Geochemical and microbial factors driving crustacean assemblages in adjacent aquifer units within the same aquifer

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Abstract. Aquifers harbor unique and highly adapted species, contributing to critical ecological processes and services. Understanding the key factors driving invertebrate assemblages in aquifers is a challenging task that, traditionally, has primarily been achieved in karst. This study aimed to uncover the factors influencing the composition and functionality of groundwater crustaceans (dimensional range: 0.036 to 1 mm) in a volcanic aquifer of central Italy. The aquifer consisted of three adjacent aquifer units (AUs) showing different geochemistry (i.e., sulfate-depleted, K-rich and earth-alkaline). We adopted a multidisciplinary approach, integrating hydrogeology, geology, microbiology, and ecology to determine whether the environmental differences that we highlighted in the three AUs were reflected in the biological assemblages. We unveiled significant differences in both the taxonomic and functional composition of groundwater crustaceans across the three AUs and these patterns remained consistent throughout the survey period. Notably, the sulfate-depleted AU lacked groundwater-obligate species, burrowers, stenothermal and moderately stenothermal species. The K-rich and earth-alkaline AUs had different species, which, however, exhibited similar functions related to locomotion, diet, and feeding habits. Stenothermal and moderately stenothermal crustacean species were only found in the K-rich AU, which lacked epigean species. Our findings suggest that major ions (SO_4^{2-}, Ca^{2+}, NO_3^-, and K^+~~SO4, Ca, NO3, and K~~), trace elements (B, Al, V, Se, and Ba), microbial factors and carbohydrate catabolic profiles might be the main descriptors of groundwater-obligate species abundances in the volcanic aquifer. Our findings revealed a correlation between the abundances of groundwater-obligate crustaceans and LNA cells, suggesting a potential selective feeding ~~behavior~~behaviour of groundwater invertebrate species on the aquatic microbial community. Our research emphasizes the need to consider diverse hydrogeological contexts within

individual aquifers. Potential avenues for future research should further consider food web dynamics in groundwater communities and their impact on carbon and nutrient cycling.

1 Introduction

The Earth's largest unfrozen reserve of freshwater is found within aquifers, geological formations responsible for storing and transmitting groundwater (Ferguson et al., 2021). They ~~harbor~~harbour highly specialized microbial and metazoan species adapted to life in permanent darkness and low energy conditions (Malard et al., 2023a). Groundwater microbes and metazoans play crucial roles in providing ecosystem services such as carbon recycling, pathogen removal, pollutant bioremediation, sediment mixing, and burrowing (Fillinger et al., 2023; Mermillod-Blondin et al., 2023). The variation in the taxonomic and functional composition of the invertebrate assemblages of a given aquifer results from a complex interplay of abiotic (e.g., hydrochemistry, porosity, flow velocity) and biotic factors (e.g., species competition, cross-kingdom interactions), acting on both evolutionary and ecological scales (Zagmajster et al., 2023). However, disentangling the combined effects of these taxonomic and functional drivers remains a difficult task (Malard et al., 2023b).

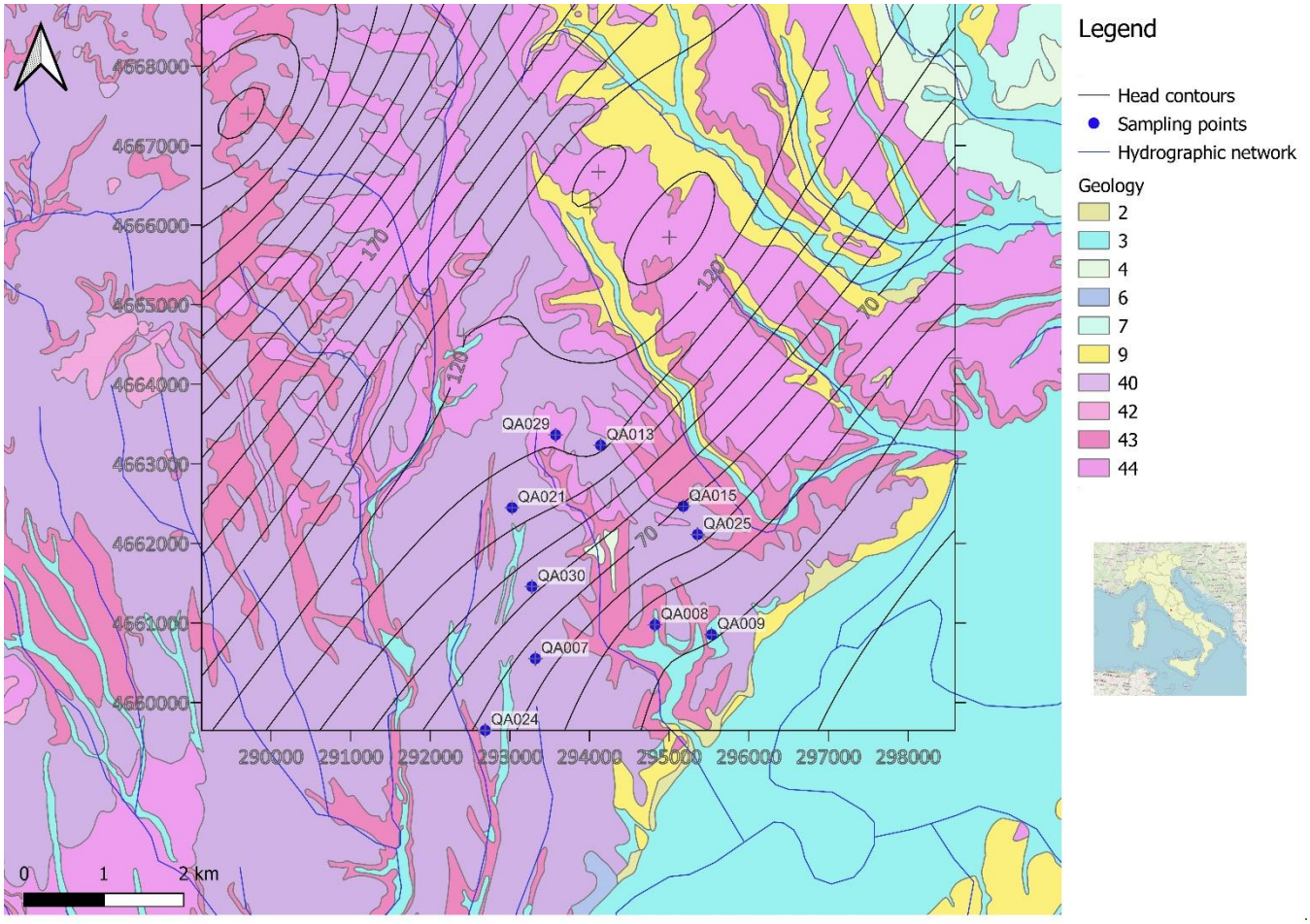
Aquifers can be subdivided into specific adjacent sections, known as aquifer units (AUs), based on geological and hydrological criteria. The species' ability to spread across different AUs depends on extrinsic factors (e.g., the degree of connectivity among AUs) and intrinsic characteristics of the species, which are mainly defined by the suitability of their functional traits (e.g., body size, shape, locomotion, and feeding habits) and the ability to adjust or adapt these traits in response to the new habitat template (Fišer et al., 2023). If a species lacks trait plasticity, it is likely to struggle to establish itself in a new environment. This phenomenon is known as habitat (or environmental) filtering (Cornwell, Schwilk, and Ackerly, 2006; Kraft et al., 2015). Groundwater crustaceans, which are the dominant metazoans in global groundwater ecosystems (Stoch and Galassi, 2010), are known to undergo environmental filtering due to abiotic factors such as pore size, flow rate, connectivity to the surface, and hydrochemistry (Trontelj, Blejec, and Fišer, 2012). Environmental filtering is known to hinder the distribution of groundwater crustaceans in adjacent AUs. The organisms tend to accumulate at the boundaries of the AUs and appear unable to disperse across them (Iannella et al., 2020; Vaccarelli et al., 2023). The role of trophic filters, which relate to the availability and type of food resources and encompass both environmental filtering and competition, has been less investigated. groundwater crustacean species in a given aquifer compete for both space and food resources (Bregović et al., 2019). Recent criticisms have pointed out challenges in accurately distinguishing between environmental filtering and competitive interactions, as both can produce similar patterns in species distribution (Cadotte and Tucker, 2017). Traditionally, research on the effect of environmental filtering on groundwater crustaceans has primarily focused on karst aquifers (Maurice et al., 2016; Culver et al., 2021). However, there is a growing recognition of the importance of non-karst aquifers, such as the volcanic ones (Wurzbacher et al., 2020). This has resulted in an intensified effort to gain a better understanding of groundwater ecosystems across a wide range of hydrogeological contexts (Hahn and Fuchs, 2009).

In this study, we aimed to unravel the factors shaping groundwater crustacean assemblages within a water table aquifer where groundwater circulates in volcanic and sedimentary formations. Preliminary analyses carried out two years prior to this study indicated the occurrence of adjacent, chemically different, AUs within the same aquifer (Ademollo et al., 2012). We adopted a multidisciplinary approach - combining hydrogeology, geology, microbiology, and ecology - to determine whether the environmental differences that we highlighted in the three AUs were also reflected on the biological assemblages. More specifically, our goal was to pinpoint the primary factors that influence the taxonomic and functional composition of crustacean assemblages across the three AUs, including: i) one hydrogeological factor (water table depth, used as proxy of the isolation of the AUs from the surface (Aquilina et al., 2023); ii) physical-chemical factors encompassing temperature, pH, dissolved oxygen, as well as major ions and trace elements; iii) microbial factors, such as microbial cell abundance and metabolic profiles. This study ventures into relatively uncharted territory of volcanic aquifers and addresses critical gaps in our understanding of the main factors shaping the groundwater invertebrate assemblages.

2 Materials and Methods

2.1 Hydrogeological aquifer settings and sampling survey

We conducted our study on the eastern flank of the Sabatini Mounts aquifer, near the valley of River Tiber, covering an area of 30 km^2 in the northeastern province of Rome (Italy). We focused on the Sabatini Volcanic complex, in particular on its eastern border, where the volcanic products overlap on the sedimentary layers of Pleistocene The sampling site is in a rural area across the Fosso Fontanalarga, a minor right-hand tributary of the River Tiber, with small-medium villages, sparse houses, agricultural fields and a few industrial activities, including a quarry for the exploitation of lapideous volcanic products (namely the "Via Tiberina Yellow Tuff" formation; Lombardi and Meucci, 2006). Pleistocene K-alkaline volcanic products of the Roman co-magmatic province (Sottili et al., 2010), mainly pyroclastic such as ignimbrites and tuffs, overlap in angular unconformity Pliocene and Pleistocene sedimentary deposits of marine and continental origin (Fig. 1).



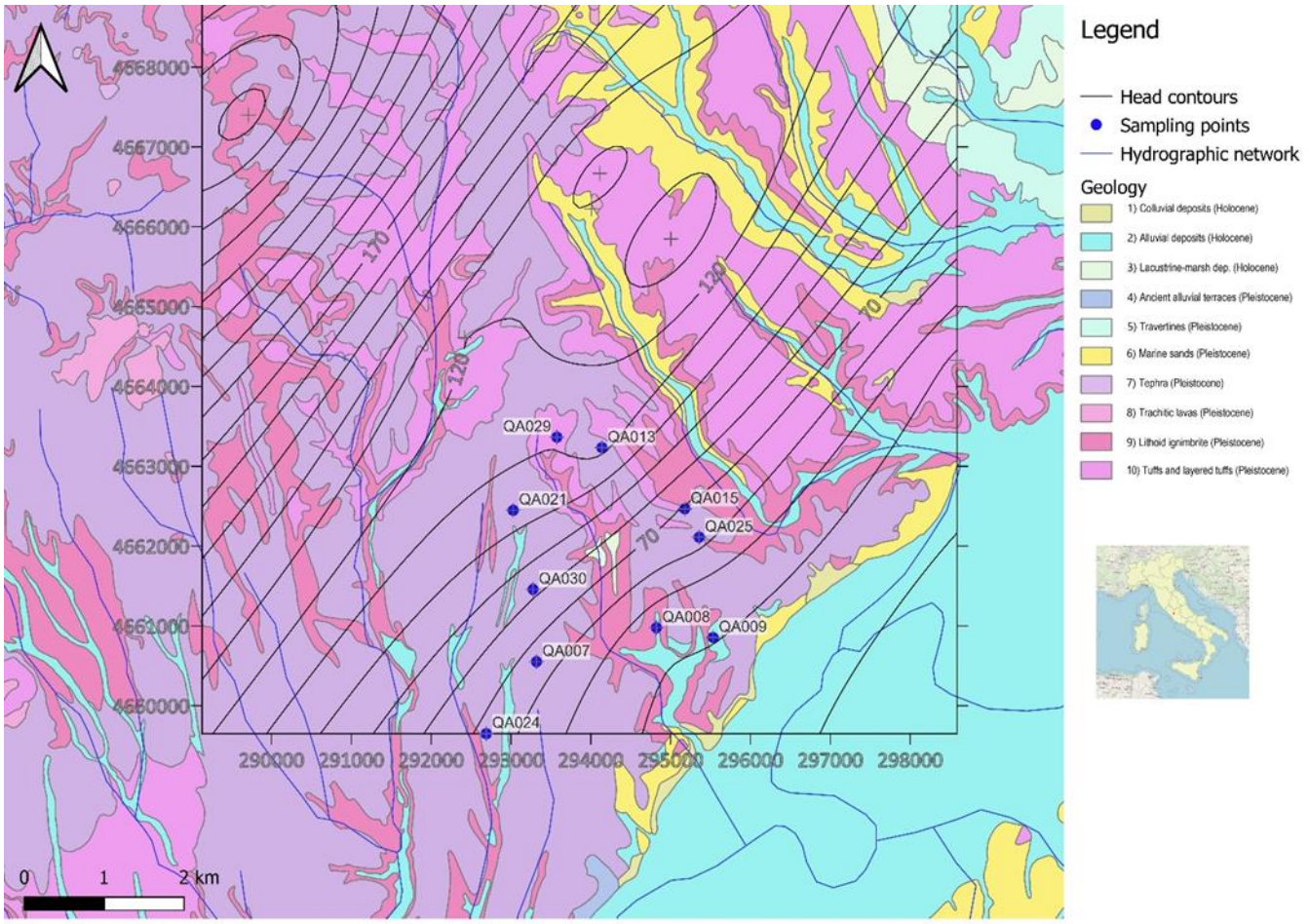


Figure 1. Geological map of the study area and sampling point locations. Head contours referring to 2014 survey are plotted (heads in m a.s.l.). Geology: ~~2~~1) Colluvial deposits (Holocene); ~~3~~2) Alluvial deposits (Holocene); ~~4~~3) Lacustrine-marsh deposits (Holocene); ~~6~~4) Ancient alluvial terraces deposits (Pleistocene); ~~7~~5) Travertines (Pleistocene); ~~9~~6) Marine sands (Pleistocene); ~~40~~7) Tephra (Pleistocene); ~~42~~8) Trachitic lavas (Pleistocene); ~~43~~9) Lithoid ignimbrite "Via Tiberina Yellow Tuff" (Pleistocene); ~~44~~10) Tuffs and layered tuffs (Pleistocene).

Holocene alluvial deposits can be found in the valley talwegs. The water table aquifer is hosted in the volcanites, with groundwater circulating mostly in the porous and fractured lithoid levels of the ignimbrites and lavas, and in the marine and continental sands and gravels below. Low permeability levels of the Pliocene marine clays and silts form the bottom of the aquifer and isolate it from the deep geothermal reservoir hosted in the thick sequence of the Meso-Cenozoic fractured limestones below (Parrone et al., 2020). The Pleistocene pyroclastic products, herein referred to as volcanites, typically exhibit a medium permeability. Since the volcanic formations outcrop more extensively (Fig. 1), aquifer recharge occurs mainly from these rocks than from the underlying sedimentary layers. Groundwater, mainly exploited for irrigation and to

supply households not connected to the public drinking water supply system, flows at depth from north-west to south-east direction through both volcanic and sedimentary layers, the latter being more important towards the edge of the volcanic system (Preziosi et al., 2013; Parrone et al., 2019).

2.2 Field and laboratory geochemical analysis

Preliminary data, collected in March 2012 (Ademollo et al., 2012), indicated the occurrence of three adjacent AUs with distinct hydrogeochemical features, characterized by sulfate-depleted, earth-alkaline, and K-rich waters, respectively. In this study, we conducted two sampling campaigns: the first was carried out between November and December 2014, and the second took place from October to December 2015. During these campaigns, we collected 20 groundwater samples from a total of 10 wells (Fig. 1; Table A1). The distribution of the wells was as follows: i) wells QA7, QA21, QA24, and QA30, situated on the western side of the aquifer, were associated with the AU characterized by sulfate-depleted groundwaters; ii) wells QA8, QA9, and QA29, situated along the valley of Fosso Fontanalarga, were within the AU with earth-alkaline groundwaters; iii) wells QA13, QA15, and QA25, located on the eastern side, belonged to the AU characterized by K-rich groundwaters. For each well, we recorded essential field data, including GPS coordinates, elevation and depth of the well, depth to water table, oxidation-reduction potential (ORP), temperature, pH, dissolved oxygen, and electrical conductivity using multiparametric portable probes (Aquaread AP 2000). To ensure accurate measurements, we purged the wells until the physical-chemical parameters stabilized before sampling. Water samples for chemical analyses were filtered on-site with 0.45-µm membrane filters and stored in HNO_3 1% treated polyethylene bottles. For major cations and trace metal determination, samples were stabilized by adding HNO_3 at pH 2. Alkalinity was determined in the laboratory by HCl titration on 50 mL of sample within 24 h from sampling. Anions were determined by ion chromatography (IC, Dionex DX-120), major cations by ICP-OES (Perkin Elmer P400) and trace elements by inductively coupled plasma mass spectrometry (ICP-MS, Agilent technologies 7500c). Samples for dissolved organic carbon (DOC) were filtered at 0.7 µm with pretreated fiber glass filters and DOC levels were determined through a Shimadzu TOC-5000 analyzer. Further analytical details are reported elsewhere (Preziosi et al., 2014; Parrone et al., 2022).

2.3 Microbial and crustacean community analyses

Samples for the microbial and crustacean community analyses were collected from all wells after purging. Aliquots (2 mL) were fixed with formaldehyde (2% final concentration), stored at 4°C, and analysed within one week from sampling by the Flow Cytometer A50-micro (Apogee Flow System, Hertfordshire, England), equipped with a solid-state laser set at 20 mV and tuned to an excitation wavelength of 488 nm. Total cell counts (TCC) were quantified using side scatter and green fluorescence measurements. The intensity of green fluorescence emitted by SYBR-positive cells facilitated the distinction of cell groups based on their nucleic acid content, namely cells with Low and High Nucleic Acid content (LNA and HNA cells) (Amalfitano et al., 2018).

Additional aliquots (15 ml) were collected, stored in sterile Falcon tubes and promptly incubated within a 6-hour timeframe to evaluate the functional diversity and metabolic preferences of the bacterial communities by measuring their degradative activity toward a set of organic carbon sources using Biolog™ EcoPlates assay (Biolog, Inc., Hayward, California, USA). The assay consists of a 96-well microplate that contains 31 common carbon sources categorized into five compound groups (i.e., carbohydrates, polymers, carboxylic acids, aminoacids, and amines), plus a control well, in triplicate. The provided substrates are labeled with the respiration-sensitive tetrazolium dye, which is reduced to formazan when microbial degradation occurs. This is a colorimetric reaction with the typical purple color development, which optical density (OD) is detected at the wavelength 590 nm by a multi-plate reader (Perkin Elmer VICTOR™ X3). OD values were measured immediately after sample inoculation (T0) and after 24 hours (T24) of incubation in dark conditions at 20°C. Microbial metabolic fingerprinting was expressed as the percentage value of each single class of compounds compared to the total OD, calculated as a fraction of the total absorbance of a single sample in the plate (Melita et al. 2019; Preziosi et al., 2019). The absorbance is proportionally related to the substrate degradation.

The sampling methodology for groundwater crustaceans was based on the manual published within the European project PASCALIS, which is considered the gold standard for groundwater biomonitoring in Europe (Malard et al., 2002). Specifically, we pumped 1000 liters of groundwater from each well using the submersible pumps already installed and filtered the extracted volume through a 63 µm sieve. Given the small size of most groundwater crustaceans (< 1 mm in length; Malard et al., 2023a), the potential concern of the animals being minced by the pump was minimized. The 1000 liters were taken during purging the well. The method is designed to capture organisms present in the well and its immediate surroundings, thereby providing a representative sample of the groundwater fauna. All boreholes were fully screened at their bottom, and the diameter of the sand filter holes was appropriate for the size of the collected animals (< 1 mm). The collected samples were preserved in a 70% ethanol solution and transported to the laboratory for further analysis. In the laboratory, we sorted the samples using a stereomicroscope at 16× magnification, identifying the taxa down to the lowest possible level of morphological taxonomic units, following established taxonomic references (e.g., Dussart and Defaye, 2006). Each specimen was categorized as either groundwater-obligate species (which complete the whole life cycle in groundwater) and epigean species (which occasionally occur in groundwater but lack adaptations for a permanent dwelling). Subsequently, we evaluated four functional traits (locomotion, diet, feeding habits and thermal tolerance) each defined by two or more categories. The trait locomotion was defined by three categories: burrowers (organisms that dig and create burrows in sediments), interstitial (organisms that live in the spaces between sediment particles) and swimmers (organisms that are adapted to swimming in the water column). The trait diet was described by two categories: fine sediments + microorganisms (organisms that feed ingesting fine sediment particles and the attached microorganisms) and living microphytes (organisms that feed on living algae). The trait feeding habits was described by three categories: deposit-feeders (organisms that consume organic matter settled on the substrate), collectors (organisms that gather particles from the water column) and grazers (organisms that feed on biofilms or algae growing on surfaces). Finally, the trait thermal tolerance was described by three categories: eurythermal (organisms that can tolerate a wide range of temperatures), moderately

stenothermal (organisms that tolerate a moderate range of temperatures) and stenothermal (organisms that tolerate a narrow range of temperatures). Each trait was assigned based on existing literature. We attributed each species to only one category per trait, except for the amphipod *Niphargus* sp., which showed a range of feeding habits and thermal tolerance (Fišer, 2019). Since we did not identify the amphipod specimens down to the species level, we preferred to share their percentages equally per each trait category to reflect this trait variability. To obtain the trait profile of each sample, we calculated the percentage of each trait category based on its abundance within the sample (Di Lorenzo et al., 2021). This means that for each trait (locomotion, feeding habits, and thermal tolerance), we determined the proportion of individuals in each category relative to the total number of individuals in the sample.

2.4 Statistical analyses

To confirm the occurrence of the three AUs, as assumed from our preliminary investigation in 2012, we performed permutational analyses of variances (PERMANOVA; Anderson, 2008) based on physical-chemical factors measured *in situ* (water table depth, temperature, pH, dissolved oxygen and electrical conductivity), major ions and elements in trace, respectively. We conducted two-way PERMANOVAs, incorporating a factor "aquifer unit" with three levels (sulfate-depleted, earth-alkaline and K-rich) and another factor "year" with two levels (2014, 2015), using resemblance matrices based on Euclidean distances calculated from normalized data. To ensure the robustness of our analysis, we examined potential multicollinearity among the variables using Draftsman's plots prior to PERMANOVAs. Variables exhibiting high collinearity ($|r| \geq 0.95$ in correlation) were considered to convey essentially identical information and were consequently removed to prevent redundancy in the analysis, in line with recommendations in Anderson et al. (2008). The variables retained for the analyses served as proxies for those that were eliminated (Anderson et al., 2008). We excluded oxygen saturation from the analyses due to > 99% linear correlation with dissolved oxygen. In line with best practices, we applied permutation of residuals under a reduced model and employed Type III sum of squares with 999 permutations. This approach offers high statistical power and more accurate type I error control for multi-factorial, unbalanced designs (Anderson et al., 2008). When appropriate, we conducted permutational post-hoc t-tests.

We evaluated the adequacy of the biological sampling effort in the whole volcanic aquifer by examining the accumulation of the total number of different observed species (Sobs) as samples were progressively added (Magurran, 2021). To assess the potential increase in species richness (S) with repeated sampling, we applied five non-parametric (Chao1, Chao2, Jackknife1, Jackknife2, and Bootstrap) and one parametric (Michaelis-Menten) estimators (Magurran, 2021). We computed the estimators at each stage as new samples were added, resulting in the generation of six curves illustrating the progression of the S with increasing sample size. We conducted the analyses through 999 randomizations without replacement (Magurran and McGill, 2011). We repeated the analyses for each AUs, separately.

To investigate potential differences in the taxonomic and functional composition of the crustacean assemblage and microbial community among the three AUs, we applied the PERMANOVA design previously outlined for the environmental variables.

We log(x+1)-transformed the crustacean abundances and microbial cell counts before generating Bray-Curtis resemblance matrices, while the percentage data pertaining to metazoan functional traits and HNA and LNA cells remained untransformed (Anderson, 2008). We added a dummy variable equal to 1 to all data to allow the analysis of values equal to zero (Clarke and Gorley, 2005). We chose not to analyze the abundances of each crustacean species due to the diminished interpretive accuracy when the abundances of individual taxa constituted less than 4% of the total abundances (Clarke and Gorley, 2005). In our study, only four species met or exceeded this specified threshold (Sect. 3.2). While PERMANOVA inherently does not necessitate explicit assumptions regarding the distributions of the original variables, we opted to conduct a Levene's test using the PERMDISP routine prior to all analyses. We focused on PERMANOVA outcomes that were not influenced by bias due to variance heterogeneity (Anderson, 2008). To provide a comprehensive overview of the significant outcomes derived from the PERMANOVAs, we utilized boxplot when considered insightful for visualization.

Finally, to assess the main hydrogeological, physical-chemical and microbial factors that influenced the composition and functionality of crustacean assemblages across the three AUs, we employed distance-based linear models (DisTLM) based on the Bray-Curtis resemblance matrix (Legendre and Anderson, 1999). We conducted both conditional and marginal tests. Conditional tests involved fitting one factor after another. We applied the BEST procedure (Legendre and Anderson, 1999) to construct models utilizing the best factor combination. We assessed the AICc value (Akaike's Information Criterion corrected for small sample sizes; Hurvich and Tsai, 1993) for all possible combinations of predictor variables, with the smallest AICc value indicating the most suitable model. Additionally, we used R^2 to evaluate the proportion of explained variation in the multivariate models. The significance of the marginal tests was determined by computing the p-values through permutations rather than traditional tables (Legendre and Anderson, 1999). For each test, we employed 999 permutations to obtain p-values testing the null hypothesis of no relationship, either for individual variables in isolation or in a conditional context (Legendre and Anderson, 1999). Factors that individually (marginal models) or together with others (conditional models) explained > 65% of the variance in the taxonomic and functional structure of the crustacean assemblage were considered robust, following the criteria established by Korbel and Hose (2011, 2017a) and Di Lorenzo et al. (2020). We applied this cut-off criterion to prevent unreliable and exaggerated claims about scientific phenomena (Kimmel et al., 2023). We performed distance-based redundancy analyses (dbRDA; Legendre and Anderson, 1999) to visualize the ordination of the fitted values from the most robust models.

Significance levels (α) were set at 0.05 for all permutational tests since they provide an exact test of each individual null hypothesis of interest (Anderson et al., 2008). All analyses were performed using E-PRIMER version 6 and PERMANOVA+ software (Anderson et al., 2008). Boxplots were generated using the libraries ggplot2 and gridExtra and the R software (R Development Core Team, 2021).

3 Results

3.1 Environmental factors and microbial community patterns

The analyses revealed no significant differences in the water table depth among the three AUs or between the two years (Table A2a and S1). However, PERMANOVA uncovered differences in temperature, pH, and electrical conductivity (Table A2a and S1). In detail, the sulfate-depleted AU exhibited a mean temperature exceeding that of the other two AUs by about 1 °C (Table 1). The earth-alkaline AU showed the lowest pH and the highest electrical conductivity values (Table 1; Fig. 2). However, no discernible distinctions were observed between the two years (Table S1).

Ammonium and phosphate exhibited concentrations below the instrumental detection limit of 0.05 mg/L in all samples and AUs and were, therefore, not included in the statistical analyses. The three AUs exhibited significant differences in the major chemical components, with variations attributed to SO_4^{2-}, HCO_3^-, Na^+, K^+~~SO4, HCO3, Na, K~~, and Si (Table A2b and S1; Fig. 2). In detail: the earth-alkaline AU displayed the highest HCO_3^-~~HCO3~~ and Si concentrations; the K-rich AU showed the highest mean concentration of Na^+ and K^+; the sulfate-depleted AU displayed the lowest mean values of SO_4^{2-}~~SO4~~ and HCO_3^-~~HCO3~~ (Tables 1 and S1; Fig. 2). Significant distinctions between the two years were only observed for Si and DOC, both of which were higher in 2014 compared to 2015 (Tables A2b and S1). Significant differences among the three AUs were also noted for trace elements, namely in the concentrations of Li, B, Rb, and U (Tables A2c and S1). The K-rich AU showed the highest concentrations of Li, B, and Rb, while the sulfate-depleted AU showed the lowest concentrations of U (Table 1 and Fig. 2). No significant differences emerged between the two years for trace elements.

Table 1: Physical-chemical and microbial characteristics of the groundwater samples in the three aquifer units of the Sabatini Mounts volcanic aquifer. Water table depth, field parameters, major components, trace elements and microbial community properties are reported as mean values. Superscript letters (a, b, c) indicate significant differences among AUs (permutational post-hoc t-tests; $p < 0.05$). * indicates statistical differences between 2014 and 2015.

	Sulfate-depleted	Earth-alkaline	K-rich
Water table depth (m)	80.01	64.26	81.82
Field parameters			
EC (µS/cm)	512.0^{b}	885.5^{a}	657.5^{c}
DO (mg/L)	7.3	5.9	7.1
ORP (mV)	183.7	156.6	211.2
pH	7.5^{b}	7.1^{a}	7.6^{b}
T (°C)	18.1^{b}	16.8^{a}	$17.2^{a,b}$
Major components			
Ca^{2+}~~Ca~~ (mg/L)	41.3	84.3	31.1
Mg^{2+}~~Mg~~ (mg/L)	8.2	12.3	10.5
Na^+~~Na~~ (mg/L)	24.6^{a}	$31.9^{a,b}$	48.4^{b}
K^+~~K~~ (mg/L)	34.9^{a}	31.9^{a}	47.4^{b}
Si (mg/L)	22.7^{a}	$22.8^{a,b}$	19.7^{b}
HCO_3^- ~~HCO_3~~ (mg/L)	231.0^{b}	377.5^{a}	279.9^{c}
Cl^- ~~Cl~~ (mg/L)	28.1	29.3	33.3
SO_4^{2-} ~~SO_4~~ (mg/L)	6.2^{b}	59.5^{a}	22.4^{a}

NO_3^- ~~NO_3~~ (mg/L)	10.2	25.8	17.2
DOC (mg/L)	0.8	0.8	0.8
Trace elements			
Al (µg/L)	10.6	11.1	14.35
As (µg/L)	20.6	22.6	26.7
B (µg/L)	64.9^{b}	89.5^{a}	117.0^{c}
Ba (µg/L)	72.3	81.5	54.9
Cr (µg/L)	1.3	1.0	1.1
Cu (µg/L)	0.3	0.7	0.5
Fe (µg/L)	23.4	22.5	17.7
Li (µg/L)	18.6^{b}	15.5^{b}	46.1^{a}
Mn (µg/L)	0.9	1.9	0.6
Ni (µg/L)	0.3	0.7	0.5
Rb (µg/L)	21.7^{b}	29.9^{a}	49.1^{c}
Se (µg/L)	0.3	0.8	0.2
Sr (µg/L)	529.4	813.3	520.7
U (µg/L)	3.1^{b}	23.4^{a}	12.0^{a}
V (µg/L)	30.9	28.4	27.1
Zn (µg/L)	37.3	10.8	14.6
Microbial community			
TCC (10^4 cells/mL)	1.0^{b}	3.1^{a}	3.7^{a}
LNA cells (% of TCC)	65.7^{b}	75.6^{a}	72.0^{a}
HNA cells (% of TCC)	34.3^{b}	24.4^{a}	27.9^{a}
HNA/LNA	0.5^{b}	0.3^{a}	0.4^{a}
Carbohydrates	21.6	38.9	15.5
Polymers*	46.0	13.2	30.7
Carboxilic acids*	24.1	23.6	43.2
Aminoacids*	5.7	19.2	8.6
Amines	2.7	5.2	1.8

Total counts of microbial cells ranged from 0.5 to 9.5 x 10^4 cells/mL, with the lowest abundances occurring in the sulfate-depleted AU (Table A2d and Fig. 2). A similar pattern was observed for the percentages of HNA and LNA cells, with the lowest percentages of LNA cells found in the sulfate-depleted AU, which was correspondingly richer in HNA cells compared to the K-rich and earth-alkaline AUs (Tables 1 and A2d; Fig. 2). The microbial pattern was consistent over the two years (Table S1).

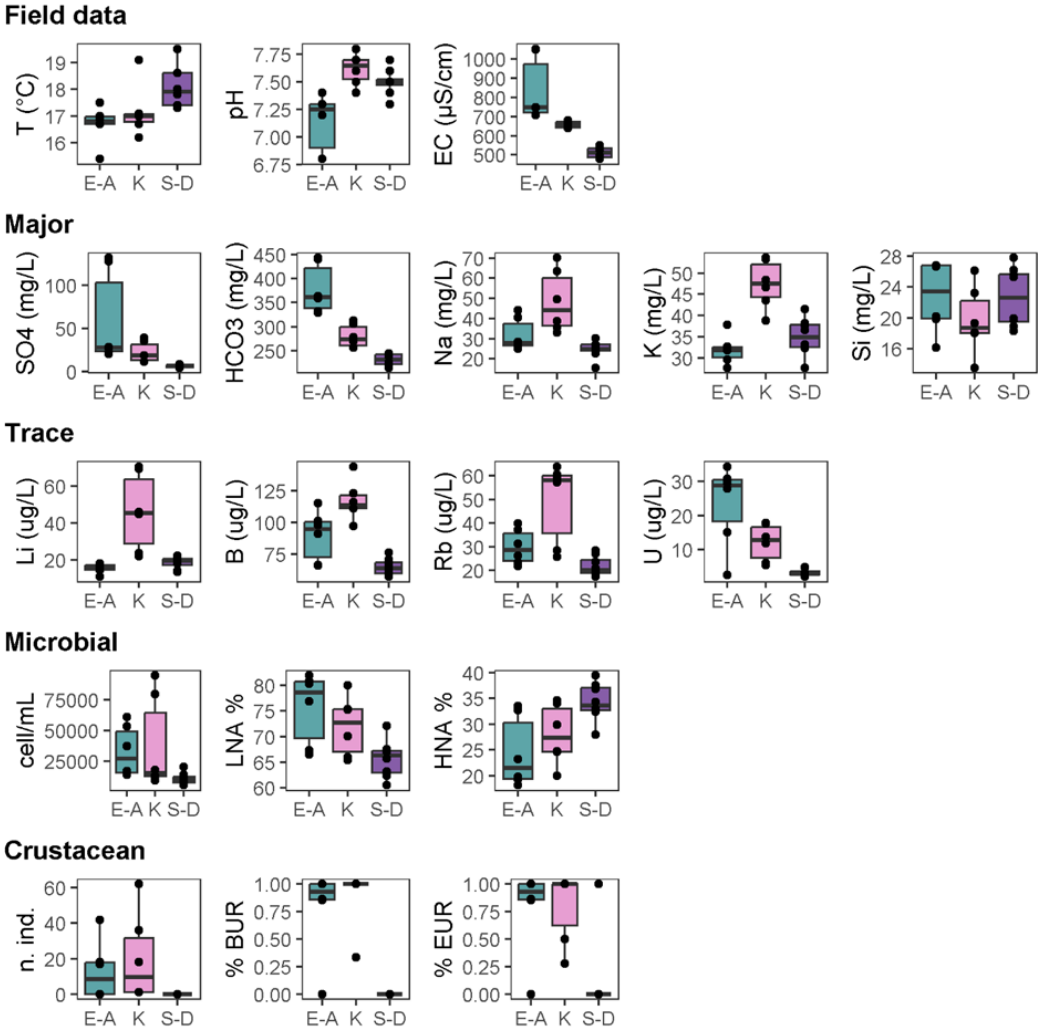


Figure 2: Boxplots showing significant differences (p < 0.05, permutational t-test) in environmental and biological parameters among the three aquifer units (earth-alkaline in green; K-rich in pink, and sulfate-depleted in purple) of the Sabatini Mounts aquifer: Boxplot are arranged into five rows: field parameters, major chemicals, trace elements, microbes, and crustaceans. Each boxplot includes median lines, 25th-75th percentile boundaries, whiskers for 10th-90th percentiles, and black dots indicating outliers. BUR: burrowers; EUR: eurythermal.

We obtained significant PERMANOVA outcomes for microbial catabolic profiles related to carbohydrates, polymers and aminoacids degradation among the three AUs, while no differences across the AUs were detected for carboxylic acids and amines utilization. However, the results for polymers and aminoacids were affected by variance heterogeneity (Table S1). Additionally, ~~the catabolic~~catabolic profiles based on carbohydrates showed inconsistencies over the two years of investigation. Overall, the catabolic profile of the microbial assembly of the three AUs seemed to be mainly based on

carbohydrates, polymers and carboxylic acids, with variation over time, while the profiles based on amines and aminoacids were much less represented (Table 1).

3.2 Crustacean assemblages

Out of the 20 biological samples analyzed, 8 (40%) did not contain any invertebrate specimens (Table A2e). We identified 210 crustacean specimens (< 1 mm in average length), belonging to 9 species (Table A2e). Seven of these taxa were groundwater-obligate dwellers (94% of the total abundances; Table A2e) showing typical morphological adaptations to dark environments (Fig. 3).

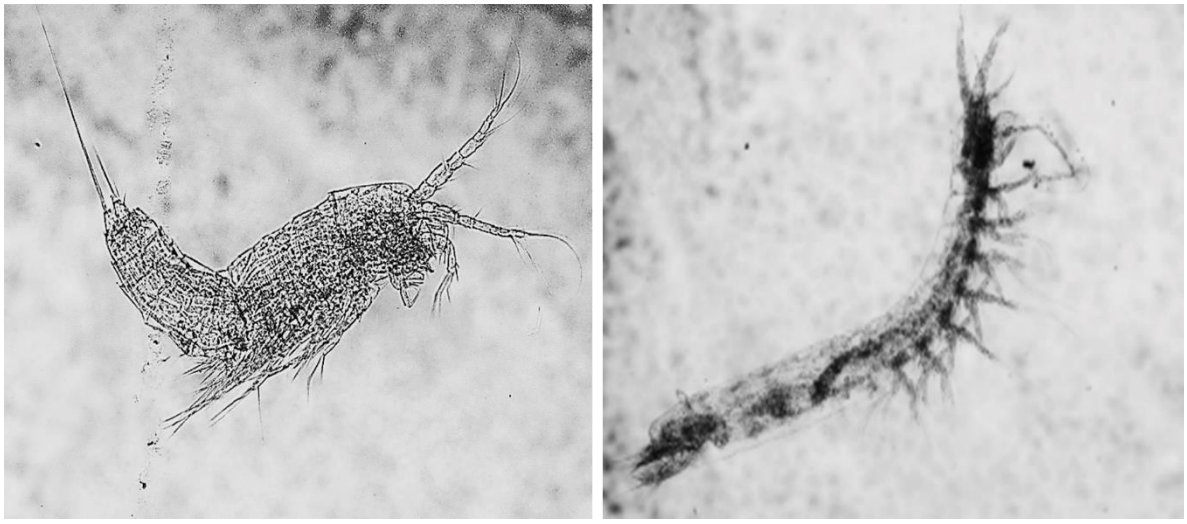


Figure 3: Groundwater-obligate crustacean species. The figure showcases two groundwater-obligate crustacean species found within the Mounts Sabatini aquifer: the harpacticoid *Parapseudoleptomesochra italica* (0.73 mm in length, on the left) and the syncarid *Meridiobathynella* sp. (0.8 mm in length, on the right). The specimens exhibit the typical morphological traits of groundwater fauna: blindness, depigmentation, and hypertrophy of sensory appendages.

Approximately 60% of the samples contained a single species only (Tables 1 and A2e). The class Crustacea Copepoda dominated with 7 species, including 5 groundwater-obligate species, 6 species belonging to the order Harpacticoida, and 1 species to the order Cyclopoida. At the aquifer level, three non-parametric estimators (out of five) and the non-parametric one indicated that 100% of the expected invertebrate biodiversity was collected during the sampling survey. However, the remaining estimators suggested that we likely missed ~~out~~ a minimum of 9% (Chao2) to a maximum of 21% (Jacknife1) of the expected biodiversity (i.e., from 1 to 3 more species; Fig. S1). Our analyses suggested that this missed biodiversity should be ~~find~~ found mainly in the earth-alkaline and K-rich aquifer units (Fig. S1).

PERMANOVA analysis indicated significant differences in the composition of crustacean assemblages; however, the outcome was affected by variance heterogeneity. On the other hand, the analyses revealed significant unbiased differences in the abundances of groundwater-obligate species among the three AUs (Table S1), with the sulfate-depleted AU lacking

groundwater-obligate species (Tables 2 and S1). Notably, the K-rich AU lacked epigean species. Remarkably, there were no significant differences observed between the two years (Table S1). Overall, the K-rich and earth-alkaline AUs exhibited comparable abundances and species richness (Table 2).

Table 2: Taxonomic and functional composition of the crustacean assemblages in the three aquifer units (AUs). Taxonomic abundances and traits are reported as n. of individuals per each aquifer unit. Superscript letters (a, b, c) indicate significant differences among AUs (permutational post-hoc t-tests; p < 0.05). + indicates groundwater-obligate species. For *Niphargus* sp. (Nsp), 0.5 individuals were counted as deposit-feeders and 0.5 as collectors, reflecting its dual functional feeding habit traits, and 0.5 as eurythermal and 0.5 as stenothermal reflecting its dual thermal tolerance traits. S-D: sulfate-depleted; E-A: earth-alkaline. Acronyms of taxa are reported in brackets. 1: Galassi (2001), 2: Galassi et al. (1999); 3: Galassi et al. (2009); 4: Galassi and De Laurentiis (2004); 5: Dussart and Defaye (2002); 6: Hose and Stampp (2019); 7: Schminke (1974); 8: Fišer (2019).

Taxon	S-D	E-A	K-rich	Ref.
+*Parapseudoleptomesochra italica* Pesce & Petkovski, 1980 (Pit)	0	43	100	
+*Parastenocaris* sp. (Psp)	0	2	0	
+*Nitocrella psammophila* Chappuis, 1954 (Nps)	0	0	5	
+*Pseudectinosoma reductum* Galassi & De Laurentiis, 1997 (Pre)	0	0	1	
+*Acanthocyclops agamus* Kiefer, 1938 (Aag)	0	2	12	
+*Meridiobathynella* sp. (Msp)	0	30	0	
+*Niphargus* sp. (Nsp)	0	0	1	
Elaphoidella gracilis (Sars, G.O., 1863) (Egr)	0	13	0	
Moraria poppei (Mrázek, 1893) (Mpo)	1	0	0	
Epigean species	1	13	0	
Groundwater-obligate species	0^{b}	77^{a}	119^{a}	
Trait locomotion				
Burrowers [Pit, Nps, Pre, Msp, Nsp, Egr]	0^{b}	86^{a}	107^{a}	1,2,3,5,6,7,8
Interstitials [Psp, Mpo]	1	2	0	1,2,3,5
Swimmers [Aag]	0	2	12	1,2,3,4,5
Trait diet				
Fine sediments + microorganisms [Pit, Psp, Nps, Pre, Aag, Msp, Nsp, Mpo]	1	77	119	1,2,3,4,5,6,7,8
Living microphytes [Egr]	0	13	0	1,2,3,5
Trait feeding habit				
Deposit feeders [Pit, Psp, Nps, Pre,-Aag, Msp, Nsp]	1^{a}	$77^{a,b}$	118.5^{b}	1,2,3,4,5,6,7,8
Collectors [Pre, Npsp]	0	0	0.5	1,2,3,5
Grazers [Egr]	0	13	0	1,2,3,5
Thermal tolerance				
Eurythermal [Pit, Nps, Msp, Nsp, Egr, Mpo]	1^{a}	$86^{a,b}$	105.5^{b}	1,2,3,5,6,7,8
Moderately stenothermal [Aag]	0	2	12	1,2,3,4,5
Stenothermal [Psp, Pre, Nsp]	0	2	1.5	1,2,3,5

However, the taxonomic composition in the two AUs was different. In detail, the groundwater-obligate harpacticoid *Parapseudoleptomesochra italica* was the most abundant species, accounting for 67% of crustacean biodiversity in the aquifer (Table A2e). The groundwater-obligate *P. italica* and *Acanthocyclops agamus* were found in both earth-alkaline and K-rich AUs (Table 2). *Nitocrella psammophila*, *Pseudectinosoma reductum* and *Niphargus* sp. (groundwater-obligate species) occurred in the K-rich AU, while *Parastenocaris* sp. and *Meridiobathynella* sp. (groundwater-obligate species) were collected in the earth-alkaline AU where the epigean *Elaphoidella gracilis* (epigean species) was also present (Table A2e).

The epigean species *Moraria poppei* was the only species found in the sulfate-depleted AU, with a single individual collected from well QA21 in 2014 (Table A2e).

Most of the species collected in this study were burrowers and eurythermal (Table 2). The locomotion and thermal tolerance traits exhibited variation among the three AUs, primarily driven by the higher percentages of eurythermal species in the earth-alkaline AU compared to the other two AUs (Tables S1 and A2f; Fig. 2). Notably, moderately stenothermal and stenothermal species were collected only from the K-rich AU and never from the other two AUs (Table A2f). PERMANOVA outcomes indicated significant differences in the diet trait among the AUs, although this result was biased by variance heterogeneity (Table S1). The deposit feeding trait was the most prevalent among various feeding habits, with collectors and grazers being less common, but the analyses did not identify any significant differences across the AUs (Tables 2 and S1). The pattern was consistent over time for all functional traits (Tables 2 and S1).

3.3 Linear models

3.3.1 Hydrogelogical factor

The linear models based on the water table depth as descriptor accounted ≤ 25% of the variance of the taxonomic and functional composition of the crustacean assemblages in the aquifer (Table S2).

3.3.2 Physical-chemical factors

Concerning field factors, the multivariate linear models accounted < 65% of the variance of the taxonomic and functional composition of the crustacean assemblages in the aquifer, with electrical conductivity, pH and ORP being the main descriptors. None of the marginal models explained > 65% of the variance (Table S2).

Multivariate models based on major ions explained < 65% of the variance of the taxonomic composition, locomotion, diet, feeding habits, and thermal tolerance traits (Table S2). However, four major ions (SO_4^{2-}, Ca^{2+}, NO_3^-, and K^+) ~~(SO4, Ca, NO3, and K)~~ together accounted for 91% of the variance in groundwater-obligate species (Table S2), with abundances significantly decreasing in samples depleted of SO_4^{2-}~~SO4~~ (Fig. 4). None of the major chemical elements individually explained more than 65% of the variance (Table S2). Concerning trace elements, multivariate linear models explained < 65% (Table S2) of the variance of the trait locomotion and thermal tolerance. On the contrary, they explained > 65% of the variance of the taxonomic composition (best model 71%: V, Cr, As, Se, and U), abundances of groundwater-obligate species (best model 89%: B, Al, V, Se, and Ba), trait diet (best model 81%: Li, B, Cr tot, Ni, As, and U) and feeding habits (best model 71%: Li, B, Cr tot, Ni, As, and U), with abundances and trait percentages increasing with increasing concentrations of all trace elements except for Cr tot (Fig. 4). The contribution of individual trace elements to marginal models was always < 35%, when significant (Table S2).

3.3.3 Microbial factors

The conditional and marginal linear models based on microbial factors (TCC, LNA, HNA cells and catabolic pathways) explained < 65% of the taxonomic and functional composition of the crustacean assemblages. However, LNA cells and the carbohydrate pathways together explained 71% of the abundances of groundwater-obligate species (Table S2), with abundances increasing with increasing LNA cells and microbes using carbohydrates as substrates (Fig. 4). The individual contribution of LNA cells to the explained variance was 66%.

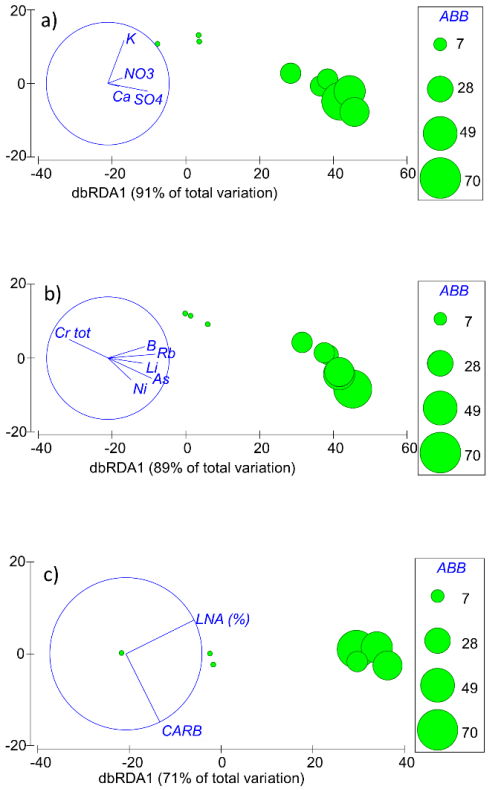


Figure 4: dbRDA plots showing the linear relations between the abundances (ABB) of groundwater-obligate species and the factors selected by the BEST procedure of the DistLM. a) major ions; (b) trace element and c) microbial factors (LNA%: percentages of Low Nucleic Acid cells; CARB: carbohydrate catabolic profile).

4 Discussion

Despite recent advancements in the field, numerous knowledge gaps persist in identifying the factors that shape the taxonomic and functional composition of groundwater crustaceans (Mammola et al., 2021). Particularly overlooked and unexplored is the analysis of groundwater assemblages within volcanic aquifers and the role of trophic factors in shaping the distribution of groundwater-obligate fauna through processes of environmental filtering (Saccò et al., 2019). To confront

these challenges, our study adopted a comprehensive, multidisciplinary approach that harnessed the collective knowledge spanning hydrogeology, geology, microbiology and ecology.

While the water table depth did not vary significantly across the aquifer, our analyses confirmed and supported the occurrence of three adjacent AUs, each characterized by distinct hydrochemical facies and microbial community patterns. The sulfate-depleted AU showed the highest mean temperature, the lowest concentrations of SO_4^{2-}~~SO4~~, HCO_3^-~~HCO3~~ and U, the lowest microbial abundances and percentages of LNA cells, and the highest percentages of HNA cells. The earth-alkaline AU showed the lowest pH values and the highest electrical conductivity and HCO_3^-~~HCO3~~ and Si concentrations, while the microbial abundances were comparable to those in the K-rich AU. Finally, the K-rich AU presented the highest concentrations of Na^+, K^+~~Na, K~~, Li, B and Rb.

We examined the crustacean assemblages of the aquifer, revealing significant variations in both taxonomic and functional composition across the three AUs. In detail, the sulfate-depleted AU lacked groundwater-obligate species, burrowers, and stenothermal or moderately stenothermal species. The K-rich and earth-alkaline AUs, which showed comparable abundances and species richness, were characterized by different species, which, however, showed the same functions concerning locomotion, diet and feeding habit. Notably, stenothermal or moderately stenothermal crustacean species occurred in the K-rich AU only, which was, however, depleted of epigean species. We used a stringent cut-off criterion (Korbel and Hose; 2011, 2017a; Di Lorenzo et al., 2020) to identify the potential predictors of these differences and concluded that water table depth was not a driving factor in shaping these assemblages. The taxonomic composition seemed to be mainly driven by trace elements such as V, Cr, As, Se, U. The main descriptors of the abundances of groundwater-obligate species appeared to be the major ions SO_4^{2-}, Ca^{2+}, NO_3^- and K^+~~SO4, Ca, NO3 and K~~, the trace elements B, Al, V, Se, and Ba, the microbial factors related to LNA cells and carbohydrate catabolic profile. Finally, the trace elements Li, B, Cr tot, Ni, As, and U seemed to be the main drivers of the traits diet and feeding habits. Water chemistry did not appear to exert a detrimental effect on the composition and functionality of crustacean assemblages in the target aquifer, consistent with previous studies (e.g., Di Lorenzo et al., 2020), except for Cr. This result may be attributed to the likelihood that the chemicals recognized as detrimental to groundwater organisms were either not detectable or existed in concentrations that were not harmful in our study. For instance, trace elements, such as Ni, Zn, As, Li, are toxic to groundwater fauna at concentrations > 150 µg/L (Di Lorenzo et al., 2023) which, however, were never measured in the study area. Ammonium (which exhibited concentrations below instrumental detection limit in our study) is lethal to groundwater-obligate species at concentrations > 12 mg/L, albeit causing cellular and physiological damage at concentrations ≥ 36 µg/L (Di Lorenzo et al., 2015). Nitrate causes no harm to groundwater fauna at concentrations < 100 mg/L (Di Lorenzo et al., 2023), which are much higher than those observed in our study. Previous research has shown that groundwater species are sensitive to pharmaceutical compounds, pesticides, and BPA (Di Lorenzo et al., 2023). In our preliminary study (Ademollo et al., 2012), chlorinated pesticides were not detected and only traces of PAHs and PCBs were found slightly above the detection limits (0.01 ng/L). High tolerance to SO_4^{2-}~~SO4~~ (which was positively correlated to groundwater species abundances in this study) is known for many groundwater-obligate species of marine origin, such as *P. italica*, *N. psammophila* and *P. reductum* (Galassi, 2001). For instance, *N. psammophila*

has been collected from the chemoautotrophic groundwater of the Frasassi cave system (Italy) where sulfate concentrations reach up to 199 mg/L (Galassi et al., 2017). *Parapseudoleptomesochra italica* has been recorded from the Movile Cave (Romania) where groundwater has high sulfate concentration and, similarly, *P. reductum*, has been discovered in the in sulfidic groundwaters of Melissotrypa Cave (Greece) (Brad et al., 2021). While prior studies have reported synergistic toxic effects of pollutant mixtures on groundwater species (Di Marzio et al., 2018), our research seems to find no significant differences in chemical parameters among the various AUs that could account for varying toxicity levels. For instance, in the sulfate-depleted AU, which lacked groundwater-obligate species, most of the tested chemical elements exhibited lower concentrations compared to the earth-alkaline or K-rich AUs. Studies on the ecotoxicology of groundwater organisms have been limited by the life history traits of groundwater fauna that make them often unsuitable for laboratory experiments (Di Lorenzo et al., 2019). Our study offered preliminary insights into the potential sensitivity of groundwater crustaceans to chemicals that have hitherto remained untested in prior research, including elements like uranium and boron. The negative correlation between the abundances of groundwater-obligate species and Cr is, in our view, not directly related to the toxicity of this element. Total chromium is present in the aquifer at concentrations ranging from 0.4 to 1.8 µg/L, which are considered harmless based on available literature data (Di Lorenzo et al., 2023). This might be a statistical artifact since lower abundances of groundwater-obligate species are found in the sulfate-depleted AU, where the average Cr concentration is slightly higher than in the other AUs. Therefore, we observe this negative correlation in Figure 4. However, we suggest that the absence of groundwater-obligate species in this AU is related to microbial factors, as we will explain later.

While the sulfate-depleted AU lacks groundwater-obligate species, the earth-alkaline and K-rich AUs differ in terms of species composition. This outcome seems to suggest that, although the AUs are hydrogeologically connected, the groundwater species collected in this study seemed not to migrate across the aquifer units. This finding aligns with literature. Iannella et al. (2020) observed that European groundwater-obligate harpacticoid species (which represent 67% of the species in our study) were unable to disperse across boundaries between two adjacent AUs. Accordingly, Vaccarelli et al. (2023) observed that the dispersal of groundwater-obligate copepod species in the Eastern Lessinian Massif (Italy) is constrained by non-fractured igneous rocks, as it appears to be in our study. Only two groundwater-obligate species, *P. italica* and *A. agamus*, were found in both the K-rich and earth-alkaline AUs. The remaining species collected in this study were unique to one AU only, such as the groundwater-obligate *P. reductum* (a Tertiary relict of ancient marine origin; Galassi et al., 1999), which was collected from the K-rich AU, along with *N. psammophila*. The genus *Nitocrella*, which has direct marine origin, serves as an indicator of ancient evolutionary events (Galassi et al., 2009). Notably, the K-rich AU showed the highest concentrations of Li, B, and Rb, which are characteristic elements of the deep geothermal facies and possibly of fossil marine waters of Neogene age (Duchi et al., 2003). The presence of stenothermal and moderately stenothermal species in this AU, and only in this one, suggests that the habitat of this K-rich aquifer unit may be conservative and, therefore, suitable for preserving ancient evolutionary lineages with no close relatives in surface environments, such as *P. reductum*.

Two out of the six indicators revealed that a small percentage of taxonomic diversity (ranging from 1 to a maximum of 3 species) was not captured in this study. This finding is commonly encountered because groundwater sampling is essentially a

blind process (Mammola et al., 2021). Wells serve as windows through which we gain insight into the subterranean biodiversity in the portions of the aquifer surrounding them, but groundwater habitats extend more extensively (Ficetola et al., 2019). However, we speculate that the absence of groundwater-obligate species in the sulfate-depleted AU might be partly due to the low percentages of LNA cells found in this AU, rather than, or in addition to, incomplete sampling. The LNA and HNA cell counts exhibited variations among the three AUs. Specifically, the sulfate-depleted AU displayed the lowest LNA cell percentages. As detected by flow cytometry, LNA and HNA cell groups are considered constitutive traits of aquatic microbial communities, typically comprising cells of varying sizes, genome content, and phylogenetic affiliations (Gasol et al., 1999; Proctor et al., 2018). Notably, HNA cells were recognized as an active fraction of the bacterioplankton community, and their abundance was reported to positively correlate with heterotrophic production rates in freshwaters of different origin (Rubbens et al., 2019). In contrast, LNA cells were traditionally thought to represent a more dormant or quiescent portion of the aquatic microbial community (Lebaron et al., 2002). They were reported as small-sized microorganisms with slower metabolic activity and a wide range of survival strategies suitable for thriving in adverse conditions, including oligotrophy (Hu et al., 2022). In previous groundwater studies, the percentage of LNA cells and the use of carbohydrates were linked at either low or high nutrient levels~~LNA cells contribution and carbohydrate utilization were associated under pristine and unbalanced nutrient conditions~~ (Melita et al. 2019). This is explained by the role played by carbohydrates as important energy-rich carbon source and storage molecules for the aquatic bacterial metabolism (Arnosti et al., 2014). These observations seem to suggest that groundwater quality can directly affect the functional properties of the aquatic microbial communities with implications on the pattern of the energy fluxes among organic matter, microbes and the organisms located in the upper levels of the food web. In this study, the high linear correlation (r = 0.80) between groundwater crustacean abundance and LNA cells (supported by an explained variance R^2> 65%) seem to suggest that groundwater crustaceans might derive significant benefits from the presence of LNA cells. Considering the high abundances of deposit-feeders and crustaceans feeding on sediments and microorganisms in the earth-alkaline and K-rich AUs~~,~~ compared to the sulfate-depleted AU, we venture to speculate that they may selectively feed on LNA cells. If confirmed by further studies, our findings would suggest that the feeding behavior of groundwater crustaceans might play a role in structuring the groundwater microbial community and biomass, with potential consequences on subterranean carbon turnover and nutrient cycling.

Total cell counts also varied across the AUs with the sulfate-depleted AU showing the lowest values. However, we observed only a moderate linear correlation (r = 0.5, R^2 = 25%; Table S2) between TCC and groundwater crustacean abundance, indicating that TCC explains a limited portion of the variation in crustacean abundance. ~~However, we did not observe a strong linear correlation (r = 0.5, R^2 = 25%; Table S2) between TCC and groundwater crustacean abundance.~~ This result might seem counterintuitive since most of the collected species are known to feed on fine sediments and microorganisms. Nevertheless, there should be a logical explanation for this outcome. The planktonic microbial community represents a fraction of total aquifer microorganisms that is found in the interstitial water volume by detaching from sediments (Flemming and Wuertz, 2019). Since the crustacean species examined in this study are not filtrators, it is probable that they

consume a portion of microbial planktonic cells smaller than the portion of the sediment-attached ones. This feeding habit could be the underlying cause of the weak correlation observed in this study. This observation raises the possibility that a more robust correlation between diet and feeding habits and LNA cells might have emerged had we included sediment-attached cells in our analysis.

On this matter, we did not observe a significant correlation between DOC and groundwater crustacean assemblages. This suggests that DOC is likely not directly utilized by the species collected in this study but needs to be processed by bacteria before becoming accessible to metazoans (Foulquier et al., 2009; Griebler and Lueders, 2009; Segawa et al., 2015). Furthermore, DOC concentrations did not significantly differ across the three AUs, being always <1 mg/L, in line with the values normally observed in groundwater systems (Foulquier et al., 2010). DOC entering subterranean environments from the surface primarily comprises stable and recalcitrant components that resist bacterial degradation, leaving only a small fraction available for microbial communities (Shen et al., 2015). Our analyses indicated that the sulfate-depleted AU had the highest proportions of microbes metabolizing polymers, which are more complex and resistant to degradation than simple carbohydrates or carboxylic acids (Oest et al., 2018; Melita et al., 2019). Additionally, we observed variations in the proportions of microbes utilizing different substrates from year to year, likely influenced by surface-produced organic matter types (Saccò et al., 2019; Melita et al., 2023). Our findings prompt a discussion about the minimum quantity of energy resources needed to sustain a resident groundwater-dependent crustacean assemblage. To provide a rough estimate, considering that a prokaryotic cell contains approximately 25 fg of carbon (Griebler et al., 2002), the sulfate-depleted AU in our study had an average microbial biomass of 2.65-4 mg C/L. Since no groundwater-dwelling species were collected in this aquifer unit during our survey, further studies are necessary to assess if this biomass level could be a limiting factor for groundwater-dependent crustacean assemblages. Finally, we acknowledge two potential limitations of our study: the incomplete sampling of crustacean biodiversity and the timing of the sampling survey. Since we missed a small percentage of the expected biodiversity, our findings should be considered preliminary. Complete biodiversity sampling in groundwater is challenging due to the many impediments associated with both the essentially blind nature of groundwater sampling (Mammola et al., 2021). Furthermore, while eDNA and metagenomics approaches currently face challenges in certain contexts, particularly with markers such as 18S (Korbel et al., 2017b), classic DNA barcoding using COI markers has been shown to be an effective tool for delineating cryptic groundwater species (Altermatt et al., 2023). ~~and the current inefficiency of genetic markers able to unveil cryptic groundwater species (Korbel et al., 2017b).~~ In addition, while seasonality in groundwater recharge may influence geochemical processes (Jasechko et al., 2014), previous studies have shown that seasonality has relatively little impact on the distribution of biota in groundwater ecosystems. For instance, Korbel et al. (2015) demonstrated that habitat structure, water quality, and site attributes are the key environmental variables influencing groundwater metazoan distribution, with only minimal variance explained by seasonality. Extending the survey timing, sampling effort and implementing the use of classic DNA barcoding and eDNA approaches ~~eDNA~~ in future studies would be helpful in confirming our results and addressing these potential limitations.

5 Conclusions

Our multidisciplinary study delved into the taxonomic and functional composition of groundwater crustaceans and attempted to unravel the intricate dynamics of the crustacean communities in three aquifer units within the same aquifer. We assessed significant variations in crustacean distribution and functional traits across the aquifer units, with the sulfate-depleted ~~groundwater body~~aquifer unit standing out as a seemingly inhospitable environment for groundwater-obligate species. Our findings pointed to the crucial role of microbial communities in driving the composition of groundwater-obligate crustacean assemblages. Additionally, the study stimulates a discussion on the sensitivity of groundwater-obligate species to aquifer settings. Our research underscores the importance of singling out diverse hydrogeological contexts within individual aquifers. Potential avenues for future research encompass metagenomic studies on specific microbial taxa that are at the base of groundwater food webs, while stable isotope analyses would help elucidate the dietary preferences and food web dynamics of groundwater-obligate crustaceans and their impact on nutrient cycling.

Data availability

The raw data have been reported in Appendix A.

Supplement

The supplement containing Figure S1, Tables S1 and S2 related to this article is available online at:

Author contribution

TDL, SA, EP: Conceptualization; TDL, SA, MM, AZ, DP, SG, DR, ATDC, EP: Methodology; MM, AZ, DP, SG, DR, DMPG: Data curation; TDL, SA, EP, DMPG: Writing- Original draft preparation. SA, EP, DMPG: Validation; TDL, SA, EP, DMPG, ATDC: Writing- Reviewing and Editing.

Competing interests

The authors declare that they have no conflict of interest.

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References

Altermatt, F., Westram, A. M., Pasinelli, G. and Burri, R.: Advances and challenges in using DNA barcoding and metabarcoding to study biodiversity, Molecular Ecology, 32(4), 881–897, doi: 10.1111/mec.16955, 2023.

Arnosti, C., Bell, C., Moorhead, D. L., Sinsabaugh, R. L., Steen, A. D., Stromberger, M., Wallenstein, M., and Weintraub, M. N.: Extracellular enzymes in terrestrial, freshwater, and marine environments: perspectives on system variability and common research needs, Biogeochemistry, 117, 5–21, doi:10.1007/s10533-013-9906-5, 2014.

Ademollo, N., Amalfitano, S., Benedetti, B., Del Bon, A., Falconi, F., Fazi, S., Gallo, S., Ghergo, S., Mastroianni, D., Patrolecco, L., Petrangeli, A.B., Pettine, M., Preziosi, E., Rossi, D., and Zoppini, A.: Indagini per una caratterizzazione di dettaglio del sito di Quadro Alto - Riano (RM), Rapporto Tecnico Finale Convenzione tra Comune di Riano e IRSA-CNR, [ID: 213341], https://intranet.cnr.it/servizi/people/prodotto/scheda/i/213341, Accessed on November 28, 2023, 2012.

Amalfitano, S., Fazi, S., Ejarque, E., Freixa, A., Romaní, A. M., and Butturini, A.: Deconvolution model to resolve cytometric microbial community patterns in flowing waters, Cytometry A, 93, 194–200, doi:10.1002/cyto.a.23304, 2018.

Anderson, M. J., Gorley, R. N., and Clarke, K. R. (Eds.): PERMANOVA+for PRIMER: Guide to Software and Statistical Methods, PRIMER-E: Plymouth, UK, pp. 214, 2008.

Aquilina, L., Stumpp, C., Tonina, D., and Buffington, J. M.: Hydrodynamics and geomorphology of groundwater environments, in: Groundwater Ecology and Evolution (Second Edition), edited by: Malard, F., Griebler, C., and Rétaux, S., Academic Press, San Diego, pp. 3–37, doi:10.1016/B978-0-12-819119-4.00014-7, 2023.

Brad, T., Iepure, S., and Sarbu, S. M.: The Chemoautotrophically Based Movile Cave Groundwater Ecosystem, a Hotspot of Subterranean Biodiversity, Diversity (Basel), 13(3), 128, doi:10.3390/d13030128, 2021.

Bregović, P., Fišer, C., and Zagmajster, M.: Contribution of rare and common species to subterranean species richness patterns, Ecol. Evol., 9, 11606–11618, doi:10.1002/ece3.5604, 2019.

Cadotte M.W., Tucker C.M.: Should Environmental Filtering be Abandoned?, Trends Ecol. Evol., 32(6),429–437, doi: 10.1016/j.tree.2017.03.004, 2017.

Clarke, K. R. and Gorley, R. N.: PRIMER: Getting started with v6, PRIMER-E Ltd: Plymouth, UK 931, 932, 2005.

Cornwell, W. K., Schwilk, D. W., and Ackerly, D. D.: A trait-based test for habitat filtering: convex hull volume, Ecology, 87, 1465–1471, doi:10.1890/0012-9658(2006)87[1465:ATTFHF]2.0.CO;2, 2006.

Culver, D. C. and Pipan, T. (Eds.): The biology of caves and other subterranean habitats, online edn., Oxford Academic, Oxford, ISBN 9780191860485, 2019.

Culver, D.C., Deharveng, L., Pipan, T., Bedos, A.: An Overview of Subterranean Biodiversity Hotspots, Diversity (Basel), 13(10), 487, doi:10.3390/d13100487, 2021.

Di Lorenzo, T., Cifoni, M., Lombardo, P., Fiasca, B., and Galassi, D. M. P.: Ammonium threshold values for groundwater quality in the EU may not protect groundwater fauna: evidence from an alluvial aquifer in Italy, Hydrobiologia, 743, 139–150, doi:10.1007/s10750-014-2018-y, 2015.

Di Lorenzo, T., Di Marzio, W. D., Fiasca, B., Galassi, D. M. P., Korbel, K., Iepure, S., Pereira, J. L., Reboleira, A. S. P. S., Schmidt, S. I., and Hose, G. C.: Recommendations for ecotoxicity testing with stygobiotic species in the framework of groundwater environmental risk assessment, Sci. Total Environ., 681, 292–304, doi:10.1016/j.scitotenv.2019.05.030, 2019.

Di Lorenzo, T., Fiasca, B., Tabilio Di Camillo, A., Murolo, A., Di Cicco, M., and Galassi, D. M. P.: The weighted Groundwater Health Index (wGHI) by Korbel and Hose (2017) in European groundwater bodies in nitrate vulnerable zones, Ecol. Indic., 116, 106525, doi:10.1016/j.ecolind.2020.106525, 2020.

Di Lorenzo, T., Fiasca, B., Di Cicco, M., Cifoni, M., and Galassi, D. M. P.: Taxonomic and functional trait variation along a gradient of ammonium contamination in the hyporheic zone of a Mediterranean stream, Ecol. Indic., 132, 108268, doi:10.1016/j.ecolind.2021.108268, 2021.

Di Lorenzo, T., Avramov, M., Galassi, D. M. P., Iepure, S., Mammola, S., Reboleira, A. S. P. S., and Hervant, F.: Physiological tolerance and ecotoxicological constraints of groundwater fauna, in: Groundwater Ecology and Evolution (Second Edition), edited by: Malard, F., Griebler, C., and Rétaux, S., Academic Press, San Diego, pp. 457–479, doi:10.1016/B978-0-12-819119-4.15004-8, 2023.

Di Marzio, W. D., Cifoni, M., Sáenz, M. E., Galassi, D. M. P., and Di Lorenzo, T.: The ecotoxicity of binary mixtures of Imazamox and ionized ammonia on freshwater copepods: Implications for environmental risk assessment in groundwater bodies, Ecotoxicol. Environ. Saf., 149, 72–79, doi:10.1016/j.ecoenv.2017.11.031, 2018.

Duchi, V., Matassoni, L., Tassi, F., and Nisi, B.: Studio geochimico dei fluidi (acque e gas) circolanti nella regione vulcanica dei M.ti Vulsini (Italia Centrale), Ital. J. Geosci., 122, 47–61, 2003.

Dussart, B. and Defaye, D. (EDS.): World directory of Crustacea Copepoda of inland waters, Backhuys, 2002.

Ferguson, G., McIntosh, J. C., Warr, O., Sherwood Lollar, B., Ballentine, C. J., Famiglietti, J. S., Kim, J. -H., Michalski, J. R., Mustard, J. F., Tarnas, J., and McDonnell, J. J.: Crustal Groundwater Volumes Greater Than Previously Thought, Geophys. Res. Lett., 48, e2021GL093549, doi:10.1029/2021GL093549, 2021.

643 Ficetola, G. F., Canedoli, C., and Stoch, F.: The Racovitzan impediment and the hidden biodiversity of unexplored
644 environments, Conserv. Biol., 33, 214–216, doi:10.1111/cobi.13179, 2019.
645 Fillinger, L., Griebler, C., Hellal, J., Joulian, C., and Weaver, L.: Microbial diversity and processes in groundwater, in:
646 Groundwater Ecology and Evolution (Second Edition), edited by: Malard, F., Griebler, C., and Rétaux, S., Academic Press,
647 San Diego, pp. 211–240, doi:10.1016/B978-0-12-819119-4.00009-3, 2023.
648 Fišer, C., Brancelj, A., Yoshizawa, M., Mammola, S., and Fišer, Ž.: Dissolving morphological and behavioral traits of
649 groundwater animals into a functional phenotype, in: Groundwater Ecology and Evolution (Second Edition), edited by:
650 Fišer, C.: *Niphargus*—A model system for evolution and ecology. In Encyclopedia of caves (pp. 746-755). Academic Press,
651 2019.
652 Flemming, H. -C. and Wuertz, S.: Bacteria and archaea on Earth and their abundance in biofilms, Nat. Rev. Microbiol., 17,
653 247–260, doi:10.1038/s41579-019-0158-9, 2019.
654 Foulquier, A., Simon, L., Gilbert, F., Fourel, F., Malard, F., and Mermillod-Blondin, F.: Relative influences of DOC flux and
655 subterranean fauna on microbial abundance and activity in aquifer sediments: new insights from 13C-tracer experiments,
656 Freshw. Biol., 55, 1560–1576, doi:10.1111/j.1365-2427.2010.02385.x, 2010.
657 Galassi, D..M..P.: Groundwater copepods: diversity patterns over ecological and evolutionary scales, Hydrobiologia, 453,
658 227-253, doi:10.1023/A:1013100924948, 2001.
659 Galassi, D..M..P., De Laurentiis, P., and Dole-Olivier, M.: Phylogeny and biogeography of the genus *Pseudectinosoma*, and
660 description of *P. janineae* sp. n. (Crustacea, Copepoda, Ectinosomatidae), Zool. Scr., 28, 289–303, doi:10.1046/j.1463-
661 6409.1999.00018.x, 1999.
662 Galassi, D. M. P., De Laurentiis, P.: Little-known cyclopoids from groundwater in Italy: re-validation of *Acanthocyclops*
663 *agamus* and redescription of *Speocyclops italicus* (Crustacea, Copepoda, Cyclopoida). Vie et Milieu/Life & Environment,
664 203-222, 2004
665 Galassi, D. M. P., Fiasca, B., Di Lorenzo, T., Montanari, A., Porfirio, S., and Fattorini, S.: Groundwater biodiversity in a
666 chemoautotrophic cave ecosystem: how geochemistry regulates microcrustacean community structure, Aquat. Ecol., 51, 75–
667 90, doi:10.1007/s10452-016-9599-7, 2017.
668 Galassi, D. M. P., Huys, R., and Reid, J. W.: Diversity, ecology and evolution of groundwater copepods, Freshw. Biol., 54,
669 691–708, doi:10.1111/j.1365-2427.2009.02185.x, 2009.
670 Gasol, J. M., Zweifel, U. L., Peters, F., Fuhrman, J. A., and Hagström, Å: Significance of Size and Nucleic Acid Content
671 Heterogeneity as Measured by Flow Cytometry in Natural Planktonic Bacteria, Appl. Environ. Microbiol., 65, 4475–4483,
672 doi:10.1128/AEM.65.10.4475-4483.1999, 1999.
673 Griebler, C. and Lueders, T.: Microbial biodiversity in groundwater ecosystems, Freshw. Biol., 54, 649–677,
674 doi:10.1111/j.1365-2427.2008.02013.x, 2009.

675 Griebler, C., Mindl, B., Slezak, D., and Geiger-Kaiser, M.: Distribution patterns of attached and suspended bacteria in
676 pristine and contaminated shallow aquifers studied with an in situ sediment exposure microcosm, Aquat. Microb. Ecol., 28,
677 117–129, doi:10.3354/ame028117, 2002.
678 Hahn, H. J. and Fuchs, A.: Distribution patterns of groundwater communities across aquifer types in south-western
679 Germany, Freshw. Biol., 54, 848–860, doi:10.1111/j.1365-2427.2008.02132.x, 2009.
680 Hose, G.C., Stumpp, C.: Architects of the underworld: bioturbation by groundwater invertebrates influences aquifer
681 hydraulic properties. Aquat. Sci., 81, 20, doi: 10.1007/s00027-018-0613-0, 2019.
682 Hu, W., Zhang, H., Lin, X., Liu, R., Bartlam, M., Wang, Y.: Characteristics, Biodiversity, and Cultivation Strategy of Low
683 Nucleic Acid Content Bacteria, Front. Microbiol., 13, 900669, doi:10.3389/fmicb.2022.900669, 2022.
684 Hurvich, C. M. and Tsai, C.: A corrected Akaike information criterion for vector autoregressive model selection, J. Time
685 Ser. Anal., 14, 271–279, doi:10.1111/j.1467-9892.1993.tb00144.x, 1993.
686 Iannella, M., Fiasca, B., Di Lorenzo, T., Biondi, M., Di Cicco, M., and Galassi, D. M. P.: Spatial distribution of stygobitic
687 crustacean harpacticoids at the boundaries of groundwater habitat types in Europe, Sci. Rep., 10, 19043,
688 doi:10.1038/s41598-020-76018-0, 2020.
689 Jasechko, S., Birks, S. J., Gleeson, T., Wada, Y., Fawcett, P. J., Sharp, Z. D., McDonnell, J. J., Welker, J. M.: The
690 pronounced seasonality of global groundwater recharge. Water Resour. Res. 50, 8845e8867, doi:10.1002/2014wr015809,
691 2014.
692 Kimmel, K., Avolio, M. L., and Ferraro, P. J.: Empirical evidence of widespread exaggeration bias and selective reporting in
693 ecology, Nat. Ecol. Evol., 7, 1525–1536, doi:10.1038/s41559-023-02144-3, 2023.
694 Korbel, K. L. and Hose, G. C.: A tiered framework for assessing groundwater ecosystem health, Hydrobiologia, 661, 329–
695 349, doi:10.1007/s10750-010-0541-z, 2011.
696 Korbel, K. L., Hose, G. C.: Habitat, water quality, seasonality, or site? Identifying environmental correlates of the
697 distribution of groundwater biota. Freshw. Sci., 34(1), 329–343, https://doi.org/10.1086/680038, 2015.
698 Korbel, K. L., and Hose, G. C.: The weighted groundwater health index: Improving the monitoring and management of
699 groundwater resources, Ecol. Indic., 75, 164–181, doi:10.1016/j.ecolind.2016.11.039, 2017a.
700 Korbel, K. L., Chariton, A., Stephenson, S., Greenfield, P., Hose, G. C.: Wells provide a distorted view of life in the aquifer:
701 implications for sampling, monitoring and assessment of groundwater ecosystems, Sci. Rep., 7, 40702,
702 https://doi.org/10.1038/srep40702, 2017b.
703 Kraft, N. J. B., Adler, P. B., Godoy, O., James, E. C., Fuller, S., and Levine, J. M.: Community assembly, coexistence and
704 the environmental filtering metaphor, Funct. Ecol., 29, 592–599, doi:10.1111/1365-2435.12345, 2015.
705 Lebaron, P., Servais, P., Baudoux, A.-C., Bourrain, M., Courties, C., and Parthuisot, N.: Variations of bacterial-specific
706 activity with cell size and nucleic acid content assessed by flow cytometry, Aquat. Microb. Ecol., 28, 131–140,
707 doi:10.3354/ame028131, 2002.

Legendre, P. and Anderson, M. J.: Distance-based redundancy analysis: testing multispecies responses in multifactorial ecological experiments, Ecol. Monogr., 69, 512-512, doi:10.1890/0012-9615(1999)069[0001:DBRATM]2.0.CO;2, 1999.

Lombardi, G. and Meucci, C.: Il Tufo Giallo della Via Tiberina (Roma) utilizzato nei monumenti romani, Rend Lincei, 17, 263–287, doi:10.1007/BF02904766, 2006.

Magurran, A. E. and McGill, B. J. (Eds.): Biological diversity: Frontiers in measurement and assessment, Oxford University Press, Oxford New York, USA, pp. 345, ISBN 978-0-19-958067-5, 2011.

Magurran, A. E.: Measuring biological diversity, Curr. Biol., 31, R1174–R1177, doi:10.1016/j.cub.2021.07.049, 2021.

Malard, F., Dole-Olivier, M.-J., Mathieu, J., Stoch, F.: Sampling Manual for the Assessment of Regional Groundwater Biodiversity.

https://www.researchgate.net/publication/267567541_Sampling_Manual_for_the_Assessment_of_Regional_Groundwater_Biodiversity, Accessed on November 28, 2023, 2002.

Malard, F., Griebler, C., and Retaux, S. (Eds.): Groundwater Ecology and Evolution, Academic Press, San Diego, pp. 610, ISBN 9780128191194, 2023a.

Malard, F., Machado, E. G., Casane, D., Cooper, S., Fišer, C., and Eme, D.: Dispersal and geographic range size in groundwater, in: Groundwater Ecology and Evolution (Second Edition), edited by: Malard, F., Griebler, C., and Rétaux, S., Academic Press, San Diego, pp. 185–207, doi:10.1016/B978-0-12-819119-4.15003-6, 2023b.

Mammola, S., Lunghi, E., Bilandžija, H., Cardoso, P., Grimm, V., Schmidt, S. I., Hesselberg, T., and Martínez, A.: Collecting eco-evolutionary data in the dark: Impediments to subterranean research and how to overcome them, Ecol. Evol., 11, 5911–5926, doi:10.1002/ece3.7556, 2021.

Maurice, L., Robertson, A. R., White, D., Knight, L., Johns, T., Edwards, F., Arietti, M., Sorensen, J. P. R., Weitowitz, D., Marchant, B. P., Bloomfield, J. P.: The invertebrate ecology of the Chalk aquifer in England (UK). Hydrogeol. J., 24, 459–474, doi:10.1007/s10040-015-1334-2, 2016.

Melita, M., Amalfitano, S., Preziosi, E., Ghergo, S., Frollini, E., Parrone, D., and Zoppini, A.: Physiological Profiling and Functional Diversity of Groundwater Microbial Communities in a Municipal Solid Waste Landfill Area, Water (Basel), 11, 2624, doi:10.3390/w11122624, 2019.

Melita, M., Amalfitano, S., Preziosi, E., Ghergo, S., Frollini, E., Parrone, D., and Zoppini, A.: Redox conditions and a moderate anthropogenic impairment of groundwater quality reflected on the microbial functional traits in a volcanic aquifer, Aquat. Sci., 85, 3, doi:10.1007/s00027-022-00899-8, 2023.

Mermillod-Blondin, F., Hose, G. C., Simon, K. S., Korbel, K., Avramov, M., and Vorste, R. V.: Role of invertebrates in groundwater ecosystem processes and services, in: Groundwater Ecology and Evolution (Second Edition), edited by:

Oest, A., Alsaffar, A., Fenner, M., Azzopardi, D., and Tiquia-Arashiro, S. M.: Patterns of Change in Metabolic Capabilities of Sediment Microbial Communities in River and Lake Ecosystems, Int. J. Microbiol., 2018, 1–15, doi:10.1155/2018/6234931, 2018.

Parrone, D., Ghergo, S., and Preziosi, E.: A multi-method approach for the assessment of natural background levels in groundwater, Sci. Total Environ., 659, 884–894, doi:10.1016/j.scitotenv.2018.12.350, 2019.

Parrone, D., Ghergo, S., Frollini, E., Rossi, D., and Preziosi, E.: Arsenic-fluoride co-contamination in groundwater: Background and anomalies in a volcanic-sedimentary aquifer in central Italy, J. Geochem. Explor., 217, 106590, doi:10.1016/j.gexplo.2020.106590, 2020.

Parrone, D., Ghergo, S., Preziosi, E., and Casentini, B.: Water-Rock Interaction Processes: A Local Scale Study on Arsenic Sources and Release Mechanisms from a Volcanic Rock Matrix, Toxics, 10, 288, doi:10.3390/toxics10060288, 2022.

Preziosi, E., Petrangeli, A. B., and Giuliano, G.: Tailoring groundwater quality monitoring to vulnerability: a GIS procedure for network design, Environ. Monit. Assess., 185, 3759–3781, doi:10.1007/s10661-012-2826-3, 2013.

Preziosi, E., Parrone, D., Del Bon, A., and Ghergo, S.: Natural background level assessment in groundwaters: probability plot versus pre-selection method, J. Geochem. Explor., 143, 43–53, doi:10.1016/j.gexplo.2014.03.015, 2014.

Preziosi, E., Frollini, E., Zoppini, A., Ghergo, S., Melita, M., Parrone, D., Rossi, D., and Amalfitano, S.: Disentangling natural and anthropogenic impacts on groundwater by hydrogeochemical, isotopic and microbiological data: Hints from a municipal solid waste landfill, Waste. Manage., 84, 245–255, doi:10.1016/j.wasman.2018.12.005, 2019.

Proctor, C. R., Besmer, M. D., Langenegger, T., Beck, K., Walser, J. -C., Ackermann, M., Bürgmann, H., and Hammes, F.: Phylogenetic clustering of small low nucleic acid-content bacteria across diverse freshwater ecosystems, ISME J., 12, 1344–1359, doi:10.1038/s41396-018-0070-8, 2018.

R Development Core Team: R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing: Vienna, Austria, http://www.R-project.org/, 2008.

Rubbens, P., Schmidt, M. L., Props, R., Biddanda, B. A., Boon, N., Waegeman, W., and Denef, V. J.: Randomized Lasso Links Microbial Taxa with Aquatic Functional Groups Inferred from Flow Cytometry, MSystems, 4, 10-1128, doi:10.1128/mSystems.00093-19, 2019.

Saccò, M., Blyth, A. J., Humphreys, W. F., Kuhl, A., Mazumder, D., Smith, C., and Grice, K.: Elucidating stygofaunal trophic web interactions via isotopic ecology, PLoS One, 14, e0223982, doi:10.1371/journal.pone.0223982, 2019.

Schminke, H. K.: Adaptations of Bathynellacea (Crustacea, Syncarida) to life in the interstitial system of freshwater aquifers. Int. Rev. ges. Hydrobiol. Hydrogr., 59(4), 617–625, 1974.

Segawa, T., Sugiyama, A., Kinoshita, T., Sohrin, R., Nakano, T., Nagaosa, K., Greenidge, D., and Kato, K.: Microbes in Groundwater of a Volcanic Mountain, Mt. Fuji; 16S rDNA Phylogenetic Analysis as a Possible Indicator for the Transport Routes of Groundwater, Geomicrobiol. J., 32, 677–688, doi:10.1080/01490451.2014.991811, 2015.

Shen, Y., Chapelle, F. H., Strom, E. W., and Benner, R.: Origins and bioavailability of dissolved organic matter in groundwater, Biogeochemistry, 122, 61–78, doi:10.1007/s10533-014-0029-4, 2015.

Sottili, G., Palladino, D. M., Marra, F., Jicha, B., Karner, D. B., and Renne, P.: Geochronology of the most recent activity in the Sabatini Volcanic District, Roman Province, central Italy, J. Volcanol. Geotherm. Res., 196, 20–30, doi:10.1016/j.jvolgeores.2010.

Stoch, F. and Galassi, D. M. P.: Stygobiotic crustacean species richness: a question of numbers, a matter of scale, in: Fifty Years after the '"Homage to Santa Rosalia"': Old and New Paradigms on Biodiversity in Aquatic Ecosystems, Development in Hydrobiology, edited by: Naselli-Flores, L. and Rossetti, G., Springer Dordrecht, Netherlands, 217–234, doi:10.1007/978-90-481-9908-2_16, 2010.

Trontelj, P., Blejec, A., and Fišer, C.: Ecomorphological convergence of cave communities, Evolution (N Y), 66, 3852–3865, doi:10.1111/j.1558-5646.2012.01734.x, 2012.

Vaccarelli, I., Cerasoli, F., Mammola, S., Fiasca, B., Di Cicco, M., Di Lorenzo, T., Stoch, F., and Galassi, D. M. P.: Environmental factors shaping copepod distributions in cave waters of the Lessinian unsaturated karst (NE-Italy), Front. Ecol. Evol., 11, doi:10.3389/fevo.2023.1143874, 2023.

Wurzbacher, C., Kreiling, A. -K., Svantesson, S., Van den Wyngaert, S., Larsson, E., Heeger, F., Nilsson, H. R., and Pálsson, S.: Fungal communities in groundwater springs along the volcanic zone of Iceland, Inland Waters, 10, 418–427, doi:10.1080/20442041.2019.1689065, 2020.

Zagmajster, M., Ferreira, R. L., Humphreys, W. F., Niemiller, M. L., and Malard, F.: Patterns and determinants of richness and composition of the groundwater fauna, in: Groundwater Ecology and Evolution (Second Edition), edited by: Malard, F., Griebler, C., and Rétaux, S., Academic Press, San Diego, pp. 141–164, doi:10.1016/B978-0-12-819119-4.00006-8, 2023.

Appendix A

Table A1. Characteristics of the sampling sites within the three aquifer units (AUs).

ID	Lat	Long	Elevation (m a.s.l.)	Depth (m)	Use	AU
QA07	42.06812	12.50096	132	110	Domestic	sulfate-depleted
QA08	42.07234	12.51895	68	50	Domestic	earth-alkaline
QA09	42.07142	12.52757	49	29	Irrigation	earth-alkaline
QA13	42.09246	12.50991	187	115	Domestic	K-rich
QA15	42.08583	12.2710	146	115	Domestic	K-rich
QA21	42.08511	12.49676	176	105	Domestic	sulfate-depleted
QA24	42.05984	12.49368	107	80	Domestic	sulfate-depleted
QA25	42.08269	12.52501	126	120	Domestic	K-rich
QA29	42.09349	12.50305	145	64	Domestic	earth-alkaline
QA30	42.07626	12.50011	153	95	Domestic	sulfate-depleted

Table A2. Physical-chemical, microbial, and crustacean data (taxonomic and functional) of the groundwater samples in the three aquifer units (AUs) of Sabatini Mounts aquifer.

a) Field data (WT: water table depth in m a.s.l.; ORP: oxidation-reduction potential in mV; T: temperature in °C; DO: dissolved oxygen in mg/L; EC: electrical conductivity in µS/cm).

ID	YEAR	AU	WT	ORP	T	pH	DO	DO%	EC
QA07	2014	S-D	65.0	211	18.0	7.6	7.1	75	490
QA08	2014	E-A	41.3	206	16.9	6.8	3.8	39	1048
QA09	2014	E-A	40.6	105	16.7	7.2	6.6	69	749
QA13	2014	K	112.5	194	16.2	7.5	8.5	88	675
QA15	2014	K	68.8	208	17.0	7.7	7.5	79	641
QA21	2014	S-D	104.1	137	18.6	7.7	5.0	54	479
QA24	2014	S-D	67.4	248	17.3	7.5	7.0	73	545
QA25	2014	K	64.4	244	17.1	7.8	5.0	52	640
QA29	2014	E-A	110.8	201	15.4	7.3	8.0	82	707
QA30	2014	S-D	82.2	221	17.4	7.5	8.1	85	525
QA07	2015	S-D	66.5	170	18.6	7.4	11.3	121	497
QA08	2015	E-A	41.2	229	17.5	6.8	3.9	41	1052
QA09	2015	E-A	40.3	58	17.0	7.4	6.9	72	746
QA13	2015	K	112.9	161	16.7	7.6	8.4	88	679
QA15	2015	K	68.6	260	17.0	7.4	6.9	73	645
QA21	2015	S-D	104.8	130	19.5	7.5	3.2	35	479
QA24	2015	S-D	67.7	170	17.4	7.3	8.1	85	551
QA25	2015	K	63.8	200	19.1	7.7	6.5	71	665
QA29	2015	E-A	111.3	141	16.7	7.3	6.1	64	711
QA30	2015	S-D	82.9	180	17.8	7.5	8.5	91	530

b) Major components (in mg).

ID	YEAR	AU	F	Cl- ~~Cl~~	NO_3 ~~NO3~~	SO_4^{2-} ~~SO4~~	HCO_3^- ~~HCO3~~	Na^+ ~~Na~~	Mg^{2+} ~~Mg~~	K^+ ~~K~~	Ca^{2+} ~~Ca~~	Si	DOC
QA07	2014	S-D	1.6	20.9	7.1	4.8	237.9	30.1	8.1	41.5	39.4	25.3	1.5
QA08	2014	E-A	1.1	41.6	12.7	131.5	440.4	44.1	19.1	37.8	132.0	26.8	1.3
QA09	2014	E-A	1.0	17.6	44.6	28.3	358.7	25.1	5.2	29.6	32.7	26.7	0.8
QA13	2014	K	1.3	36.9	16.9	11.7	312.3	38.8	13.5	53.6	52.5	26.1	1.1
QA15	2014	K	2.1	31.0	17.5	18.6	269.6	49.5	10.7	53.3	39.5	23.2	0.8
QA21	2014	S-D	1.6	22.7	7.3	7.8	214.7	24.5	6.7	36.6	43.3	25.5	1.2
QA24	2014	S-D	0.9	32.9	20.5	7.6	241.6	27.2	10.4	32.3	51.4	27.8	0.8
QA25	2014	K	3.2	34.6	18.0	35.3	256.2	70.1	8.6	43.6	31.4	18.1	0.7
QA29	2014	E-A	0.6	29.6	25.1	21.9	331.8	28.3	11.9	31.8	81.7	26.8	0.9
QA30	2014	S-D	1.4	36.5	7.1	4.8	225.7	27.2	9.8	37.7	43.3	26.2	0.7
QA07	2015	S-D	1.6	20.4	7.0	4.6	244.0	15.6	7.1	38.2	33.7	18.9	0.5
QA08	2015	E-A	1.1	40.1	13.6	127.7	442.9	40.6	17.1	32.7	113.0	19.8	0.8
QA09	2015	E-A	1.0	17.0	33.3	27.2	363.6	27.2	10.2	31.9	77.8	16.1	0.9
QA13	2015	K	1.1	35.1	16.6	11.4	306.2	35.5	12.3	46.7	46.1	19.3	0.6
QA15	2015	K	2.1	30.7	16.7	18.5	278.2	32.9	10.1	48.4	36.1	18.0	0.8
QA21	2015	S-D	1.7	22.6	6.9	8.3	223.3	22.7	5.9	32.6	38.5	19.7	0.7
QA24	2015	S-D	0.9	29.4	18.2	7.2	242.8	24.7	8.9	27.7	43.5	19.9	0.7
QA25	2015	K	3.1	31.6	17.3	39.0	257.4	63.5	7.6	38.8	28.5	13.5	0.8
QA29	2015	E-A	0.6	29.5	25.8	20.4	328.2	26.5	10.4	27.7	68.8	20.2	0.5
QA30	2015	S-D	1.4	39.5	7.9	4.7	218.4	24.9	8.7	33.2	37.5	18.3	0.5

c) Trace elements (in µg).

ID	YEAR	AU	Li	B	Al	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Rb	Sr	Ba	Pb	U
QA07	2014	S-D	20.1	76.1	11	40.2	1.8	2.7	34.8	0.4	0.5	17.8	20.1	0.52	28	560	74.1	0.1	2.9
QA08	2014	E-A	18.1	101	7.8	35.1	0.7	0.5	21.1	1.9	1.5	14.1	29.6	1.5	40	1396	104	0.2	30.7
QA09	2014	E-A	11.1	65.8	3.2	38.6	1.1	0.4	9.2	0.3	0.2	8.4	32.6	0.2	31	127	9.4	0.2	2.5
QA13	2014	K	23.6	111	13	38.1	1.1	0.9	23.2	0.5	0.8	24.7	13.2	0.2	29	849	102	0.2	6.1
QA15	2014	K	45.9	116	14	23.1	1.4	0.3	15.1	0.3	0.6	21.8	26.7	0.4	61	416	25.4	0.2	13.6
QA21	2014	S-D	22.3	65.4	2.9	29.5	1.3	0.9	21.2	0.9	0.4	13.1	31.7	0.4	19	489	68.5	0.2	4.8
QA24	2014	S-D	14.1	67.5	3.4	31.1	1.2	0.3	8.9	0.3	0.4	84.2	13.3	0.5	24	599	88	0.2	3.2

QA25	2014	K	70.5	144	12	24.5	0.6	1.4	26.4	59.4	0.7	22.4	43.1	0.2	64	331	35.7	0.3	17.8
QA29	2014	E-A	16.2	115	11	25.5	1.5	0.2	10.8	2.1	0.6	28.1	13.3	0.6	26	831	79.1	0.2	34.3
QA30	2014	S-D	19.7	71	5.1	30.1	1.2	0.2	11.5	0.9	0.3	32.2	20.3	0.2	19	593	72.1	0.2	2.5
QA07	2015	S-D	19.1	62.5	9.9	35.5	1.3	1.8	18.8	0.1	0.2	5.5	17.5	0.1	27	493	62.8	0.1	2.7
QA08	2015	E-A	17.7	91	9.7	31.3	0.6	0.4	15.2	0.2	1.3	2.2	31.9	1.5	37	1233	90.9	0.1	29.6
QA09	2015	E-A	14.5	66.2	4.1	18.3	0.6	9.6	110	0.1	0.2	3.6	15.7	0.7	22	578	129	0.1	15.1
QA13	2015	K	22.1	97	31	34.4	1.5	0.6	24.6	0.1	0.3	7.4	13.1	0.1	26	767	96	0.2	5.5
QA15	2015	K	44.9	111	9.6	22.1	1.1	0.2	11.2	0.1	0.6	6.4	27.9	0.3	57	401	25.1	0.1	11.8
QA21	2015	S-D	22.1	60.3	31	26.6	1.6	0.7	14.8	0.1	0.3	3.3	32.9	0.2	18	454	65.7	0.2	4.7
QA24	2015	S-D	13.6	57.3	16	27.8	1.1	0.5	61	0.1	0.3	104	12.1	0.1	21	527	82.5	0.6	2.8
QA25	2015	K	69.4	123	7.1	20.6	0.4	0.1	5.9	0.1	0.2	5.3	36.2	0.1	59	360	45.5	0.1	17.6
QA29	2015	E-A	15.5	98	31	22	1.8	0.3	11.2	0.1	0.4	8.9	12.3	0.1	23	717	76.5	0.2	27.9
QA30	2015	S-D	18.2	58.8	5.9	27.1	0.9	0.1	8.5	0.1	0.1	38.4	16.8	0.1	17	520	64.7	0.1	2.1

d) Microbial community characteristics. Total cell count (TCC in cell/mL), Low Nucleic Acidic and High Nucleic Acids cells are expressed as percentages of total absorbance (CARB: carbohydrates, POL: polymers; CARB_A: carboxylic acids, AM: amino acids, AMIN: amines).

ID	YEAR	AU	TCC	LNA	HNA	HNA/LNA	CARB	POL	CARB_A	AM	AMIN
QA07	2014	S-D	9896	66	34	0.5	40	25	20	11	4
QA08	2014	E-A	13950	80	20	0.2	31	14	27	23	6
QA09	2014	E-A	53423	81	19	0.2	37	7	26	25	6
QA13	2014	K	17705	65	35	0.5	21	41	20	18	0
QA15	2014	K	9315	70	30	0.4	31	27	28	14	0
QA21	2014	S-D	9283	68	32	0.5	40	25	20	11	4
QA24	2014	S-D	5409	63	37	0.6	40	25	20	11	4
QA25	2014	K	94954	80	20	0.2	31	27	28	14	0
QA29	2014	E-A	15265	67	33	0.5	46	13	25	17	0
QA30	2014	S-D	6291	61	39	0.7	52	18	19	12	0
QA07	2015	S-D	20307	67	33	0.5	0	95	0	0	5
QA08	2015	E-A	17182	82	18	0.2	36	13	25	18	8
QA09	2015	E-A	37164	77	23	0.3	40	11	23	22	4
QA13	2015	K	13148	66	34	0.5	10	25	55	6	3
QA15	2015	K	12447	75	25	0.3	0	34	58	0	8
QA21	2015	S-D	13822	72	28	0.4	0	69	29	0	2
QA24	2015	S-D	11843	62	38	0.6	0	51	47	0	2
QA25	2015	K	79904	75	25	0.3	0	30	70	0	0
QA29	2015	E-A	61110	67	33	0.5	44	22	16	10	8
QA30	2015	S-D	8175	67	33	0.5	0	60	40	0	0

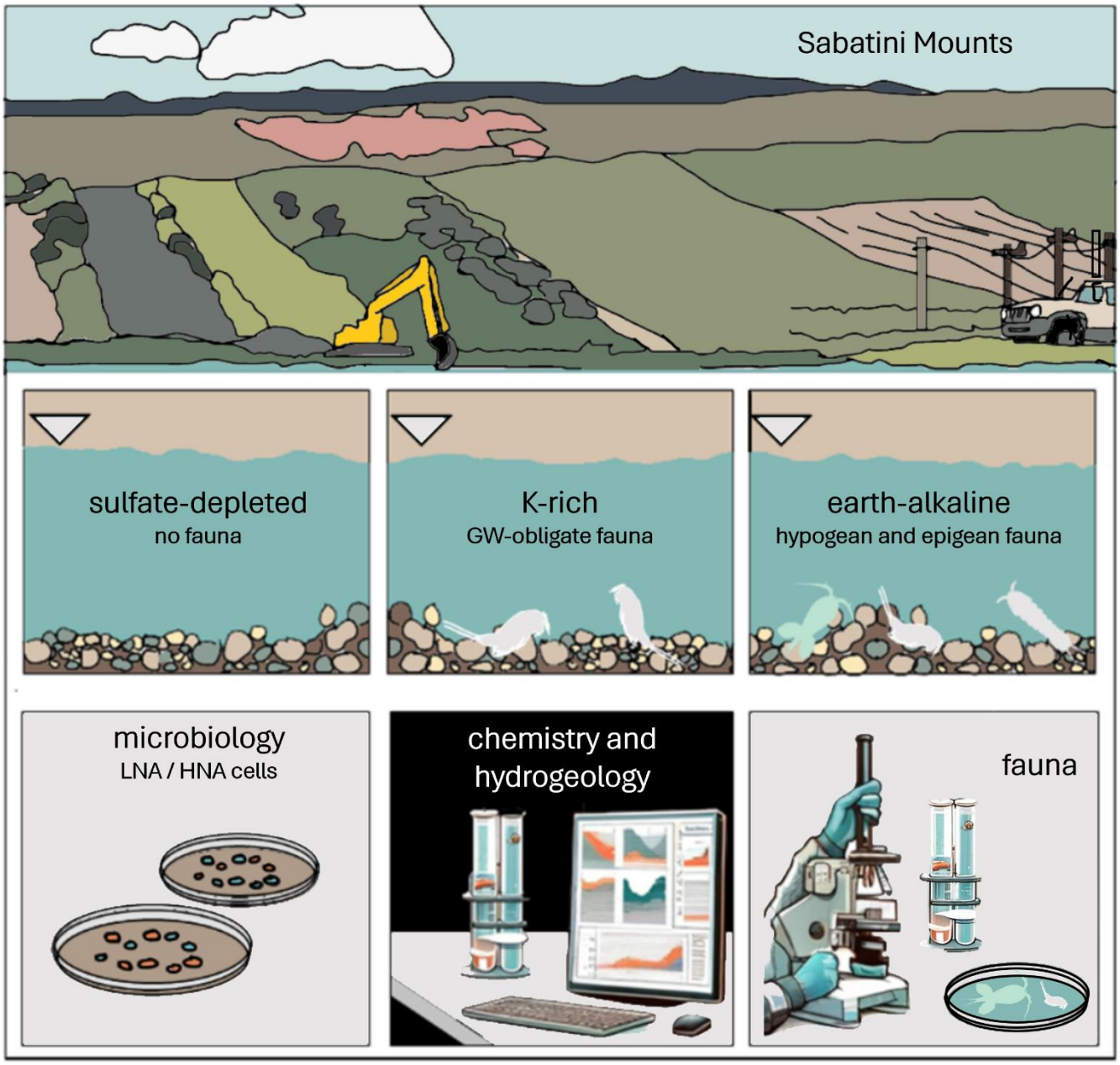
e) Taxonomic composition. Pit: *Parapseudoleptomesochra italica*; Psp: *Parastenocaris* sp. Nps: *Nitocrella psammophila*; Pre: *Pseudectinosoma reductum*; Egr*: Elaphoidella gracilis*; Aag: *Acanthocyclops agamus*; Mpo: *Moraria poppei*; Msp: *Meridiobathynella* sp.; Nsp: *Niphargus* sp.; SB: cumulative abundances of groundwater-obligate crustacean species. In each sample (ID), abundances are reported as number of individuals per 1000 L of groundwater.

ID	YEAR	AU	Pit	Psp	Nps	Pre	Egr	Aag	Mpo	Msp	Nsp	SB
QA07	2014	S-D	0	0	0	0	0	0	0	0	0	0
QA08	2014	E-A	12	2	0	0	0	0	0	3	0	17
QA09	2014	E-A	18	0	0	0	0	0	0	0	0	18
QA13	2014	K	1	0	0	0	0	0	0	0	0	1
QA15	2014	K	0	0	0	0	0	0	0	0	1	1
QA21	2014	S-D	0	0	0	0	0	0	1	0	0	0
QA24	2014	S-D	0	0	0	0	0	0	0	0	0	0
QA25	2014	K	62	0	0	0	0	0	0	0	0	62
QA29	2014	E-A	0	0	0	0	9	0	0	0	0	0
QA30	2014	S-D	0	0	0	0	0	0	0	0	0	0
QA07	2015	S-D	0	0	0	0	0	0	0	0	0	0
QA08	2015	E-A	13	0	0	0	0	2	0	27	0	42
QA09	2015	E-A	0	0	0	0	0	0	0	0	0	0
QA13	2015	K	1	0	0	0	0	0	0	0	0	1
QA15	2015	K	0	0	5	1	0	12	0	0	0	18
QA21	2015	S-D	0	0	0	0	0	0	0	0	0	0
QA24	2015	S-D	0	0	0	0	0	0	0	0	0	0
QA25	2015	K	36	0	0	0	0	0	0	0	0	36
QA29	2015	E-A	0	0	0	0	4	0	0	0	0	0
QA30	2015	S-D	0	0	0	0	0	0	0	0	0	0

f) Functional composition (in percentage): BUR: burrowers, INT: interstitial; SWI: swimmers; FS-M: fine sediments + microorganisms; LM: living microphytes; D-F: deposit-feeders; COL: collectors; GRA_ grazers; EUR: eurythermal; MST: moderately stenothermal; STE: stenothermal.

			LOCOMOTION			DIET		FEEDING HABITS			THERMAL TOLERANCE		
ID	YEAR	GWB	BUR	INT	SWI	FS-M	LM	D-F	COL	GRA	EUR	MST	STE
QA07	2014	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA08	2014	E-A	0.9	0.1	0.0	1.0	0.0	1.0	0.0	0.0	0.9	0.0	0.1
QA09	2014	E-A	1.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
QA13	2014	K	1.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
QA15	2014	K	1.0	0.0	0.0	1.0	0.0	0.5	0.5	0.0	0.5	0.0	0.5
QA21	2014	S-D	0.0	1.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
QA24	2014	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA25	2014	K	1.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
QA29	2014	E-A	1.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	1.0	0.0	0.0
QA30	2014	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA07	2015	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA08	2015	E-A	0.9	0.0	0.1	1.0	0.0	1.0	0.0	0.0	0.9	0.1	0.0
QA09	2015	E-A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA13	2015	K	1.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
QA15	2015	K	0.3	0.0	0.7	1.0	0.0	0.9	0.1	0.0	0.3	0.6	0.1
QA21	2015	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA24	2015	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
QA25	2015	K	1.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	0.0	0.0
QA29	2015	E-A	1.0	0.0	0.0	0.0	1.0	0.0	0.0	1.0	1.0	0.0	0.0
QA30	2015	S-D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

877 **Graphical abstract**



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