ORIGINAL ARTICLE

Differences between groundwater fauna in shallow and in deep intergranular aquifers as an indication of different characteristics of habitats and hydraulic connections

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ABSTRACT

The fauna in the hyporheic zones of rivers has been relatively well studied but that from the phreatic zone remains comparatively unknown and there are few investigations into deeper intergranular aquifers (over 30 m in depth) due to technical difficulties. Two shallow boreholes of 29 m depth and two deep boreholes of 100 m depth, both near Ljubljana (Slovenia), were sampled more than 30 times between 14 January 2008 and 3 March 2009. On each occasion 14.4 to 18.0 m³ of water were abstracted using a high-capacity pump, then filtered by means of a plankton net with a mesh size of 60 µm. Organisms larger than 2 mm were damaged by the pump rotors, but their identification was still possible, while smaller representatives of the Copepoda (Crustacea) passed the rotors without damage. A near-by artesian borehole was sampled on 6 occasions. Water chemistry, physical properties and faunal composition analyses were carried out for each borehole. A total of 32 taxa, 24 of which were stygobites, were identified. Copepoda alone were represented by 16 species, 15 of which were stygobites. The shallow boreholes differ from the deep boreholes in their higher temperatures and higher concentrations of K^+, Na^+, Ca^{2+}, Mg^{2+} and SO_4^{2-} ions. The copepod communities in samples from the shallow boreholes differ sharply from those from the deep boreholes. There were also clear differences between shallow boreholes in two aquifers located a few kilometres apart, in physical and chemical characteristics as well as in fauna composition. Taxa with different ecological affinities, collected from groundwater, are indicators of hydraulic connections between different parts of an aquifer as well as of communication between surface and subsurface water bodies. The present study suggests that subterranean fauna, as well as epigean fauna, can provide effective support for classical dye/salt tracing experiments.

Key words: Ecology; karst; sampling methods; Slovenia; unconsolidated sediments.

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INTRODUCTION

Groundwater fauna from fissured/fractured and inter-granular aquifers have been investigated for more than 250 years (Botosaneanu, 1986). More than 6700 stygobites (*i.e.* obligate groundwater dwellers) worldwide have been described to date (Galassi, 2001; Galassi *et al.*, 2009a). In Europe, there are approximately 1800 known stygobitic species (Botosaneanu, 1986; Gibert and Culver, 2009), of which 1570 are Crustacea (Zagmajster *et al.*, 2014). In the European PASCALIS project (Protocols for the ASessment and Conservation of Aquatic Life In the Subsurface) about a 1000 stygobites were identified in only 6 countries (Deharveng *et al.*, 2009; Stoch and Galassi, 2010). Although these numbers appear large, most records are based on a single sampling at single locations and sporadic sampling over long time intervals. This situation has changed over the last three decades, as groundwater ecology research has intensified (Gibert *et al.*, 1994; Schmidt *et al.*, 2007; Gibert and Culver, 2009; Hahn and Fuchs, 2009; Larned, 2012). Ecological studies

of groundwater ecosystems, in intergranular aquifers in particular, developed rapidly during the 1990's (Gibert *et al.*, 1990; Danielopol *et al.*, 2001; Gibert, 2001; Gibert and Deharveng, 2002; Hancock *et al.*, 2005; Danielopol and Griebler, 2008). Moreover, the important functional role of epigean, as well as hypogean fauna and microbes, in groundwater, in transformation, decomposition, and re-cycling of organic material and nutrients, has been widely recognised (Vervier *et al.*, 1992; Claret *et al.*, 1999; Griebler and Lueders, 2009). An important element that determines the distribution of groundwater fauna is hy-drogeology, particularly pore spaces (Maurice and Bloomfield, 2012). The hyporheic zone has continued to be studied intensively (Danielopol and Rouch, 1991; Rouch 1992; Boulton *et al.*, 2003; Di Lorenzo *et al.*, 2013), while, in contrast, the deeper aquifer zones, the phreatic zone, have received comparably little attention and still constitute a *research frontier for freshwater ecol-ogy* (Larned, 2012). The few faunistic and ecological studies carried out to date have revealed that the deeper areas of the phreatic zone are habitats with very specific

fauna (Marmonier *et al.*, 1993; Stoch *et al.*, 2009; Di Lorenzo *et al.*, 2015) but detailed knowledge is still lacking. As summarized by Larned (2012), this is mainly due to manifold limitations such as: i) difficult accessibility, ii) lack of information about ecosystem boundaries, iii) lack of information regarding spatial heterogeneity, iv) lack of taxonomic and biogeographic information and v) the limited number of field investigations that have been conducted.

Sampling is an important issue. The use of powerful submersible pumps that allow the abstraction of large amounts of water from various depths causes bias due to the destruction of larger organisms during their passage through the pump. However, fauna from deeper boreholes and piezometers can be collected from open boreholes (*i.e.* boreholes without installed pumps) using the Cvetkov net or modified plankton nets/phreatic nets (Malard *et al.*, 2002; Karanovic, 2006; Schmidt *et al.*, 2007; Eberhard *et al.*, 2009; Hahn and Fuchs, 2009). Air-lift pumps can be utilised in deep boreholes (Malard *et al.*, 1994) but, for effective collection, a high-capacity, compact air-compressor is required. In consequence, sampling of fauna in intergranular aquifers has rarely been carried out at depths exceeding 20 to 30 m (Datry *et al.*, 2005; Bork *et al.*, 2008; Hahn and Fuchs, 2009).

In 2008 and 2009, four boreholes used for public-water supply and located in intergranular aquifers near Ljubljana (Slovenia), and a nearby artesian borehole have been sampled on several occasions. Three of the boreholes provide water from a depth between 10 and 28 m below ground level (bgl) and two from a depth between 55 and 100 m bgl. The aims of the study were: i) to determine the abundance and composition of groundwater fauna over several months, ii) to systematically address similarities/ differences in fauna between shallow and deep aquifer zones, iii) to test the hypothesis of a strong connection between the intergranular and adjacent karstic aquifer, and finally iv) to evaluate the efficiency of groundwater fauna sampling using submersible, high-capacity pumps. The underlying hypothesis was that differences in water characteristics and faunal compositions are smaller between sites within the same aquifer and greater between shallow and deep aquifers.

METHODS

The study area

The main study area was focussed on the public water supply station near the village of Brest (45° 57' 39.66" N, 14° 29' 42.12" E, 300 m above sea level), about 10 km south of Ljubljana (Fig. 1). The study area, hereafter termed the Brest aquifer, has an area of about 6 km². It is underlain by alluvial deposits of the River Iška which has the mean annual discharge rate of 0.8 m³ s⁻¹. The river

flows from the karstic Krim Massif, which has an area of 150 km², a mean altitude of about 800 m and a dominant geology of limestone and dolomite (Pleničar, 1970). A secondary study area was located in the Bevški Mah area, approximately 4 km east of the main study area (45° 58' 16.71" N, 14° 32' 39.41" E, 289 m above sea level), on the alluvial deposits of the River Želimeljščica (hereafter termed the Mah aquifer). This river catchment is underlaid predominantly by dolomite and has a mean annual discharge of 0.4 m³ s⁻¹. Both aquifers are part of the area previously studied during the PASCALIS project (Gibert and Culver, 2009).

Alluvial deposits of the Brest aquifer are up to 108 m thick, while those of the Mah aquifer are about 30 m thick. The Brest aquifer consists of two defined aquifer horizons separated by an aquitard (Fig. 2). The upper horizon consists of Holocene deposits (the shallow Brest aquifer or sBa) and is about 28 m thick. The water table occurs at a
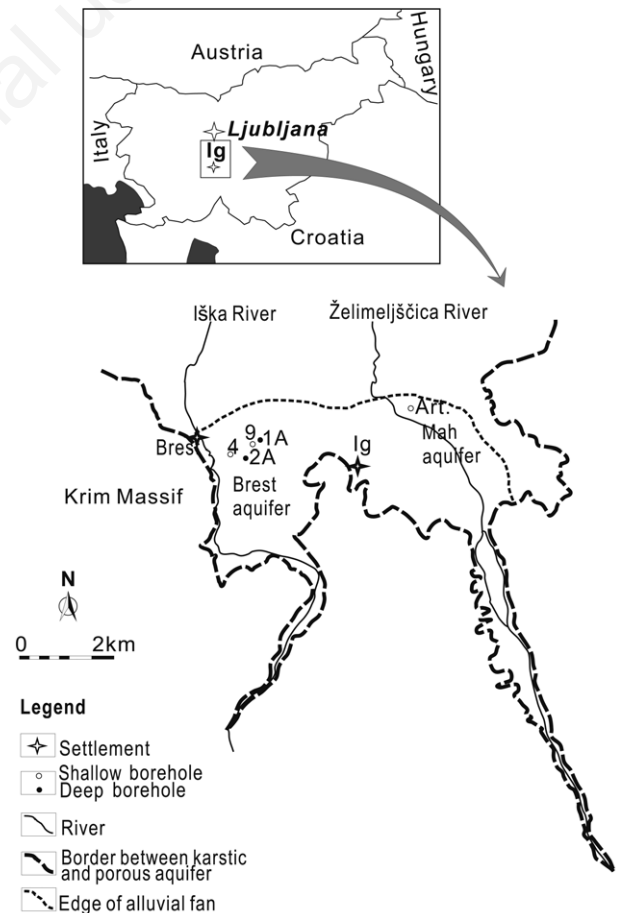

Fig. 1. The study area location, showing the areas underlain by the Brest aquifer (with sampling boreholes Brest 1A, Brest 2A, Brest 4 and Brest 9) and Mah aquifer (borehole Art.) south of Ljubljana, Slovenia.

depth of approximately 10 m bgl. A two meter thick, low permeability layer composed of red silt and silt loam, acts as an aquitard, that separates the Holocene (sBa) from the deeper Pleistocene deposits (the deep Brest aquifer or dBa). The deep aquifer is between 57 and 59 m thick and contains a number of often discontinuous, less permeable horizons of gravel with loam, silt and sand. A fractured dolomite (fissured karstic aquifer) is present beneath the Pleistocene deposits at a depth of 100 to108 m (Mencej, 1988/89).

The Mah aquifer, which is partially overlain by up to 10 m of impermeable gray lacustrine clay (*clay with snail shells*), consists of 16 m thick Holocene gravel, sand and silt deposits. At a depth of between 28 and 30 m the aquifer is underlain by the same low permeable layer, composed of red silt and silt loam, as in the Brest aquifer. The low permeability layer directly overlies the fractured dolomite in the vicinity of the artesian sampling borehole (hereafter Art.) where water overflows at ground level.

The shallow Brest aquifer is predominantly recharged by the nearby River Iška and by local infiltration from precipitation. The deep aquifer is predominantly recharged by groundwater from the karstic Krim Massif, with only a relatively small contribution from the River Iška (Urbanc and Jamnik, 2002). In contrast, the shallow Mah aquifer is recharged almost exclusively by the River Želimeljščica.
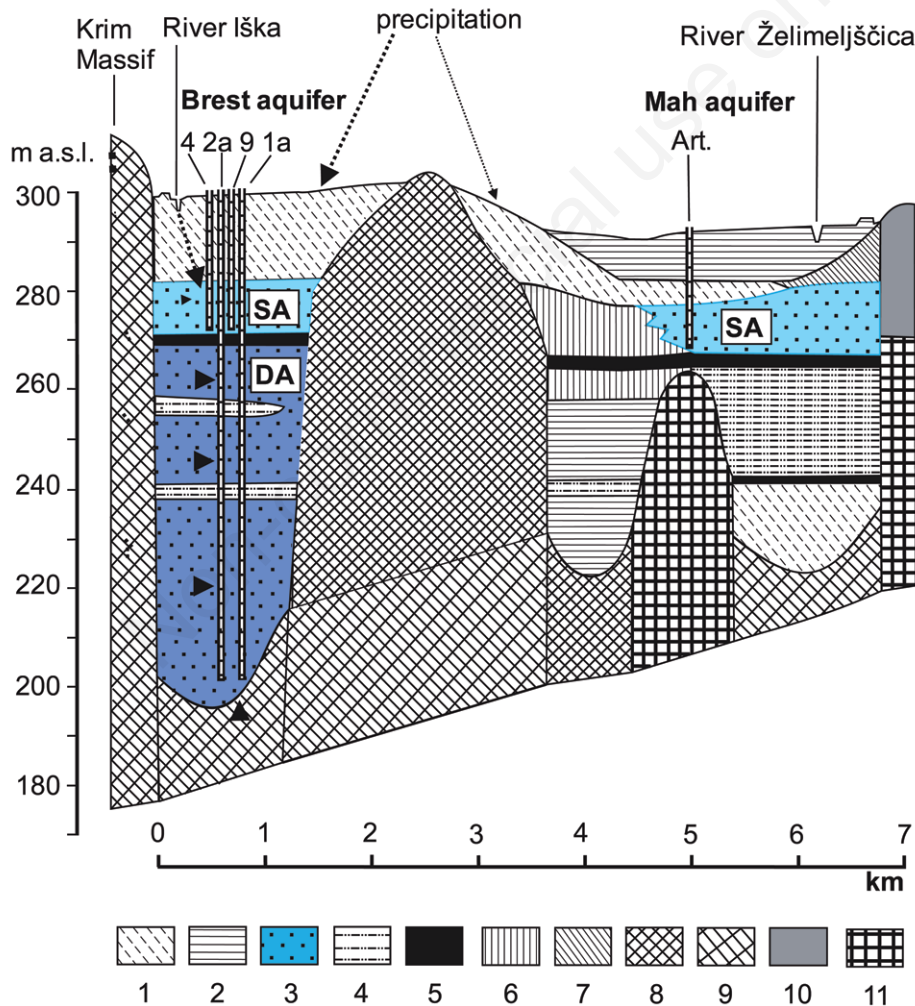

Fig. 2. Cross section of the Brest and Mah aquifers (after Mencej, 1988/89) with indicated position and depth of sampled boreholes. 1: gravel with sand mixed with some silt; 2: silt loam (the "clay with snails' shells"); 3: gravel with silt and sand filled with water; 4: gravel with loam, silt and sand; 5: red silt and silt loam (the border layer); 6: loamy gravel and gravel with organic loam; 7: rubble, poorly rounded gravel of non-carbonate rocks and loam; 8: limestone and dolomite (Jurassic); 9: dolomite (Upper Triassic); 10: limestone, tuff and sandstone (Upper Triassic); 11: crystalline dolomite; SA - shallow aquifer; DA - deep aquifer. Arrows indicate relative contribution of inflow water into the aquifers from different sources.

Sampling boreholes

The boreholes in the Brest aquifer are positioned along a line extending between 320 and 850 m to the east of the River Iška and perpendicular to it (Fig. 1). Nine boreholes, that abstract water from the sBa, (boreholes Brest 1 to Brest 9) are positioned equidistantly, approximately 50 m apart. The two boreholes collecting water from the dBa (boreholes Brest 1A and Brest 2A) are close to Brest 9 and Brest 4, respectively. The characteristics of the sampled boreholes are summarized in Tab. 1. A single borehole (Art.) was used to obtain samples from the shallow Mah aquifer.

Boreholes Brest 1A, Brest 4 and Brest 9 were sampled on 34, 21 and 31 occasions respectively, between 14 January 2008 and 3 March 2009 at 7 to 21 day intervals. Brest 2A was sampled on 8 occasions between 16 January and 23 March 2009. On each occasion, water from each borehole was pumped for 30 minutes at a rate of 8 to10 L s^{-1} (total volume 14.4 to18.0 m^3). Water was filtered through 1.5 m long plankton net with a mesh size of 60 µm, equipped at the end with an additional filtering bottle (Brancelj, 2003). The artesian borehole in Mah aquifer was sampled on 6 occasions between 14 January 2008 and 3 March 2009, for a duration of one hour on each occasion (total volume 7.2 m^3 per event) using the same filtering device.

Materials and data processing

Water for physical and chemical analyses was collected at the end of each pumping event, together with on-site measurements of temperature and conductivity. Major ions (Ca^{2+}, Mg^{2+}, K^+, Na^+, SO_4^{2-}, $N-NO_3^-$) were analysed by means of ion chromatography (Compact IC Methrom 761) in the laboratory.

Specimens were sorted and preserved in 70% alcohol within 4 h from collection. Copepods were counted and identified to the species level. Information on the population structure was collected: gender, developmental stage (adult/juvenile), and reproductive status [mating (=interconnection of male and female)/females with attached spermatophore/unfertilised females]. Individuals of other taxa (>2 mm length) were frequently found damaged by the pump rotors. In order to estimate the number of individuals only the best preserved parts were counted and determined to the lowest possible taxonomic level.

Statistical analyses, except species richness accumulation curves, were performed using R (R Core Team, 2013) and package vegan (Oksanen *et al.*, 2013). Average values for environmental variables (major ions, pH, temperature, electric conductivity) and their standard deviations were calculated separately for each borehole and environmental data were normalized using the Box-Cox transformation (Osborne, 2010). Since the variables were on different scales (*e.g.*, chemical parameters in mg L^{-1}, electric conductivity in µS cm^{-1}) they were standardized to a range of 0-1, using the formula: Xi - min(X)/max(X) - min(X). Aquifer depths were dummy coded and used together with the standardized environmental variables in a distance-based redundancy analysis (db-RDA) (Legendre and Anderson, 1999). Zero-adjusted Bray-Curtis (za-BC) dissimilarities were calculated on square-root transformed species abundances data in order to avoid the effects of an overabundance of zero values (Clarke *et al.*, 2006). The data matrix was subsequently utilised for a principal coordinate analysis, and the resulting components were applied as species data in the db-RDA, in order to avoid negative eigen-values when principal coordinates were calculated on abundance data. Since such results cannot be included in the db-RDA, the za-BC matrix was also square-root transformed (Legendre and Legendre, 1998).

To perform the Similarity Percentage Analysis (SIM-

Tab. 1. Characteristics of the sampling boreholes in the Brest and Mah aquifers.

Parameter (unit) \ Borehole	Brest 1A	Brest 2A	Brest 4	Brest 9	Art.
Ground level elevation (m asl)	299	299	299	300	291
Borehole depth (m)	100.5	100.5	27.8	27.8	26.1
Borehole diameter (mm)	500	500	500	500	50
Casing type	Inox steel	Inox steel	Inox steel	Inox steel	Inox steel
Borehole screened intervals (m bgl)	55.0-95.0	68.5-94.5	9.8-25.8	9.8-25.8	24.0-26.0
Screen perforation size (mm)	100x5	100x5	100x5	100x5	Ø10
Pump capacity (L s^{-1})	8-10	8-10	8-10	8-10	2
Filteration duration (h)	0.5	0.5	0.5	0.5	1
Water volume filtered per sampling event (m^3)	14.4-18.0	14.4-18.0	14.4-18.0	14.4-18.0	7.2
Number of sampling events	34	8	21	31	6
Total filtered volume (m^3)	544	128	336	496	43.2

bgl, below ground level.

PER) and Permutational Multivariate Analyses of Variance (PERMANOVA) on invertebrate abundances as input data, the za-BC dissimilarity matrix was used. The procedure provides two main benefits: i) it allows the use of a distance matrix, and ii) it performs random permutations of the input data, thus providing a normal distribution (Anderson, 2001; McArdle and Anderson, 2001).

The SIMPER was used to evaluate the contribution of each species to the differences found between aquifers (Clarke, 1993). The method calculates the average BC dissimilarity between all pairs of sample groups (*i.e.* habitats). The species that consistently contribute significantly to the average dissimilarity between sites was considered characteristic of the habitat. The PERMANOVA was used to statistically evaluate significant differences between aquifers (Anderson, 2001).

Species richness accumulation curves (SRAC) were calculated for the three boreholes rich in taxa, using EstimateS 7.5. S_{obs} was calculated according to the Mao-Tau function. ACE, ICE, and Chao 2 were calculated on randomised samples without replacement and presented as averages of 50 runs (Chao, 1984, 1987; Chazdon *et al.*, 1998; Chao *et al.*, 2000; Colwell *et al.*, 2004). For each of the boreholes sampled most (Brest 1A, 4 and 9), SRACs were calculated separately using all the stygobites collected or only stygobitic Copepoda.

RESULTS

Physical and chemical characteristics of the aquifers

Groundwater samples from the various boreholes differed in their physical and chemical characteristics according to sampling depth and distance from the river (Tab. 2).

Electric conductivity was lower in the deep groundwater. In samples from the sBa boreholes, it increased with distance from the river. Groundwater temperature in the dBa boreholes (Brest 1A and 2A) and in the sBa located remotely from the River Iška (Brest 9) had mean annual temperatures of less than 12°C (11.3-11.8°C) but the other two sBa boreholes (Brest 4 and Art.) had mean annual temperatures higher than 12°C (12.1-12.2°C) (Tab. 2). Values of pH were invariably higher in groundwater from the deep boreholes. Mean concentrations of cations and anions were lower in samples of deep than of shallow groundwater (Ca^{2+}: 54.6 mg L^{-1} *vs* 79.3 mg L^{-1}; Mg^{2+}: 27.6 mg L^{-1} *vs* 42.6 mg L^{-1}; K^+: 0.43 mg L^{-1} *vs* 0.50 mg L^{-1}; Na^+: 0.71 mg L^{-1} *vs* 1.75 mg L^{-1}; SO_4^{2-}: 3.81 mg L^{-1} *vs* 6.43 mg L^{-1}) (Tab. 2). Nitrate, chloride and bicarbonate showed no significant differences with depth or with the geographical location of the boreholes relative to the river.

Fauna composition

A total of 32 taxa (species, genus, and family or order level) were collected. As individuals in some groups (*i.e.* Nematoda, Oligochaeta, and Tardigrada) that were not identified to the species level belonged to more than one species, the number of species was actually higher. Representatives of some taxa were found only once and 8 of them were represented by only one individual (*i.e.* singletons) (Tab. 3). Most singletons belong to the group of Copepoda (5 species). Taxa were grouped into three defined ecological categories: *stygobites* (*i.e.* obligate groundwater dwellers), *riverine benthos* and *aquatic insects* (as a special group within benthos). A fourth, *mixed group*, contained taxa with undefined ecology owing to their approximate level of identification.

Tab. 2. Mean values±SD of physical and chemical characteristics of groundwater samples from five boreholes penetrating the deep (dBa) and shallow (sBa) Brest and Mah aquifers in 2008/2009.

Parameter (unit) \ Borehole / Aquifer	Brest 1A (dBa)	Brest 2A (dBa)	Brest 4 (sBa)	Brest 9 (sBa)	Art. (Mah)
Screen zone (m bgl)	60-100	60-100	10-29	10-29	29-29
El. cond. (µS cm^{-1})	467±25	425±27	514±35	696±18	570±13
Temp. (°C)	11.7±0.1	11.3±0.1	12.1±0.3	11.8±0.1	12.2±0.1
pH	7.56±0.03	7.59±0.20	7.44±0.20	7.19±0.13	7.43±0.11
Ca_2^+ (mg L^{-1})	57.0±2.2	52.2±0.8	62.0±2.8	87.8±1.8	88.1±3.0
Mg_2^+ (mg L^{-1})	28.8±1.0	26.3±0.6	30.0±1.4	49.7±1.5	48.1±1.5
K^+ (mg L^{-1})	0.44±0.1	0.41±0.1	0.62±1.0	0.53±0.1	0.34±0.1
Na^+ (mg L^{-1})	0.71±0.1	0.70±0.1	0.85±0.1	1.40±0.2	3.01±0.2
Cl^- (mg L^{-1})	3.11±0.1	2.27±1.3	1.88±0.1	4.42±0.2	2.19±0.4
$N-NO_3^-$ (mg L^{-1})	5.35±0.1	1.74±0.3	1.58±0.8	3.07±1.7	0.87±0.9
SO_4^{2-} (mg L^{-1})	4.13±0.1	3.48±01	4.52±0.1	5.39±0.5	9.37±0.1
HCO_3^- (mg L^{-1})	289±9.1	273±5.2	280±4.9	440±8.6	—

bgl, below ground level; El. cond., electric conductivity.

Tab. 3. List of organisms collected from the five boreholes in 2008/2009.

Taxon	Brest 1A dBa ind. m^{-3}	Brest 2A dBa ind. m^{-3}	Brest 4 sBa ind. m^{-3}	Brest 9 sBa ind. m^{-3}	Art. Mah ind. m^{-3}	TOT. All sum
COPEPODA						
CYCLOPOIDA						
Acanthocyclops hispanicus* Kiefer, 1937					0.208	9
Acanthocyclops sambugarae* Kiefer, 1981	0.002					1
Austriocyclops sp.*				0.003		1
Diacyclops languidoides* (Lilljeborg, 1901)	0.002		0.033	0.306		169
Graeteriella unisetigera* (Graeter, 1908)	0.061			0.002		34
HARPACTICOIDA						
Bryocamptus pyrenaicus* (Chappuis, 1923)	0.002					1
Ceuthonectes serbicus* Chappuis, 1924	0.180			0.002		99
Chappuisius sp.*		0.031				4
Elaphoidella charon* Chappuis, 1936	0.004		0.036	0.250		144
Elaphoidella millennii* Brancelj, 2008	0.002		0.006			3
Nitocrella hirta* Chappuis, 1923				0.002		1
Nitocrella omega* Hertzog, 1936					0.486	21
Paracamptus schmeili# (Mrazek, 1893)	0.007					4
Paramorariopsis brigitae* Brancelj, 2011	0.118					67
Parastenocaris nolli alpina* Kiefer, 1969	0.011					6
Parastenocaris gertrudae* Kiefer, 1968			0.003			1
OTHER TAXA						
Rotatoria (several taxa)	0.002		0.060	0.002		22
Nematoda (several taxa)	0.059		0.027	0.109		95
Bryozoa (statoblast)				0.002		1
Gastropoda (shells only) (2 taxa)*			0. 009	0.022		14
Oligochaeta (5 taxa)§	0.064	0.164	0.016	0.031	0.764	135
Tardigrada (several taxa)	0.007		0.083	0.177		120
Cladocera (*Alona* sp.)			0.003			1
Ostracoda - sp. A*			0.004			4
Ostracoda - sp. B*					0.093	2
Syncarida (cf. *Bathynella* sp.)*	0.004			0.012		8
Isopoda (*Microcharon* sp.; 2? taxa)*				0.006	0.046	5
Amphipoda (*Niphargus* sp.; 2 taxa)*§	0.037	0.003	0.020	0.234		61
Ephemeroptera (larva)	0.002					1
Odonata (larva)				0.003		1
Chironomidae - larvae			0.051		0.116	24
Hydrachnida (2 taxa)				0.006	0.010	7
Total no. of taxa	17	3	16	15	6	32

Oligochaeta:
 - *Marionina* group *argentea*
 - *Nais variabilis* Piguet, 1906
 - *Marionina* sp.
 - *Enchytraeus* sp.
 - Enchytraeidae gen. sp.
Amphipoda:
 - *Niphargus* cf. *multipennatus*
 - *Niphargus* cf. *aquilex*

TOT, absolute number of individuals collected in all boreholes; underlined taxa: collected only once. Deep Brest aquifer (dBa): boreholes 1A and 2A; shallow Brest aquifer (sBa): boreholes 4 and 9; Mah aquifer: shallow aquifer, artesian borehole (Art.); *stygobitic taxa; #stygophilic taxa; §determined subsequently.

At least 23 taxa of stygobites were recorded, the Copepoda being the most commonly represented group (15 stygobites out of 16 species). Additional stygobites belonged to the Gastropoda, Ostracoda, Syncarida, Isopoda and Amphipoda (Tab. 3). Aquatic insects found in groundwater were represented by larvae from two epigean groups: Odonata and Diptera (Chironomidae). All were early instars and found in March/April and at the end of September.

The mixed group contained three taxa: Rotatoria, Nematoda and Oligochaeta, all of which probably contained both epigean and stygobitic taxa. Individuals of Nematoda and Oligochaeta were particularly common, while those of Rotatoria were not (Tab. 3).

Species distribution in shallow and deep aquifers

Cumulative numbers of taxa per borehole in the sBa ranged from 6 (Art.) to 16 taxa (Brest 4) and in dBa from 3 (Brest 2A) to 17 (Brest 1A) (Tab. 3). Sampling efficiency (the percentage of empty samples and the average number of taxa per sample) was not related to borehole depth or geographical location (Tab. 4). The average number of taxa per sample was similar in four of the boreholes (Brest 1A, 4, 9 and Art.), ranging from 3.00 to 3.71 taxa (median=3 and 4, respectively), while in Brest 2A the number was significantly lower (0.75 taxa, median=0) (*U*-test: P<0.05, n=56). Data pertaining solely to stygobites ranged from 1.19 to 3.00 in Brest 1A, 4, 9 and Art., and was low in Brest 2A (0.38 taxa).

Species rarefaction curves and estimation curves did not reach a plateau in three of the boreholes rich in taxa, either for Copepoda or stygobites (Fig. 3). The highest values for Copepoda were recorded in Brest 1A with an S_{obs} value of 10, but still increased after 33 samplings. Copepoda values of S_{obs} in the two shallow boreholes were 5 taxa, while mean values for the Chao 2 estimators were higher by two and three taxa, respectively. For stygobites only Brest 4 approached the asymptote with 8 stygobitic taxa observed and 10 predicted by the mean Chao 2.

Copepoda were the most abundant stygobites. In Brest 9, *Diacyclops languidoides* and *Elaphoidella charon* were represented by 157 and 130 individuals, respectively, collected from a total of 496 m³ of groundwater. Another set of 3 abundant species (*Ceuthonectes serbicus* (98 individuals), *Paramorariopsis brigitae* (67 individuals) and *Graeteriella unisetigera* (33 individuals), was collected from 544 m³ of water from the well Brest 1A. *Nitocrella omega* (21 individuals) was collected only from the well Art., where the population density of Copepoda was the highest (0.486 individuals m⁻³).

Representatives of Amphipoda (two species: *Niphargus* cf. *multipennatus*, *Niphargus* cf. *aquilex*) were collected from both sBa (40 individuals) and dBa (21 individuals). As most of them were heavily damaged, the affinity of the particular species to either shallow or deep aquifers could not be determined. Nematoda (95 individuals) and Oligochaeta (135 individuals) were found in shallow and deep groundwater but their affinity for a particular aquifer (shallow *vs* deep) or water body (surface *vs* underground) was unclear. Representatives of other groups (Gastropoda (shells only), Tardigrada, Isopoda, and Chironomidae) prevailed or were found exclusively in samples from sBa and Art.

Differences in community composition between aquifers were confirmed by a SIMPER analysis (Tab. 5). *Diacyclops languidoides* and *E. charon* were the most discriminating species, accounting for 47.4% of the dissimilarity between sBa, Art. and dBa. They were characteristic for the sBa, while *P. brigitae* and *C. serbicus* were representatives of the dBa (together accounting for 24.5%). At a finer resolution, *Chappuisius* sp. was characteristic of Brest 2A, while *G. unisetigera*, *C. serbicus* and *P. brigitae* were typical for Brest 1A. The presence of *Nitocrella omega* was characteristic of (and unique for) Art.

Patterns revealed in the SIMPER analysis were confirmed by the distance-based redundancy analysis (Fig. 4). *G. unisetigera*, *P. brigitae* and *C. serbicus* were present in dBa, with lower conductivity, SO_4^{2-} and Na^+ concentra-

Tab. 4. Sampling efficiency in five boreholes south of Ljubljana, Slovenia in a period 2008/2009. Numbers in brackets are values for stygobites.

Borehole Aquifer	Brest 1A dBa	Brest 2A dBa	Brest 4 sBa	Brest 9 sBa	Art. Mah
No. of samples collected	34	8	21	34	6
No. of empty samples	4 (6)	5 (6)	6 (7)	3 (3)	0 (0)
% of empty samples	11.8 (17.6)	62.5 (74.4)	28.5 (33.3)	8.8 (8.8)	0.0 (0.0)
Max. no. of taxa per sample	7 (6)	3 (2)	7 (3)	7 (5)	4 (4)
Average no. of taxa per sample	3.09 (2.06)	0.75 (0.38)	3.00 (1.19)	3.71 (2.76)	3.33 (3.00)
Standard deviation	±1.88 (±1.58)	±1.16 (±0.74)	±2.32 (±1.08)	±1.70 (±1.26)	±0.52 (±0.89)
Median no. of taxa per sample	3 (2)	0 (0)	3 (1)	4 (3)	3 (3)

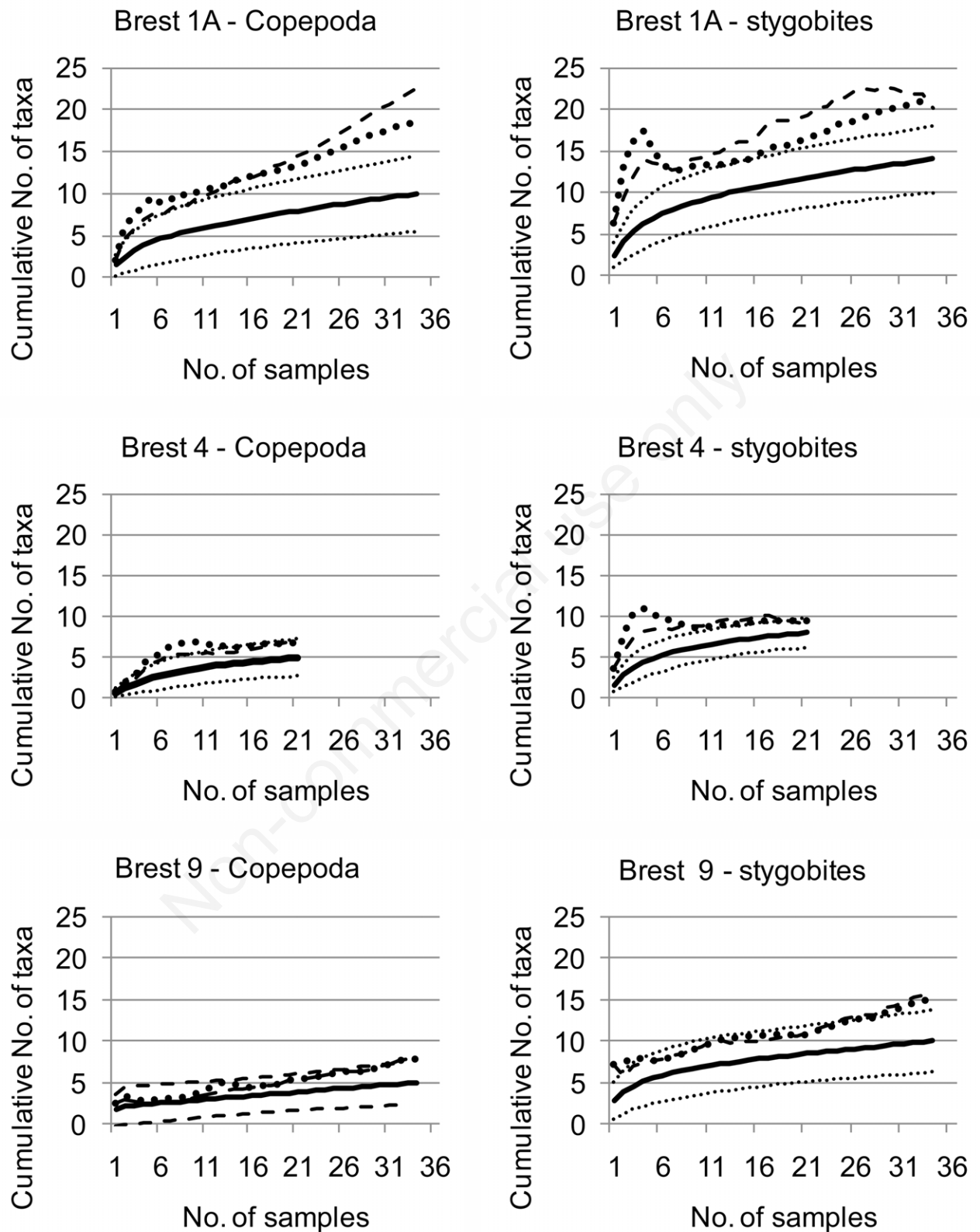*dBa, deep Brest aquifer; sBa, shallow Brest aquifer.*

Fig. 3. Species richness accumulation curves for Copepoda and stygobites collected from three boreholes between February 2008 and March 2009 from the Brest aquifer. Note: Brest 1A=deep aquifer; Brest 4 and 9=shallow aquifer). Lines: heavy solid - S_{obs} (Mao Tau); light dotted - S_{obs} 95% Cl (upper and lower); dashed - Chao 2_{mean}; heavy dotted - ICE $_{mean}$

tions but higher NO_3^{2-} concentrations. The shallow boreholes were distributed along two gradients. The first, a conductivity gradient, showed that Brest 9 had the highest values and Art. the lowest. The second was a K^+, Na^+ and SO_4^{2-} gradient, with Brest 9 having the lowest and Art. the highest values. *Elaphoidella charon* and *D. languidoides* were predominantly associated with higher conductivity values, while *N. omega* was distributed along lower conductivity values, but with higher K^+ and Na^+ concentrations. Both axes explained 33% of the variance, inherent faunal data. A Monte-Carlo test showed that all canonical axes were significant (Trace=0.331, F-ratio=4.445, P<0.001).

DISCUSSION

Water quality and its origin

Water, chemical and physical characteristics, together with faunal composition, differed between the deep and shallow Brest aquifers (Tab. 2; Fig. 4). Rather low $\delta^{18}O$ values in the deep aquifer indicated that the origin of that water is likely to be at higher elevations (Krim Massif) and the age of water (11 TU) supported this. Thus, the close proximity of the karstic aquifer may provide an explanation for this anomaly.

The shallow and deep Brest aquifers are separated by a low permeable layer of clay that acts as an aquitard. Isotope $\delta^{18}O$ analyses revealed that the shallow aquifer near the River Iška (incl. Brest 4) and the deep aquifer (Brest 1A and 2A) received most recharge from the river and precipitation from the karstic Krim Massif ($\delta^{18}O$=-9.6 / -9.4 ‰) while shallow boreholes far from the river (incl. Brest 9) received most water from local precipitation ($\delta^{18}O$=-9.2‰). Measurements of tritium concentration indicate that shallow boreholes contained recent water (with 5.8-7.5 TU) while, in Brest 1A and Brest 2a (11 TU) a water age of between 10 and 50 years was indicated (Urbanc and Jamnik, 2002; Urbanc *et al.*, 2012).

Biodiversity in porous aquifers

Few groundwater studies on invertebrates have sampled numerous sites or samplings on the same place have been frequent (Karanovic, 2006; Hahn and Fuchs, 2009; Galassi *et al.*, 2009b). When one sampling event on several sites in a given area has been done, it usually resulted in low α-diversity at the local spatial scale (*i.e.,* the single well) but high γ-diversity on a regional scale has been observed (Gibert, 2001; Stoch and Galassi, 2010). With repeated samplings, information on α-diversity at the local scale usually increases, but could lead to reduction of local populations being observed. However, repeated samplings provide additional information on the composition, abundance and dynamics of groundwater fauna assemblages in the aquifers.

Tab. 5. Symmetrical matrix of SIMPER analysis results. Above the diagonal are the Copepoda species contributing most to observed differences, below the diagonal are their respective contributions. Only species contributing more than 10% are listed. Right hand side: results of PERMANOVA (total SS=23.75, F=17.25).

	1A	2A	4	9	Art.	1A	2A	4	9	Art.
1A		Chans Graun Ceuse Parbr Diala	Graun Elach Diala Ceuse Parbr	Diala Elach Graun	Nitom Graun Ceuse Parbr		*	**	***	***
2A	25.9 24.7 13.6 10.9 10.9		Chans Elach Diala	Elach Diala Chans	Nitom Chans Diala			**	***	***
4	21.4 17 15.7 12.4 10.2	29.9 22.6 21.2		Diala Elach	Nitom Elach Diala				*	*
9	29.1 27.4 14.1	35.3 30.8 18.9	24.9 22.9		Diala Elach Nitom					**
Art.	39.1 23.4 13.2 10.8	53.2 33.1 13.7	45.1 21.4 19.6	35.7 34.2 28.3						

*Deep aquifer: Brest 1A, Brest 2A; shallow aquifer: Brest 4 and Brest 9, Art. Abbreviations of the species are: the first three letters from genus name and the first two letters from species name; see names in full in Tab. 3. *P<0.05; **P<0.01; ***P<0.001.*

The effect of high frequency of sampling on a small number of sites *vs* low frequency on a high number of sites can be illustrated in two contrasting case studies. A total of 23 stygobitic taxa were collected during this study of the Brest and Mah aquifers, a low number if compared to the 60 taxa collected in wells in Baden-Würtenberg (South-western Germany) (Hahn and Fuchs, 2009). However, the number of taxa from Slovenia is proportionally high, when some other numbers are compared. In Germany 304 boreholes/wells were sampled twice on an area of 38.000 km^2, while in Slovenia 5 boreholes were sampled 8 to 34 times on an area of 10 km^2 (Tab. 1). The average number of taxa per sample was also higher in Slovenia (1.2 to 3.7) than in Germany (0.5 to 1.8). Although the two studies employed different strategies, including sampling equipment, frequency of sampling and volumes of water filtered, the comparison revealed that high sampling frequency and the pumping of large volumes of water yielded more information on the composition, biodiversity, dynamics and abundance of fauna in intergranular aquifers on a small scale.

Both studies actually reflect habitat diversity (*i.e.,* β-diversity) as well, since different geological or hydrological units were sampled. In a case from the Slovenian study, sBa, dBa and Art. represent three different habitats on a small geographical, km, scale, each of them with specific fauna composition, not only within a single aquifer but also between aquifers in the same area at different depths. Site-specific fauna on a small scale was also observed in the karstic spring system of the River Pescara in the Abruzzi region (central Italy) (Stoch *et al.,* 2016).

The indication of high biodiversity of stygobitic taxa in our study was reflected in the SRACs (Fig. 3). For stygobitic taxa, in particular the Copepoda, the S_{obs} species rarefaction curve did not attain a plateau following 34 samplings. Nevertheless, raising SRACs in the Brest aquifers supports the results obtained by Zagmajster *et al.,* (2014) who concluded that Slovenia is a *hot spot* of groundwater biodiversity, as a result of the small, median range sizes of stygobitic species and thus a high β-diversity. Pumps with high pumping capacity can theoretically collect fauna from more than one habitat and are appropriate for study of α- and β-diversity. Other, less costly sampling techniques, like Cvetkov net or Bou-Rouch pump, can produce reliable results when γ-diversity is evaluated on a regional scale, if the number of samples is high enough. However in some regions studied during the PASCALIS project, γ-diversity continued to increase even after 1800 sampling events (Deharveng *et al.*, 2009, Dole-Olivier *et al.*, 2009).

Fig. 4. Distance-based redundancy analysis on zero-adjusted Bray-Curtis dissimilarity: a) distribution of samples; b) environmental variables and species. Copepod species were plotted as independent variables. Species abbreviations: first three letters of genus name and first two letters of the species name (for full names see Tab. 3). Cond=electric conductivity, Art.=borehole at Mah, B1A=borehole Brest 1A, B2A=borehole Brest 2A, B4=borehole Brest 4, B9=borehole Brest 9.

Horizontal and vertical connectivity within intergranular aquifers

The similarity between the shallow Brest and Mah aquifers (Fig. 3; Tab. 3) appears to be a result of the low level of identification of some taxa (Oligochaeta, Amphipoda, Isopoda, Chironomidae) but, for the Copepoda that were identified to species level, there were marked differences. For the two most common species, *D. languidoides* and *E. charon*, there was an overlap between boreholes Brest 4 and Brest 9 but the number of individuals of both stygobitic taxa was lower in Brest 4 which lies closer to the River Iška (at a distance of 500 m com-
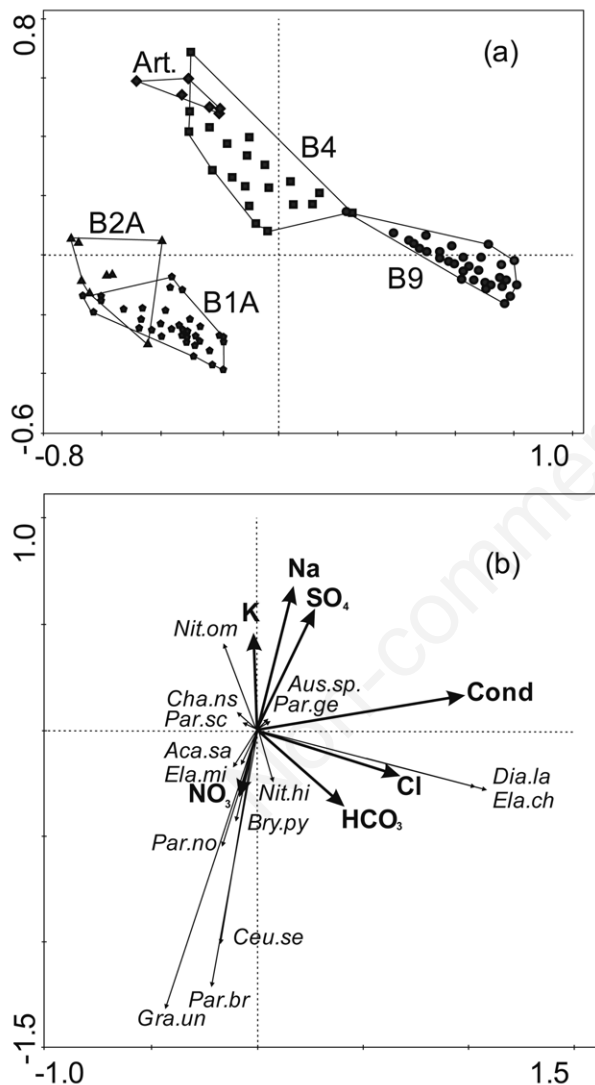
pared to Brest 9 at 800 m from the river). In addition, more stygoxene and stygophile taxa and individuals were present in Brest 4 (Tab. 3). The presence of stygoxenes or stygophiles is probably the result of intensive water pumping, which induced water flow from the river at a rate of 500 m over about 10 to 14 days. Tracing experiments have demonstrated water flow rates in the shallow Brest aquifer of between 42 and 50 m per day (Urbanc *et al.*, 2012).

While Stanford and Ward (1993) proposed the *hyporheic corridor* concept for the exchange of groundwater fauna along the rivers, Ward and Palmer (1994) introduced the hypothesis of *interstitial highways* in order to draw attention to the large potential for faunal exchange between the epigean and hypogean sections of groundwater systems. Transport of riverine benthos during this study indicated that such highways (actually interstitial conduits) existed also in the shallow Brest aquifer. As physical (electric conductivity) and chemical (pH, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^-; Tab. 2) parameters differed between boreholes Brest 4 and Brest 9, it is expected that each has its own system of interstitial conduits which communicate with the river. At the same time, differences in abundance of *D. languidoides* and *E. charon* in the two boreholes also indicate isolation between boreholes.

Low communication between shallow and deep aquifers/boreholes was well demonstrated by data from the two adjacent boreholes (shallow borehole Brest 9 and deep borehole Brest 1A) that are located only 30 m apart. Both wells were populated with highly mobile copepod species (*D. languidoides* and *E. charon* in the shallow aquifer and with *C. serbicus*, *P. brigitae* and *G. unisetigera* in the deep aquifer). Representatives of taxa that were abundant in the shallow borehole were rare in the deep borehole and *vice versa* (Tab. 3).

Thus, knowledge about the ecological requirements of a particular species could be useful in hydrological studies as they can be used as bioindicators (Stoch *et al.*, 2016).

Connectivity between porous and karstic aquifers

Karstic and intergranular aquifers interface with each other (Gibert, 2001). During the PASCALIS project and other studies on stygobitic fauna, the hyporheic zone of the rivers, caves and springs on Krim Massif and adjacent areas have been sampled several times to record the fauna composition in different habitats (Brancelj, 2002, 2009, 2011). When intensive exchange between karstic and porous aquifers occurs no significant differences have been observed in faunal compositions (Hahn and Fuchs, 2009). However, a question arises concerning the ecological conditions that allow the presence of stygobitic taxa from one type of aquifer to be present within another aquifer type over a longer period. Four copepod species (*B. pyrenaicus*, *E. millennii*, *P. brigitae* and *C. serbicus*),

found in deep borehole Brest 1A, are an example that confirms the entrainment hypothesis based on species population structure and morphology. In *P. brigitae* the sex ratio was 51 females to 19 males, while in *C. serbicus* the ratio was 96 females to 3 males. No juveniles or egg-carrying females were found and only one female with spermatophore. An almost total absence of any indication of sexual activity supports the hypothesis of entrainment from the Krim Massif karstic aquifer.

Morphological adaptations that are characteristic for karstic/epikarstic species, as described by Brancelj (2009, 2011), are present in *B. pyrenaicus*, *E. millennii* and *P. brigitae*. Such adaptations (strong setae and spines on furcal rami, short appendages) have not been previously found in species living in intergranular aquifers. *Ceuthonectes serbicus* has no such morphological adaptations, as it was always found in small karstic springs (Petkovski, 1984; Brancelj, unpublished data). Since the screened section of borehole Brest 1A comes close to the dolomite bedrock (laterally ca. 800 m and vertically ca. 10 m), it is likely that the four species mentioned above were entrained in recharge from the karstic aquifer of the Krim Massif.

Efficiency of sampling techniques

Shallow aquifers, where groundwater levels are no deeper than a few metres, can be sampled by using piston-pumps (*i.e.* the Bou-Rouch method) (Bou and Rouch, 1967). In large wells, a traditional plankton net or Cvetkov net (Cvetkov, 1968) can be used to sample the fauna. Both sampling strategies have considerable, known limitations. The first has depth limitation and a lateral range of sampling, whilst the second can only be used to collect fauna which selectively or accidentally entered the monitoring well or borehole (Karanovic, 2006; Hahn and Fuchs, 2009). Competitive and/or predatory exclusion can induce a bias in faunal composition, as has been observed in pools in karstic caves (Brancelj, 2002; Cottarelli *et al.*, 2012).

An alternative option that can be used to collect small quantities of groundwater is a small mobile pump working at low pumping rates (Malard *et al.*, 2002). However, active swimming fauna (Copepoda Cyclopoida, Amphipoda) or fauna that are tightly anchored to the substrate (Turbellaria, Gastropoda) are likely to resist the pump suction. It is probable that samples obtained using nets and small mobile pumps reflect only qualitative and semi-quantitative compositions of groundwater fauna and cannot be considered to be quantitative.

Pumps with high pumping capacity (in excess of 10 L s^{-1}) are likely to achieve sufficient force to *extract* fauna from the porous matrix and may return more realistic quantitative data on the composition of groundwater fauna. The major disadvantages of this method are that these pumps have high-speed rotors that damage many or-

ganisms. They induce a high water flow velocity at the outlet pipe, resulting in problems of resistance, clogging and instability of sampling nets. These disadvantages, apart from costs for electricity, were the main reasons that high-capacity pumps have not been used more frequently for sampling groundwater fauna in intergranular aquifers.

During this study it has been demonstrated that a 1.5 m long plankton net with a mesh size of 60 μm, retained inorganic and organic particles efficiently without clogging, even when large quantities of water were filtered (*i.e.* up to 18 m^3). Larger animals (>2 mm long) are damaged. Although their fragments are quite easily collected there are problems with accurate identification of the fragments to species level, while copepods passed through the pumps undamaged. As they represent the majority of groundwater fauna, the method with high-capacity pumps could provide good results on quality and quantity of groundwater fauna.

CONCLUSIONS

The groundwater system to the south of Ljubljana, Slovenia, consisting of two intergranular aquifers that are connected to a karstic aquifer, is very complex, diverse and dynamic. There is a considerable degree of hydraulic continuity between the rivers and the adjacent shallow aquifers, but there was no detectable communication between the shallow Brest and Mah aquifers, which lie only 4 km apart. The shallow and deep Brest aquifers are separated by a low permeable clay layer. The shallow aquifer received recharge from the nearby river, while the deep aquifer receives recharge predominantly from the nearby karstic aquifer. Hydrogeological separation has also resulted in differing species community structures, which are specific to each aquifer.

To study groundwater biodiversity on a small scale, (*i.e.* α- and β-diversity), it is important to replicate sampling on several occasions. At the same time, ecological affinities of taxa can provide additional information for studies of hydrological connectivity between different parts of an aquifer or among water bodies (*i.e.* surface *vs.* sub-surface). Taxa with different ecological requirements could be used as "biotracers" on fine-scale studies on structure and dynamic of aquifers.

ACKNOWLEDGMENTS

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