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Mayfly ecological traits in a European karst spring: species, microhabitats and life histories

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Key words: Life cycle, Low species richness, Microhabitats, New species data.

Abstract: Despite the recent increase in the number of mayfly studies in karst freshwater habitats, their biology and ecology in springs are still poorly characterized. Therefore, we studied mayfly assemblages in a European karst rheocrene spring at five microhabitats monthly over a one-year period. Three species were recorded: *Baetis alpinus* (Pictet, 1843), *Baetis rhodani* (Pictet, 1843) and *Rhithrogena braaschi* (Jacob, 1974). The latter species represents a new record for the fauna of Bosnia and Herzegovina. All three species inhabited all studied microhabitats but with varying abundance. Individual species were associated with a specific substrate type and/or water velocity and/or water depth. The grazer/scraper *Rh. braaschi* was most common at microhabitats with inorganic substrate (cobbles, mixture of pebbles and sand), moderate water velocity and higher water depth. The rheophilic grazer/scraper and gatherer/collector *B. alpinus* was most common at microhabitats with mosses and highest water velocity. The grazer/scraper and gatherer/collector *B. rhodani* was recorded at all microhabitats, yet due to its preference for moderate water velocity, the highest number of individuals were collected from cobbles. We recorded movements of mayfly nymphs among the available microhabitats during their life cycles, due likely to their dietary requirements and search for suitable refugia. *Baetis alpinus* has a bivoltine, *B. rhodani* polyvoltine and *Rh. braaschi* univoltine life cycle with a long emergence period. The results presented here contribute to the knowledge of spring and mayfly ecology.

Abbreviations: HCA–Hierarchical cluster analysis, NMDS–Non-metric multidimensional scaling, UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

Nomenclature: Bauernfeind and Soldán (2012).

Introduction

Springs are isolated freshwater habitats formed at places where groundwater wells to the land surface. These complex ecotones are characterized by the interaction of groundwater, surface water and terrestrial ecosystems, resulting in stable environmental conditions and high heterogeneity of the aquatic, semi-aquatic and terrestrial microhabitats (e.g., Cantonati and Ortler 1998, Barquín and Scarsbrook 2008, Kløve et al. 2011). Due to thermal constancy, relative hydrological and physicochemical stability of spring systems, many biological processes (e.g., life histories, evolutional processes, microhabitat preferences, behaviour, biogeographical traits) can be studied under naturally controlled conditions. These conditions are also repeatable in the laboratory, making springs natural laboratories (Odum 1957, Likens 2010). Many studies showed that springs harbour a unique and diverse aquatic fauna, and therefore could be considered as important freshwater biodiversity hotspots (Ferrington 1995, Smith et al. 2003, Cantonati et al. 2006, Staudacher and Füreder 2007). Humans have historically benefited from springs, given their considerable cultural significance (Scarsbrook et al. 2007), but even more importantly, they represent a crucial source of drinking water. Yet, due to their isolation and small dimensions, spring habitats are particularly sensitive and endangered by numerous anthropogenic activities, such as water abstraction, sedimentation, removal of the surrounding vegetation, nutrient deposition and the effects of climate change (Cantonati et al. 2006). Despite the peculiarity, high ecological value and biological complexity of these habitats, springs have not been sufficiently included in freshwater ecological studies.

This study was conducted in a rheocrene spring located in the largest continuous karst landscape in Europe, the Dinaric Karst, extending over approximately 60,000 km^2 (Mihevc et al. 2010). The karst landscape is formed by a complex of morphological, hydrological and hydrogeological terrain features shaped by water soluble rock. Due to the specificity of karst geology and hydrology (e.g., specific physical, chemical, thermal conditions, distinctive water circulation and storage both on the surface and underground), the inhabiting organisms had to adapt to survive in such a challenging environment (Bonacci 2009). Therefore, Dinaric Karst habitats have been recognized as biodiversity hotspots, with high levels of endemism (e.g., Bonacci 2009, Ivković and Plant 2015, Previšić et al. 2014). However, many of these habitats and their biota remain insufficiently investigated and a potential and interesting subject of research.

Mayflies are an aquatic insect order widely used as indicators in bio-monitoring assessments (e.g., Lenat and Penrose 1996, Ferro and Sites 2007). They represent a large proportion of the aquatic ecosystems biomass, contributing up to 25% of total zoobenthos production (Elliott et al. 1988). Due to changes in environmental conditions (i.e., habitat morphology, substrate type, physical and chemical water properties), the composition and structure of mayfly assemblages change downstream in the lotic habitats (e.g., Moog and Hartmann 2017, Vilenica et al. 2016a, b, 2017a, b). The most diverse assemblages are typical for the upper reaches of fast flowing streams and rivers, while springs and high mountain habitats usually have low diversity (Bauernfeind and Soldán 2012). Systematic studies providing detailed data on mayfly ecological traits, such as life cycles, microhabitat, habitat and environmental preferences, are critical for understanding freshwater ecosystem functioning (e.g., Brittain 1991, Raddum and Fjellheim 1993, Erba et al. 2003). Despite the recent increase in research examining mayfly ecology in karst freshwater habitats (Vilenica et al. 2014, 2016ab, 2017ab, Petrović et al. 2015), their assemblages in springs remained poorly characterized (Savić et al. 2016). Therefore, rare, unusual, new and taxonomically interesting findings are expected.

The main goal of this study was to fill the existing knowledge gap concerning the ecological traits of mayflies in Southeast Europe. The research questions asked were: a) What are the composition and structure (longitudinal and trophic) of mayfly assemblages at different microhabitats? b) Which are the preferred microhabitats (i.e., substrate type, water depth and velocity) for the recorded species? c) Do microhabitat preferences change during the life cycle? and d) What are the mayfly life cycles in the investigated karst spring?

Material and methods

Study area

The Bistrica River is a typical watercourse in the deep and well-developed Dinaric Karst. The river flows over three km through Southwestern Bosnia and Herzegovina, through the largest karst field or *polje*, Livanjsko polje. The rheocrene spring of the river is located in the town of Livno (N 43°49’55.76”; E 17°00’31.21”, altitude 777 m) on the slopes of the Bašajkovac hill. The spring is partially encased in concrete, and several houses stand near the spring (see Fig. 1) (Malez 1963, Magdalenić 1971). The climate is transitive from sub-Mediterranean to continental. The mean annual air temperature during the study period was 10.4°C (mean minimum 4.2°C; mean maximum 17.1°C), and the annual rainfall was 1090.7 mm (FHMZ BIH 2013).

Mayfly sampling

Mayfly nymphs were collected together with other macroinvertebrates on a monthly basis in the period from September 2007 to August 2008. Five dominant microhabitats were recognized and sampled (with a share of at least 5% coverage): cobbles (microlithal) (M1), mixture of pebbles and sand (akal) (M2), mosses on macrolithal (M3), mosses on technolithal (M4) and mosses on mesolithal (cobbles) (M5). Inorganic substrate categories were defined using the Wentworth scale (Wentworth 1922).

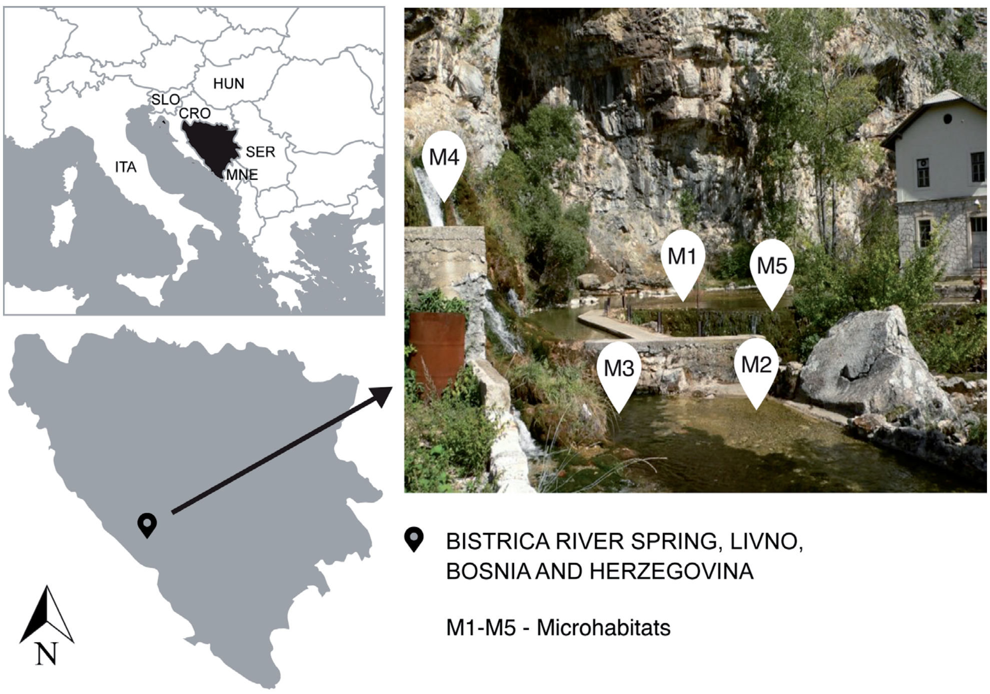


Figure 1. Geographical position of the Bistrica River Spring, located in Livno, Bosnia and Herzegovina, with sampled microhabitats. Legend: M1: cobbles (microlithal), M2: mixture of pebbles and sand (akal), M3: mosses on macrolithal, M4: mosses on technolithal, M5: mosses on mesolithal (cobbles).

Samples were collected using Surber samplers (mesh size: 0.5 mm; surface area 14 cm × 14 cm at microhabitat with mosses on technolithal (M4) (due to very high macroinvertebrate abundance) and 25 cm × 25 cm at all other microhabitats). Samples were preserved in 80% ethanol. Abundance was expressed as individuals m^{-2} to allow comparison of different sized samples.

Mayflies were identified to the lowest possible taxonomical level (depending on the nymphal stage; i.e., very small individuals of the *Baetis* genus were identified only to the genus level) using Müller-Liebenau (1969), Jacob (1974) and Bauernfeind and Humpesch (2001). After identification, total nymphal body length without cerci and antennae was measured using the micrometre on a dissecting stereomicroscope (Stemi 2000-C, Carl-Zeiss). A total of 4436 mayfly nymphs were measured. All voucher specimens are deposited at the Department of Biology, Faculty of Science, University of Zagreb, Croatia.

Abiotic parameters

Measurement of abiotic parameters was determined by two important facts: the small size of the studied spring and the fact that the spring is a protected water area. Therefore, the study was conducted in an 8 m transect, always at the same spot in the spring. Additionally, during each sampling event (monthly), all parameters were measured at the same time of day. Preliminary study indicated that physicochemical parameters, except water depth and water velocity, did not differ along the transect. Hence, at each sampling event, the following physicochemical water properties were measured (Table 1): water temperature, oxygen concentration, oxygen saturation (using the oximeter WTW Oxi 330/SET), pH (using the pH-meter WTW pH 330), conductivity (with the conductivity meter WTW LF 330), alkalinity (by titration with 0.1 M HCl) and nutrients (ammonium by HRN ISO 70-3:1998 method, nitrates by HRN ISO 7890-3:2001 method and orthophosphates by HRN ISO 6878:2001 method). Additionally, at each microhabitat, water velocity (with P-670-M velocimeter; Dostmann electronic) and water depth (with handheld meter) were measured.

Data analysis

One-way ANOVA test with the Tukey HSD *post hoc* test was used to identify differences in water velocity and depth between microhabitats.

Hierarchical cluster analysis (HCA - UPGMA) and non-metric multidimensional scaling (NMDS) ordination based on a Bray-Curtis similarity matrix were used to examine variability in mayfly assemblage composition among microhabitats. Prior to analysis, data were log transformed.

The composition of mayfly assemblages in terms of trophic structure and longitudinal zonal associations of species at various microhabitats was analysed using the classification given by Moog and Hartmann (2017), combined with Bauernfeind and Soldán (2012).

The non-parametric Kruskal-Wallis H test, followed by the multiple-comparison *post hoc* test, was used to determine differences among microhabitats in species richness, total abundance and the abundance of individual mayfly species.

Spearman's rank correlation coefficient was used to assess the correlation of species richness, total abundance and the abundance of individual species with water velocity and water depth.

Mayfly life cycles were analysed by grouping the nymphs into 1 mm body size classes and observing dark wing pads for mature nymphs.

Bray-Curtis similarity index, HCA and NMDS analyses were conducted in Primer 6 (Clarke and Gorley 2006). One-way ANOVA, Kruskal-Wallis H test and Spearman's rank correlation coefficient were calculated in Statistica 13.0 (Dell Inc. 2016). All figures were processed with Adobe Illustrator CS6.

Results

Abiotic parameters

Most physicochemical water properties showed relatively small oscillations during the study period (Table 1). Ammonia values were elevated between February and July compared to the rest of the study period.

Water velocity (one-way ANOVA; $F = 30.15$, $df = 4.56$, $p < 0.001$) and water depth ($F = 25.27$, $df = 4.56$, $p < 0.001$) differed among microhabitats in the spring.

Microhabitats with mosses had higher water velocity compared to the microhabitat with cobbles (microlithal; M1) (Tukey HSD *post hoc* test; mosses on macrolithal (M3; $p < 0.01$), mosses on technolithal (M4; $p < 0.001$), mosses on mesolithal (cobbles) (M5; $p < 0.001$)), and compared to the microhabitat with a mixture of pebbles and sand (akal; M2) (Tukey HSD *post hoc* test; mosses on macrolithal (M3; $p < 0.001$), mosses on technolithal (M4; $p < 0.001$), mosses on mesolithal (cobbles) (M5; $p < 0.001$)). Among microhabitats with mosses, velocity was higher on mosses on technolithal (M4) compared to mosses on macrolithal (M3; $p < 0.01$). Overall, mosses on technolithal (M4) had the highest water velocity, while the mixture of pebbles and sand (akal; M2) had the lowest velocity (Fig. 2a).

Depth was greater at the cobbles (microlithal; M1) microhabitat compared to other microhabitats (Tukey HSD *post hoc* tests; mosses on mesolithal (cobbles) (M5; $p < 0.001$), mosses on macrolithal (M3; $p < 0.01$), mosses on technolithal (M4; $p < 0.05$) and mixture of pebbles and sand (akal; M2; $p < 0.05$)). Furthermore, mosses on mesolithal (cobbles; M5) had lesser depth compared to mosses on macrolithal (M3; $p < 0.001$), mosses on technolithal (M4; $p < 0.001$) and a mixture of pebbles and sand (akal) (M2; $p < 0.001$). Overall, the microhabitat with a mixture of pebbles and sand (akal; M2) had the greatest depth, while mosses on mesolithal (cobbles; M5) were the shallowest (Fig. 2b).

Table 1. Mean values of physical and chemical water properties at the Bistrica River Spring measured from September 2007 to August 2008.

Abiotic factor / study period	IX 07	X 07	XI 07	XII 07	I 08	II 08	III 08	IV 08	V 08	VI 08	VII 08	VIII 08
Water temperature (°C)	8.8	8.3	7.9	8.0	8.0	8.1	8.0	8.1	9.0	8.2	8.0	8.4
O_2 (mg L^{-1})	12.11	12.45	11.18	11.24	10.98	13.45	11.22	12.57	12.30	11.48	13.24	10.70
O_2 (%)	107.6	110.7	97.2	98.0	114.0	139.2	100.5	109.9	105.7	100.6	119.6	94.2
pH	8.23	7.57	7.86	7.36	8.21	8.09	8.19	8.34	7.00	7.85	8.62	8.53
Conductivity (µS cm^{-1})	368	342	337	365	357	355	357	327	358	360	369	380
Alkalinity ($CaCO_3$ mg L^{-1})	205.2	195.2	180.2	185.2	175.2	180.2	175.2	185.2	155.1	160.1	195.2	175.2
Nitrates (mg L^{-1})	0.15	0.42	0.48	0.60	0.60	0.42	0.59	0.37	0.61	0.61	0.13	0.36
Ammonium (mg L^{-1})	0.005	0.005	0.005	0.005	0.005	0.020	0.015	0.020	0.017	0.017	0.005	0.012
Phosphates (mg L^{-1})	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005
Water velocity (cm s^{-1})	26.2	44.6	21.4	36.4	54.8	41.0	39.4	37.6	49.4	39.4	24.4	33.0
Water depth (cm)	16.6	23.8	18.0	14.2	19.8	23.4	21.2	19.8	20.5	22.6	21.8	16.8

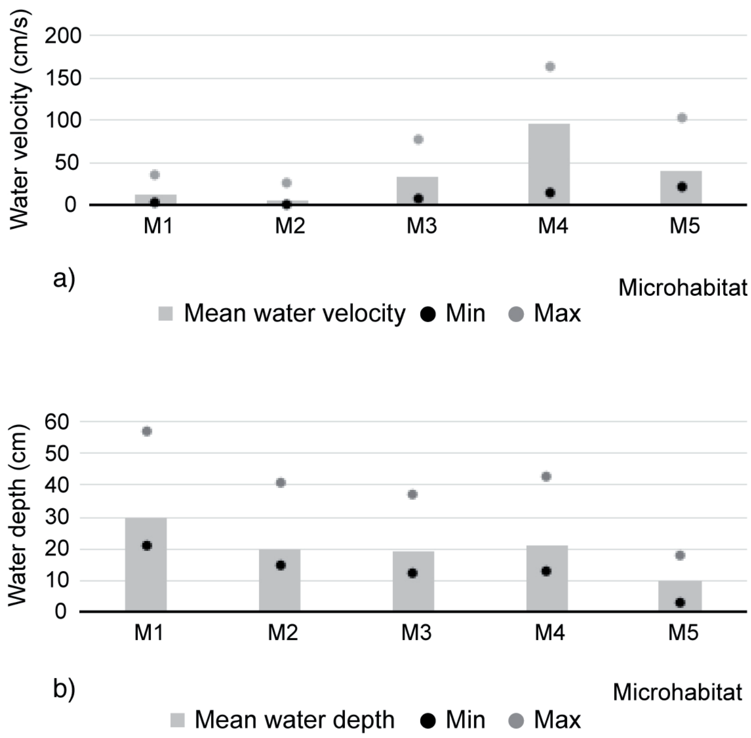


Figure 2. Mean, minimum and maximum values of a) water velocity and b) water depth at different microhabitats in the Bistrica River Spring measured from September 2007 to August 2008. Legend: M1: cobbles (microlithal), M2: mixture of pebbles and sand (akal), M3: mosses on macrolithal, M4: mosses on technolithal, M5: mosses on mesolithal (cobbles).

Mayfly assemblages

We collected and identified a total of 4436 mayfly nymphs belonging to three species: *Baetis alpinus* (Pictet, 1843), *Baetis rhodani* (Pictet, 1843) and *Rhithrogena braaschi* (Jacob, 1974).

NMDS ordination (Fig. 3) showed that mayfly assemblages did not group according to microhabitat type. Species richness and abundance were comparable among microhabitats (Kruskal-Wallis H test, multiple comparisons *post hoc* test, H (2, N = 36) = 3.89, $p > 0.05$). Both *Baetis* species were recorded at all microhabitats, while *Rh. braaschi* was recorded at all microhabitats with the exception of mosses on the technolithal (M4) (Table 2). Species richness correlated negatively with water velocity (Spearman's rank correlation, R = –0.29, $p < 0.05$).

All microhabitats were characterized by the highest share of rhithral elements (species preferring upper and middle reaches), while potamal elements (species preferring lower reaches) were the least represented (Fig. 4a). Compared to microhabitats with mosses, microhabitats with inorganic substrates (cobbles, mixture of pebbles and sand) had a slightly higher percentage of crenal elements (spring loving species). Only grazers/scrapers and gatherers/collectors were present

Table 2. Abundance (N, individuals m^{-2}) and share (%) of mayfly taxa in various microhabitats in the Bistrica River Spring collected from September 2007 to August 2008. Legend: M1: cobbles (microlithal), M2: mixture of pebbles and sand (akal), M3: mosses on macrolithal, M4: mosses on technolithal, M5: mosses on mesolithal (cobbles); indet. - unidentified individuals due to very juvenile stage.

Mayfly taxa / Microhabitat	M1 (N)	M1 %	M2 (N)	M2 %	M3 (N)	M3 %	M4 (N)	M4 %	M5 (N)	M5 %
Baetis sp. indet.	0.0	0.0	0.0	0.0	196.0	36.2	3336.3	43.0	1365.0	64.0
Baetis rhodani (Pictet, 1843)	200.0	21.3	94.7	28.4	56.0	10.4	42.5	0.6	109.3	5.1
Baetis alpinus (Pictet, 1843)	581.3	62.0	72.0	21.6	286.7	53.0	4381.8	56.5	654.8	30.7
Rhithrogena braaschi (Jacob, 1974)	156.0	16.6	166.7	50.0	2.7	0.5	0.0	0.0	2.7	0.1
Σ	937.3		333.3		541.3		7760.5		2131.8	

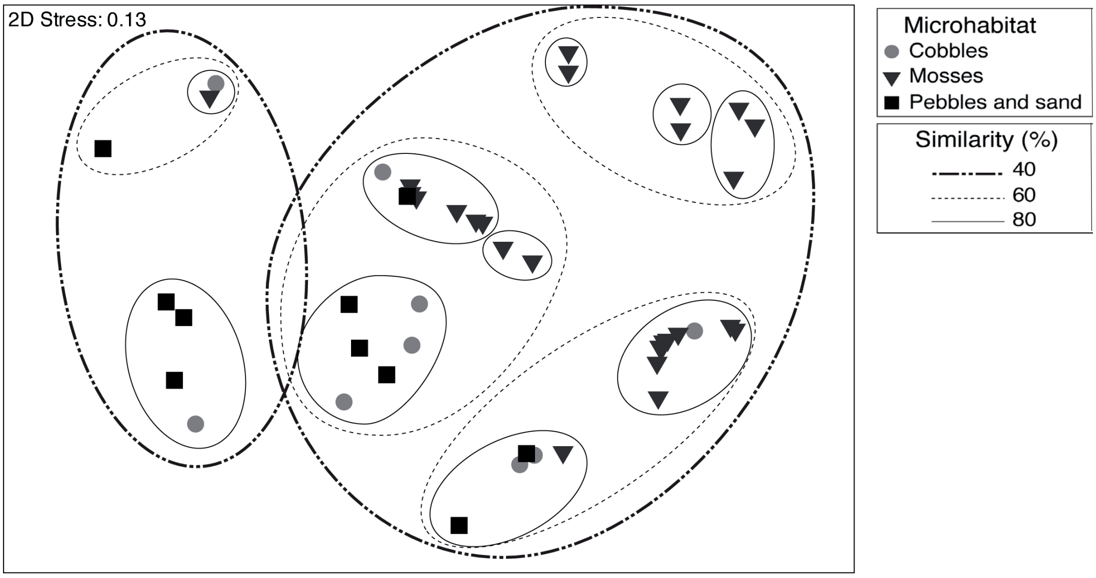


Figure 3. Non-metric multidimensional scaling (NMDS) ordination of mayfly assemblages based on the Bray-Curtis similarity coefficient (clusters obtained by group average linking, UPGMA) and their log-transformed abundances based on monthly samples taken from various microhabitats in the Bistrica River Spring.

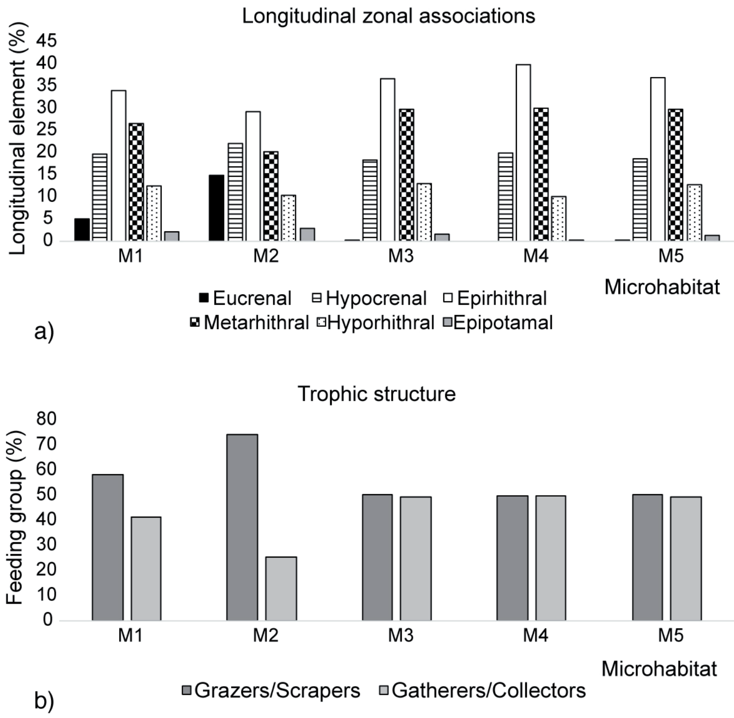


Figure 4. a) Mayfly assemblage structure according to longitudinal zonal associations, and b) trophic structure of mayfly species recorded at five microhabitats in the Bistrica River Spring. Microhabitats: M1: cobbles (microlithal), M2: mixture of pebbles and sand (akal), M3: mosses on macrolithal, M4: mosses on technolithal, M5: mosses on mesolithal (cobbles).

in the spring and were evenly represented at microhabitats with mosses, while microhabitats with inorganic substrates had a higher percentage of grazers/scrapers (Fig. 4b).

Mayfly microhabitat selection

Baetis alpinus was more abundant in mosses compared to mixture of pebbles and sand (Kruskal-Wallis H test, multiple comparisons *post hoc* test, H (2, N = 36) = 7.53, $p < 0.05$), while abundances of *B. rhodani* were comparable among microhabitats (H (2, N = 36) = 0.73, $p > 0.05$) (Table 2). Differences in the abundance of *Rhithrogena braaschi* were significant among microhabitats (H (2, N = 36) = 6.54, $p < 0.05$), though the Multiple comparisons *post hoc* test could not determine which groups differed. Yet, the abundances were markedly higher in microhabitats with inorganic substrates (cobbles, mixture of pebbles and sand) compared to microhabitats with mosses (Table 2).

The abundance of *Baetis alpinus* positively correlated (Spearman correlation coefficient, $\rho = 0.33$, $p < 0.05$) with water velocity, while abundances of *B. rhodani* ($\rho = -0.28$, $p < 0.05$) and *Rh. braaschi* correlated negatively ($\rho = -0.42$, $p < 0.05$). Moreover, abundance of *Rh. braaschi* positively correlated with water depth ($\rho = 0.25$, $p < 0.05$).

Mayfly life cycles

Baetis alpinus was recorded during the entire study period. The highest number of individuals was recorded between September 2007 and January 2008, when the highest number of juvenile nymphs with body length up to 3 mm was also collected. Mature nymphs (with dark wing pads) with body length between 7 and 10 mm were collected in September and October 2007 and between February and August 2008 (Fig. 5a).

Baetis rhodani was collected between September 2007 and January 2008, in March 2008 and between June and August 2008, when both juvenile and mature nymphs (with dark wing pads) were recorded (Fig. 5b).

Rhithrogena braaschi was recorded during the majority of the study period, with the highest number of both juvenile and mature nymphs (with dark wing pads) collected in

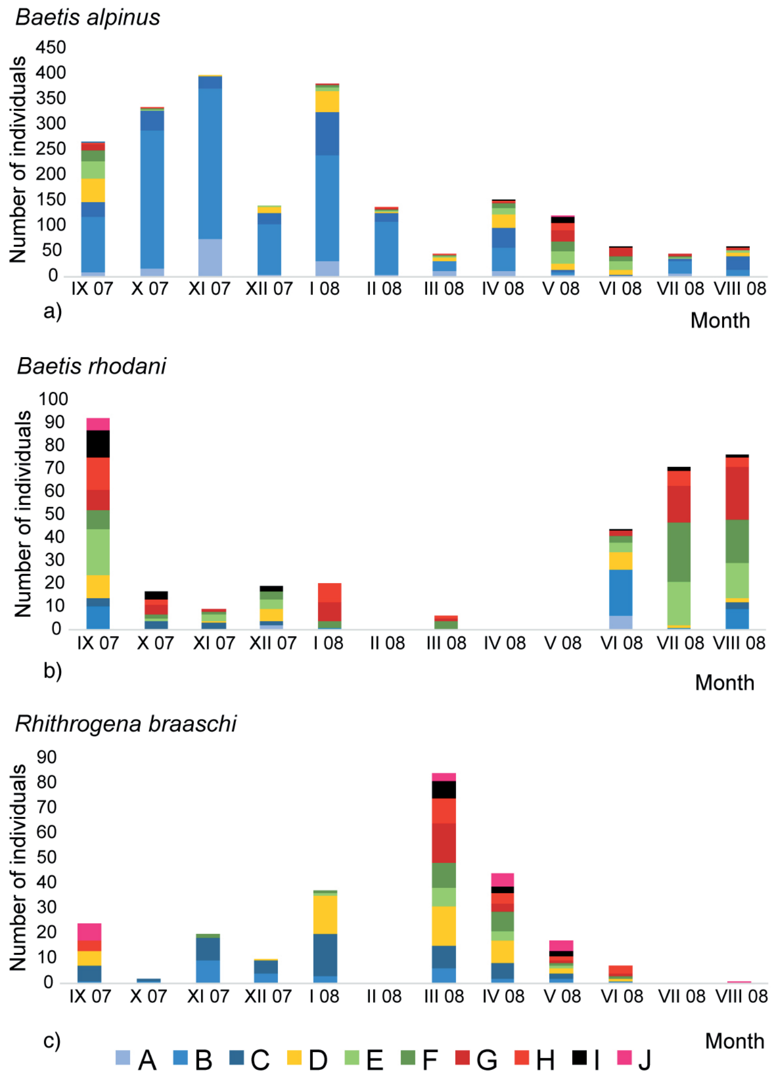
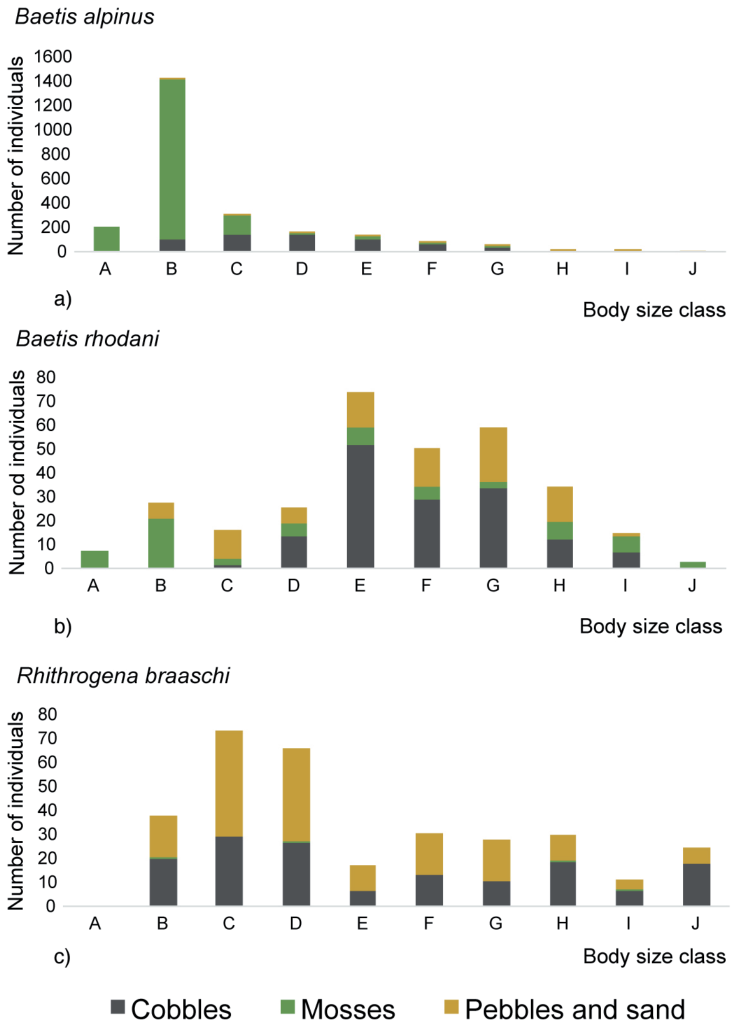


Figure 5. Seasonal dynamics of a) *Baetis alpinus*, b) *Baetis rhodani* and c) *Rhithrogena braaschi* at the Bistrica River Spring from September 2007 to August 2008. Body size classes: A = 0.01–0.99 mm; B = 1.00–1.99 mm; C = 2.00–2.99 mm; D = 3.00–3.99 mm; E = 4.00–4.99 mm; F = 5.00–5.99 mm; G = 6.00–6.99 mm; H = 7.00–7.99 mm; I = 8.00–8.99 mm; J = 9.00–9.99 mm.

Figure 6. Abundances of different nymph size classes at microhabitats of the Bistrica River Spring from September 2007 to August 2008 for a) *Baetis alpinus*, b) *Baetis rhodani* and c) *Rhithrogena braaschi*. Body size classes: A = 0.01–0.99 mm; B = 1.00–1.99 mm; C = 2.00–2.99 mm; D= 3.00–3.99 mm; E = 4.00–4.99 mm; F = 5.00–5.99 mm; G = 6.00–6.99 mm; H = 7.00–7.99 mm; I = 8.00–8.99 mm; J = 9.00–9.99 mm.



March 2008. Juvenile nymphs with body length up to 4 mm were present in most samples except in August 2008. Mature nymphs with body length between 8 and 10 mm were collected during spring and summer, i.e., in September 2007 and between March and August 2008 (Fig. 5c).

Microhabitat selection during the life cycle

Juvenile nymphs (with body length of 1 and 2 mm) of *Baetis alpinus* were most numerous at microhabitats with mosses. Middle size nymphs (with body length between 3 and 6 mm) were most numerous at the microhabitat with cobbles, while the abundances of mature nymphs (with body length between 8 and 10 mm) were comparable between microhabitats on mosses and mixture of pebbles and sand (Fig. 6a).

Juvenile nymphs of *Baetis rhodani* were also most numerous at microhabitats with mosses. Middle sized and mature nymphs (with body length between 3 and 7 mm) were most numerous at the microhabitat with cobbles. The microhabitat with a mixture of pebbles and sand had comparable abundances of nymphs belonging to all size classes. The smallest (body length up to 1 mm) and largest (body length between 9 and 10 mm) individuals were recorded only at microhabitats with mosses (Fig. 6b).

Juvenile and middle-sized nymphs (with body length up to 8 mm) of *Rhithrogena braaschi* were most abundant at the microhabitat with a mixture of pebbles and sand. Mature nymphs (body length between 8 and 10 mm) were mostly recorded from cobbles. Mosses had similar numbers of juvenile and mature nymphs, yet these abundances were very low (Fig. 6c).

Discussion

Mayfly assemblages

With only three recorded species, this study confirmed low species richness of mayfly assemblages in spring habitats (e.g., Bauernfeind and Moog 2000, Bottová and Derka 2013, Vilenica et al. 2014, 2016a, 2017a). Two of these species, *Baetis alpinus* and *B. rhodani,* are widely distributed in Europe, while the studied spring also contains *Rhithrogena braaschi,* which is restricted to the Balkan Peninsula

(Bauernfeind and Soldán 2012). This is also the first record of this species for the fauna of Bosnia and Herzegovina (Bauernfeind and Soldán 2012, Savić et al. 2016). Such low diversity could be a result of the specific environmental factors, such as oligotrophic water quality, high alkalinity, and low and constant water temperature year round (Moog and Hartmann 2017, Harper and Peckarsky 2006, Vilenica et al. 2017a, b). Therefore, the domination of the cold stenotherm *B. alpinus* is not surprising (Buffagni et al. 2017). *Baetis rhodani* is an eurytherm species, while the temperature preferences of *Rh. braaschi* have not yet been systematically studied. However, the species has previously been recorded at similar habitats (Vilenica et al. 2016ab, 2017ab, Buffagni et al. 2017), indicating its preferences for colder water conditions. A high share of rhithral elements in the mayfly assemblage structure is due to the presence of spring and upper reaches loving species, *B. alpinus* and *Rh. braaschi* (Bauernfeind and Soldán 2012, Vilenica et al. 2016 a, b, 2017a, b, Buffagni et al. 2017). The presence of the potamal element is due to the eurytopic *B. rhodani* that inhabits a wide range of freshwater habitats (Bauernfeind and Soldán 2012, Buffagni et al. 2017). Similar composition and structure of mayfly assemblages was recorded in several springs of the Dinaric Karst in neighbouring Croatia (Vilenica et al. 2014, 2016ab, 2017ab). Moreover, such an assemblage structure is in partial agreement with some other studies conducted in European springs [e.g., Mori and Brancelj 2006 (with records of *Baetis melanonyx* (Pictet, 1843)*, B. rhodani, Ecdyonurus picteti* (Meyer-Dür, 1864), *Ecdyonurus zelleri* Eaton, 1885*, Rhithrogena* gr. *semicolorata*), Maiolini et al. 2011 (with records of *B. alpinus, B. rhodani, Ecdyonurus* gr. *helveticus, Rhithrogena* gr. *hybrida, Rh.* gr. *loyolaea* and *Serratella ignita* (Poda, 1971), Bottová and Derka 2013 (with records of B. *alpinus, B. rhodani* and *Rhithrogena semicolorata* (Curtis, 1834)]. However, the results differ markedly from some other studies [e.g., Barquín and Death 2009 (with records of *Baetis* spp., *Ecdyonurus* spp., *Rhithrogena* spp. and *S. ignita*), Savić et al. 2016 (with records of *B. rhodani, Ecdyonurus* sp., *Electrogena* sp., *Habroleptoides confusa* Sartori & Jacob, 1986, *Ephemera danica* Müller, 1764 and *Caenis* sp.)].

High mayfly abundance in the studied spring could be a consequence of anthropogenic impact, i.e., inflow of organic matter into the habitat, which could have enhanced periphyton development providing more food resources for mayfly nymphs. Firstly, the spring is closely surrounded by several houses. Moreover, it is also connected with Blidinje Lake, an anthropogenic reservoir (Radoš 2017) relatively rich in nutrients and organic matter (Ivanković et al. 2011), and its waters drain into the spring. Such high mayfly abundance was also recorded in the anthropogenically impacted Ruda Spring in neighbouring Croatia (Vilenica et al. 2016a), while unimpacted karst springs in the same area had markedly lower abundances (Vilenica et al. 2016a, 2017a).

Microhabitat selection

Freshwater microhabitats are characterized by the combination of substrate type, water velocity, water depth, organic matter deposits, turbulence and hydraulic parameters (Lampert and Sommer 1997, Gordon et al. 2002, Miliša et al. 2006). Mayfly nymphs use a wide range of microhabitats to search for food resources and for their activities (resting, movement, refuge from predators). Habitat specialists depend on certain microhabitats during essential parts of their nymphal stages, e.g., stable substrates for filter-feeders or lithal with epilithic diatoms for grazers (Moog and Hartmann 2017, Schmidt-Kloiber and Hering 2015). The Bistrica River Spring has an open canopy that enables the growth of algae and macrophytes, important food resources for the nymphs. Therefore, similar shares of grazers/scrapers and gatherers/collectors within the assemblages were not surprising. All species were recorded at all available microhabitats, though each species showed a certain level of microhabitat preference. Buffagni et al. (2017) found that feeding strategies and food availability are closely related to microhabitat selection. Moreover, although all three of the recorded species are rheophilic, they were confined to microhabitats with different water velocity, likely in relation to food availability. *Rhithrogena braaschi* is a grazer/scraper that feeds on periphyton overgrowth on stony substrates (Bauernfeind and Soldán 2012, Buffagni et al. 2017), which is why it was most numerous at inorganic substrates characterized with lower water velocity and higher water depth compared to microhabitats with mosses. Both *Baetis* species can feed by grazing/scraping or gathering/collecting using both inorganic substrates overgrown by periphyton and mosses with trapped organic matter as food resources (Bouchard 2004, Buffagni et al. 2017). Thus, *B. alpinus* was mainly collected from microhabitats with mosses associated with the highest water velocity that brings the required quantities of organic matter into the microhabitat (Ciborowski 1983). These results only partially corroborate previous studies where the species was recorded at microhabitats with megalithal and macrolithal (Buffagni et al. 2017). Our results could be related to interspecific competition with the other two species at microhabitats with lithal substrates. The eurytopic *B. rhodani* inhabits a wide range of microhabitats (Buffagni et al. 2017), which was also the case in our study. Yet, as the species was associated with moderate water velocity, it was somewhat more numerous at microhabitat with cobbles.

In habitats with stable environmental conditions, such as springs, competition (McAuliffe 1984, Feminella and Resh 1990) and predation (Flecker and Allan 1984, Teague et al. 1985) can play an important role in the population dynamics of aquatic macroinvertebrates. The movements of nymphs among the available microhabitats during different stages of their growth was also recorded. Juvenile nymphs of the two *Baetis* species were most numerous at microhabitats with mosses, where they could find large quantities of organic matter to feed on and refuge from predators (e.g., Marshall and Westlake 1990, Schulz et al. 2003, Miliša et al. 2006). On the other hand, larger and mature nymphs also visited inorganic substrates where they could find additional food resources in the periphyton. Juvenile and mid-sized nymphs of *Rh. braaschi* were more numerous at inorganic microhabitats with smaller particle size (i.e., mixture of pebbles and sand), while mature nymphs used larger inorganic particles, likely to graze on. This shift in microhabitat selection could be a result of intraspecific competition (Hart 1983). Water velocity also has an important influence on the distribution of nymphs related to size, i.e., older nymphs were recorded to relocate at microhabitats with higher velocity or to gather in shoals near river banks (Kovalek 1978). In our study, all microhabitats had a relatively fast water current and were relatively shallow, and more mature nymphs shifted from microhabitats with higher to lower water velocity. Only *B. alpinus* remained at microhabitats with the highest velocities during the entire life cycle, as their nymphs can tolerate a strong water current (up to 1.5 ms^{-1}) (Bauernfeind and Soldán 2012). Together with competition for food resources (Buffagni et al. 1995), movements of mayfly nymphs among microhabitats could also be a result of seeking an appropriate emergence spot (Elliott and Humpesch 1983, Wagner et al. 2011).

Life cycles

The life cycle of *B. alpinus* has been determined as variable (univoltine or bivoltine) depending on the environmental conditions and altitude (Sowa 1975, Clifford 1982, Kukuła 1997, López-Rodríguez et al. 2008). Due to lower water temperatures, previous studies reported a univoltine life cycle at higher (upstream) sites, while at lower (downstream) sites, the life cycle was bivoltine (e.g., Landa 1969, Kukuła 1997). In the Bistrica River Spring, the species had a bivoltine life cycle, which corroborates the study of Bottová and Derka (2013) conducted in a karst spring in Slovakia. Due to stable thermal conditions, the life cycle of *B. rhodani*, a species with a flexible life cycle (Clifford 1982, Bauernfeind and Humpesch 2001), was determined as polyvoltine, as the most commonly reported type of life cycle (e.g., Buffagni et al. 2002, Erba et al. 2003). Yet, our results are not in accordance with Bottová and Derka (2013) who determined its life cycle as univoltine. The biology and ecology of *Rh. braaschi* is still insufficiently investigated, though its life cycle is considered to be univoltine with a short emergence period in spring and early summer (Bauernfeind and Soldán 2012). In the Bistrica River Spring, the life cycle was univoltine, though the emergence period was prolonged due to the stable thermal conditions, corroborating the study of Vilenica et al. (2017b) in karst lotic habitats in Croatia.

Conclusions

With a newly recorded species for Bosnia and Herzegovina, *Rhithrogena braaschi*, this study contributes to our knowledge of the insufficiently investigated mayfly fauna of the Balkan Peninsula. Due to specific environmental conditions, especially low and constant water temperatures, mayfly assemblages in karst springs could be characterized as species poor (e.g., Vilenica et al. 2016a, 2017a). The Dinaric Karst habitats have long been recognized as a global biodiversity hotspot (Bănărescu 2004, Ivković and Plant 2015). Yet, at the same time they are strongly threatened by numerous anthropogenic pressures (Freyhof 2012, Schwarz 2012). Since mayflies are widely used as bio-indicators of freshwater ecosystems (Landa and Soldán 1991), new insights into mayfly ecology, i.e., their microhabitat preferences and life histories, could contribute to the classification and protection of karst freshwater habitats in the Balkan Peninsula and form an important basis for further research and conservation practices for European mayflies and their habitats.

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