four replicates: water temperature, dissolved oxygen concentration and saturation (using the WTW Oxi 330/SET oximeter), conductivity (using the WTW LF 330 conductivity meter), pH (using the WTW pH 330 pH-meter), water depth (using a handheld meter), and water velocity (using the SonTek Flow Tracker).

In addition, we collected four replicate 1-litre water samples at the same points at each study site for the laboratory analysis of water chemical properties (alkalinity, chemical oxygen demand, nitrite, nitrate, and orthophosphate concentrations) using standard analytical procedures [43].

Dominant substrates (covering more than 5% of the study site) consisted mainly of fine sediments (sand, silt, mud), lithal (stones, gravel), and aquatic vegetation (submerged and emergent); detailed substrate composition at each study site is shown in [41].

### 2.3. Mayfly Sampling and Identification

Mayfly nymphs, along with other representatives of the macrozoobenthos, were collected in 15–16 April 2021 using a Surber sampler (25 cm × 25 cm, mesh size 500 µm). Four replicate samples were collected at each study site, proportional to the microhabitats present (i.e., a total of 12 samples were collected at each river). Right away after the sampling, all collected samples were preserved in 70% ethanol and later sorted and identified in the laboratory. Identification of mayfly nymphs was performed using standard identification tools [44,45]. Taxonomy follows [23,46].

### 2.4. Data Analysis

Kruskal–Wallis H test with pairwise comparisons of average ranks *post hoc* test was used to analyze differences in the environmental variables and assemblage metrics (abundance, species richness, Shannon, and Simpson diversity indices) among the four intermittent rivers. Mayfly assemblage metrics were calculated for each study site at each investigated intermittent river, based on the four replicates collected at each site.

We assessed similarities in the composition of mayfly assemblages among the four studied rivers using the hierarchical cluster analysis (HCA) based on the Bray–Curtis similarity matrix. Prior to analysis, species abundance data were log (x + 1) transformed. Replicates without mayfly specimens were not included in the HCA.

We tested the relationship between mayfly assemblages and environmental variables using the canonical correspondence analysis (CCA). In this analysis, we included a total of 12 species and nine environmental variables (those that differed significantly among the four rivers; oxygen saturation was excluded due to its correlation with concentration of dissolved oxygen in water). Rare species were downweighed. Prior to the analysis, mayfly abundances were log (x + 1) transformed. The relationship between species composition and environmental variables was tested using the Monte Carlo test with 499 permutations ($p < 0.05$).

The relationships between individual mayfly species and environmental variables in the four rivers was analysed using the Spearman correlation coefficient. The same analysis was used to evaluate the relationships of mayfly assemblage metrics and individual species with the physical variables of the microhabitats, i.e., water velocity and water depth. To analyze the relationships of mayfly assemblage metrics and individual specieswith substrate types as the microhabitat component, Kruskal–Wallis H test with pairwise comparisons of average ranks was used as a *post hoc* test.

Each data set was tested for normality using the Shapiro–Wilk W test Statistica 13.0 [47]. Kruskal–Wallis H test with pairwise comparisons of average ranks *post hoc* test and Spearman correlation coefficient were performed in Statistica 13.0 [47]. Bray–Curtis similarity index and HCA were conducted in the Primer 6.0 software package [48]. CCA and Monte Carlo test were performed in the CANOCO package version 5.11 [49].

## 3. Results

### *3.1. Environmental Variables*

The Kruskal–Wallis H test (df = 3, N = 48) showed significant differences in the following environmental variables among the four intermittent rivers: conductivity (H = 40.14, $p < 0.001$), water temperature (H = 40.12, df = 3, $p < 0.001$), the concentration of nitrites (H = 38.57, $p < 0.001$), dissolved oxygen concentration (H = 33.70, $p < 0.001$), water velocity (H = 29.48, $p < 0.001$), oxygen saturation (H = 28.36, $p < 0.001$), pH (H = 21.77, $p < 0.001$), chemical oxygen demand (H = 14.07, $p < 0.01$), the concentration of orthophosphates (H = 19.73, $p < 0.01$), and alkalinity (H = 22.11, $p = 0.01$).

Pairwise comparisons of the mean ranks showed that water temperature was significantly higher in the Miljašić Jaruga and Guduča rivers than in the Krčić and Čikola rivers ($p < 0.05$, Figure 2a). Water pH was significantly higher in the Čikola River than in the Miljašić Jaruga and Guduča rivers ($p < 0.05$, Figure 2b). Furthermore, water velocity was significantly lower in the Miljašić Jaruga River than in the Krčić and Guduča rivers ($p < 0.05$, Figure 2c). On the other hand, water depth did not differ significantly among the four intermittent rivers ($p > 0.05$, Figure 2d). Dissolved oxygen concentration was significantly higher in the Krčić and Miljašić Jaruga rivers than in the Čikola and Guduča rivers ($p < 0.05$, Figure 2e), whereas oxygen saturation was significantly higher in the Miljašić Jaruga River than in the other three rivers ($p < 0.05$, Figure 2f).

Conductivity was significantly lower in the Krčić River than in the other three rivers ($p < 0.05$). It was also lower in the Čikola and Guduča rivers compared with the Miljašić Jaruga River ($p < 0.05$, Figure 3a). Likewise, alkalinity was significantly lower in the Krčić River than in the other three rivers ($p < 0.05$, Figure 3b). Chemical oxygen demand was significantly higher in the Guduča River than in the Krčić and Miljašić Jaruga rivers ($p < 0.05$, Figure 3c). Additionally, the concentration of ortho-phosphates was significantly lower in the Čikola River than in the Miljašić Jaruga and Guduča rivers ($p < 0.05$, Figure 3d), while the concentration of nitrites was significantly lower in the Čikola than in the Guduča River, with no nitrites recorded in the Krčić River ($p < 0.05$, Figure 3e). In contrast, nitrate concentrations did not differ significantly among the four investigated rivers ($p > 0.05$, Figure 3f).

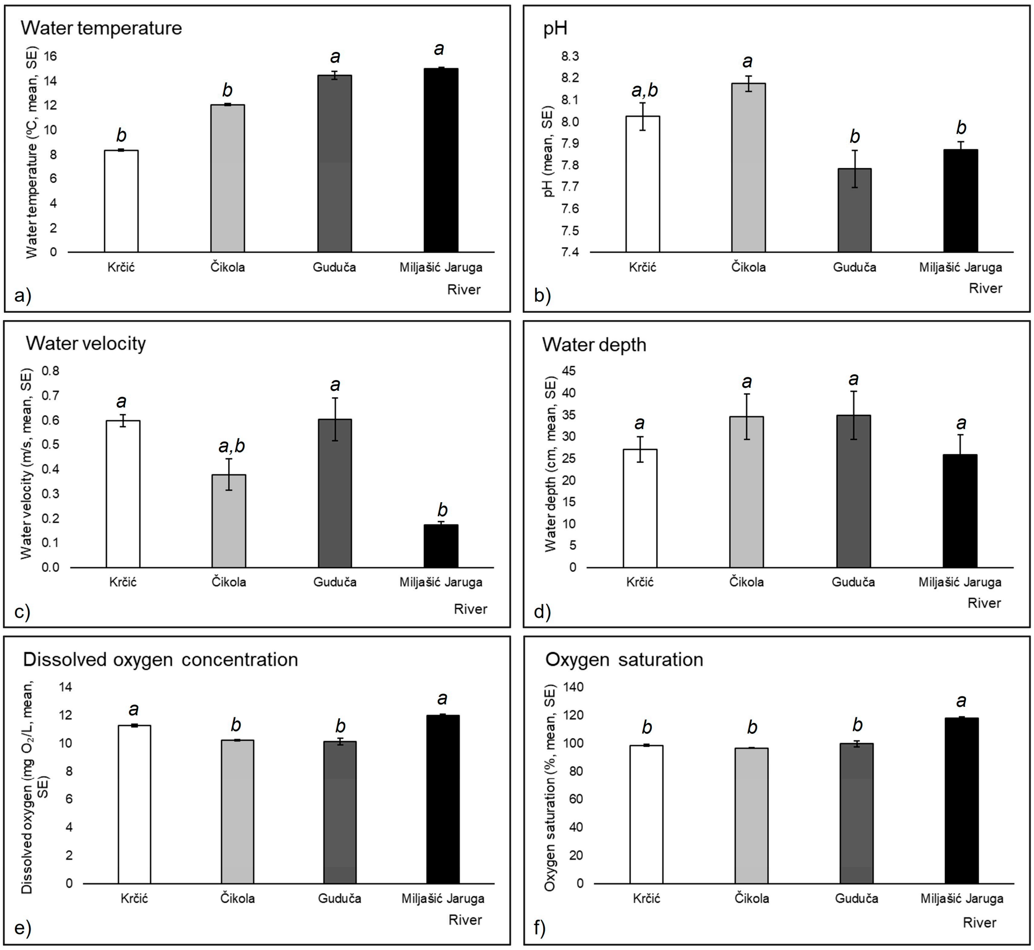


**Figure 2.** Physico-chemical parameters of the water measured at four intermittent rivers in the karst Mediterranean region of the Balkans shown as mean with standard error (SE): (**a**) water temperature, (**b**) pH, (**c**) water velocity, (**d**) water depth, (**e**) dissolved oxygen concentration, (**f**) oxygen saturation.

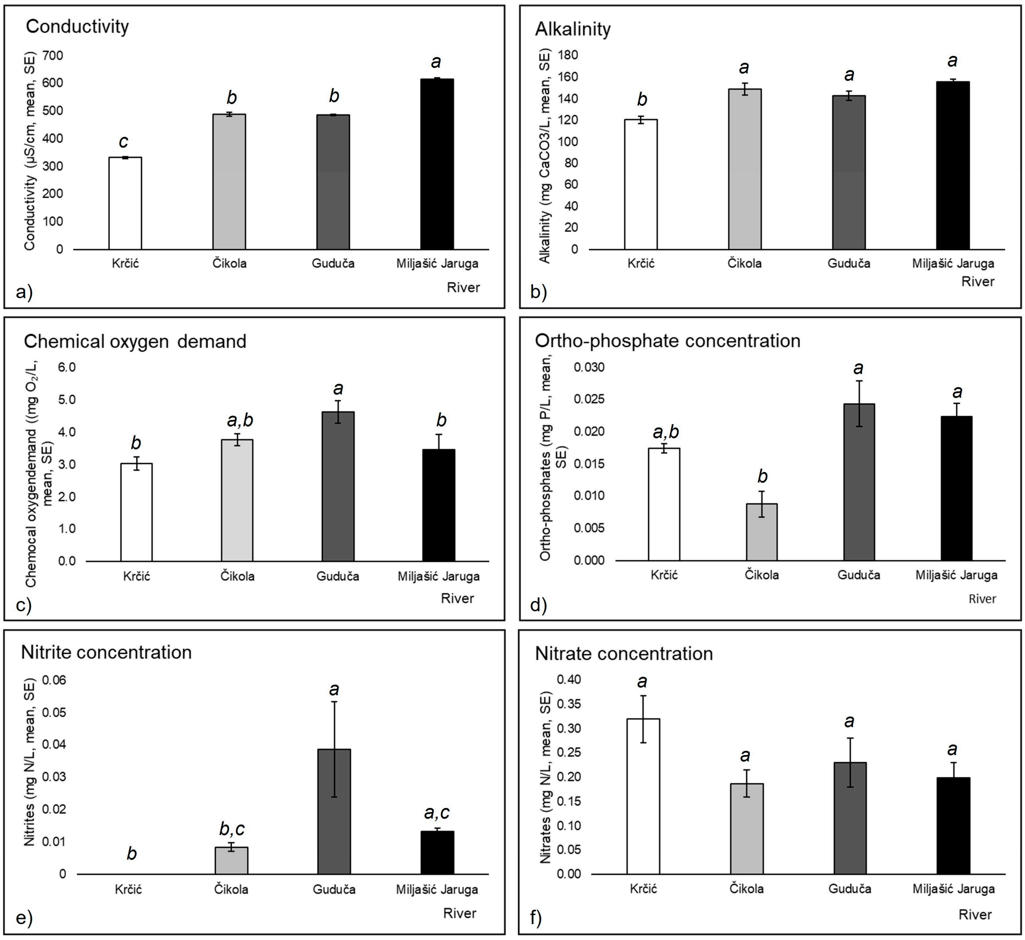


**Figure 3.** Physico-chemical parameters of the water measured at four intermittent rivers in the karst Mediterranean region of the Balkans shown as mean with standard error (SE): (**a**) conductivity, (**b**) alkalinity, (**c**) chemical oxygen demand, (**d**) orthophosphate concentration, (**e**) nitrite concentration, (**f**) nitrate concentration.

### *3.2. Mayfly Assemblages*

At the four investigated Mediterranean intermittent rivers, a total of 12 mayfly species was recorded (Table 1). The most widespread species were *Nigrobaetis niger* and *Centroptilum luteolum*, recorded at three out of four rivers, while the most abundant was *Baetis rhodani* (Table 1). Although seven of the recorded species occurred in a single river, *Caenis luctuosa* was recorded in the lowest abundance at the Miljašić Jaruga River (Table 1).

Species richness was lowest at the Krčić River (with three species), while both Čikola and Guduča rivers had a total of six species (Table 1). However, species richness was significantly higher at the Čikola River than at the Miljašić Jaruga River (Kruskal–Wallis H test, df = 3, $n$ = 48; H = 11.59, $p < 0.01$, Figure 4a). Mayfly abundance did not differ significantly among the rivers (H = 7.28, $p > 0.05$), but it ranged from 431.7 individuals per square meter in the Miljašić Jaruga River to as many as 1888.7 individuals per square meter in the Krčić River (Figure 4b). Both diversity indices differed significantly among the four rivers, with the Čikola and Guduča rivers exhibiting higher values compared with the Krčić River (Shannon diversity index, H = 15.06, $p < 0.05$, Figure 4c; Simpson diversity index, H = 15.67, $p = 0.001$, Figure 4d).

**Table 1.** Mayfly species and their abundance (number of individuals per m$^2$) recorded at four intermittent rivers in the karst Mediterranean region of the Balkans, Croatia. Species codes are used in the CCA.

| Mayfly Species/ River | Species Codes | Krčić | Čikola | Guduča | Miljašić Jaruga |
|---|---|---|---|---|---|
| *Baetis liebenauae* Keffermüller, 1974 | *B lie* | | 42.3 | | 346.7 |
| *Baetis rhodani* (Pictet, 1843) | *B rho* | 1872.7 | | 198.7 | |
| *Nigrobaetis niger* (Linnaeus, 1761) | *N nig* | 1.3 | 61.2 | 210.7 | |
| *Centroptilum luteolum* Müller, 1776 | *C lut* | | 93.8 | 20.0 | 4.0 |
| *Procloeon pennulatum* (Eaton, 1870) | *P penn* | | 208.0 | | |
| *Serratella ignita* (Poda, 1761) | *S ign* | | 87.2 | 36.0 | |
| *Caenis horaria* (Linnaeus, 1758) | *C hor* | | 35.7 | | |
| *Caenis luctuosa* (Burmeister, 1839) | *C luc* | | | | 2.3 |
| *Habrophlebia lauta* Eaton, 1884 | *H lau* | | | 32.0 | |
| *Paraleptophlebia werneri* Ulmer, 1919 | *P wer* | | | | 78.7 |
| *Ecdyonurus starmachi* Sowa, 1971 | *E sta* | | | 217.3 | |
| *Rhithrogena braaschi* Jacob, 1974 | *R bra* | 14.7 | | | |
| Abundance (ind/m$^2$) | | 1888.7 | 528.2 | 714.7 | 431.7 |
| Species richness | | 3 | 6 | 6 | 4 |

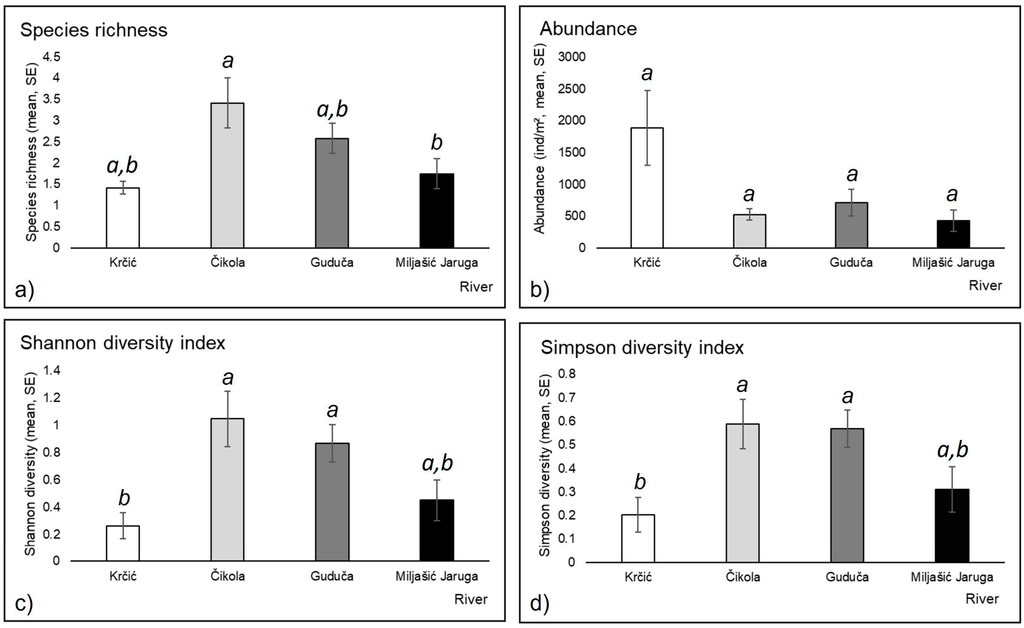


**Figure 4.** Mayfly assemblage metrics at four intermittent rivers in the karst Mediterranean region of the Balkans, shown as mean with standard error (SE): (**a**) species richness, (**b**) abundance (individuals per m$^2$), (**c**) Shannon, and (**d**) Simpson diversity indices.

The HCA revealed low similarity among the investigated rivers at less than 10% (Figure 5), i.e., most rivers harbor unique mayfly assemblages. The Miljašić Jaruga River differed the most from the other three rivers (only 5% similarity) based on its mayfly assemblages (Figure 5). The spring area of the Guduča river (study site 1) was grouped with the Krčić River (with 45% similarity), while the two downstream sites were grouped with the Čikola River (with 30% similarity) (Figure 5).

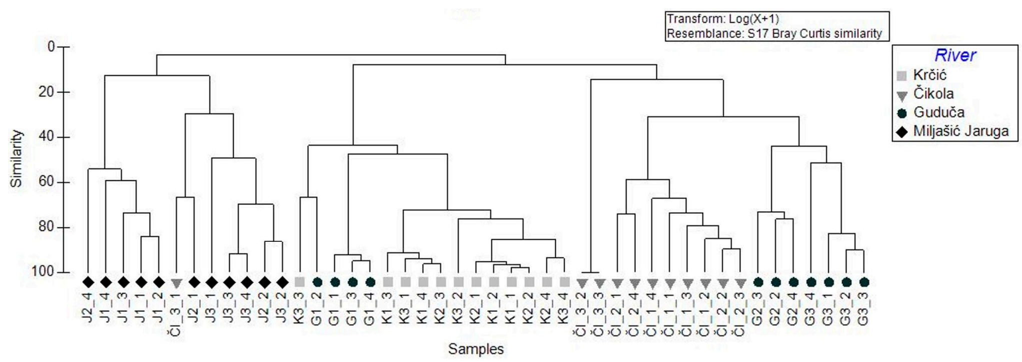


**Figure 5.** Cluster analysis showing similarity of mayfly assemblages among the four intermittent rivers in the karst Mediterranean region of the Balkans, Croatia. The analysis is based on Bray–Curtis similarity coefficient and species' log (x + 1)-transformed abundances. Study sites without mayfly records were not included in the analysis.

### *3.3. Mayfly Assemblages and Environmental Variables*

The F1 × F2 ordination plot shows the ordination of the species and environmental data included in the CCA (Figure 6). The eigenvalues for the first two CCA axes were 0.84 and 0.62, explaining 37.5% of the species–environment relationships. According to the Monte Carlo permutation test, the species–environment ordination is significant (first axis: F-ratio = 9.68, $p = 0.002$; overall: trace = 2.32, F = 5.77, $p = 0.002$), which indicates a significant relation of mayfly assemblages to the environmental variables examined. Axis 1 was associated with conductivity (R = −0.78) and water velocity (R = 0.71), and Axis 2 was associated with dissolved oxygen concentration (R = 0.56), indicating that these were the most important parameters in explaining mayfly assemblage patterns (Figure 6).

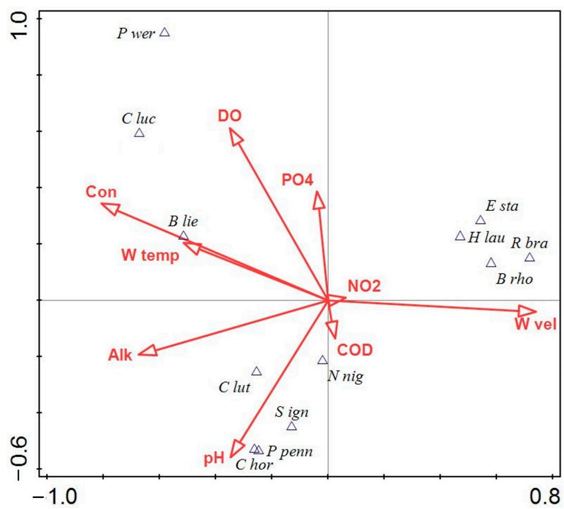


**Figure 6.** F1 × F2 plane of the Canonical correspondence analysis (CCA) based on 12 mayfly species and nine environmental variables. Abbreviations of the species codes (black triangle symbols) are in Table 1. Legend: Environmental variables (red arrow symbols): W temp—water temperature (°C), W vel—water velocity (m/s), DO—dissolved oxygen content (mg/L), Con—conductivity (µS/cm), pH—pH, NO2—nitrite concentration (mgN/L), PO4—orthophosphates (mgP/L), COD—chemical oxygen demand (mg$O_2$/L), Alk—alkalinity (mg $CaCO_3$/L).

### *3.4. Mayfly Species and Environmental Variables*

*Baetis liebenauae* abundance showed positive correlations with conductivity (R = 0.44, $p < 0.01$) and alkalinity (R = 0.31, $p < 0.05$) and negative correlations with water velocity (R = −0.55, $p < 0.001$) and nitrates (R = −0.30, $p < 0.05$). *Baetis rhodani* abundance was positively correlated with water velocity (R = 0.62, $p < 0.001$), while negative correlations were documented for conductivity (R = −0.69, $p < 0.001$), alkalinity (R = −0.59, $p < 0.001$), water temperature (R = −0.49, $p < 0.001$), and nitrites (R = −0.38, $p < 0.01$). Positive correlations were recorded between *C. luteolum* abundance and water depth (R = 0.37, $p = 0.01$), alkalinity (R = 0.32, $p < 0.05$), and chemical oxygen demand (R = 0.29, $p < 0.05$), while negative correlations were recorded with water velocity (R = −0.34, $p < 0.05$) and nitrate concentration (R = −0.32, $p < 0.05$). *Serratella ignita* abundance was positively correlated with water depth (R = 0.29, $p < 0.05$), and alkalinity (R = 0.32, $p < 0.05$) while negative correlations with dissolved oxygen (R = −0.27, $p < 0.05$) and phosphate concentration in the water were found (R = −0.32, $p < 0.05$). Positive correlations were found between the abundance of *Caenis horaria* and water depth (R = 0.31, $p < 0.05$) and pH (R = 0.29, $p < 0.05$), while a negative correlation with phosphate concentration in the water was found (R = −0.45, $p = 0.001$). The abundance of *Habrophlebia lauta* was positively correlated with the concentration of nitrites in the water (R = 0.36, $p = 0.01$), water velocity (R = 0.30, $p < 0.05$) and chemical oxygen demand (R = 0.30, $p < 0.05$) while a negative correlation was recorded with the concentration of dissolved oxygen (R = −0.45, $p = 0.001$) and pH (R = −0.39, $p < 0.01$). Positive correlations were recorded between the abundance of *Ecdyonurus starmachi* and the concentration of nitrites in the water (R = 0.37, $p = 0.01$) and water velocity (R = 0.35, $p < 0.05$), while negative correlations were recorded with the concentration of dissolved oxygen (R = −0.51, $p < 0.001$) and pH (R = −0.47, $p < 0.001$). *Rhithrogena braaschi* abundance was negatively correlated with conductivity (R = −0.39, $p < 0.01$), the concentration of nitrites in the water (R = −0.37, $p = 0.01$), water temperature (R = −0.36, $p = 0.01$), chemical oxygen demand (R = 0.−31, $p < 0.05$), and alkalinity (R = −0.31, $p < 0.05$).

Correlations of abundances of *N. niger*, *P. pennulatum*, and *P. werneri* with physicochemical water parameters are shown in [41].

### 3.5. Mayflies and Microhabitats

Mayfly assemblage metrics (abundance, species richness, Shannon, and Simpson diversity) did not differ significantly among the investigated microhabitats (Kruskal–Wallis H test, Spearman correlation differ coefficient $p > 0.5$).

Among the identified species, significant results for substrate selection were found only for *B. rhodani* (Kruskal–Wallis H test, H (df = 7, N = 24) = 13.68, $p < 0.05$). Pairwise

Comparisons of mean ranks showed a significant difference in the species' abundance between mosses and angiosperms, *B. rhodani* being most abundant in microhabitats with mosses. *Baetis liebenauae* abundance negatively correlated with water velocity in the present microhabitats (i.e., it showed preference for microhabitats with lower water velocity) (R = −0.46, $p < 0.05$), similar to *P. pennulatum* (R = −0.61, $p < 0.05$) and *S. ignita* (R = −0.48, $p = 0.01$). *Centroptilum luteolum* abundance positively correlated with water depth in the present microhabitats (i.e., it showed preference for microhabitats with greater water depth) (R = 0.41, $p = 0.01$).

## 4. Discussion

In the investigated intermittent rivers in the karst Mediterranean region of the Balkans, we recorded fourteen percent of Croatian mayflies [50], which can be considered a rather high species richness for such hydrologically extreme freshwater habitats [51]. Nevertheless, mayfly species richness per river was low, between three and six species, with each river harboring unique mayfly assemblages. Such results suggest a high influence of the hydrological and habitat characteristics of each river, in line with the specific habitat requirements of individual mayfly species [23,52–54].

A higher species richness and a greater diversity of mayflies were recorded at the Čikola and Gudušča rivers compared with the other two rivers. This is likely due to a higher habitat heterogeneity, both in different river reaches and different sections of the study sites, that are characterized by segments with both slower and faster water currents, and a variety of microhabitats. Consequently, these rivers were inhabited by species with different flow preferences. This is best illustrated by the presence of either rheo-to limnophile (e.g., *B. liebenauae*, *N. niger*, *S. ignita*, *P. pennulatum*) or limno- to rheophile species (e.g., *E. starmachi*, *H. lauta*), known to inhabit both slow flowing streams and stagnant waters [52–55]. In addition, a higher water velocity in the Guduča River compared with the Čikola River allows the occurrence of some rheophile species (e.g., *B. rhodani*) in the former, while the latter is inhabited by some true limnophiles (*C. luteolum*, *C. horaria*) [52–54]. The lowest species richness recorded in the Krčić River could be related to the uniformity of microhabitats and water parameters along its relatively short course.

However, the highest mayfly abundance was recorded in the Krčić River, due to the dominance of a rheophile generalist, *B. rhodani* [52–54]. In contrast, the Miljašić Jaruga River exhibited the lowest abundance and greatest difference in mayfly assemblages from the other three rivers, most likely due to the comparatively higher level of anthropogenic impacts on this river. This river is, along its entire course, a channel type, hydromorphologically altered habitat, with a slow water current, and high amounts of muddy sediment, detritus, and xylal (plant debris). It is also characterized by densely developed algae and emergent aquatic vegetation. Additionally, a high share of the land-cover classes surrounding this river are of anthropogenic origin [42]. Therefore, an interplay of anthropogenic disturbances, i.e., water pollution and hydromorphological alterations along the river's course, might have negatively impacted mayfly assemblages inhabiting this river. This is supported by previous studies which show that many mayfly species disappear rapidly when faced with habitat alteration and pollution [51,56–58].

*Centroptilum luteolum* and *N. niger* were the most widespread species, recorded at three rivers each. Limnophile *C. luteolum* [52–54], a species found primarily in lentic habitats and slow flowing streams with moderate and warm water temperatures (>10 °C) [52–54], was absent from the Krčić River, most probably due to the low water temperature and the river's fast current, without sections with pools and lower water velocity. Rheo- to limnophile *N. niger* [52–54] was not recorded at the Miljašić Jaruga River. This is most probably the result of unfavorable habitat conditions (e.g., low water velocity, muddy substrate, altered morphology), as this species was most commonly recorded in the rhithralic parts of running waters [23,24,59]. Nevertheless, this river provided habitat for the rarest species recorded in the study area, *C. luctuosa*. In previous studies, this species has been reported from anthropogenic lotic and lentic waterbodies in the region [29,30], indicating that anthropogenically modified and channel-type habitats could be suitable for this species.

The most interesting mayfly record in the investigated intermittent rivers was rheophile *Rh. braaschi* [52–54], a Balkan endemic previously reported by [60], also from the Krčić River. Similar to other *Rhithrogena* species from the *semicolorata* group, this species is considered to be typical for fast flowing perennial lotic habitats [13,23,61]. The unexpected presence of *Rh. braaschi* in the Krčić River is most probably due to the suitably high water velocity and high share of the species' preferable microhabitat, lithal [23,26]. A certain flexibility, i.e., variations in emergence period duration [26,62], could provide it some advantage in such hydrologically extreme habitats. Nevertheless, the species' life cycle and survival strategies in intermittent rivers should be inspected in more detail in future studies, as its biology and ecology are still insufficiently investigated [23]. In addition, within this research we recorded several species rare in Croatian freshwater habitats, *N. niger*, *P. pennulatum*, and *P. werneri* [41], indicating the potential conservation value of the studied intermittent rivers.

Conductivity, water velocity, and dissolved oxygen concentration in water were the main environmental variables structuring the composition of mayfly assemblages in intermittent karst Mediterranean rivers, similar to the results observed for perennial lotic habitats [25,58,63]. Mayfly assemblages were more diverse in rivers with lower conductivity, higher water velocity, and higher dissolved oxygen concentrations in the water. The Miljašić Jaruga River, characterized by various anthropogenic pressures (e.g., hydromorphological alteration, surrounding land-use) [42] had the highest conductivity values among the studied intermittent rivers. This is most probably related to substrate composition encompassing mainly fine sediments (e.g., mud, detritus), but also to the inflow of nutrients from the surrounding agricultural fields. The high sensitivity of mayflies to morphological and physico-chemical habitat alteration (see above) [29,57,58] is probably responsible for the negative response to the increased conductivity values in the study area. On the other hand, mayfly assemblages showed a positive response to higher values of water velocity and dissolved oxygen concentration in the water, in accordance with the biology of most of the recorded species [23,52–54]. Mayflies occur in both running and standing waters, although a greater number of species prefer lotic habitats [23,59]. In addition, most mayfly species require well-oxygenated water [23,25,58], which is often associated with a higher water velocity in running freshwater habitats.

Associations with abiotic environmental variables were also observed at the species level, e.g., the occurrence of *B. liebenauae* and *P. werneri* [41] was positively correlated with conductivity; these species were most abundant or present only at the Miljašić Jaruga River. This could be related to their preferences for substrate type, i.e., *B. liebenauae* was previously reported mostly from macrophytes [52–54], which are very well developed in this river. *Paraleptophlebia werneri* was previously found on fine sediment and particulate organic matter, which are among the dominant substrates in the Miljašić Jaruga River [52–54]. On the other hand, rheophile *B. rhodani* and *Rh. braaschi* [52–54], negatively associated with conductivity, were mostly or exclusively present at the Krčić River, characterized by fast water current and an abundance of lithal substrates [52–54]. Rheophile *B. rhodani* was associated with habitats with higher water velocities, while limnophile *C. luteolum* and rheo- to limnophile *B. liebenauae* were most abundant in habitats with a lower water velocity, which is in line with their preference for water current [52–54]. Species' associations with dissolved oxygen concentrations in water were most probably related to their preferences for some other environmental variable. For instance, the association of rheo-to limnophile *S. ignita* and limno- to rheophile *H. lauta* and *E. starmachi* with rivers/sites characterized by lower concentrations of dissolved oxygen in water, could be related to their preference for slow flowing habitats [52–54], such as the sites (at the Čikola and/or Guduča rivers) where they were recorded. Similarly, as reported by [41], rheo- to limnophile *P. pennulatum* was most abundant at sites (at the Čikola River) with lower concentrations of dissolved oxygen which could also be related to its preference for slow flowing streams or standing waters with warm water ($\geq$18 °C) and well-developed aquatic vegetation, the species' preferred substrate type [41,52–54]. As shown by [41], *P. werneri* was associated with higher values of dissolved oxygen in water, as it was recorded only at the well-oxygenated Miljašić Jaruga River, which could be related to its preference for fine sediment substrates that were abundant in this river.

We also found some inconsistencies, such as the preference of limno- to rheophile *H. lauta* and *E. starmachi* [52–54] to habitats (at the Guduča River) with higher water velocity and rheophile *P. werneri* [52–54] to habitats (at the Miljašić Jaruga River) with a lower water velocity [41]. The recorded inconsistencies are most probably influenced by the preferences of those species for some other environmental variable, such as the substrate composition or food availability. Moreover, the ecological preferences of *P. werneri* and *P. pennulatum* in karst Mediterranean lotic habitats are still rather scarce and should, together with the previously mentioned inconsistencies, be inspected in more detail in future studies.

At the studied intermittent rivers, mayfly assemblages and most constituent species did not show a clear preference for a particular substrate type (i.e., they were found on different substrates), but rather showed associations with water depth and/or flow velocity in the available microhabitats, in line with previous studies showing higher preferences for abiotic microhabitat parameters [58]. Only *B. rhodani* showed a preference for microhabitats with mosses, consistent with its known preferences for phytal and lithal microhabitats [52–55]. The generalist *S. ignita*, as well as *B. liebenauaue* and *P. pennulatum*, showed a preference for microhabitats with a lower water velocity. This is in accordance with the lack of the substrate preference, in the case of *S. ignita*, or the preference of the other two species for microhabitats with phytal substrate (angiosperms) [52–54], often associated with lower water velocity. The generalist *C. luteolum* was most abundant at microhabitats with a higher water depth (associated with lower water velocity), which can be explained by its preference for lentic habitats [52–54]. Together with abiotic water properties, microhabitat heterogeneity represents an important factor in shaping mayfly assemblages [27,64]. Various microhabitats provide mayfly nymphs with both food resources and refuge from predators [23,65].

## 5. Conclusions

Our study shows that although a rather high number of mayfly species can inhabit intermittent rivers in the studied region, species richness per river is low, and is highly dependable on river morphology, the physico-chemical environment, and anthropogenic pressures. Some endemic or locally rare mayfly species were documented, indicating the potential conservation value of the studied intermittent rivers. The results presented here contribute to our knowledge of the habitat preferences and ecology of aquatic insects in karst Mediterranean freshwater habitats in the Balkans with extreme hydrological conditions. Therefore, our results could contribute to the mayfly species protection in karst Mediterranean intermittent freshwater habitats, as well as to the development of conservation measures for these threatened ecosystems.

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