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A MULTIPARAMETER ANALYSIS OF ENVIRONMENTAL GRADIENTS RELATED TO HYDROLOGICAL CONDITIONS IN A BINARY KARST SYSTEM (UNDERGROUND COURSE OF THE PIVKA RIVER, SLOVENIA)

MULTIPARAMETRSKA ANALIZA OKOLJSKIH GRADIENTOV, POVEZANIH S HIDROLOŠKIMI RAZMERAMI V BINARNEM KRAŠKEM SISTEMU (PODZEMNI TOK REKE PIVKE, SLOVENIJA)

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Abstract UDC 551.444:556.114(497.471)

Janez Mulec, Metka Petrič, Alenka Koželj, Clarissa Brun, Erika Batagelj, Aleš Hladnik & Ladislav Holko: A multiparameter analysis of environmental gradients related to hydrological conditions in a binary karst system (underground course of the Pivka River, Slovenia)

Chemical and bacterial gradients under different hydrological conditions were studied in a well-developed underground karst system. Water samples were collected from the main underground drainage conduit of the Pivka River from October 2013 until June 2016. The system responds quickly to external pulses (precipitation events), and is also impacted by human interventions, as is demonstrated mainly by fluctuations of sulphates, chlorides, and occasionally elevated concentrations of organic and faecal pollutants. Chemical and bacterial parameters showed a monotonous trend of decreasing concentrations from the ponor towards the interior of the karst massif during stable hydrological conditions, and a significant change during high water conditions. High flow events tend to equilibrate chemical and bacterial parameters in the underground river. Concentrations of chlorides, TOC (total organic carbon) and nitrates were the most indicative parameters describing the formation of the gradient. Stable isotopes of hydrogen and oxygen in water indicated that the main karst conduit collects isotopically different waters from the aquifer. The river water collected

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Janez Mulec, Metka Petrič, Alenka Koželj, Clarissa Brun, Erika Batagelj, Aleš Hladnik & Ladislav Holko: Multiparametrska analiza okoljskih gradientov, povezanih s hidrološkimi razmerami v binarnem kraškem sistemu (podzemni tok reke Pivke, Slovenija)

V dobro razvitem podzemnem kraškem sistemu smo pri različnih hidroloških pogojih preučevali kemijske in bakterijske gradiente. Vzorce vode smo odvzeli iz glavnega podzemnega toka reke Pivke med oktobrom 2013 in junijem 2016. Sistem se hitro odziva na zunanje impulze (padavinski dogodki) in je tudi podvržen človekovim posegom, kar dokazujejo predvsem nihanja v koncentraciji sulfatov in kloridov ter občasno povišane koncentracije organskih in fekalnih onesnaževal. Spremljanje kemijskih in bakterijskih parametrov v stabilnih hidroloških razmerah je pokazalo monotoni trend zniževanja koncentracij od ponora proti notranjosti kraškega masiva. Razmere se izrazito spremenijo v času visokih vod, ko pride v podzemnem vodotoku do izenačenja tako kemijskih kot bakterijskih parametrov. Kloridi, TOC (skupni organski ogljik) in nitrati so bili najbolj indikativni parametri za opis nastanka gradienta. Stabilni izotopi vodika in kisika v vodi so pokazali, da vodotok glavnega kraškega kanala zbira izotopsko različne vode iz vodonosnika. Voda podzemne reke po devetih kilometrih toka v podzemlju je bila vedno izotopsko lažja kot vode iz gorvodno vzorčevanih

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after nine kilometres of underground flow was always isotopically lighter than the waters collected from the upstream sites. Multiparameter analysis proved to be a useful tool for providing a more comprehensive understanding of the dynamics of the underground water, which influence both the underground environment and the ecology of the biome.

Key words: karst, hydrology, water chemistry, nutrients, stable isotopes, PCA, bacteria.

mest. Multiparametrska analiza se je izkazala kot uporabno orodje za celovitejše razumevanje dinamike podzemnih voda, ki vpliva tako na podzemno okolje kot ekologijo bioma.

Ključne besede: kras, hidrologija, kemija vode, hranila, stabilni izotopi, PCA, bakterije.

INTRODUCTION

For management of water resources for human use and for the well-being of dependent aquatic ecosystems the understanding of groundwater and surface water interactions at all scales is necessary. This is especially important for binary karst systems that are fed by both autogenic (diffuse infiltration into a karst aquifer) and allogenic recharge (sinking water from surrounding non-karst areas). Streams coming from non-karst watersheds commonly show large discharge fluctuations, have low mineral content and carry dissolved or particulate organic matter, especially during flood events (Bailly-Comte *et al*. 2009). At the contact with karst or a short distance beyond it, they sink into the ground, travel through conduits in the aquifer and eventually discharge downstream through caves and springs. As a result, groundwater and surface water constitute a single hydrodynamic system (Katz 2002; Koit *et al*. 2017). The defining characteristics of karst aquifers are rapid throughput times, localization of flow along essentially one-dimensional flow paths within the conduit system, and the presence of deposits of clastic sediments in many of the conduits. Peak flows in karst systems may be up to 100 times the low flows, and flow velocities are also far higher during high flows. The ability of the conduit system to store and release contaminants is dependent both upon the nature of the contaminants and upon the storm flow characteristics of the system (Vesper *et al*. 2001).

Karst waters are significant sources of water supply in many regions of the world. In the context of a global shortage of good quality water, they are particularly vulnerable to eutrophication resulting either from natural processes, e.g., toxic algal blooms, human intervention, such as intense agriculture production, or water pollution from industry and urbanization (Jianhua *et al*. 2016; Long *et al*. 2012; Shi *et al*. 2009; Vallejos *et al*. 2015; Wang *et al*. 2018). Impact of pollution on underground ecosystems is particularly detrimental in well-karstified aquifers, because they generally react rapidly to changes in hydrological conditions (Ender *et al*. 2018; Hartmann *et al*. 2014). Once in the light-deprived underground environment, different materials, particulate matter and partly and/or completely dissolved compounds of natural and anthropogenic origin form concentration gradients towards the interior of the karst massif. These gradients are useful in providing a better understanding of the processes affecting the hydrological cycle in the karst. Formation of a gradient in the underground is generally challenged by dilution and/or concentration of compounds, and details of abiotic and/or biotic conversions of the material should be further explored. Many aquatic organisms adapted to such environments rely upon nutrients that are either introduced from the surface or result from *in situ* chemolithoautotrophy-based primary production (Hutchins *et al*. 2016). Contamination of groundwater with pathogenic organisms is associated with an introduction of faecal material of human and animal origin (Heinz *et al*. 2009). Noteworthy contributors to potentially hazardous faecal pollution are point sources such as failed septic systems, leaking sewer lines and cesspools, animal-fattening areas, dairy farms and other intensive animal-husbandry operations (Macler & Merkle 2000). Water quality in karst systems can be monitored using several physical, chemical and microbial parameters. Bacterial indicator groups that respond to organic pollution represent an additional estimator of the health of underground systems (Pronk *et al*. 2006). Monitoring the fate of natural tracers, e.g., stable isotopes that are directly related to the water cycle, and compounds subjected to chemical and biochemical conversions can reveal the complexity of underground water quality, particularly in cases with an evident impact from eutrophication.

The objective of the present study was to analyse and correlate the geochemical and bacterial gradients in a well-developed underground karst system during different hydrological conditions. Such a study of transport properties that demonstrate well-defined flow characteristics within the system can add important information to the results obtained at a broader scale, when only the input and output points are known, with limited data on water chemistry and artificial tracers (Gabrovšek *et al*. 2010).

Study was undertaken in a binary karst system comprising 9 km-long underground course of the Pivka River, which is occasionally affected by the pollution events in the catchment area. The watercourse within the system is readily accessible at various locations by way of natural cave entrances, and the cave system harbours a diverse stygobitic (groundwater-adapted) fauna (Pipan & Culver 2007). Physical, chemical and bacterial parameters were used simultaneously to assess the water quality during different hydrological (flow) conditions relative to the input values in a complex karst aquifer, and to relate dilution/concentration effects and (bio)chemical conversions. Along with the multiparameter approach, monitoring was carried out at several points along the underground watercourse, adding additional information to the studies where only the input and output environmental values of the karst system were considered.

MATERIAL AND METHODS

SITE DESCRIPTION

The Pivka Basin with the Pivka River (southwestern Slovenia) is slightly to moderately impacted by agriculture, industry and urbanization. In its upper part the Pivka River is recharged from a karst aquifer within the Javorniki Mountains, which are composed mostly of Cretaceous limestone (Buser *et al*. 1967). In the lower part of the river course, limestone is overlain by low-permeability Eocene flysch deposits, upon which the surface drainage networks of the Pivka River and its tributary the Nanoščica River have developed (Fig. 1A). Discharges of the Pivka River range from 0.001 to 66 m^3/s, and the mean discharge is 5.3 m^3/s (Archive hydrological data assessed 26 January 2015, http://vode.arso.gov.si/hidarhiv/pov_arhiv_tab.php). The Pivka River provides the main allogenic recharge to the famous Postojna Cave system (Postojnska jama), which comprises several caves: from Postojna Cave at the ponor site (E 14.2037° N 45.7827°, 529 m a.s.l.) to the Pivka Cave (Pivka jama), which provides the ultimate access to the underground Pivka River course within this system (Fig. 1A). Some of the system's dry passages are open for tourist visits, attracting approximately 700,000 visitors per year.

The known underground course of the Pivka River is a continuous and almost completely accessible water passage with different channel geometries. Upstream from the Pivka Cave, the flow is obstructed by several collapses that cause significant ponding of water, giving rise to flow cascades along a series of lakes impounded upstream of each collapse zone. Evidently braided channels are present and which flow paths are followed by the river flow depends upon the flow rates. The channel slope in this part of the system is about 0.01, i.e., about twice the slope in the first part of the cave system (Gabrovšek *et al*. 2010). Underground flow continues through unexplored channels towards Planina Cave (Planinska jama) at the southern edge of the Planina polje (Planinsko polje). Here the Pivka cave stream flows along the western branch of the cave and converges with the Rak cave stream, which occupies the eastern branch of the cave. The latter passage carries most of the water from the Rak River, which sinks into Tkalca Cave (Tkalca jama). Downstream from the underground confluence, the water flows out of Planina Cave as the Unica spring (E 14.2457° N 45.8199°, 453 m a.s.l.) (Fig. 1A). A subsurface connection between the Pivka River in Postojna Cave and the Pivka cave stream in Planina Cave has been confirmed by several tracer tests. Tracers injected into the Javorniki karst plateau were also detected in the Pivka cave stream, which confirms that there is additional autogenic recharge from the karst aquifer (Gabrovšek *et al*. 2010; Kogovšek & Petrič 2004; Ravbar *et al*. 2012).

SAMPLING

The underground Pivka River was sampled at four sites: (Site 1) immediately downstream of the ponor in Postojna Cave (Veliki Dom, 0.1 km inside the cave), (Site 2) 0.9 km from the ponor (Spodnji Tartar), (Site 3) at the siphon in Pivka Cave (4.1 km from the ponor), and (Site 4) just upstream of the confluence with the Rak cave stream in Planina Cave, at a distance of 9.0 km from the ponor (Figs. 1A and 1B).

Hydrological conditions are the most important factor that determine the underground water flow and water chemistry. Seven sampling campaigns were carried out during the period from October 2013 to June 2016 under different hydrological conditions (27 June 2013, 21 October 2013, 14 July 2014, 25 November 2014, 1 September 2015, 15 December 2015, and 28 June 2016). Because of the extensive nature of the cave system, sampling was time consuming and lasted for up to 5 hours (between the first and the last sampling sites). This time difference should be taken into account when interpreting the data collected during changeable hydrological conditions.

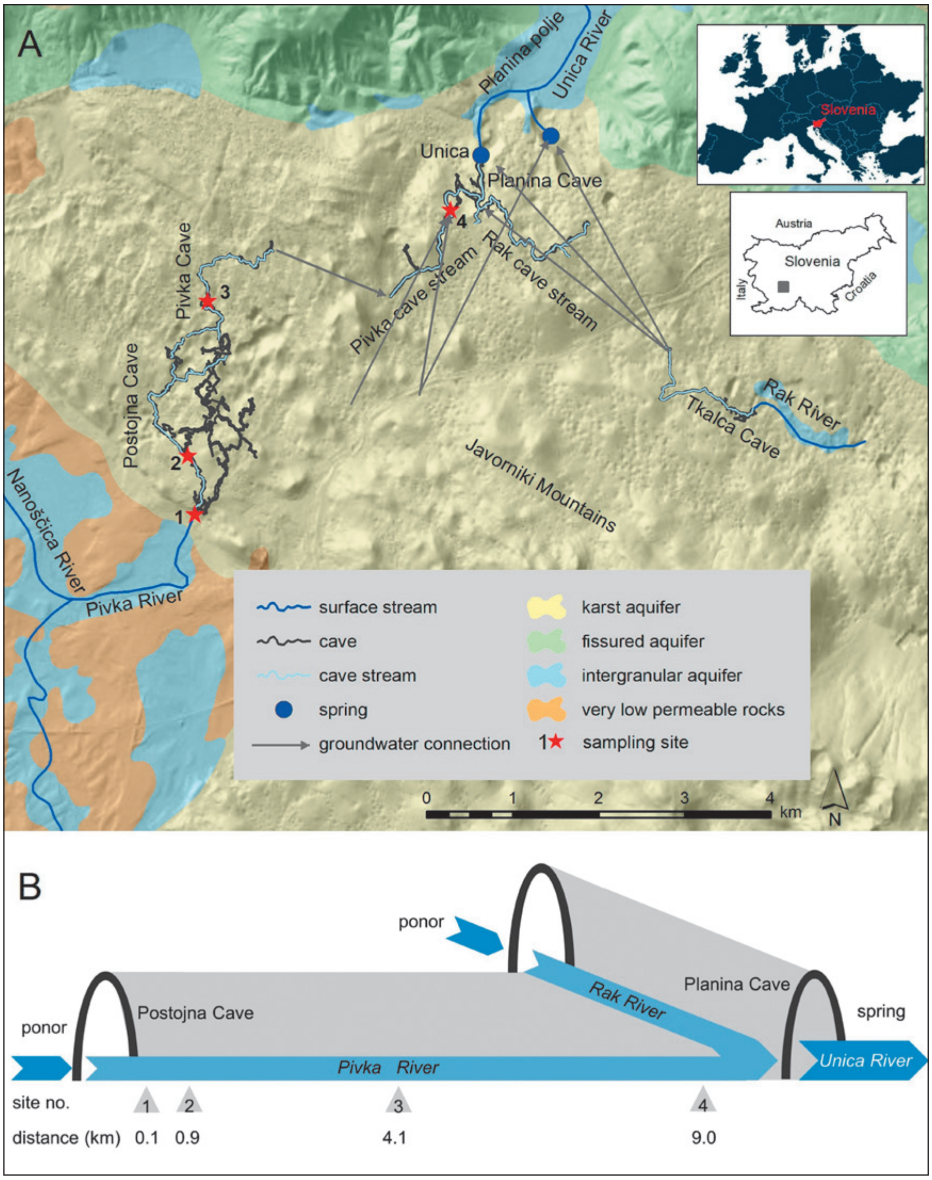

Fig. 1: Study site: A – hydrogeological map of the area with designated sampling sites along the underground Pivka River, B – schematic representation (not to scale) of sampling sites (1-4) along the underground Pivka River, with distances from the ponor in Postojna Cave; sources: Lidar (Slovenian Environment Agency); Hydrogeological map (Geological Survey of Slovenia); Cave cadastre (Karst Research Institute ZRC SAZU; Speleological Association of Slovenia).

On-site measurements of water temperature (T), electrical conductivity (EC), pH and dissolved oxygen (DO) were performed using a WTW Multiline 3420 portable meter (WTW, Germany) during collection of water samples for chemical and microbiological analyses. Water samples were subdivided for selected analyses in the caves at each individual site: water from two 1.5 litre plastic bottles was analysed for true colour, turbidity, ammonium, chloride, fluoride, nitrite, nitrate, sulphate, orthophosphate and total phosphorus. Water from two 1 litre glass bottles was analysed for anionic surfactants, and water from 250 millilitre glass bottles without air was analysed for TOC. A 20 ml glass bottle was filled directly from the source for analysis of isotopes of oxygen and hydrogen. For microbiological analyses, water samples were collected aseptically in 0.5 litre sterile plastic bottles. All samples were transferred to the laboratory in a cool box.

High-frequency monitoring of physico-chemical parameters contributes to better understanding of the hydrogeological functioning of karst systems (e.g., Pronk *et al*. 2006; Tissier *et al*. 2013). In the periods from August 2013 to August 2014 and from November 2015 to August 2016, T and EC were measured every 30-minutes by Onset HOBO Conductivity data loggers at the ponor site (150 m upstream from Site 1) and in the Pivka cave stream in Planina Cave (Site 4).

Precipitation data collected at the Postojna meteorological station (E 14.1928° N 45.7661°, 533 m a.s.l) were obtained from the Slovenian Environment Agency. Discharges of the Pivka River at the ponor in Postojna

Cave (E 14.2037° N 45.7827°, 529 m a.s.l.) were defined as sums of the discharges of the Nanoščica and Pivka rivers, measured by the Slovenian Environment Agency at the two hydrological stations (E 14.1812° N 45.7800°, 517 m a.s.l and E 14.1861° N 45.7299°, 529 m a.s.l, respectively) upstream of their confluence. The confluence is 2.1 km upstream the ponor in Postojna Cave.

ANALYSES OF PHYSICAL AND CHEMICAL PARAMETERS

Laboratory analyses were performed at the National Laboratory of Health, Environment and Food, Koper, and Karst Research Institute ZRC SAZU, Postojna, according to the following methods: true colour (SIST EN ISO 7887-2012), turbidity (SIST EN ISO 7027:2000), ammonium (ISO 7150-1:1984), nitrite (SIST EN 26777:1996), nitrate (HM075-HPLC), anionic surfactants (SIST ISO 7875-1:2004), total organic carbon – TOC (ISO 8245:1999), chloride (ISO 10304-1- 2007), sulphate (ISO 10304-1- 2007), fluoride (ISO 10304-1- 2007, HM052/UV-VIS), orthophosphates (SIST EN ISO 6878:2004), total phosphorus (SIST ISO 6878-7:2004).

Water samples were analysed for their stable isotopes of oxygen and hydrogen using a Thermo Delta Plus isotope-ratio mass spectrometer (Thermo Fischer Scientific). The samples were placed in 10 ml screw-top vials, and for D/H, a platinum catalyst was added. The vials were sealed with septa and all air was removed from the sample vials by an automated, autosampler-assisted flushing procedure that uses a mixture of either H_2 or CO_2 in He. After the required equilibration time (D: 40 min, ^{18}O: 20 h), the whole batch of samples was analysed. The results were elaborated by a five points calibration curve linear regression algorithm. For the upper and lower points of the calibration curve the following reference materials were used: VSMOW (Vienna Standard Mean Ocean Water, $\delta^{18}O$ 0 ‰; δ^2H 0 ‰); SLAP (Standard Light Antarctic Precipitation, $\delta^{18}O$ -55.5 ‰; δ^2H -428 ‰); for the intermediate points a mix of the two was made, while the control for the accuracy of the calibration was the GISP (Greenland Ice Sheet Precipitation, $\delta^{18}O$ -24.78 ‰; δ^2H -189.7 ‰). The data were expressed in the conventional δ notation in per mille with respect to Vienna Standard Mean Ocean Water. Analytical accuracy was ±0.1‰ for $\delta^{18}O$ and ±1‰ for δ^2H. Evolution of the isotopic composition of water along the river course was used to identify possible contribution of different waters to the river.

BACTERIAL INDICATOR GROUPS

Several indicator bacterial groups were taken to trace the external impact in the cave system. The total concentration of heterotrophic aerobic bacteria, coliforms, *Escherichia coli* and enterobacteria were estimated using RIDA®COUNT plates (Mulec *et al*. 2012). The commercially available RIDA®COUNT test plates contain standard nutrients and a specific chromogenic detection system to detect cultivable microorganisms or a selected group. The specific microbial enzymes will change the originally colourless substrate to a distinctively coloured colony (Morita *et al*. 2003). Plates were inoculated with one millilitre of the water sample and cultivated for 48 hours at 37°C, and grown colonies were expressed as Colony-Forming Units (CFU) per ml. Enterococci were determined following the ISO 7899-2:2000 membrane filtration method. Bacterial analyses were performed at the National laboratory of health, environment and food, Koper, and Karst research institute ZRC SAZU, Postojna.

For statistical analyses the following bacterial indicator groups were used: total bacterial counts (BAC), concentration of enterococci (ENCOC), *E. coli* (ECO), coliforms (COL), non-coliform bacteria (NCOBA – represented bacterial group that excludes coliform bacteria), non-enteric bacteria (NENBA – represented bacterial group which excludes enteric bacteria), non-*E. coli* coliforms (NECCO – calculated as the number of *E. coli* colonies subtracted from the total coliform counts), non-*E. coli* enterobacteria (NECEN – calculated as the number of *E. coli* colonies subtracted from the total enterobacterial counts (Oarga *et al*. 2012).

PRINCIPAL COMPONENT ANALYSIS

Results of bacterial and physico-chemical analyses were interpreted by means of a multivariate statistical tool, Principal Component Analysis (PCA). PCA is widely used for data exploration and/or reduction purposes: by plotting data in the coordinate system of two PCs one can visualize trends and patterns present in the samples. These trends are deduced from the scores diagram. Similarities and differences among the original variables are investigated by means of the loadings plot (Golež & Hladnik 2013; Hladnik & Muck 2002). Mathematically, PCA can be performed either by eigenvalue decomposition of a data covariance (or correlation) matrix or by singular value decomposition of a data matrix, usually after mean centring the data matrix for each original variable (Abdi & Williams 2010). PCA was performed using singular value decomposition of the data matrix after standardization - subtraction of the mean and division by the standard deviation of each attribute (parameter).

RESULTS AND DISCUSSION

HYDROLOGICAL CONDITIONS

Sampling campaigns to investigate water chemistry and bacterial load were carried out under various hydrological conditions. On 27 June 2013, 1 September 2015, and 15 December 2015, during a period of low flows, the hydrological conditions were stable, which means that during the sampling campaign no significant changes in the discharge and physical parameters were detected at the sampling points along the underground watercourse. Hydrological conditions were more unstable during the sampling on 25 November 2014, in high flow conditions at the end of the strongest flood pulse of the monitored period. Three samplings were carried out during flood pulses following lesser precipitation events: on 21 October 2013 during an increase of discharge, and on 14 July 2014 and 28 June 2016 during recessions (Fig. 2).

Time series of measured EC values at sampling sites 1 and 4 were compared to provide a more detailed assessment of hydrological characteristics at the time of sampling. Fig. 3 presents an example from the sampling campaign in October 2013. At Site 1, the sample was collected 13 hours after the peak of the precipitation event, at the time when the EC decreased at the ponor indicating the beginning of water-quality changes induced by the event. The sample at Site 4 was taken 4 hours earlier, when stable EC values at this site were indicative of the pre-event conditions. Thus the two samples are not directly comparable. The October 2013 sampling campaign

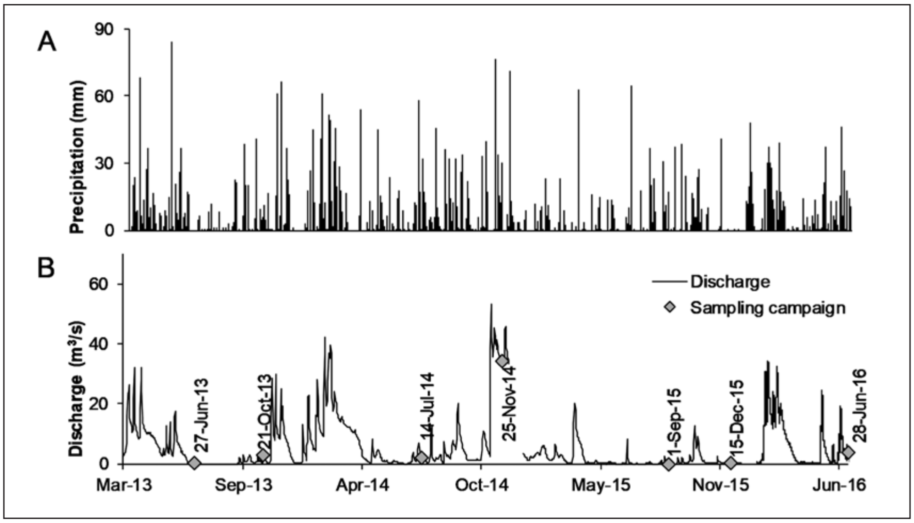

Fig. 2: (A) Daily precipitation in Postojna and (B) discharges of the Pivka River at the ponor during the sampling campaigns.

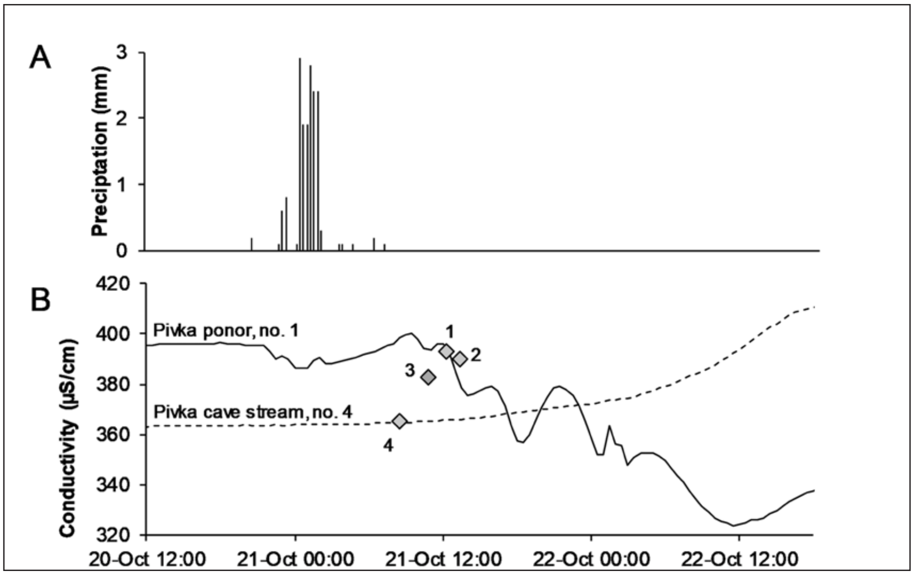

Fig. 3: An example of unstable hydrological conditions in October 2013: A – precipitation, B – on-site measured EC at sampling sites 1 and 4, with designated sampling times at four sites along the groundwater course of the Pivka River.

represents the most extreme difference in flow conditions during the sampling period. Interpretation of chemical and microbiological data during unstable hydrological conditions must be based upon an understanding of what the individual samples represent.

STABLE ISOTOPES

Stable isotopes have long been used to elucidate the sources of water (Clark & Fritz 1997; Kendall & Caldwell 1998). Isotopic compositions of the samples at Sites 1 and 2 were quite similar, i.e., the differences were close to the limits of analytical accuracy (Fig. 4). This indicates the same source of water at both sampling sites. Water at Site 3 was lighter isotopically than at Sites 1 and 2, but its isotopic composition in July and November 2014 differed significantly from those at Sites 1 and 2. The difference in isotopic composition of river waters between Sites 1, 2 and 3 indicated contributions of isotopically-lighter water along the river course. A reverse pattern was observed in July 2014, when the water at Site 3 was isotopically very heavy compared to samples from all other sites. The July 2014 sampling was conducted during flow recession following a minor rainfall–runoff event. It is therefore possible that other flow paths delivering isotopically-heavier water were activated.

Water at Site 4 is noticeably depleted in heavy isotopes compared to the upstream sites. Samples collected in September 2015 are plotted below the Global Meteoric Water Line, which indicates evaporated water (Fig. 5). However, the pattern was similar to that observed on other sampling days, i.e., isotopically-similar waters at Sites 1 and 2, different (usually lighter) water at Site 3 and noticeably lighter water at Site 4.

The analyses showed that along the course of the

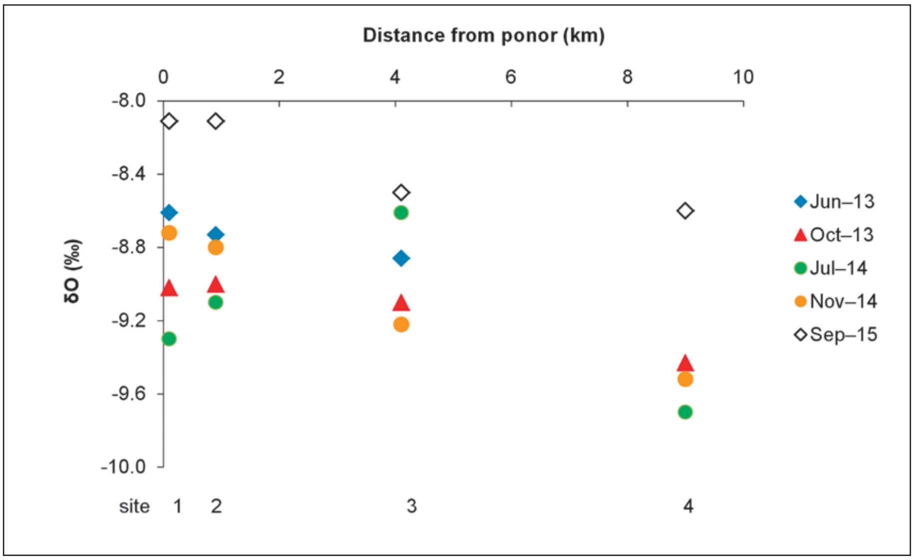

Fig. 4: $\delta^{18}O$ (‰) at sampling sites (1–4) during the study period; the blue and green symbols display summer values, orange and red symbols show the autumn values and white symbols indicate the evaporated water sampled in September 2015.

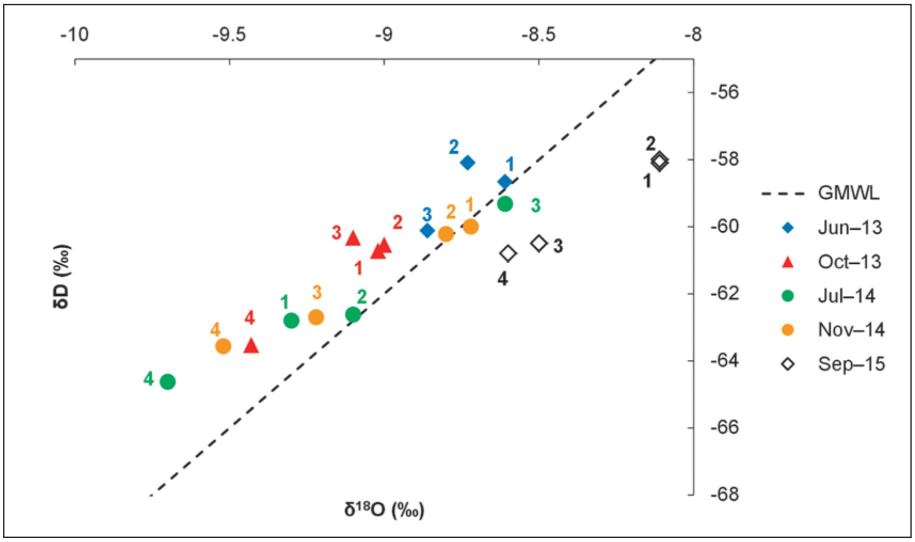

Fig. 5: Comparison of the waters from different sampling campaigns with indicated sampling sites (1–4) relative to the Global Meteoric Water Line (GMWL, $\delta^2H=8\delta^{18}O+10$).

Pivka River water becomes isotopically lighter, which is the opposite of the gradient known from surface streams in non-karst areas (Holko *et al*. 2015). Change in the isotopic composition of the river waters occurs mainly between Sites 3 and 4. The isotopic gradient existing in the river between the ponor and Site 4 indicates an increasing contribution of water percolating through the rocks of the massif. A very clear gradient was also observed during the flood conditions on 25 November 2014, when the isotopic composition of water at Site 3 was significantly lighter than at Sites 1 and 2. This indicates the mobilization of flow paths that are less active between the two river sections during low flow conditions. This is in accordance with the results of another study, which showed activation of additional flow paths during high flow conditions (Gabrovšek *et al*. 2010).

CHEMICAL AND MICROBIOLOGICAL PARAMETERS AND THEIR INTERRELATIONSHIPS

Stable isotopes indicated activation of other flow paths during high flows. In addition, the chemical composition of the water provided information on interactions with the surroundings, e.g., rocks and sediments, and even the impact of (bio)chemical conversions. The measured parameters showed differences between sites, some of them being directly related to changes in hydrological conditions. Levels of dissolved oxygen were usually lower at the ponor (Fig. 6A); this can be attributed partly to enhanced microbial consumption and higher temperature at this site. Levels of nitrogen (maximum concentration of ammonium at 0.15 mg/l and nitrates at 6.76 mg/l) and phosphorus (< 0.5 mg/l) compounds were surprisingly low considering that the upstream area is subjected to ag-

Tab. 1: Ranges of physico-chemical and bacterial parameters of the underground Pivka River at sampling sites, with designated codes for PCA analysis.

SAMPLING CAMPAIGN								
Parameter	Code (PCA)	27-Jun-13	21-Oct-13	14-Jul-14	25-Nov-14	1-Sep-15	15-Dec-15	28-Jun-16
T (°C)	TEMP	11.9–16.6	10.2–11.3	13.0–15.6	6.7–7.8	13.6–18.9	2.3–7.2	14.8–15.6
EC (µS/cm)	COND	407–458	357–393	317–337	393–394	366–405	427–454	353–420
pH		7.75–8.16	7.76–8.06	7.55–7.72	7.99–8.08	7.56–7.75	7.91–8.26	7.68–7.88
DO (mg/l)	DO	5.15–9.66	7.80–10.54	7.93–10.27	10.67–11.38	5.90–9.87	11.78–12.77	8.12–9.93
Colour (m^{-1})	COLOR	0.20–0.50	0.10–0.20	1.00–1.40	0.20–0.23	0.59–1.22	nd	nd
Turbidity (NTU)	TURBID	0.49–3.40	2.40–6.00	3.80–15.10	1.10–1.40	1.20–7.00	nd	nd
NH_4 (mg/l)	NH4	0.007–0.087	0.022–0.072	0.031–0.153	0.009–0.015	0.011–0.063	nd	0.030–0.074
NO_2 (mg/l)		<0.001–0.130	0.010–0.055	0.011–0.056	0.004–0.008	0.008–0.061	nd	0.010–0.061
NO_3 (mg/l)	NO3	3.04–5.88	3.45–6.37	2.60–4.18	2.82–3.12	4.20–6.57	2.05–6.76	nd
F (mg/l)		<0.100	<0.100–0.120	0.112–0.127	0.022–0.051	0.046–0.111	nd	0.037–0.172
Cl (mg/l)	Cl	8.0–20.7	10.4–17.0	4.9–8.6	3.4–3.7	24.0–28.3	9.9–16.8	9.8–13.0
SO_4 (mg/l)	SO4	4.3–6.5	11.6–13.3	3.5–7.0	2.7–3.1	10.3–10.9	8.4–12.5	4.3–4.8
o-PO_4 (mg/l)	o-PO4	0.075–0.186	0.270–0.310	0.114–0.171	0.016–0.021	0.095–0.109	0.090–0.120	0.037–0.172
Tot-PO_4 (mg/l)	TOT-PO4	0.105–0.342	0.370–0.460	0.215–0.327	0.041–0.057	0.111–0.184	nd	0.117–0.228
Anionic surfactants (mg/l)		<0.010–0.015	0.011–0.017	<0.010–0.015	0.002–0.007	0.005–0.012	nd	nd
TOC (mg/l)	TOC	4.57–7.21	3.27–5.00	3.56–4.69	1.24–1.34	3.96–6.79	nd	1.21–3.32
Bacteria (CFU/ml)	BAC	84–835	163–2520	398–2020	106–172	191–531	102–274	274–7030
Coliforms (CFU/ml)	COL	29–206	40–389	109–443	35–60	54–262	26–67	147–1020
Enterococci (CFU/100 ml)	ENCOC	0–20	7–530	80–820	37–72	12–57	nd	nd
Enterobacteria (CFU/ml)	ENBAC	40–294	40–627	138–795	34–72	57–332	29–83	125–1300
E. coli (CFU/ml)	ECO	0–6	1–24	3–48	1–4	1–4	0–1	1–78
Non-coliforms (CFU/ml)	NCOBA	55–633	121–2131	289–1657	71–122	137–391	75–207	167–3520
Non-enterics (CFU/ml)	NENBA	44–557	116–2385	216–1335	62–110	133–365	73–204	149–5730
Non-*E. coli* coliforms (CFU/ml)	NECCO	29–200	40–366	106–395	34–57	52–258	26–66	146–942
Non-*E. coli* enterics (CFU/ml)	NECEN	40–289	39–609	135–747	33–69	56–328	29–83	124–1231

ricultural and industrial pressure and occasional pollution impact. High concentrations of cultivable microbial indicators (Tab. 1) indicated that at least some of the nitrogen, phosphorus and sulphur compounds were fixed in microbial biomass.

Sulphate and chloride (Fig. 6B) exhibited greater fluctuations in concentration (Tab. 1). The presence of anionic surfactants, even in low concentrations, indicated pollution related to human activities upstream of the ponor. Water samples from 27 June 2013, 21 October 2013, 14 July 2014, 25 November 2014 and 1 September 2015 were also screened for the presence of chloroform, trichloroethane, tetrachloromethane, trichloroethane, bromodichloromethane, tetrachloroethene, dibromochloromethane, and bromoform. Quantities of these compounds were below detection limits.

Concentrations of bacterial indicators varied among sites. They were higher in the upper part of the underground water flow (Sites 1 and 2, Fig. 6C), and particularly in the samples from 21 October 2013, 14 July 2014 and 28 June 2016. The proportions of different bacterial indicators within the community did not remain constant across the sampling sites. The lowest concentrations of bacterial indicators were encountered during the high discharge of the Pivka River on 25 November 2014. They were accompanied by the lowest recorded values of TOC and other chemical parameters, which were caused by water dilution due to precipitation (Tab. 1).

PCA analyses were run to investigate further the relationships between the measured parameters across the sampling campaigns and to explain the contributions of individual original variables (Fig. 7). The first three extracted PCs account for more than 83% of data variance, i.e., of parameters' variability: PC1 – 52.7 %, PC2 – 19.2 % and PC3 – 11.5 %. The remaining 18 PCs (designated as Residuals in Fig. 7) therefore capture only a tiny percentage of the data variability.

Several of the investigated parameters showed similar behaviour with respect to the monitored sampling sites. Each of the eleven variables that are situated close to each other on the right-hand side of the PC1–PC2 loadings diagram (Fig. 8A) – NECCO, COL, BAC, NCOBA, NECEN, ENCOC, TURBID, ENBAC, NH_4, ECO and NENBA (see also Fig. 7) – exhibits a rather monotonous trend of decreasing values in the direction of river flow, i.e., between sites 1 > 2 > 3 > 4 (or in some rare cases 2 > 1 > 3 > 4). This is true for four of the five sampling campaigns. Sampling performed on 25 November 2014 is evidently an exception to this rule, because here most of the measurements for all four sites show only a minor fluctuation; this fact is also evident when looking at the PC1–PC2 scores diagram (Fig. 8B), where all four corresponding data points, i.e., sampling sites, are located in close proximity in the bottom-left part of the plot. This can be explained by specific hydrological conditions. The samples were taken during high water conditions at the

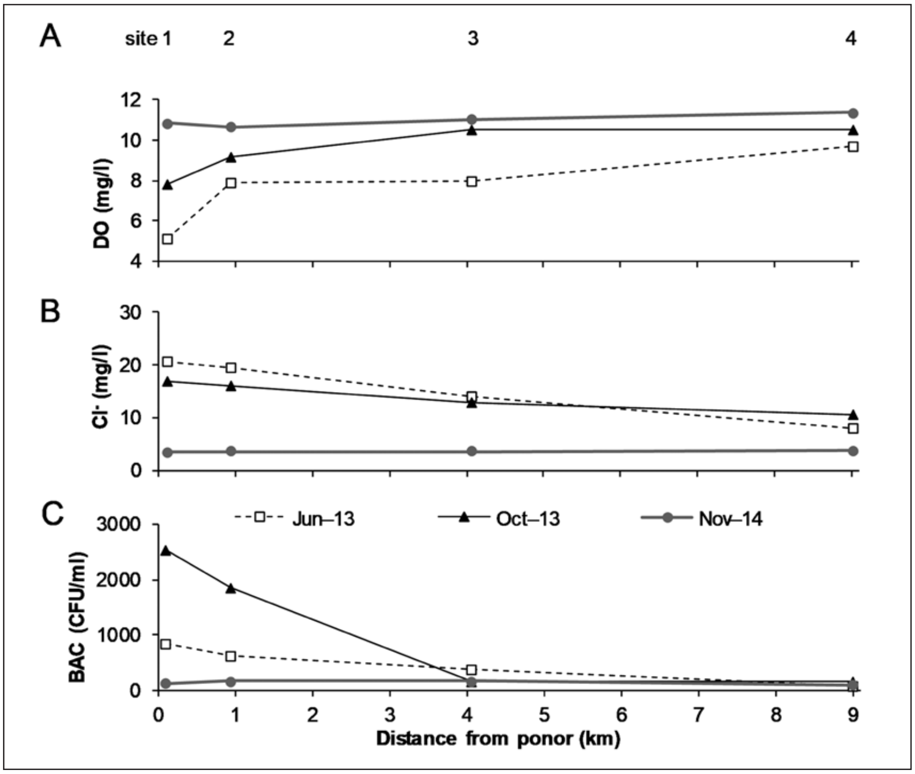

Fig. 6: Gradients of (A) oxygen, (B) chlorides and (B) bacteria between sites 1–4, with respect to the distance from the ponor of the underground Pivka River during different hydrological conditions.

Fig. 7: PCA results show the contribution of measured parameters to the first three PCs.

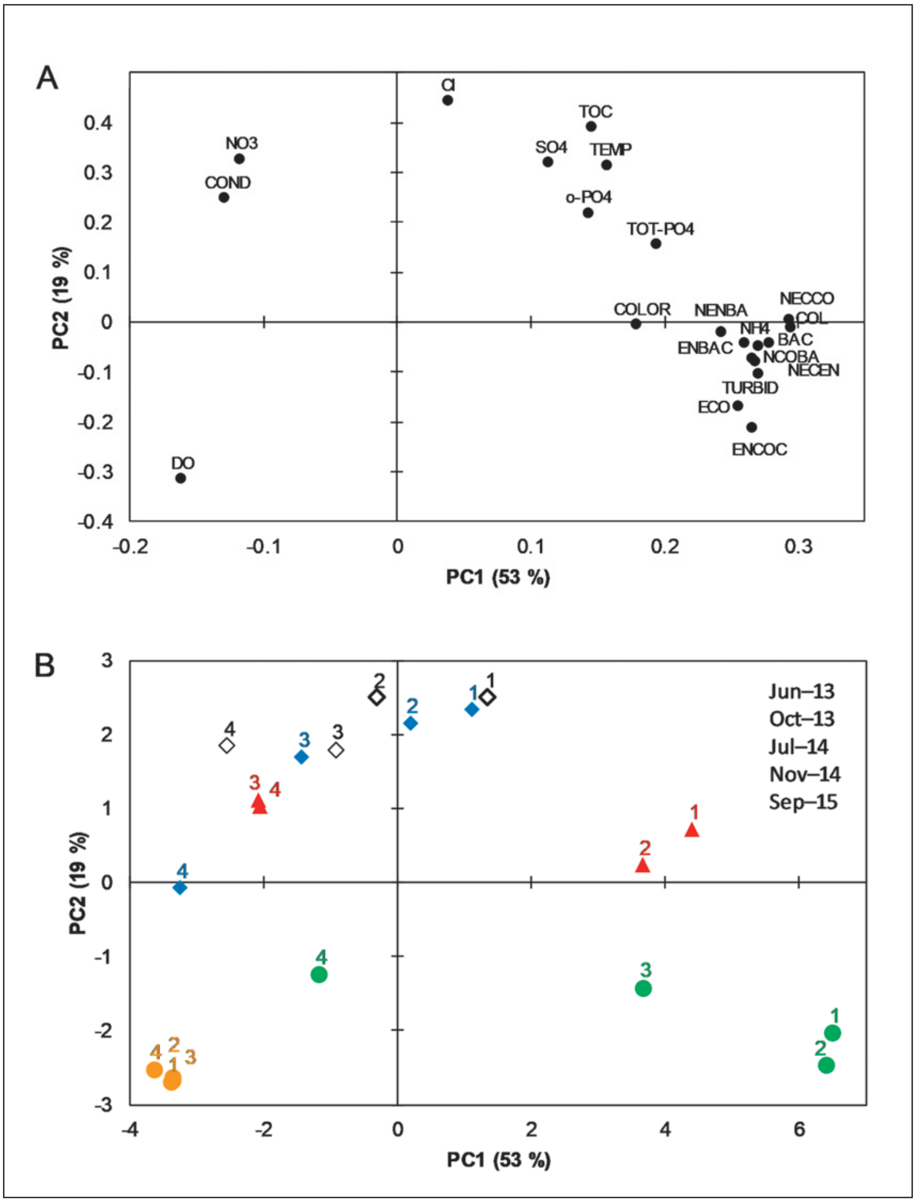

Fig. 8: PCA results: A – PC1-PC2 loadings plot, B – PC1-PC2 scores plot.

end of the strongest flood pulse in the observed period, when a high dilution by rainwater and fast transport within the cave system balanced the chemical and bacterial composition of the groundwater.

On the other hand, variables located on the left-hand side of the loadings plot are generally characterized by an increase in their values along the river course 1< 2< 3< 4. This is true for DO in particular, and to some extent also for nitrate. In addition, pronounced negative projection of the variable DO onto the horizontal axis – PC1 – revealed that this parameter was high during sampling in November 2014, because all four corresponding data points (1–4) in the scores diagram are also marked by significant negative projections onto PC1.

Chloride and to some extent TOC and nitrate are, according to Fig. 7 and 8A, the variables that are mainly responsible for the variability between the four sampling dates. Measured chloride values were highest in September 2015 and lowest in July and November 2014 (see locations of the corresponding data points in Fig. 8B). In contrast to TOC and nitrate, which are subjected to dilution and biochemical processes, chloride does not suffer microbial conversions.

The underground Pivka River is an example of a well karstified and dynamic underground karst system. During periods of high discharge there are no great differences in physical, chemical and bacterial parameters between the sampling sites, whereas during times of low discharge clear gradients are apparent. Similar values of chemical and bacterial parameters during the high flow in November 2014 confirmed the expected behaviour, which can be attributed to a very high dilution by rainwater and a homogenous, well-mixed and fast pulse of water with no distinguished underground gradient. However, stable isotope analyses showed a clear gradient during that event (Fig. 4), which indicates the activation of flow paths delivering isotopically lighter percolation waters from the karst massif.

Chloride, TOC and nitrate represented the most indicative parameters describing the formation of a chemical gradient. Not only these parameters, but also others are subjected to dilution effects in the underground (Tab. 1, Fig. 8A). Some parameters also reflect microbial metabolism, e.g., TOC and heterotrophic decomposition of organic matter, and nitrates and nitrification. The impact of nitrification, indicated by an increase in nitrate concentrations downstream from the ponor, should not be underestimated in the Pivka River and similar karst systems. The bacterial groups used in the study, which reflect mostly human impact, are not crucial for biogeochemical cycling of elements and matter. They represent only a minor part of the underground microbiome, but their importance lies rather in their potential pathogenic nature for humans and animals.

In comparison to the conditions of surface aquatic ecosystems, karst ground waters are exposed to more stable environmental conditions. Hydrological conditions and the microbiome direct the fate of organic loads and pollution. Continuous measurements of physical parameters and regular monitoring of chemical parameters and bacterial indicators, in this case particularly *E. coli* and enterococci, are crucial to monitor the health of the ecosystem particularly during varying hydrological conditions.

CONCLUSIONS

Underground karst systems with natural accessibility, such as the underground Pivka River, provide excellent study sites to monitor the quantitative changes of chemical and biological factors. Concentration gradients of physicochemical and bacterial parameters towards the interior of the karst massif were common during stable hydrological conditions, with chloride, TOC and nitrate concentrations being the most indicative parameters. Impact of rainwater and fast transport are responsible for equilibrium of chemical and bacterial parameters in the underground. Environmental pollution reflected in faecal bacteria can be used as an additional natural tracer in order to provide better understanding of underground water dynamics and formation of concentration gradients. Simultaneous analysis of stable isotopes gives a more comprehensive prospect on underground karst hydrology. Analyses of stable isotopes of hydrogen and oxygen in the underground Pivka River indicated mixing with isotopically lighter percolation water along the underground river, which can be explained by an important share of autogenic recharge in the observed binary karst system. Additionally, the results indicated the mobilization of otherwise less active flow paths during high-flow conditions. More data and long-term monitoring are needed to confirm whether this is a common mechanism for the studied karst system. In order to understand water dynamics and health of the underground ecosystem, the chemical compounds that enter microbial metabolism, e.g., nitrate, ammonium, sulphates and organic compounds, should be carefully interpreted and correlated with microbial biomass and activity.

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Appendix: Measured parameters of the underground Pivka River at sampling sites, part 1.

Sampling date	Sampling site	T (°C)	EC (µS/cm)	DO (mg/l)	Colour (m^{-1})	Turbidity (NTU)	NH_4 (mg/l)	NO_2 (mg/l)	NO_3 (mg/l)	F (mg/l)	Cl (mg/l)	SO_4 (mg/l)	o-PO_4 (mg/l)	TOT-PO_4 (mg/l)	Anionic surfactants (mg/l)
27-Jun-13	1	16.6	458	5.15	0.50	3.40	0.087	0.130	3.04	< 0.1	20.7	6.5	0.186	0.314	0.013
27-Jun-13	2	16.4	456	7.88	0.40	3.20	0.036	0.032	4.08	< 0.1	19.5	6.2	0.171	0.342	0.015
27-Jun-13	3	15.0	436	8.00	0.30	1.30	0.011	<0.001	5.88	< 0.1	14.1	5.5	0.186	0.226	<0.01
27-Jun-13	4	11.9	407	9.66	0.20	0.49	0.007	0.004	4.78	< 0.1	8.0	4.3	0.075	0.105	<0.01
21-Oct-13	1	11.2	393	7.80	0.20	4.50	0.072	0.055	3.56	0.120	17.0	13.2	0.290	0.440	0.017
21-Oct-14	2	11.3	390	9.14	0.20	6.00	0.034	0.035	3.45	0.120	16.0	13.3	0.270	0.400	0.012
21-Oct-15	3	10.2	383	10.54	0.20	2.40	0.024	< 0.010	5.46	< 0.1	12.8	12.1	0.310	0.370	0.012
21-Oct-16	4	10.3	357	10.49	0.10	2.50	0.022	0.019	6.37	< 0.1	10.4	11.6	0.290	0.460	0.011
14-Jul-14	1	15.5	317	7.93	1.40	15.10	0.153	0.043	2.60	0.126	6.8	7.0	0.155	0.321	0.015
14-Jul-14	2	15.6	319	8.79	1.40	14.60	0.084	0.040	2.77	0.127	7.1	6.7	0.155	0.327	0.012
14-Jul-14	3	15.5	336	9.40	1.20	8.30	0.070	0.056	3.21	0.113	8.6	5.8	0.171	0.313	0.009
14-Jul-14	4	13.0	337	10.27	1.00	3.80	0.031	0.011	4.18	0.112	4.9	3.5	0.114	0.215	nd
25-Nov-14	1	6.7	393	10.84	0.20	1.40	0.010	0.008	2.86	0.022	3.4	2.7	0.021	0.041	0.004
25-Nov-14	2	6.7	394	10.67	0.20	1.40	0.009	0.008	3.04	0.039	3.6	2.7	0.016	0.048	0.007
25-Nov-14	3	7.0	394	11.04	0.20	1.40	0.012	0.006	2.82	0.044	3.6	3.1	0.017	0.047	0.004
25-Nov-14	4	7.8	394	11.38	0.23	1.10	0.015	0.004	3.12	0.051	3.7	3.0	0.016	0.057	0.002
1-Sep-15	1	18.9	389	5.90	1.04	7.00	0.063	0.061	4.20	0.111	27.6	10.9	0.098	0.153	0.008
1-Sep-15	2	18.7	393	7.52	0.99	4.80	0.023	0.015	4.74	0.065	28.3	10.4	0.109	0.184	0.005
1-Sep-15	3	15.8	366	9.19	1.22	4.00	0.014	0.010	5.62	0.072	24.0	10.3	0.098	0.123	0.007
1-Sep-15	4	13.6	405	9.87	0.59	1.20	0.011	0.008	6.57	0.046	28.3	10.3	0.095	0.111	0.012
15-Dec-15	1	2.3	454	12.49	nd	nd	nd	nd	nd	nd	16.8	12.5	0.098	nd	nd
15-Dec-15	2	3.1	454	12.77	nd	nd	nd	nd	nd	nd	15.9	11.9	0.094	nd	nd
15-Dec-15	3	5.5	444	12.32	nd	nd	nd	nd	nd	nd	14.5	12.0	0.110	nd	nd
15-Dec-15	4	7.2	427	11.78	nd	nd	nd	nd	nd	nd	9.9	8.4	0.124	nd	nd
28-Jun-16	1	14.8	357	8.12	nd	nd	0.074	0.061	2.80	nd	11.0	4.5	0.142	0.209	nd
28-Jun-16	2	14.9	354	8.85	nd	nd	0.059	0.055	2.90	nd	9.8	4.3	0.145	0.222	nd
28-Jun-16	3	15.6	353	9.50	nd	nd	0.057	0.043	3.30	nd	10.0	4.5	0.172	0.228	nd
28-Jun-16	4	15.0	420	9.93	nd	nd	0.030	0.010	4.60	nd	13.0	4.8	0.037	0.117	nd

Appendix: Measured parameters of the underground Pivka River at sampling sites, part 2.

Sampling date	Sampling site	TOC	Bacteria (CFU/ml)	Coliforms (CFU/ml)	Enterococci (CFU/ml)	Enterobacteria (CFU/ml)	*E. coli* (CFU/ml)	Non-coliforms (CFU/ml)	Non-enterics (CFU/ml)	Non-*E. coli* coliforms (CFU/ml)	Non-*E. coli* enterics (CFU/ml)
27-Jun-13	1	6.75	835	202	20	278	5	633	557	197	274
27-Jun-13	2	7.21	630	206	7	294	6	424	336	200	289
27-Jun-13	3	5.04	381	148	3	221	0	234	160	148	221
27-Jun-13	4	4.57	84	29	0	40	0	55	44	29	40
21-Oct-13	1	5.00	2520	389	530	136	24	2131	2385	366	112
21-Oct-14	2	4.71	1860	332	380	627	18	1529	1233	314	609
21-Oct-15	3	3.55	166	40	10	40	1	126	127	40	39
21-Oct-16	4	3.27	163	43	7	48	1	121	116	42	47
14-Jul-14	1	4.69	2020	363	680	685	27	1657	1335	337	659
14-Jul-14	2	4.55	1590	443	820	795	48	1147	795	395	747
14-Jul-14	3	4.12	1510	320	570	558	10	1190	953	310	548
14-Jul-14	4	3.56	398	109	80	138	3	289	261	106	135
25-Nov-14	1	1.34	133	59	72	72	3	74	62	56	69
25-Nov-14	2	1.34	172	50	74	72	4	122	100	46	69
25-Nov-14	3	1.24	168	60	61	58	3	108	110	57	55
25-Nov-14	4	1.26	106	35	37	34	1	71	72	34	33
1-Sep-15	1	6.79	531	262	57	332	4	269	199	258	328
1-Sep-15	2	5.93	520	129	12	155	1	391	365	128	154
1-Sep-15	3	6.26	353	120	16	152	2	233	201	118	150
1-Sep-15	4	3.96	191	54	22	58	2	137	133	52	56
15-Dec-15	1	nd	208	65	nd	83	0	144	126	65	83
15-Dec-15	2	nd	274	67	nd	71	1	207	204	66	70
15-Dec-15	3	nd	102	27	nd	29	0	75	73	27	29
15-Dec-15	4	nd	147	26	nd	42	0	121	105	26	42
28-Jun-16	1	3.32	7030	760	nd	1300	69	6270	5730	691	1231
28-Jun-16	2	3.07	6750	1020	nd	1180	78	5730	5570	942	1102
28-Jun-16	3	2.68	2050	860	nd	1180	39	1190	870	821	1141
28-Jun-16	4	1.21	274	147	nd	125	1	127	149	146	124

nd- no data