Key drivers of microbial abundance, activity, and diversity in karst spring waters across an altitudinal gradient in Slovenia

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ABSTRACT: In aquatic ecosystems, the biomass, activity and composition of microorganisms are determined to a large extent by local and regional environmental conditions. While karst aquifers are an important source for drinking water, the ecology of microbial communities in karst waters has hardly been studied. This study examined the regional variations and seasonal dynamics of microbial communities in pristine karst spring waters of Slovenia (Central Europe). Fifteen springs distributed across 5 eco-regions exhibiting a strong altitudinal gradient were sampled 4 times a year. Evaluation of the microbial communities included quantification of prokaryotic biomass via total cell counts and microbial activity estimated via measurements of electron transport system activity. The taxonomic structure of the bacterial communities was analysed by terminal restriction fragment length polymorphism fingerprinting. Biological measures were complemented by a set of physico-chemical parameters, including dissolved organic carbon, nutrients, major ions, temperature, electrical conductivity, pH, and dissolved oxygen. Bacterial community structure differed significantly with seasons and eco-regions, with the latter causing greater variation. While the overall power of the environmental variable tested was a moderate factor (15%) in explaining the variability in microbial community structure, catchment altitude was a key determinant. Prokaryotic cell density and microbial activity in spring water decreased with an increase in catchment altitude and were significantly positively correlated. For a better understanding of karst ecosystems and the ecosystem service of water purification, future investigation should address karst microbial communities at a higher phylogenetic and functional resolution.

KEY WORDS: Karst springs · Microbial diversity · Total cell counts · TCC · Electron transport system activity · ETSA · Terminal restriction fragment length polymorphism · T-RFLP

1. INTRODUCTION

Groundwater from karst aquifers supplies a quarter of the world's population with drinking water (Smart & Worthington 2004a). At the same time, karst aquifers are unique ecosystems that are populated by a vast variety of microorganisms (Farnleitner et al. 2005, Griebler & Lueders 2009) and groundwater meiofauna (Botosaneanu 1986, Danielopol et al. 2000, Gibert & Culver 2009). These organisms provide essential eco-

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system services such as water purification processes through the mineralisation of carbon and other nutrients (Griebler & Avramov 2015). In particular, microbes are crucial in maintaining good chemical and hygienic groundwater quality (Griebler & Avramov 2015, Feichtmayer et al. 2017, Griebler et al. 2019).

In Slovenia, about half of the country is covered by karst (Gams 1974), and karst water contributes up to 50% of the drinking water supply (Brečko Grubar & Plut 2001). Karst springs are 'open windows' into the karst systems, which are otherwise difficult to access for research. Microbial communities are discharged with karst water to the surface, making them accessible for detailed analysis (Smart & Worthington 2004b). To date, karst springs, even when used to supply drinking water, are regularly monitored only for (1) the quantity of discharge, (2) basic physico-chemical parameters (temperature, pH, electrical conductivity [EC], dissolved oxygen [DO], major ions, and nutrients), (3) specific groups of chemicals (e.g. pesticides), and (4) the presence of indicators for faecal contamination (*E. coli* and coliform bacteria). No measures are routinely taken to monitor ecological parameters, including the abundance, biomass, activity, and diversity of microbial communities (Griebler et al. 2010, Korbel & Hose 2011). As a consequence, our knowledge about the microbiology of karst aquifers and springs is rather limited (Farnleitner et al. 2005, Pronk et al. 2009, Wilhartitz et al. 2009, Shabarova et al. 2014), including the Slovenian karst.

Groundwater ecosystems have classically been considered to be stable environments that undergo relatively little variations in physico-chemical conditions over time compared to other ecosystems on the land surface (Goldscheider et al. 2006, Griebler & Lueders 2009). Consequently, compared to microbial communities in other aquatic or terrestrial ecosystems (Comte et al. 2017, Langenheder & Lindström 2019), the mechanisms that shape microbial community structure across space and time in groundwater have been understudied (see e.g. Fillinger et al. 2019, Zelaya et al. 2019, Yan et al. 2020). However, more recent studies on single aquifers, including alluvial (Lin et al. 2012, Zhou et al. 2012, Graham et al. 2016, 2017, Zelaya et al. 2019), fractured (Yan et al. 2020), and karst aquifers (Shabarova et al. 2013, 2014, Savio et al. 2019), showed that groundwater microbial communities can indeed display considerable temporal variation in response to changes in hydrochemistry, composition of the carbon pool, and organic matter (OM) availability, as well as hydrodynamics caused by changes in groundwater discharge. Changes in discharge can be especially

important for the temporal variation in microbial pattern, as they affect the dispersal of taxa across aquifer zones via passive transport and can partially override the effect of physico-chemical conditions on microbial community composition (Yan et al. 2020). In karst ecosystems, this can be of particular importance, because in times of high discharge, microbial taxa can be spread across karst pools that are otherwise hydrologically separated over longer time periods. During periods of low discharge and hydrological separation, divergent microbial communities may develop within individual pools due to site-specific changes in hydrochemistry, quantity and quality of the OM, and priority effects (Shabarova et al. 2013, 2014, Svoboda et al. 2018). Studies comparing microbial communities across aquifers are so far rare (Ben Maamar et al. 2015, Danczak et al. 2018). However, Fillinger et al. (2019) demonstrated that differences in microbial community composition between aquifers could be explained in large part by differences in environmental conditions, and to a lesser extent by limited exchange of taxa across aquifers via dispersal. To this point, there are no studies that have compared temporal variations in microbial communities in groundwater across multiple aquifers — which would be an important step in identifing general mechanisms underlying these variations.

In this study, we investigated spatio-temporal dynamics of prokaryotic cell numbers, microbial respiratory activity, and bacterial community structure from 15 karst springs that belong to geographically distinct aquifers in 5 eco-regions of Slovenia (Central Europe). Although similar in geology (a mixture of limestone and dolomite), the springs in the selected eco-regions differ in mean altitude of the catchment and altitude of spring outlets, catchment sizes, as well as mean annual precipitation and air temperature. We hypothesized that (1) assuming a significant influence of environmental conditions on groundwater microbial community structure (Stegen et al. 2013, Graham et al. 2016, 2017, Fillinger et al. 2019, Zelaya et al. 2019, Yan et al. 2020), the bacterial communities within eco-regions and seasons would be more similar to each other than between eco-regions and seasons, (2) regional differences would have a greater impact on bacterial community structure than seasonality; and (3) microbial abundance and activity in spring water would decrease with higher altitudes (Wilhelm et al. 2015). To account for seasonal dynamics, all springs were sampled once in autumn, winter, spring, and summer between October 2014 and August 2015. To elucidate the driving forces involved in shaping bacterial communities in

karst spring waters, we applied a set of simple measures, i.e. total prokaryotic cell counts (TCC), microbial respiratory activity (as electron transport system activity, ETSA), and assessment of microbial community structure based on terminal restriction fragment length polymorphism (T-RFLP) fingerprinting of 16S rRNA gene amplicons. In addition, a comprehensive set of physico-chemical parameters of the spring water was determined at each sampling event.

2. MATERIALS AND METHODS

2.1. Study sites

We investigated 15 karst springs within 5 eco-regions in Slovenia (Central Europe) (Fig. 1). Two regions belong to the Alpine karst (the Kamnik-Savinja-Karavanke Alps and the Julian Alps), 2 regions to the high Dinaric karst (the Trnovo plateau and the Ljubljanica karst), and one region to the low Dinaric karst (the Dolenjska karst). In each eco-region, 3 hydrologically discrete springs were selected for sampling. Their main hydrogeological characteristics were previously studied by Mezga (2014) and Cerar (2016) and are summarized in Table 1.

2.2. Sample collection

We conducted 4 sampling campaigns between October 2014 and August 2015, once per season (October 2014, February 2015, May 2015, and August 2015). Sampling was performed only in dry weather conditions, when no or minimal precipitation occurred 7 d before, to avoid sampling predominantly surface-derived microorganisms washed out from the upper soil layers. Sampling campaigns were planned based on data from the nearest hydrological station to each spring, which recorded river discharge and precipitation (ARSO 2014a,b).

We filled three 5 l sterile glass bottles (sterilised at 121°C for 15 min, rinsed with 96% ethanol, and then thoroughly rinsed on-site with spring water before sample collection) per sampling site with spring water for microbial analyses. We carefully filled bottles to avoid contamination. Samples were transported to the laboratory within 8 h (cooled in the dark) and stored at 4°C until further processing.

2.3. Physico-chemical measurements

Spring water temperature (T_{water}), pH, EC, and DO concentration were measured using field sensors (WTW Multi set 3430) on-site. For further chemical analyses, we filtered spring water on-site using 0.45 and 0.22 μm membrane filters (Sartorius Minisart® NML Syringe Filters 16555-K and 16534-K, respectively) and stored the samples in 50 ml PVC plastic bottles. Samples were transferred cooled and in the dark into the laboratory and stored at 4°C until analyses. We performed analyses within 24 h after sampling.

Eco-regions
1 - the Kamnik-Savinja-Karavanke Alps
2 - the Julian Alps
3 - the Trnovo plateau
4 - the Ljubljanica karst
5 - the Dolenjska karst

Karst regions
1&2 - the Alpine karst
3&4 - the high Dinaric karst
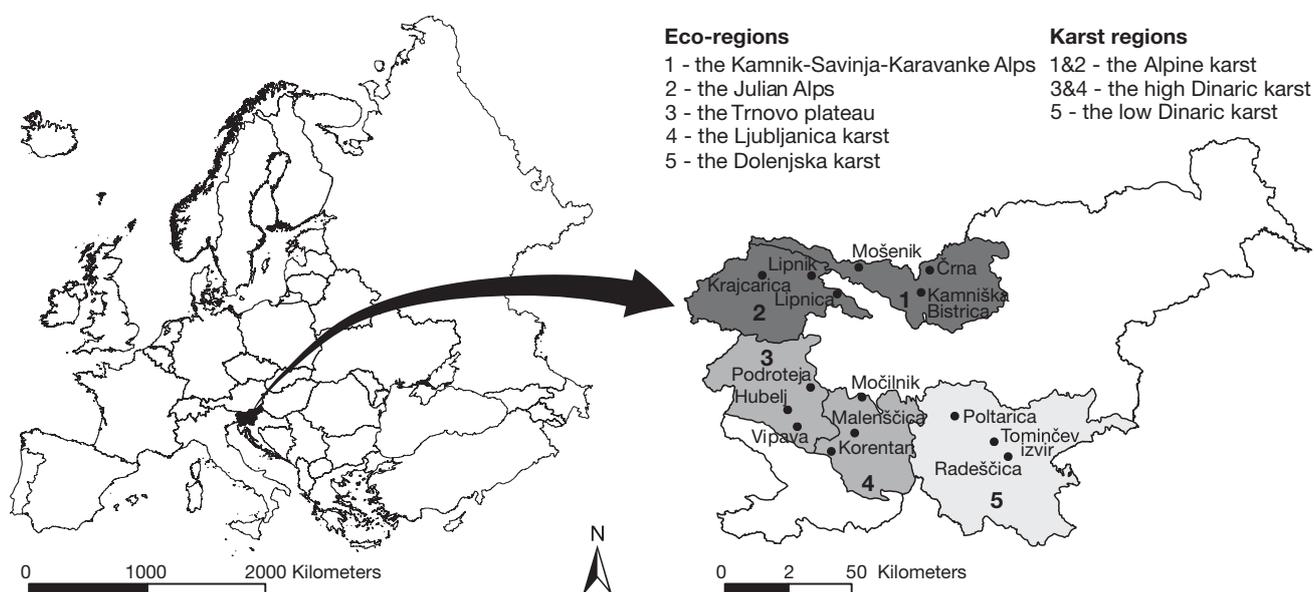5 - the low Dinaric karst

Fig. 1. Europe (left) and Slovenia (right) with studied karst eco-regions (1 to 5) and springs (black points)

Table 1. Main characteristics of studied springs from 5 karst eco-regions in Slovenia (data extracted from Mezga 2014 and Cerar 2016)

Eco-region	Spring	Altitude of spring outlet (m)	Mean altitude of catchment (m)	Size of catchment area (km^2)	Mean annual amount of precipitation (1971–2000) (mm)	Mean annual air temperature (1971–2000) (°C)	Litho-logical unit
1. The Kamnik-Savinja-Karavanke Alps	Mošenik	840	1360.4	2.11	2178	4.3	Limestone
	Kamniška Bistrica	592	1683.5	22.12	2208	3.7	Limestone
	Črna	744	1361.8	22.01	2077	4.5	Dolomite
2. The Julian Alps	Krajcarica	706	1310.4	0.87	2537	5.2	Limestone
	Lipnik	648	1444.4	20.39	2302	4.8	Dolomite
	Lipnica	494	784.8	21.26	2278	5.2	Limestone
3. The Trnovo plateau	Podroteja	330	602.3	51.05	2300	8.2	Dolomite
	Hubelj	229	1009.6	52.16	2483	6.9	Limestone
	Vipava	103	809.9	100.9	2276	7.9	Limestone
4. The Ljubljanica karst	Močilnik	295	672.8	1054	1941	7.9	Limestone
	Malenščica	450	595.2	22.81	1962	8.6	Limestone
	Korentan	537	572.0	6.34	1900	9.0	Limestone
5. The Dolenjska karst	Poltarica	272	420.6	261.3	1422	9.0	Limestone
	Tominčev izvir	173	517.2	278.2	1524	8.6	Limestone
	Radeščica	176	580.0	194.5	1630	8.0	Limestone

Major cations (Ca^{2+}, Mg^{2+}, Na^+, K^+, NH_4^+) and anions (SO_4^{2-}, NO_3^-, NO_2^-, Cl^-) were determined by ion chromatography (Metrohm, 761 Compact IC), and dissolved organic carbon (DOC) as non-purgeable organic carbon in a total organic carbon analyzer (Analytik Jena Multi C/N 3100).

2.4. Analyses of prokaryotic cell abundances

To determine microbial abundance, we measured TCC by flow cytometry, as described by Hammes et al. (2008). In the laboratory, water samples (50 ml) were fixed with formaldehyde (3.6% final concentration) in triplicate and stored at 4°C until further analyses. A sample volume of 500 µl was stained with 6 µl SYBR-Green I working solution (10 µl SYBR Green I nucleic acid stain [10 000×, Invitrogen-Thermo Scientific] and 990 µl Tris buffer [10 mM; pH 8.0]), immediately mixed on a vortex, and incubated for 13 min at 37°C in the dark before adding 100 µl of a reference beads solution (Trucount Tubes, Becton Dickinson) as an internal standard. We counted stained cells in a CYTOMICS FC 500 Flow Cytometer (Beckman Coulter) using a 488 nm blue light laser for excitation (Bayer et al. 2016). Cells were distinguished from inorganic and organic particles based on side scatter and fluorescence measured at 569 ± 15 nm (±SD). To

obtain technical replicates, each of the triplicate samples was measured twice.

2.5. Analyses of microbial respiratory activity

We measured microbial ETSA according to Packard (1971) with modifications made by G.-Tóth (1999). ETSA is a measure of the metabolic activity of the microorganisms, which is reflected in the transport of electrons through the respiratory chain in the plasma membrane. We collected microbial biomass from 1 l of spring water on a sterile 0.2 µm cellulose nitrate filter (Sartorius Cellulose Nitrate Membrane Filter). Filters were stored at −80°C until analysis. Filters were homogenised in 3 ml of ice-cold homogenisation buffer (0.1 M sodium phosphate buffer at pH 8.4; 75 µM $MgSO_4$; 0.15% w/v polyvinyl pyrrolidone; 0.2% v/v Triton-X-100) with an ultrasonic homogeniser (Cole-Parmer, 4710 Series) for 3 min. Samples were centrifuged for 4 min at 0°C and 8500 × g (2K15, Sigma). A 1.5 ml aliquot of substrate solution (0.1 M sodium phosphate buffer at pH 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% v/v Triton-X-100) and 0.5 ml of reagent solution (2.5 mM 2-(p-iodo-phenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride [INT]) were added to 0.5 ml of supernatant from the homogenate and incubated for 45 min at 20°C. The reaction was stopped by adding 0.5 ml of stop-

ping solution (formalin:H_3PO_4 = 1:1 v/v). For control, blanks (substrate and reagent solution) were incubated and stopped as in the samples, and then 0.5 ml of supernatant from the homogenate was added. Formazan production was measured spectrophotometrically (WTW, PhotoLab Spektral) from the absorbance of the sample at 490 nm against the blank. The ETSA was measured as the rate of tetrazolium dye reduction and expressed in µl of oxygen l^{-1} h^{-1}, as described by Kenner & Ahmed (1975). We measured all samples in triplicate.

2.6. DNA extraction, amplification of bacterial 16S rRNA gene, and T-RFLP analyses

We used T-RFLP to analyse microbial community structure. For each sampling site, we collected microbial cells from 2 l of spring water (in triplicate) on sterile 0.22 µm filters by vacuum filtration (Corning 250 ml Vacuum Filter/Storage Bottle System with Cellulose Acetate Membrane). Afterwards, we stored the filters at −80°C until further processing.

We extracted bacterial DNA from one-half of each filter (corresponding to 1 l of filtered water) using the PowerSoil®DNA Isolation Kit according to manufacturer's instructions (MO BIO Laboratories). The DNA was used as a template for PCR amplification of 16S rRNA gene fragments using universal bacterial primers 27f (FAM-5′-AGA GTT TGA TCC TGG CTC AG-3′) labelled with 6-FAM (6-Carboxyfluorescein) and 927r (5′-CCG TCA ATT CCT TTR AGT TT-3′), which are used to study bacterial community structure from different environments (Heuer & Smalla 1997). PCR was performed on 3 independent samples per spring and time point. We performed amplification in 50 µl reaction mixtures, containing 2 µl of DNA template, 10 µl of 5× PCR GoTaq® Flexi Buffer (Promega), 4 µl of 25 mM $MgCl_2$ solution (Promega), 0.5 µl of 100% deionised formamide, 0.5 µl of 20 mg ml^{-1} non-acetylated BSA, 1 µl of 10 mM deoxynucleotide triphosphate (dNTP), 1 µl of a 10 µM of each primer, 0.8 µl of 5 units $µl^{-1}$ GoTaq® DNA Polymerase (Promega), and 29.2 µl of nuclease-free Milli-Q water. The PCR was performed in a TProfessional Basic Thermocycler (Biometra) with the following conditions: initial denaturation for 5 min at 94°C followed by 30–35 cycles (depending on DNA template concentration) with a denaturation for 1 min at 94°C, annealing for 1 min at 53°C and an extension for 1.5 min at 72°C, and the final extension for 10 min at 72°C. PCR products were checked on a 0.8% (w/v) agarose gel and stained with GelRed (Nucleic Acid Stain, Biotium).

We purified PCR products using the Pure Link® PCR Purification Kit (Invitrogen) according to the manufacturer's instructions and then quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Purified PCR products (100 ng) were digested with FastDigest™ enzyme *AluI* (Thermo Scientific) in a 30 µl reaction mixture according to the manufacturer's instructions and purified by ethanol precipitation. Briefly, samples were mixed with 3 µl of 3 M sodium acetate (pH 5.2) and 66 µl of 96% (v/v) ethanol and incubated for 30 min on ice. After incubation, samples were centrifuged at 15 000 × g and 4°C for 30 min. Supernatants were discarded, 500 µl cold 70% (v/v) ethanol was added, and samples were immediately centrifuged at 15 000 × g and 4°C for 10 min. Supernatants were discarded, and samples were air-dried and stored at −20°C for further analyses.

For T-RFLP analysis, digested and purified products were mixed with 3 µl of sterile Milli-Q water, 0.4 µl of GeneScan™ 500 ROX™ Size Standard (Applied Biosystems), and 10 µl of deionised formamide. Prior to fragment analysis, DNA samples were denatured at 95°C for 4 min and then frozen on ice. The T-RFLP analyses were performed with an automated 3130*xl* Genetic Analyzer (Applied Biosystems).

2.7. Data analyses

We used multi-class linear discriminant analysis (LDA) to separate eco-regions in classes based on physico-chemical data, with the program Paleontological Statistics (PAST) version 3.16 (Hammer et al. 2001). Before analysis, physico-chemical parameters were log(x + 1) transformed to approximate normal data distribution. We applied the Kruskal-Wallis non-parametric test to test for differences in physico-chemical parameters between seasons and eco-regions, using SPSS version 20.0 (IBM Corporation 2011).

Raw T-RFLP data were imported into the program Peak Scanner version 1.0 (Applied Biosystems), which identifies peaks and fragment sizes. In the analyses, DNA fragments smaller than 50 and larger than 500 bp were removed and the size standard GS500 was used. Data exported from Peak Scanner were imported into the web based tool T-Align (Smith et al. 2005), where multiple T-RFLP profiles could be aligned and compared. Relative abundances of T-RFs were used to calculate differences in community structure between samples (β-diversity) based on Bray-Curtis dissimilarity, using PAST. Differences

in community structure between season and eco-region were visualized by non-metric multi-dimensional scaling (nMDS). Prior to the nMDS analysis, data were normalized and square root transformed. ANOSIM with 9999 randomizations was conducted to determine significant differences between seasons and eco-regions, using Bray-Curtis dissimilarity in PAST.

We used canonical correspondence analysis (CCA) to relate bacterial communities to the environmental variables and to test how much variability they explain. Before the analysis, the output data from T-Align were normalised and square rooted and environmental variables were $\log(x + 1)$ transformed. The CCA forward selection method was used to reduce the number of explanatory variables in the canonical ordination model. The analyses were conducted in CANOCO 5 (ter Braak & Šmilauer 2012).

The TCC and ETSA data were tested for normal distribution and separated to eco-regions and seasons. Data were $\log(x + 1)$ transformed, and univariate ANOVA with post-hoc Tukey test for multiple pairwise comparisons within seasons and eco-regions were performed. The analyses and the box-and-whisker plots were done in SPSS.

In addition, Spearman's rank-order correlation and hierarchical multiple regression analyses with curve fitting were performed in SPSS to test for changes in prokaryotic cell densities and microbial respiratory activities, respectively with increasing altitude of the catchment. Before regression analyses, data were $\log(x + 1)$ transformed to approximate normal distribution.

3. RESULTS

3.1. Differences in physico-chemical parameters between eco-regions and seasons

LDA revealed that karst spring waters from the different eco-regions generally showed distinct physico-chemical parameters, except for Ljubljanica karst and Dolenjska karst, which exhibited similar characteristics to one another (Fig. 2; a detailed overview of individual parameters is shown in Table 2). Nitrate (NO_3^-) concentrations explained most of the variance on the first axis, which separated

the Alps from other eco-regions, indicating that NO_3^- concentrations decrease with higher altitudes. Conversely, concentrations of major ions (Cl^-, Na^+, Ca^{2+}) and DOC, as well as EC, separated eco-regions on the second axis. We found the highest concentrations of major ions in the Julian Alps compared to the Kamnik-Savinja-Karavanke Alps, and in the Ljubljanica and Dolenjska karst compared to the Trnovo plateau. While statistically significant differences between eco-regions were detected for all individual physico-chemical parameters (pooling all seasons together) (Kruskal-Wallis test; all $p < 0.05$), we only revealed significant differences between seasons for DOC ($p < 0.001$) and pH ($p = 0.001$) when pooling all regions together.

3.2. Spatial–temporal variations in bacterial community structure

The nMDS and ANOSIM analyses indicated that the structure of the bacterial communities changed with seasons in each eco-region (Fig. 3). The largest seasonal changes in bacterial community structure occurred in the Dolenjska karst and the smallest in the Trnovo plateau (1-way ANOSIM; Dolenjska karst: R = 0.688, $p < 0.001$; Trnovo plateau: R = 0.364, $p < 0.001$). Bacterial community structures also var-
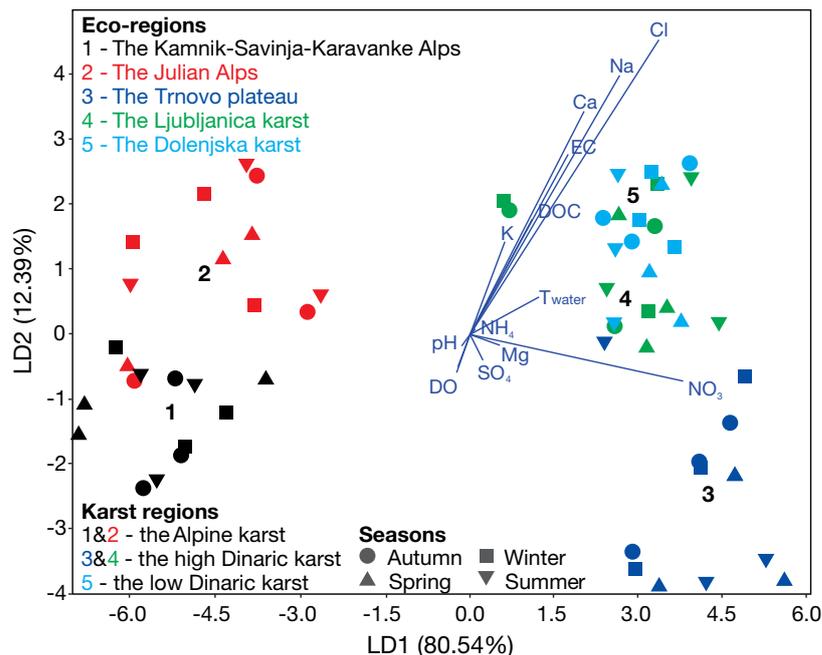

Fig. 2. Linear discriminant analysis (LDA) biplot of physico-chemical water parameters combined for 15 springs from 5 karst eco-regions in Slovenia, sampled across 4 seasons (autumn, winter, spring, summer) in the period 2014−2015

ied regionally within seasons, despite a certain degree of overlap of samples from different regions, mainly between the Ljubljanica and Dolenjska karst eco-regions (Fig. 4). Results of the 1-way ANOSIMs also showed that the most prominent difference between bacterial community structures across eco-regions occurred in autumn (R = 0.645, p < 0.001) and the least prominent in winter (R = 0.426, p < 0.001). In summary, the comparison between seasons and eco-regions with the 2-way ANOSIM indicated a greater variation in bacterial community structure between eco-regions (R = 0.543, p < 0.001) compared to seasonal variations (R = 0.440, p < 0.001).

3.3. Relationship between bacterial community structure and environmental parameters

CCA was used to further explore the effects of changes in physico-chemical parameters of the spring water on the observed differences and dynamics in bacterial community structure between seasons and eco-regions (Fig. 5). Environmental variables explained 14.8% of the variation in bacterial community structure (eigenvalue 1 = 0.247, eigenvalue 2 = 0.093) and total inertia was 3.789. The environmental variables with the strongest individual effect on bacterial community structure were the catchment altitude (6.1% explained variation), sulphate concentration (2.1%), oxygen concentration (1.8%), NO_3^- concentration (1.7%), precipitation within 7 d before sampling (1.6%), and pH (1.5%). The altitude of the catchment together with the NO_3^- concentration disentangled samples from different eco-regions along the first CCA axis; the Kamnik-Savinja-Karavanke Alps and part of the Julian Alps, with the highest altitude and lowest NO_3^- concentrations, versus the Ljubljanica and Dolenjska karst on the opposite side of the axis representing a relatively lower altitude and higher NO_3^- concentrations. On the other hand, no separation of eco-regions was observed along the second CCA axis, which was mainly influenced by precipitation within 7 d before sampling and the sulphate concentration.

3.4. Differences in prokaryotic abundance and microbial activity between eco-regions and seasons

When evaluated across all seasons, the lowest TCC in spring water on average was detected in the Kamnik-Savinja-Karavanke Alps ($2.3 \times 10^4 \pm 2.4 \times 10^4$ cells ml^{-1}), and the highest in the Dolenjska karst ($1.6 \times 10^5 \pm$

Table 2. Environmental parameters for 15 karst spring waters from 5 eco-regions from Slovenia (Central Europe) once per season between October 2014 and August 2015 (values are mean ± SD of 4 samplings of the 3 springs per eco-region)

| Eco-region	Temp. (°C)	O_2 conc. (mg l^{-1})	O_2 satur. (%)	EC (µS cm^{-1})	pH	Cl^- (mg l^{-1})	NO_3^- (mg l^{-1})	SO_4^{2-} (mg l^{-1})	Na^+ (mg l^{-1})	NH_4^+ (mg l^{-1})	K^+ (mg l^{-1})	Ca^{2+} (mg l^{-1})	Mg^{2+} (mg l^{-1})	DOC (mg l^{-1})
1. The Kamnik-Savinja-Karavanke Alps	6.7 ± 1.0	11.2 ± 0.7	100.0 ± 3.6	239 ± 52	8.1 ± 0.2	0.6 ± 0.5	1.7 ± 0.1	9.6 ± 10.8	0.6 ± 0.5	0.05 ± 0.01	0.2 ± 0.1	47.5 ± 6.7	9.3 ± 3.7	1.7 ± 0.9
2. The Julian Alps	6.4 ± 0.7	11.3 ± 0.4	99.0 ± 2.7	261 ± 54	8.1 ± 0.2	1.3 ± 1.4	1.9 ± 0.5	2.1 ± 0.7	0.9 ± 0.7	0.08 ± 0.03	0.2 ± 0.2	58.9 ± 15.8	5.6 ± 1.6	2.8 ± 1.6
3. The Trnovo plateau	8.8 ± 0.8	11.1 ± 0.6	98.0 ± 4.0	311 ± 45	7.9 ± 0.3	2.1 ± 0.7	6.9 ± 1.0	3.2 ± 0.7	1.5 ± 0.5	0.07 ± 0.02	0.3 ± 0.1	69.6 ± 17.3	9.2 ± 6.1	2.9 ± 1.1
4. The Ljubljanica karst	9.3 ± 1.7	10.1 ± 0.7	91.9 ± 2.8	401 ± 28	7.8 ± 0.2	4.6 ± 2.4	7.2 ± 2.9	4.3 ± 0.7	3.3 ± 1.7	0.08 ± 0.02	0.5 ± 0.2	96.6 ± 11.0	7.5 ± 5.3	2.5 ± 0.8
5. The Dolenjska karst	9.8 ± 0.4	10.2 ± 0.4	91.8 ± 2.5	452 ± 29	7.6 ± 0.4	4.0 ± 2.1	4.6 ± 0.7	4.7 ± 0.9	2.9 ± 1.4	0.10 ± 0.03	0.6 ± 0.1	103.1 ± 12.2	12.8 ± 4.2	3.3 ± 1.5
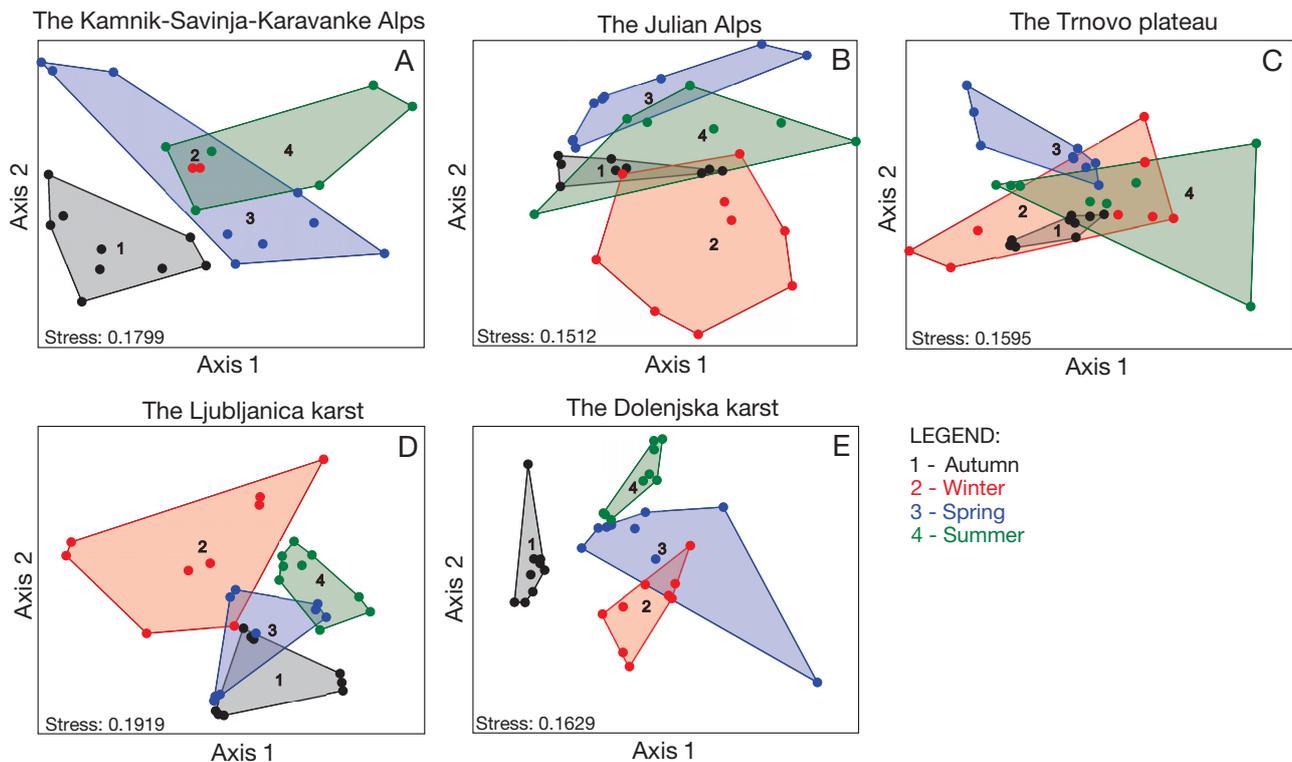
Fig. 3. Non-metric multi-dimensional scaling analyses based on Bray-Curtis similarities of bacterial community structure of 15 springs from 5 karst eco-regions in Slovenia sampled across seasons (autumn, winter, spring, and summer) in the period from October 2014 to August 2015. Each enclosed shape with 9 points represents 3 springs in triplicate (as some samples did not have enough DNA for analyses, there are fewer points in some frames)

1.4×10^5 cells ml^{-1}), which represent the eco-regions with the highest and the lowest catchment altitude, respectively (detailed results in Table 3, Fig. 6). Significant differences for TCC were found between eco-regions (by average seasonal values; ANOVA; $F_{4,40}$ = 15.953, p < 0.001) and between seasons (by average regional values; $F_{3,40}$ = 6.643, p = 0.001). When tested in pairwise comparisons, TCC was neither statistically different between the Dolenjska and the Ljubljanica karst, nor between the Trnovo plateau and the Julian Alps considering values from all seasons (Tukey's post-hoc test; all p > 0.050). Regarding differences between seasons, TCC values in winter were the most significantly different from TCC values in other seasons (Tukey's post-hoc test; all regions combined; all p < 0.012).

The seasonal average ETSA ranged from 0.185 ± 0.096 μl O$_2$ l^{-1} h^{-1} in the Kamnik-Savinja-Karavanke Alps (eco-region with the highest catchment altitude) to 0.735 ± 0.606 μl O$_2$ l^{-1} h^{-1} in the Dolenjska karst (eco-region with the lowest catchment altitude; detailed results in Table 4, Fig. 6). There were significant differences between eco-regions (ANOVA; $F_{4,40}$ = 13.681, p < 0.001) and seasons ($F_{3,40}$ = 9.090, p < 0.001). According to the Tukey's post-hoc test

based on the ETSA values, eco-regions clustered into 2 groups (by combining all seasons): the Kamnik-Savinja-Karavanke Alps, the Julian Alps, with the Trnovo plateau; and the Ljubljanica and Dolenjska karst (all p > 0.050). The largest differences in ETSA among seasons (by combining all regions) were found between autumn and spring (p < 0.001).

3.5. Influence of catchment area characteristics on prokaryotic cell density and microbial respiratory activity

The relationships between catchment area characteristics and prokaryotic cell density (i.e. TCC), microbial respiratory activity (i.e. ETSA) as well as DOC and pH were tested with Spearman's rank-order correlation analysis. Prokaryotic cell density and microbial respiratory activity were significantly correlated. For TCC, a significant negative correlation was found with the altitude of the catchment, and significant positive correlation with water temperature and DOC concentrations. Additionally, EC and concentration of Ca^{2+} ions were significantly positively correlated with TCC and ETSA and neg-
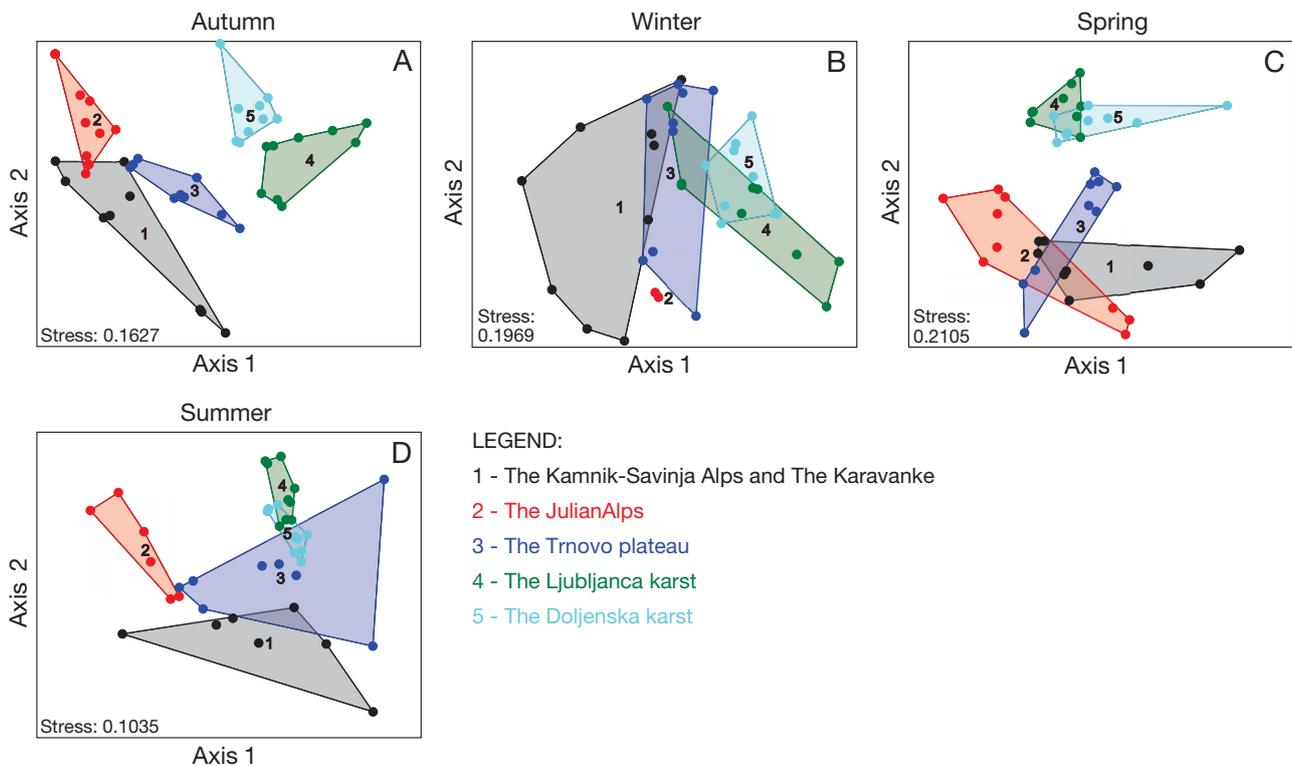
Fig. 4. Non-metric multi-dimensional scaling analyses based on Bray-Curtis similarities of bacterial community structure of 15 springs from 5 karst eco-regions in Slovenia (the Kamnik-Savinja-Karavanke Alps, the Julian Alps, the Trnovo plateau, the Ljubljanica karst, and the Dolenjska karst) sampled across 4 seasons in the sampling period from October 2014 to August 2015. Each enclosed shape with 9 points represents 3 springs in triplicate (as some samples did not have enough DNA for analyses, there are fewer points in some frames)

atively correlated with pH (detailed results in Table 5).

We tested our third hypothesis, i.e. a decrease in microbial abundance and activity with increasing altitude of the catchment, using hierarchical multiple regression analysis. Both TCC and ETSA decreased significantly with increasing catchment altitude (on a log–log scale; Fig. 7), explaining 43.5 and 28.4% of the variation in TCC and ETSA, respectively (i.e. adjusted R^2; detailed results in Table 6). No additional significant effect of season was observed when including season as additional categorical co-variable in the models.

4. DISCUSSION

To date, most of the studies on groundwater microbial communities have addressed contaminated aquifers, with only a few that looked at pristine groundwater ecosystems, and even fewer into karst aquifers (Farnleitner et al. 2005, Goldscheider et al. 2006, Pronk et al. 2009, Zhou et al. 2012, Shabarova et al. 2013, 2014, Wilhartitz et al. 2013, Sinreich et al.

2014). The aim of this study was to provide an improved understanding of the effect of individual environmental conditions and seasonal variation on prokaryotic abundance, microbial activity, and bacterial community structure in karst aquifers. We studied the spatio-temporal dynamics of microbial communities in waters from 15 springs situated in 5 geographically distinct karst eco-regions in Slovenia, spanning about 50 km in north–south direction and 1200 m in altitude.

Previous studies have reported an influence of environmental conditions on groundwater microbial community biomass, activity, and composition (Stegen et al. 2013, Sirisena et al. 2014, Beyer et al. 2015, Graham et al. 2016, 2017, Fillinger et al. 2019, Yan et al. 2020), and as the environmental characteristics of the catchments differed between the 5 studied eco-regions, we hypothesized that the bacterial communities within eco-regions would be more similar to each other than between eco-regions. With nMDS and ANOSIM analyses, this hypothesis was confirmed; our results were in line with the findings by Danczak et al. (2018) and Fillinger et al. (2019), who reported significant variations for microbial commu-
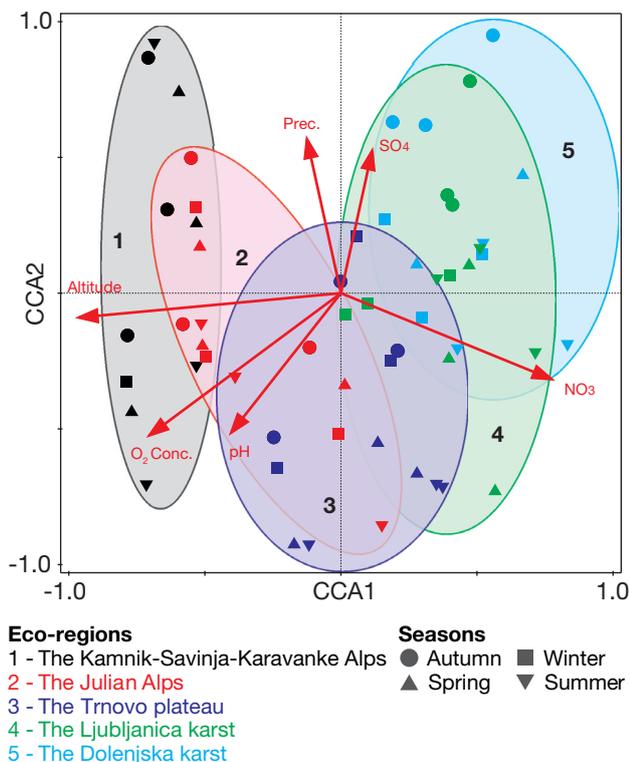
Fig. 5. Canonical correspondence analysis (CCA) ordination biplot of bacterial community structure across 5 karst eco-regions. Arrows indicate the magnitude and direction of the most significant environmental variables associated with bacterial community structure. Altitude: altitude of catchment; O_2 Conc.: oxygen concentration; Prec.: precipitation within 7 d before sampling

Maamar et al. 2015, Graham et al. 2016, 2017, Fillinger et al. 2019, Zelaya et al. 2019, Yan et al. 2020); water pH especially has been reported as a key factor, outweighing even the contribution of high temperatures to the biogeographic distribution of microbial communities in geothermal springs across an area of ~8000 km^2 (Power et al. 2018). Results of our study are further comparable to boreal aquatic ecosystems (Niño-García et al. 2016), where a clear separation into regional groups was obtained based on differences in the water chemistry of surface lakes and rivers; however, in terms of bacterial community structures, smaller regional differences were shown, with a significant overlap between sites from different regions. They showed that pH and water residence time were the most important environmental variables shaping bacterial community structure in the selected research area (Niño-García et al. 2016). In our study, environmental conditions only explained a small fraction of the variation in microbial community structure (~15%), which might point towards a strong influence of other factors, such as stochastic dispersal of taxa via groundwater discharge as has previously been observed for other karst systems (Shabarova et al. 2013, 2014, Savio et al. 2019). Unfortunately, during this study it was not possible to test bacterial community data with the groundwater residence time and hydrological regime.

It is important to note that the present observations on microbial community structure based on T-RFLP fingerprints might not be directly comparable with those other studies, which assessed microbial community composition at a higher resolution using high-throughput amplicon sequencing. The T-RFLP fingerprinting was implemented here only to detect the most abundant fraction of taxa in the communities while missing rare, low-abundant taxa that often actually comprise the bulk of the total taxon richness in microbial communities (Jia et al. 2018). Although it is still unresolved whether the assembly of rare

nities of different aquifers. We identified catchment altitude, which showed an inverse relationship with NO_3^- concentrations in the spring water, as well as the amount of precipitation, pH, and concentrations of DO and sulphate as the main environmental factors determining bacterial community structure across the 5 eco-regions. The importance of environmental conditions in shaping subsurface microbial communities has been previously underlined (Ben

Table 3. Prokaryotic abundance (total cell counts; cells ml^{-1}) of 5 karst eco-regions (mean ± SD of 3 springs per eco-region) for 4 seasons during the sampling period from October 2014 to August 2015. Regional and seasonal averages are included

| Eco-region	Autumn	Winter	Spring	Summer	Regional average
1. The Kamnik-Savinja-Karavanke Alps	$4.1 \times 10^4 \pm 4.8 \times 10^4$	$1.1 \times 10^4 \pm 1.6 \times 10^3$	$1.9 \times 10^4 \pm 1.2 \times 10^4$	$2.0 \times 10^4 \pm 4.4 \times 10^3$	$2.3 \times 10^4 \pm 2.4 \times 10^4$
2. The Julian Alps	$1.6 \times 10^4 \pm 8.7 \times 10^3$	$1.8 \times 10^4 \pm 7.2 \times 10^3$	$1.2 \times 10^5 \pm 1.1 \times 10^5$	$1.0 \times 10^5 \pm 1.1 \times 10^5$	$6.4 \times 10^4 \pm 8.2 \times 10^4$
3. The Trnovo plateau	$8.0 \times 10^4 \pm 4.9 \times 10^4$	$2.7 \times 10^4 \pm 1.5 \times 10^4$	$6.6 \times 10^4 \pm 4.8 \times 10^4$	$5.3 \times 10^4 \pm 2.1 \times 10^4$	$5.6 \times 10^4 \pm 3.7 \times 10^4$
4. The Ljubljanica karst	$9.5 \times 10^4 \pm 4.0 \times 10^4$	$5.5 \times 10^4 \pm 1.8 \times 10^4$	$1.0 \times 10^5 \pm 2.9 \times 10^4$	$1.6 \times 10^5 1 \pm 6.6 \times 10^4$	$1.0 \times 10^5 \pm 5.2 \times 10^4$
5. The Dolenjska karst	$3.9 \times 10^5 \pm 3.4 \times 10^4$	$4.8 \times 10^4 \pm 1.7 \times 10^4$	$8.1 \times 10^4 \pm 1.9 \times 10^4$	$1.2 \times 10^4 \pm 2.4 \times 10^4$	$1.6 \times 10^5 \pm 1.4 \times 10^5$
Seasonal average	$1.2 \times 10^5 \pm 1.4 \times 10^5$	$3.2 \times 10^4 \pm 2.1 \times 10^4$	$7.7 \times 10^4 \pm 5.9 \times 10^4$	$9.0 \times 10^4 \pm 7.0 \times 10^4$	$8.1 \times 10^4 \pm 9.0 \times 10^4$
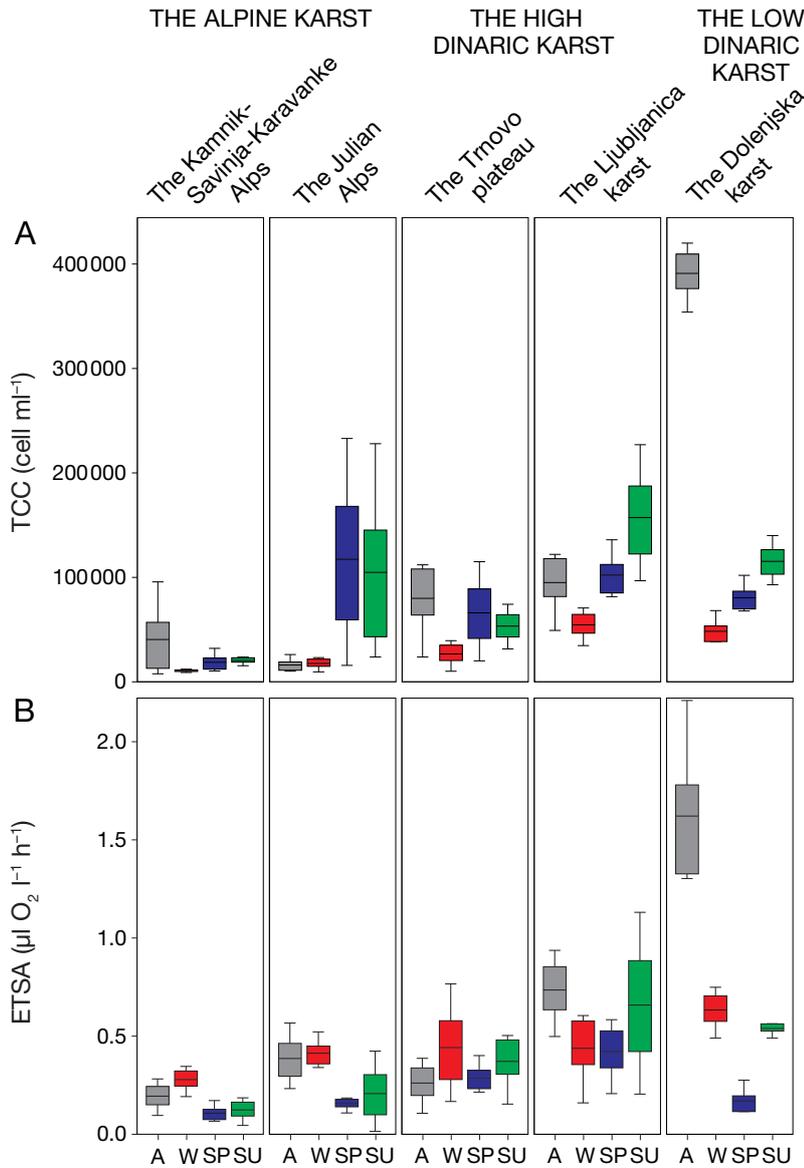
Fig. 6. Total cell counts (TCCs) and electron transport system activity (ETSA) for 15 springs from 5 karst eco-regions in Slovenia (Central Europe) across the 4 sampling seasons from October 2014 to August 2015. Middle horizontal lines: mean values; boxes: lower and upper quartiles; whiskers: minimum and maximum values; A: autumn; W: winter; SP: spring; SU: summer

and abundant taxa underlies similar mechanisms (Liao et al. 2017, Jia et al. 2018, Mo et al. 2018, Nyirabuhoro et al. 2020), we have to take into account that the factors that were identified to influence microbial community structure based on T-RFLP in our study likely only reflect the impact on the most abundant taxa and thus might not be representative of the microbial communities as a whole. Considering this potential bias, our conclusions based on the effects of environmental conditions and regional features of the catchments need to be treated with the necessary caution.

We confirmed the hypothesis that the bacterial communities were more similar within than between seasons; but in association with the second hypothesis, we further confirmed that differences in eco-regional patterns have a stronger impact on bacterial community structure than seasonality. While the temporal variability in groundwater chemical composition has historically been regarded to be comparably low (Nelson 2002), seasonal dynamics of groundwater chemistry and microbiology strongly depend on the type of aquifer (e.g. alluvial versus karst; Griebler et al. 2010) as well as its geographical location and altitude (Farnleitner et al. 2005). The largest seasonal variations in groundwater chemistry have been observed and are expected in karst systems due to high variability in the quantity of discharge and flow velocities, which depend on the position,

Table 4. Electron transport system activity (ETSA; $\mu l\ O_2\ l^{-1}\ h^{-1}$) values of 5 karst eco-regions (mean ± SD of 3 springs per eco-region) for 4 seasons during the sampling period from October 2014 to August 2015. Regional and seasonal averages are included

Eco-region	Autumn	Winter	Spring	Summer	Regional average
1. The Kamnik-Savinja-Karavanke Alps	0.194 ± 0.093	0.279 ± 0.079	0.107 ± 0.057	0.124 ± 0.072	0.185 ± 0.096
2. The Julian Alps	0.386 ± 0.169	0.413 ± 0.096	0.155 ± 0.041	0.208 ± 0.206	0.294 ± 0.168
3. The Trnovo plateau	0.261 ± 0.142	0.441 ± 0.303	0.289 ± 0.099	0.372 ± 0.190	0.345 ± 0.185
4. The Ljubljanica karst	0.735 ± 0.222	0.438 ± 0.243	0.420 ± 0.193	0.658 ± 0.463	0.566 ± 0.293
5. The Dolenjska karst	1.621 ± 0.511	0.633 ± 0.132	0.169 ± 0.092	0.538 ± 0.042	0.735 ± 0.606
Seasonal average	0.639 ± 0.590	0.441 ± 0.200	0.228 ± 0.150	0.380 ± 0.292	0.422 ± 0.374

Table 5. Spearman's rank-order correlation analysis of total cell counts (TCC), electron transport system activity (ETSA), dissolved organic carbon (DOC), and pH correlated with catchment area characteristics. T_{water}: water temperature; EC: electrical conductivity; T_{air}: air temperature; *p < 0.05; **p < 0.01 (2-tailed). The strongest correlations are in **bold**

Variable	Spearman's rho	TCC	ETSA	DOC	pH
TCC	r_s	1.000	0.521**	**0.495****	−0.251
	Sig. (2-tailed)		0.000	0.000	0.053
	N	60	60	57	60
ETSA	r_s	0.521**	1.000	0.181	−0.398**
	Sig. (2-tailed)	0.000		0.177	0.002
	N	60	60	57	60
DOC	r_s	0.495**	0.181	1.000	0.034
	Sig. (2-tailed)	0.000	0.177		0.803
	N	57	57	57	57
pH	r_s	−0.251	−0.398**	0.034	1.000
	Sig. (2-tailed)	0.053	0.002	0.803	
	N	60	60	57	60
T_{water}	r_s	**0.606****	0.514**	**0.418****	−0.443**
	Sig. (2-tailed)	0.000	0.000	0.001	0.000
	N	60	60	57	60
O_2 concentration	r_s	−0.483**	−0.393**	−0.315*	0.339**
	Sig. (2-tailed)	0.000	0.002	0.017	0.008
	N	60	60	57	60
EC	r_s	**0.610****	**0.655****	0.315*	**−0.639****
	Sig. (2-tailed)	0.000	0.000	0.017	0.000
	N	60	60	57	60
Cl^-	r_s	0.401**	0.417**	0.159	−0.398**
	Sig. (2-tailed)	0.002	0.001	0.238	0.002
	N	60	60	57	60
NO_3^-	r_s	0.499**	0.385**	0.287*	−0.276*
	Sig. (2-tailed)	0.000	0.002	0.031	0.033
	N	60	60	57	60
SO_4^{2-}	r_s	0.132	0.298*	0.027	−0.304*
	Sig. (2-tailed)	0.314	0.021	0.845	0.018
	N	60	60	57	60
Na^+	r_s	0.433**	0.486**	0.168	−0.438**
	Sig. (2-tailed)	0.001	0.000	0.211	0.000
	N	60	60	57	60
NH_4^+	r_s	0.349*	0.325	0.102	−0.324
	Sig. (2-tailed)	0.043	0.061	0.587	0.061
	N	34	34	34	34
K^+	r_s	0.542**	0.560**	0.135	−0.442**
	Sig. (2-tailed)	0.000	0.000	0.323	0.000
	N	59	59	56	59
Ca^{2+}	r_s	**0.605****	**0.623****	0.367**	**−0.612****
	Sig. (2-tailed)	0.000	0.000	0.005	0.000
	N	60	60	57	60
Mg^{2+}	r_s	0.114	0.174	−0.032	−0.216
	Sig. (2-tailed)	0.388	0.183	0.813	0.097
	N	60	60	57	60
Precipitation within 7 d before sampling	r_s	0.264*	−0.040	0.006	−0.063
	Sig. (2-tailed)	0.042	0.761	0.966	0.631
	N	60	60	57	60
Altitude of catchment	r_s	**−0.615****	−0.567**	−0.299*	−0.249
	Sig. (2-tailed)	0.000	0.000	0.024	0.436
	N	60	60	57	12
Altitude of spring	r_s	−0.550**	−0.447**	**−0.425****	0.495**
	Sig. (2-tailed)	0.000	0.000	0.001	0.000
	N	60	60	57	60
Catchment area	r_s	0.546**	0.427**	0.292*	0.397**
	Sig. (2-tailed)	0.000	0.001	0.027	0.002
	N	60	60	57	60
Mean amount of precipitation	r_s	−0.463**	−0.424**	−0.145	−0.418**
	Sig. (2-tailed)	0.000	0.001	0.281	0.001
	N	60	60	57	60
Mean annual T_{air}	r_s	0.590**	0.532**	0.252	0.395**
	Sig. (2-tailed)	0.000	0.000	0.059	0.002
	N	60	60	57	60

structure, and size of voids and fissures in the karst rock massive (Pronk et al. 2009, Zhou et al. 2012). However, our study provides evidence that the differences between eco-regions in Slovenia, with respect to groundwater composition, exceed the seasonal oscillations; exceptions were DOC and pH. DOC in groundwater mainly derives from terrestrial ecosystems, i.e. direct leaching after leaf fall and from decomposing OM in the soil layer (Gooddy & Hinsby 2008), which depends on the season that determines surface temperature, hydrological conditions, and litterfall decomposition (Kirschbaum 2006). In our study area, air temperature peaks in summer, hydrological discharge and flow peak in spring and autumn (from heavy rain events and snow melting), while litterfall, leaching, and decomposition peak in autumn. Consequently, significant seasonal variations of DOC in groundwater occurred (DOC_{max} in autumn, DOC_{min} in winter). Besides seasons, DOC is significantly correlated with the altitude of the spring, water temperature, EC, and calcium and oxygen concentrations. The median concentration of DOC in the investigated spring waters (2.4 mg l^{-1}) is comparable to DOC concentrations from other European groundwaters, with median concentrations of about 2 mg l^{-1} (Gooddy & Hinsby 2008). The variations in pH values can be explained by local environmental temperature, mineral composition of the geological strata, hydrological dynamics, and soil composition (Nelson 2002). Thus in our study, EC and calcium concentration showed significant correlation with pH, as well as the altitude of the spring, water temperature, sodium and potassium concentrations, and mean annual amount of precipitation. Accordingly, water pH was highest in summer and lowest in autumn. The median groundwater pH value from our study sites in Slovenia (8.0 ± 0.3) was slightly higher than other European limestone groundwaters (7.2–7.8)
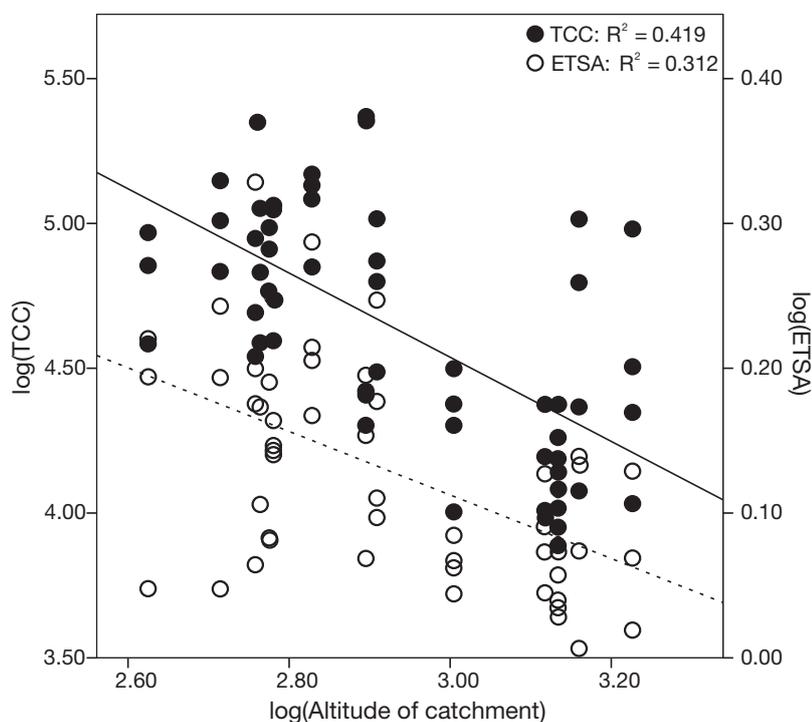
Fig. 7. Log of total cell count (TCC; solid black line) and log of electron transport system activity (ETSA; dashed black line) versus log altitude of catchment

(2018), who also highlighted the crucial role of the composition and quality of OM. In our study, TCC was significantly correlated with EC, water temperature, calcium concentration, mean annual air temperature, as well as with the altitude of spring and size of the catchment area. The results of TCC from Slovenian karst aquifers showed strong seasonal variation in comparison to other pristine alluvial aquifers (Stein et al. 2010, Zhou et al. 2012), which was expected due to the more dynamic karst systems (Shabarova et al. 2013, 2014, Savio et al. 2019). The ETSA values of spring water in our study were on average lower than the ETSA of the surface and interstitial waters of a prealpine river in Slovenia (Simčič & Mori 2007), which was predicted due to lower microbial biomass in the groundwater (Griebler & Lueders 2009). In our study, significant correlations with catchment area characteristics were found, especially strong with EC, concentration of calcium and potassium ions, and mean annual air temperature and water temperature; however, in interstitial waters, researchers found no significant correlation between ETSA and basic physico-chemical variables (Simčič & Mori 2007), which could be a result of shorter sampling period and smaller sampling area.

(Wendland et al. 2008). Seasonality was also reflected in the dynamics of abundances of prokaryotic cells (i.e. TCC) and microbial respiratory activities (i.e. ETSA) in spring water, which again showed a relationship with DOC dynamics (e.g. the highest values in autumn, the lowest in winter). The correlation between DOC and prokaryotic abundance was previously mentioned by Hofmann & Griebler

Table 6. Summary of hierarchical multiple regression results for 2 models (without and with season) including significant variable, total and adjusted R^2 (variance explained), F and regression slope (β) with p-values explaining log(TCC) (n = 60) and log(ETSA) (n = 57). TCC: total prokaryotic cell counts; ETSA: electron transport system activity

Result explained	Model	Significant variable	Total R^2	Adjusted R^2 (%)	F and p	β and p
log(TCC)	1	Log(altitude of catchment)	0.457	43.5	$F_{1,25} = 21.029$ p < 0.001	β = −0.676 p < 0.001
	2	Log(altitude of catchment) Season	0.469	42.5	$F_{2,24} = 10.614$ p < 0.001	β = −0.676 p < 0.001 β = 0.112 p = 0.459
log(ETSA)	1	Log(altitude of catchment)	0.312	28.4	$F_{1,25} = 11.317$ p = 0.002	β = −0.558 p = 0.002
	2	Log(altitude of catchment) Season	0.330	27.4	$F_{2,24} = 5.912$ p = 0.008	β = −0.558 p = 0.003 β = −0.136 p = 0.424

Significant regional differences were also shown for prokaryotic abundance and microbial respiratory activities. A negative correlation was found between prokaryotic abundance and microbial activity with catchment altitude (Hypothesis 3). A similar altitudinal pattern was observed for the richness and diversity in stream bacteria (epilithic biofilms) (Wilhelm et al. 2015), which could be due to an increase in DOC diversity downstream. Similar results were shown for soil bacteria (Bryant et al. 2008); the authors of that study suggested a combined influence of soil temperature, pH, and total nitrogen as the most important predictors of bacterial community composition. However, Wang et al. (2011) showed contrasting results of bacterial biofilms in the streams, where a monotone increasing pattern with altitude was demonstrated, and Fierer et al. (2011) showed no significant altitudinal pattern in soil bacteria at all. The eco-regions addressed in our study showed an increasing surface area covered by meadows and forests with lower catchment altitude, which resulted in an increase in thickness of soil cover and seasonal leaf mass as well as increasing DOC concentration down-gradient. At the same time, areas at lower altitudes harbour larger human population densities than higher alpine regions. This may explain higher NO_3^- concentrations, released from diffusive (agricultural land) and point sources (waste water discharge), which in turn could have contributed to an elevation of microbial abundance and activity in the aquifers at lower altitudes. We propose that future studies should address microbial community composition at a higher resolution while also considering further important key variables describing the hydrological cycle, such as water age and residence time. Moreover, focus should be on the energetic basis of the microbes and their functions, addressing the metagenome and transcriptome, as well as the biodegradability of the DOM.

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