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RESEARCH ARTICLE OPEN ACCESS

Use of Microbial Biofilms to Assess Groundwater Quality in Karstic Ecosystems

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ABSTRACT

Karstic aquifers are particularly vulnerable to contaminations but, unlike surface waters, biological indicators of their groundwater quality are lacking. We propose a methodology based on microbial biofilms developed on artificial substrates (clay beads) to evaluate groundwater quality. Clay beads were incubated every 2 months over 18 months in three karstic stations characterised by contrasting nutrient and organic matter inputs from their catchments. After every 2-month incubation, microbial biofilms on clay beads were analysed for biomass, hydrolytic and dehydrogenase activities, and prokaryotic community structure. NH_4^+ , NO_3^- , PO_4^{3-} and dissolved organic carbon (DOC) concentrations were also measured in groundwater during the experiment. Biofilm biomass and activities were not positively correlated with nutrient and DOC concentrations in groundwater probably due to biofilm growth inhibition by antibiotics in the station having the highest nutrient and DOC concentrations. In contrast, the diversity of prokaryotes in biofilms was positively correlated with nutrient and DOC availability. In the studied heterotrophic karstic stations, the quantity of resources originating from the catchments determined the diversity but also the prokaryotic community structures of biofilms. We selected several microbial taxa as potential indicators of groundwater quality. The next step will be to test their applicability in other karstic ecosystems.

1 | Introduction

Karst aquifers are critically important for drinking water supply in many areas throughout the world (Ford and Williams 1989; Chen et al. 2017; Stevanović 2019). These aquifers are characterised by complex geological and hydrological structures with networks of cavities and fractures which facilitate water and solute transports but limit self-purification processes (Goldscheider 2005; Jiang and Yan 2010; Personné et al. 1998). Consequently, the groundwater quality of karsts is particularly vulnerable to pollutions from human activities like intensive agriculture, urbanisation, and industrial practices (Griebler and Avramov 2015). Anthropogenic pressures

can degrade these ecosystems through inputs of organic matter, nutrients and various organic, inorganic and emerging contaminants (pesticides, hydrocarbons, endocrine disruptors, nanoparticles, micro-plastics...) which impact groundwater quality (e.g., Lukač Reberski et al. 2022). Currently, the monitoring and management of groundwater resources are based essentially on chemical and hydrogeological parameters, as well as the monitoring of a limited set of bacteria, indicators of faecal contamination, detected from grab samples of groundwater (Gao et al. 2020; Gao et al. 2023). However, these chemical and microbiological approaches only provide snapshots of contaminations at a given time and, therefore, may not be truly representative of the environmental

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conditions occurring over time (Pronk et al. 2009; Wendland et al. 2005). In this context, use of indicators based on biological communities are recognised as promising tools to monitor the quality of groundwater ecosystems (Korbel and Hose 2017; Saccò et al. 2022; Steube et al. 2009; Voisin et al. 2020; Hellal et al. 2021).

Groundwater ecosystems are relatively stable habitats characterised by complete darkness, nutrient scarcity (oligotrophy), and limited physicochemical fluctuations (e.g., temperature) (Goldscheider et al. 2006; Griebler and Lueders 2009). Thus, they harbour biological communities well adapted to their environment which, in turn, would be highly vulnerable to environmental changes (Grösbacher et al. 2016; Morse et al. 2021; Shabarova et al. 2014). In this context, the hydrological connectivity between surface and groundwater ecosystems determines the fluxes of organic matter and contaminants in groundwater and consequently has a key influence on the abundances, activities and compositions of local groundwater biological communities (Datry et al. 2005; Foulquier et al. 2011; Taylor et al. 2013; Voisin et al. 2020). This assumption, predominantly reported from alluvial aquifers, paves the way for the use of biological communities as indicators of the organic contamination of karstic aquifers. Amongst the biological communities living in groundwater ecosystems, microorganisms are ubiquitous and constitute the main actors in the functioning of these ecosystems, making them relevant candidates for groundwater quality monitoring (e.g., Griebler and Avramov 2015). The diversity and activity of microorganisms in aquifers have been already used to detect disturbances and to evaluate the ecological status of these groundwater ecosystems (Fillinger et al. 2019; Korbel et al. 2022; Mermillod-Blondin et al. 2013; Smith et al. 2012; Steube et al. 2009). Nevertheless, most studies using microbial communities as indicators of environmental conditions in groundwater were mainly conducted in alluvial aquifers (Fillinger et al. 2019; Griebler and Lueders 2009). In comparison, karstic aquifers remain poorly studied (Pronk et al. 2009; Savio et al. 2019) although these fractured aquifers are more vulnerable to contaminations than porous aquifers due to their high connectivity with surface ecosystems (Kačaroğlu 1999; Malard et al. 2023).

Moreover, microbial studies performed in karstic ecosystems have essentially focused on planktonic bacteria (e.g., Farnleitner et al. 2005; Guan et al. 2023; Shabarova et al. 2014), whilst the majority of microorganisms in aquifers live attached to rock surfaces and sediment particles and constitute biofilms that play fundamental roles in biogeochemical processes (Alfreider et al. 1997; Flemming and Wuerzt 2019; Griebler and Lueders 2009; Harvey et al. 1984). Thus, the sampling of groundwater and associated free-living bacteria would not be pertinent to obtain reliable assessments of groundwater ecosystem functioning. The analysis of biofilms developed on groundwater sediments would be a better and more integrative way to evaluate microbial communities in karstic environments. Nevertheless, the large variability in the available mass, size, and mineralogy amongst sites made them poorly suitable for comparison of microbial communities amongst different karstic environments (Cuadros 2017; Zada et al. 2021). To circumvent these methodological issues, a growing number of studies used artificial

substrates such as glass beads, gravel particles or clay beads to assess microbial biomass, activities and diversity in groundwater ecosystems (Iribar et al. 2015; Lebon et al. 2023; Mermillod-Blondin et al. 2013; Williamson et al. 2012; Yu et al. 2014). Based on this approach, it has been demonstrated that biofilms developed on artificial substrates responded significantly to enrichments in dissolved organic carbon (DOC) of groundwater ecosystems impacted by artificial recharge with stormwater runoff in urban areas (e.g., Mermillod-Blondin et al. 2013). More precisely, Voisin et al. (2020) and Lebon et al. (2023) highlighted that the biomass, activity and bacterial richness of biofilms developed on clay beads were significantly stimulated by DOC enrichments associated with aquifer recharge practices. The bacterial community structure of biofilms was also impacted by these practices. For example, it has been observed that the relative proportion of 16S rRNA gene reads of bacteria belonging to the phylum Bacteroidetes in bacterial communities increased with groundwater recharge (Voisin et al. 2020; Lebon et al. 2021).

Based on these promising results obtained in sedimentary aquifers, the present study aims to test the pertinence of microbial biofilms developed on artificial substrates to detect the contamination of groundwater with organic matter and nutrients in karstic aquifers. Three karstic stations characterised by contrasting inputs of organic matter and nutrients from the surface were selected to evaluate the influence of these inputs on the biomass, activity, and prokaryotic community structure of microbial biofilms developed on artificial substrates. We developed an innovative approach, combining the study of biofilms and a long-term monitoring period including several seasons to assess whether biofilm characteristics measured in each station could be significantly influenced by meteorological/hydrological variations. To cover a full hydrological year, we established an 18-month experimental monitoring setup using clay beads as artificial substrates (Mermillod-Blondin et al. 2019; Voisin et al. 2016). These substrates were incubated in three stations draining catchments characterised by distinct land covers (cattle farming, alpine meadows, forest areas, residential areas, roads), with regular sampling (every 2 months) to analyse seasonal variations in biofilm variables (biomass and activities) and prokaryotic community structure (16S metabarcoding).

Based on literature from groundwater environments (e.g., Voisin et al. 2020), we hypothesised that the station with the highest nutrient and dissolved organic carbon concentrations would support the highest microbial biomass, activities and prokaryotic diversity (H1). Nevertheless, this expected result could be blurred by the presence of antibiotics under cattle farming areas (e.g., Huang et al. 2019). Second, prokaryotic community structure would diverge amongst stations due to the selection of taxa the most adapted to their specific chemical conditions (H2). In association with this hypothesis, each study station would reveal indicator taxa that reflect the availability of nutrients and organic matter sources (e.g., Fillinger et al. 2021; Shabarova 2013; Xiao et al. 2023). For example, taxa such as Gammaproteobacteria would proliferate in ecosystems affected by additional inputs of nutrients, organic matter or pollutants, particularly following flood events (Fillinger et al. 2021; Shabarova 2013).

2 | Materials and Methods

2.1 | Study Sites

The study was conducted in three karstic stations (A, B and C) located in the western part of the Bauges massif in Savoie (French Alps, Figure 1). The Bauges massif is a limestone formation. This 217 km² karstic massif includes around 150 springs fed by groundwater flowing through networks. Groundwater constitutes an essential drinking water resource for the population and the activities in the massif with more than 50,000 inhabitants and 7000 heads of livestock (Hobléa et al. 2008), with population peaks during touristic seasons. In this mountainous context, the karstic groundwater recharge and the groundwater contamination risks vary throughout the year depending on meteorological dynamics and anthropogenic activities such as Nordic activities in winter and agricultural practises (pastoralism) in summer. The three stations (A, B and C) were located in two karstic networks: Doria (45.6098346 N; 5.9813690 E) and Tettaz (45.6721736 N; 5.9734805 E). They were selected because they collect water from contrasting catchments in terms of land use and they are easily accessible. Moreover, the Doria network has been already studied in numerous research projects (Hobléa et al. 2008; Perrette et al. 2013; Wilhelm 2007).

The stations A and B are located in the Doria network, which drains water from the Feclaz plateau (14 km², Wilhelm 2007).

Station A is a cave stream acting as the main collector of water drained from the plateau. It collects water from areas characterised by several land uses (% of the area from cadastral data: 65%

forestry, 25% grasslands, 3% urban areas including ski resort). This station drains the majority of the watershed and is exposed to various organic carbon sources, such as soil organic matter, slurry, and debris. These organic inputs mainly originate from debris accumulated at the bottom of sinkholes and sporadic events facilitating the transfer of soil and vegetal debris in the karstic system (Wilhelm 2007).

Station B is a gallery draining water from a small catchment located in a forested area. Although stations A and B are located in the same karstic network, they are not hydrologically connected. Station B collects water from a less diverse catchment area in terms of land uses than station A. Consequently, groundwater collected in station B is expected to be less rich in organic matter sources than groundwater in station A.

Station C is located in the Tettaz cave on the Revard plateau. It collects water from a small surface runoff beneath a dairy and heifer farming operation. The study of Quiers et al. (2015) performed on fluorescence and geochemical signatures of speleothems in this karstic system indicated that this station was significantly impacted by organic matter supplies associated with agricultural activities (cattle breeding). Due to these agricultural activities in the catchment, the water collected in station C can be also contaminated with antibiotics used to treat cattle (like tetracycline, neomycin, bacitracin, prednisolone, penicillin, streptomycin, personal communication from the farmer).

In summary, the three selected stations were characterised by distinct inputs of organic matter and nutrients. Station A is exposed to multiple organic sources depending on hydrology,

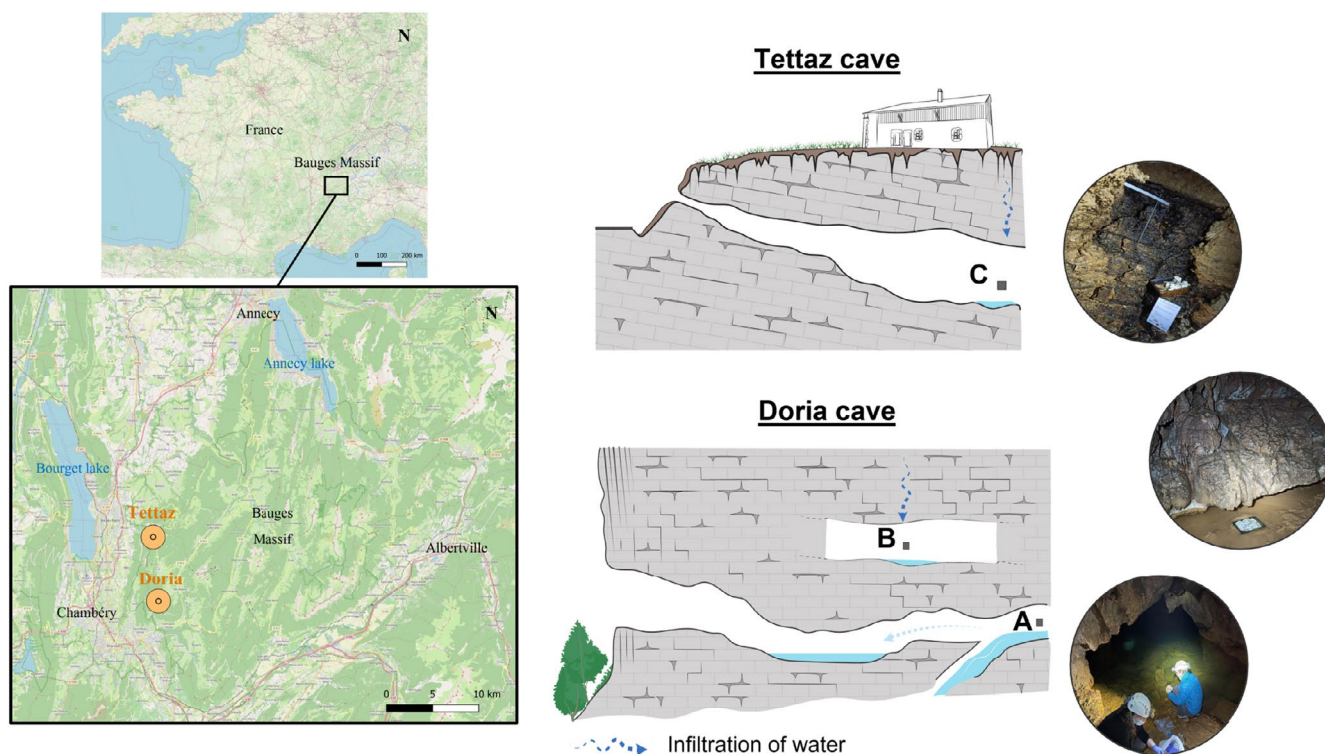


FIGURE 1 | Map showing the locations of the studied sites and schematic representations of the Tettaz and Doria caves. Left: Map of the Bauges massif (France) showing the location of the caves studied (Tettaz and Doria). Right: Schematic profiles and pictures of the Tettaz and Doria caves with the locations of the stations A, B and C. (Picture credits: Lina Fabre).

station B remains largely unaffected by organic inputs, and station C is directly influenced by agricultural activities.

2.2 | Sampling Design

The experiment was conducted from September 2022 to January 2023 in the three stations (Table 1). To evaluate the temporal variability in each station, microbial communities were sampled by incubation of artificial substrates (clay beads) according to previous experiments performed in sedimentary aquifers (Voisin et al. 2020; Lebon et al. 2021). Artificial substrates rather than natural sediments were used because sediments collected in the three stations exhibited large differences in terms of grain size distribution, specific area of colonization for bacteria, and content of organic carbon and nitrogen (Supporting Information S1). As these sediment characteristics can have key roles on biofilm development (Cuadros 2017; Zada et al. 2021), comparisons of sedimentary biofilms to evaluate differences in groundwater quality amongst stations appeared less pertinent than using standardised artificial substrates in the three stations. In addition, sediments could be considered as natural archives (e.g., Pearman et al. 2022) potentially harbouring ancient microbial communities that would not inform about the actual groundwater quality in each station. Thus, clay beads as artificial substrates were incubated during periods of two months in the three selected stations. A total of 8 incubation periods were studied during the course of the experiment. For each incubation period, three sets of 30 clay beads were incubated per station. After incubation, we assessed microbial biomass (using protein content as proxy of microbial biomass), two microbial activities (esterase and dehydrogenase activities), and prokaryotic community structures (using a 16S metabarcoding approach) from biofilm developed on clay beads. In addition, water samples were collected to quantify nutrients (ammonium, nitrate and phosphate) and dissolved organic carbon (DOC) concentrations. To evaluate the potential influence of hydrological conditions during incubation periods on the temporal dynamics of nutrients and microbial communities, we used meteorological data as a proxy of hydrological changes in the stations. The best way to monitor hydrological changes would have been to

monitor discharge rates in each station but the complex geometry of the three karstic stations did not permit this implementation. Nevertheless, discharge rates measured in the stream at the outlet of the Doria karstic system allowed validating the significant link between precipitations and discharge rate in the studied karstic system (Supporting Information S2).

2.3 | Preparation and Incubation of Artificial Substrates

According to previous studies (Mermillod-Blondin et al. 2019; Voisin et al. 2016), we used sterilised clay beads with a diameter of 8 mm as artificial substrates for biofilms in groundwater. Prior to deployment on-site, the clay beads were calibrated and burned at 550°C to eliminate traces of organic matter. They were then placed in PA12 nylon autoclave bags (9.5 cm x 3.5 cm x 2 cm with a 3.3 mm mesh size) that had been pre-cleaned with 5% bleach. Thirty beads were placed in each bag to measure protein content (5 beads), esterase (5 beads) and dehydrogenase (5 beads) activities of biofilms developed on clay beads, and to assess prokaryotic community structure (15 beads) through a metabarcoding approach. Once prepared, the bags were autoclaved for 40 min at 120°C before being incubated in groundwater at the three stations for incubation periods of 2 months. In order to limit the variability in hydrodynamics amongst stations on biofilms, bags of clay beads were incubated in stainless steel boxes (previously sterilised by heating at 550°C) to cut the water current. This procedure allowed to reduce the variability in hydrological condition amongst stations, by limiting the potential influence of shear stress on biofilms (e.g., Wang et al. 2022).

After incubation, artificial substrates were collected on-site in 50 mL Falcon tubes filled with water from the sampled station. Upon retrieval from the stations, beads intended for microbiological analyses (protein quantification and microbial activities) were stored at 4°C to prevent biofilm development before analysis. Microbial activities were performed within 24 h after clay bead collection using 10 beads per set of 30 beads (3 sets per station and period). The remaining 5 beads dedicated to protein quantification were frozen at -20°C until analysis. For

TABLE 1 | Meteorological variables calculated for the 8 incubation periods (P1 to P8).

Code	Incubation period	Cumulative precipitation (mm)	Maximum precipitation in 1 day (mm)	Number of rainfall events > 20 mm
P1	20/09/2022 to 21/11/2022	307.2	47.4	4
P2	22/11/2022 to 23/01/2023	315	26.8	3
P3	24/01/2023 to 28/03/2023	204.1	36.6	3
P4	29/03/2023 to 23/05/2023	273.4	53.5	3
P5	24/05/2023 to 20/07/2023	144.8	27	3
P6	21/07/2023 to 20/09/2023	228.6	54.8	3
P7	21/09/2023 to 22/11/2023	467.5	53.5	10
P8	23/11/2023 to 24/01/2024	445.3	49	7

Note: Dates in bold correspond to sampling dates (T1–T8) of clay beads. Minimum and maximal values of the three variables are indicated in green and red, respectively.

metabarcoding analysis, 15 beads were frozen at -20°C after suppressing free water from the sample.

2.4 | Meteorological Data

Meteorological data were obtained from the weather station at La Féclaz (Feclaz_SAPC). This weather station is located at an altitude of 1328 m (coordinates: 45.647167 N, 5.983667 E) and is representative of meteorological conditions occurring in the catchments of the three studied stations. Daily rainfall records were obtained from Météo France during the experimental period from the 20th of September 2022 to the 24th of January 2024. For each incubation period of 2 months, cumulative precipitation (in mm), the maximum amount of rainfall recorded in a single day, and the number of rainfall events higher than 20 mm were calculated. These three meteorological variables were selected as proxies of the hydrological regime (flow rates, floods) in the three stations for each incubation period.

2.5 | Chemical Analysis on Water Samples

Water samples were filtered on site (Whatman GF/F glass fibre membranes, porosity: $0.7\mu\text{m}$) and stored at 4°C until analysis. Nitrate, ammonia, and phosphate concentrations were measured on filtered water samples using classical colorimetric methods with a sequential analyser (Smartchem 200, AMS Alliance, France). For each filtered water sample, 30 mL were used to measure its dissolved organic carbon (DOC) concentration. This volume was acidified with $350\mu\text{L}$ of 2N HCl to remove inorganic carbon. Then, the DOC measurement was performed using infrared detection of CO_2 produced by the thermocatalytic oxidation (850°C) of organic carbon, with a total organic carbon analyser (multi N/C 3100, Analytik Jena, Jena, Germany).

2.6 | Biofilm Biomass

A protein assay was used to estimate the biomass of biofilm developed on the artificial substrates. The protocol used follows the simplified Lowry method as described by Peterson (1977). For each sample, 5 clay beads were incubated for 20 min with 4 mL of a 50% Lowry reagent solution. After the reaction, 1 mL of Folin reagent was added, followed by a 30-min incubation in the dark. This incubation produced a reaction leading to a blue-coloured solution. After incubation, the absorbance of the solution was measured at 750 nm with a spectrophotometer (UV-1900i UV-Vis Spectrophotometer, Shimadzu Corporation., Japan). The optical density values were compared to a calibration curve made with BSA (bovine serum albumin) to determine the total protein content. Results were expressed in μg of protein per cm^2 of clay bead, after subtracting the values obtained from a control batch of five sterile clay beads.

2.7 | Biofilm Activities

The hydrolytic activity of biofilms was estimated using the fluorescein diacetate (FDA) hydrolysis method (Fontvieille

et al. 1992), as described in Voisin et al. (2016). For each sample, 5 clay beads were incubated in 3 mL of a phosphate buffer solution (pH 7.6), with the addition of 0.1 mL of a 4.8 mM FDA solution. The incubation was carried out at 15°C until the appearance of a green coloration. The reaction was stopped by adding 3 mL of acetone to the sample, followed by storage at -20°C . The concentration of fluorescein was estimated by measuring the absorbance of the supernatant at 490 nm (UV-1900i UV-Vis Spectrophotometer, Shimadzu Corporation., Japan), filtered through Whatman GF/F glass fibre membranes ($0.7\mu\text{m}$). The hydrolytic activity of the biofilms was then expressed in μmol of FDA hydrolyzed per hour per cm^2 of clay bead, after subtracting the values obtained from a batch of five sterile clay beads.

The chloride of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium (INT) was used to measure dehydrogenase activity (respiratory activity), as modified by Hourri-Davignon et al. (1989) based on Mermillod-Blondin et al. (2019). For each sample, 5 clay beads were incubated in a 0.02% INT solution at 15°C . The reaction was stopped at the same time as the samples for hydrolytic activity. The supernatant was then filtered through a nylon membrane ($0.22\mu\text{m}$, Whatman, Maidstone, UK) to recover the floating microbial cells. The extraction of INT-formazan from the clay beads and the nylon membrane containing the microbial cells was performed with 4 mL of methanol for 12 h. The concentration of INT-formazan was determined by measuring the absorbance at 480 nm (UV-1900i UV-Vis Spectrophotometer, Shimadzu Corporation., Japan). The dehydrogenase activity of the biofilms was then expressed as μmol of INT-formazan produced per hour per cm^2 of clay bead, after subtracting the values obtained from a batch of five sterile clay beads.

2.8 | Metabarcoding Data Acquisition, Processing and Analyses

Upon return from sampling campaigns, microbial communities developed on clay beads were stored at -20°C until DNA extraction. To optimise DNA extraction from clay beads, an initial chemical lysis step was performed. For each sample, the 15 clay beads were distributed in three 50 mL Falcon tubes with five beads per tube. In the first Falcon tube, 2.1 mL of a lysis buffer containing TNES (90 mM Tris, 90 mM EDTA, 750 mM NaCl), 2% SDS, and 0.1 mg/mL proteinase K was added to the beads, followed by an incubation at 56°C for 24 h with shaking at 125 rpm. After this incubation, the entire lysate (without beads) was transferred to the second Falcon tube containing five beads and incubated identically to the first tube. This step was repeated with the third tube containing the remaining five clay beads. After incubation, the final Falcon tube containing the lysate from the 15 clay beads was stored at -20°C .

DNA was then extracted from $500\mu\text{L}$ of lysate using the DNeasy PowerSoil Pro Kit, following the manufacturer's instructions (Qiagen, Germany). At the end of the protocol, DNA was eluted in $70\mu\text{L}$ of TE buffer (10 mM Tris-HCl, 1 mM EDTA). Extracted DNA concentrations ($\text{ng}/\mu\text{L}$) were quantified using a Qubit dsDNA High Sensitivity Kit (Qiagen, Germany). To avoid cross-contamination amongst samples, extraction plans were drawn up to group samples according to the expected amount of DNA

they contained. This procedure reduced the risk of contamination between samples with high DNA contents and samples with low DNA contents. In addition, the three replicates of a given condition were placed in such a way that they were never positioned twice next to samples from another given condition (e.g., for a condition A, if A1 is processed next to B1, then A2 and A3 cannot be positioned next to B2 or B3). By this way, the running order of the samples could not create artifactual biological-like patterns.

Sequencing libraries were prepared with two-step PCR. PCR1 was carried out in a 25 μ L total reaction volume with 1X PCR buffer including 12.5 μ L of Platinum II Hot Start Master Mix, (Invitrogen, USA), 0.2 μ M of each primer (515F-Y 5'-GTGYCAGCMGCCGCGGTAA-3' and 926R 5'-CCGYCAATYMTTTRAGTTT-3'; Parada et al. 2016), and 10 μ L of DNA (dilution at 0.2 ng/ μ L or initial extraction when the DNA concentration was too low). PCR amplification targeted the V4-V5 hypervariable regions of the 16S rRNA gene. As preconized by Weinroth et al. (2022), these V4-V5 regions were selected because of their classical use for studying microbial communities in groundwater ecosystems (e.g., Hong et al. 2013; Lehosmaa et al. 2021; Zhang et al. 2024). These regions allow to cover the bacterial domain but can also partially cover the archaeal domain, as observed by Fadeev et al. (2021) in sediments of the arctic ocean. Thermocycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 5 s and annealing/extension at 55°C for 7 s. For each sample, PCR1 was performed in triplicate. After validation of PCR1 products on an electrophoresis gel, the replicates for each sample were pooled and sent to the GeT-Genotoul sequencing platform (Toulouse, France) for PCR1 purification, followed by PCR2 for sample indexing and ligation of Illumina P5/P7 adapters and sequencing on an Illumina MiSeq platform (Flowcell v3, Paired-End 2*250 bp).

Four types of negative controls were incorporated throughout the protocol to monitor potential contamination due to the products used, the experimenters and the working environment: 'field controls' consisting in 15 sterilised clay beads in a Falcon tube opened during sampling collection at each sampling campaign; 'protocol controls' with 15 sterilised clay beads undergoing the entire chemical lysis and DNA extraction protocol; 'extraction controls' with 500 μ L of ultrapure water in place of lysate; and 'PCR controls' with ultrapure water instead of DNA.

The bioinformatics processing of paired-end raw reads (reads are deposited in NCBI database under the BioProject accession number PRJNA1390062, Supporting Information S3 for sample name information) was conducted in R using the DADA2 package (Callahan et al. 2016) following the dada2 tutorial (except for additional steps 1 and step 8; see below) to infer exact amplicon sequence variants (ASVs) and their relative abundances. Reads processing followed these steps: (1) filtering and trimming with cutadapt (Martin 2011) to eliminate primer sequences whilst accounting for potential primer slippage (Elbrecht et al. 2018), (2) quality profile inspection and trimming, (3) learning of error rates, (4) sequence inference, (5) merging of paired-end reads, (6) ASV table construction, (7) chimaera removal, (8)

cross-contamination elimination (see below) and (9) taxonomic assignment.

Since samples were sequenced in three separate runs, each run was processed independently up to step (7) to account for distinct error models, after which the three sequence tables were merged using the mergeSequenceTables function. During step (2), R1 and R2 reads from each sequencing run were trimmed based on their quality profiles, i.e., removing the last 5 (R1) or 11 to 17 (R2) bases. An additional cross-contamination removal step (8) was applied after chimaera removal: considering the three replicates of a given condition, ASVs present in only one of the three replicates were removed for this condition. Taxonomic assignment was performed with RDP classifier (Wang et al. 2007) using the Silva database (v138.1; Quast et al. 2013). As recommended in the dada2 tutorial, taxonomic assignment was performed in two successive steps, with the assignTaxonomy and addSpecies functions. A phyloseq object (McMurdie and Holmes 2013) was built for subsequent analyses. All ASV assigned to 'Chloroplast' or 'Mitochondria' were removed from this object.

After creating the phyloseq object, the composition of the negative controls was investigated to identify potential contaminant ASV. There was no clear pattern of contamination and very few ASV were shared amongst negative controls (Supporting Information S4). Ten low-abundance ASV were considered as contaminants and removed from the dataset. Once the negative controls were excluded from the dataset, sequencing depth was examined for each sample (see the rarefaction curves on Supporting Information S5). One replicate obtained for station B during P7 with significantly lower depth (19,764 reads) than other samples was excluded to allow dataset rarefaction at 35,243 reads. This rarefaction was performed using the rarefy_even_depth function from the phyloseq package.

2.9 | Calculation of Alpha and Beta Diversity Indices

Alpha diversity indices (ASV richness and Shannon index) were calculated using the vegan package (Oksanen et al. 2001) in R. A phylogenetic tree of the 7277 ASV was built using the DECIPHER (Wright 2016) package for sequence alignment and the phangorn (Schliep 2011) package for phylogenetic reconstruction (model 'GTR+G(4)+I'), allowing the calculation of the Faith phylogenetic diversity index (PD index). Finally, beta diversity was assessed using weighted Unifrac distances, calculated with the vegan and phyloseq packages.

2.10 | Statistical Analyses

For chemical and microbiological measurements, we considered as outlier values the replicates which diverged from the two other replicates by more than 2-fold. Then, outlier values were removed from the dataset. Overall, this procedure led to the suppression of 12 values out of the 525 values present in the dataset.

Nutrient concentrations (NH_4^+ , NO_3^- , PO_4^{3-}), dissolved organic carbon, microbial biomass, microbial activities and

alpha diversity metrics (ASV Richness, Shannon index and PD index) were compared across stations and incubation periods using two-way ANOVA followed by Tukey post hoc tests for pairwise comparisons. Prior to analysis, the assumptions of normality and homoscedasticity were tested using Shapiro–Wilk’s and Levene’s tests from package car (Fox and Weisberg 2019), respectively. When these assumptions were not met (for NO_3^- concentration, PO_4^{3-} concentration and hydrolytic activity), data were square root-transformed to achieve homoscedasticity.

A correlation matrix was generated for the entire dataset using non-parametric Spearman’s tests to identify potential correlations between environmental and microbial variables. This analysis included nutrient and DOC concentrations, biofilm biomass, biofilm activities, and alpha diversity metrics of prokaryotic communities, but also meteorological variables (total precipitation, maximal daily precipitation, and number of rainfall events > 20 mm).

For the beta diversity analysis, a PCoA was performed with weighted Unifrac distances to visualise differences in prokaryotic community composition amongst stations and incubation periods. A PERMANOVA test was performed in R with vegan package (Oksanen et al. 2001) to assess the significance of these differences. In addition, pairwise comparisons were conducted using pairwiseAdonis package (Martinez Arbizu 2020) to identify which stations and incubation periods harboured statistically distinct communities.

For each station, prokaryotic community structure was visualised using Krona charts (Ondov et al. 2011). Indval tests from package labdsv (Roberts 2023) were performed to identify indicator taxa (at order and family level) for each station, using the different incubation periods as replicates. We selected indicator taxa for each station when their significant IndVal indexes were equal to or greater than 0.7, indicating a very marked specificity according to Dufrêne and Legendre (1997) and McCune et al. (2002). The significance of these values was tested using permutation tests corrected by the Bonferroni method.

All these tests were performed using R Statistical Software (v4.4.1; R Core Team 2024).

3 | Results

3.1 | Meteorological Conditions During the 18-Month Study

Meteorological data showed that cumulative precipitation varied between 145 mm and 467 mm during the eight incubation periods of the artificial substrates (Table 1). At least 3 heavy rainfall events (i.e., with daily amounts exceeding 20 mm) occurred during every incubation period. However, the most numerous heavy rainfall events and the highest cumulative precipitations were observed at the end of the study, during incubation periods P7 and P8. Conversely, incubation period P5 was characterised by the driest conditions with low cumulative precipitations.

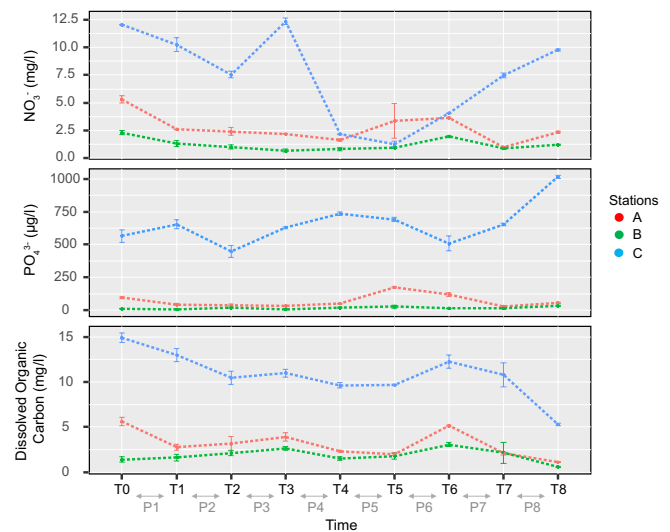


FIGURE 2 | NO_3^- , PO_4^{3-} , and DOC concentrations measured in the three stations at all sampling dates during the course of the experiment (mean \pm SD, $n = 3$ per date and station). Points represent means, and error bars indicate standard deviations. Incubation periods (P1 to P8) and sampling dates (T0 to T8) are displayed on the horizontal axis.

3.2 | Dissolved Organic Carbon and Nutrient Concentrations Differences Amongst Stations and Temporal Dynamics

Significant differences in NO_3^- , PO_4^{3-} , and DOC concentrations in groundwater (Figure 2) were measured amongst stations (p -values < 0.0001, see Table 2 for details of statistical analyses). Groundwater collected in station C exhibited the highest nutrient concentrations, with values approximately four times higher than those measured in station A for NO_3^- and DOC concentrations, and six times higher for PO_4^{3-} concentrations. Station B was characterised by the lowest nutrient concentrations measured in groundwater with values close to 0 $\mu\text{g/L}$ for PO_4^{3-} and below 2.5 mg/L for both DOC and NO_3^- , which were around two-fold lower than concentrations measured in groundwater collected in station A. In comparison with NO_3^- , PO_4^{3-} , and DOC concentrations, NH_4^+ concentration in groundwater was not significantly different amongst stations (p -value = 0.95, Table 2) despite concentrations varying between 0 and 63 $\mu\text{g/L}$ during the course of the experiment (Supporting Information S6).

Statistical tests also revealed significant statistical interactions between the factors ‘period’ (sampling date) and ‘station’ for nutrient and DOC groundwater concentrations (p -values < 0.0001, Table 2), indicating that the temporal dynamics of the groundwater chemistry varied amongst stations. This interaction was especially observed for NO_3^- concentrations in groundwater because higher temporal changes of this variable were measured in station C than in the two other stations (Figure 2).

3.3 | Biofilm Biomass and Activities

Significant differences amongst stations were observed for the three biofilm variables (p -values < 0.0001, Table 2). The biofilm

TABLE 2 | Results of analyses of variance for testing the effect of the factors ‘station’ and ‘period’ (sampling time for chemical variables) on the concentrations of NO_3^- , NH_4^+ , PO_4^{3-} , dissolved organic carbon (DOC), biofilm biomass, hydrolytic and dehydrogenase activity, bacterial richness, Shannon and phylogenetic diversities of prokaryotic communities.

Variable		Station	Period	Interaction (Station * Period)	Error
NO_3^-	df	2	8	16	53
	<i>F</i>	1338	104	72	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
NH_4^+	df	2	8	16	53
	<i>F</i>	0.05	3.24	5.6	
	<i>p</i>	0.94850	<0.01	<0.0001	
PO_4^{3-}	df	2	8	16	50
	<i>F</i>	9323	64.7	42	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
DOC	df	2	8	16	50
	<i>F</i>	2104	66.3	17.44	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
Biofilm biomass	df	2	7	13	43
	<i>F</i>	171	12.6	5.6	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
Hydrolytic activity	df	2	6	12	42
	<i>F</i>	1097	127	87.9	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
Dehydrogenase activity	df	2	7	13	46
	<i>F</i>	130	23.5	20.1	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
Bacterial richness	df	2	7	13	45
	<i>F</i>	2190	38.7	16.9	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
Shannon diversity	df	2	7	13	45
	<i>F</i>	5166	38.1	58.5	
	<i>p</i>	<0.0001	<0.0001	<0.0001	
Phylogenetic diversity	df	2	7	13	45
	<i>F</i>	4565	57.5	24.6	
	<i>p</i>	<0.0001	<0.0001	<0.0001	

Note: df=degree of freedom; F= F statistic for ANOVA.

collected in station A exhibited significantly higher values of biomass, hydrolytic activity, and dehydrogenase activity than biofilms collected in stations C and B (Figure 3). The average biomass of biofilms of station A ($7.96 \mu\text{g}/\text{cm}^2$) was around six and two times higher than the biofilm biomasses measured in station B ($1.36 \mu\text{g}/\text{cm}^2$) and station C ($4.13 \mu\text{g}/\text{cm}^2$), respectively. The highest biofilm activities measured in station A were reported from P1 and P8, with values exceeding by around 15

times and 10 times the highest microbial activities of biofilms collected in station B and C, respectively. Statistical tests revealed significant differences in biofilm biomass and activities amongst incubation periods (p -values < 0.0001, Table 2) but no clear common temporal trend for the three stations was identified. Significant statistical interactions between the factors ‘station’ and ‘period’ were also reported (p -values < 0.0001, Table 2), indicating that the temporal variation of biofilm variables varied

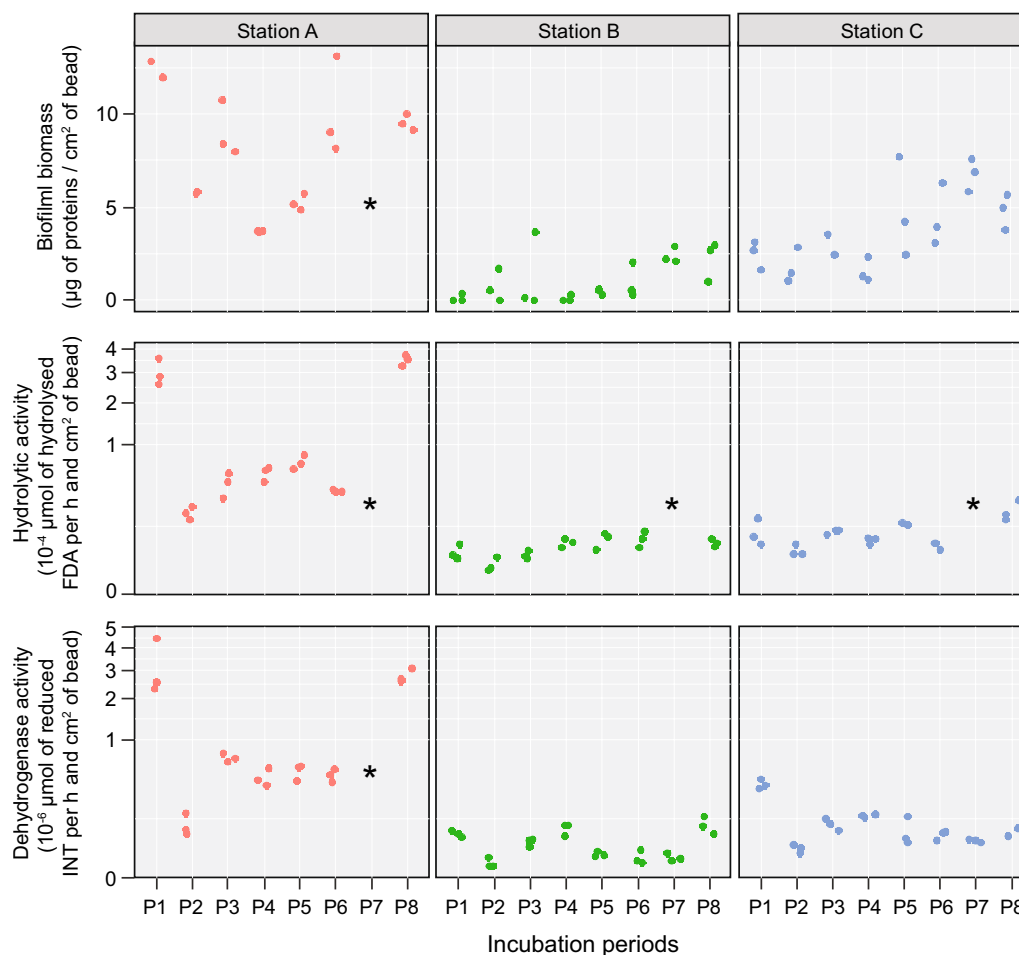


FIGURE 3 | Microbial biomasses and activities (hydrolytic activity and dehydrogenase activity) measured on clay beads incubated in the three stations for the 8 periods. The y-axes are on a cubic root scale for hydrolytic and dehydrogenase activities. The * symbol indicates missing data.

amongst stations. Nevertheless, *F*-statistics values reported in Table 2 showed that the factor ‘station’ had more influence on biofilm variables than the factor ‘period’ or the interaction between both factors (station and period).

3.4 | Prokaryotic Community Richness and Diversity

After reads processing and rarefaction at 35,243 reads per sample, the metabarcoding dataset comprised a total of 7277 exact amplicon sequence variants (ASV). After taxonomic assignment, 0.3% of all reads were not assigned (‘NA’) at the Phylum level, 1.2% at the Class level, 3.9% at the Order level, 11.8% at the Family level, 42.7% at the Genus level, and 81.7% at the Species level. Therefore, all representations and analyses based on ASV taxonomy were performed at the Family and/or Order levels.

Significant differences in alpha diversity metrics were observed amongst stations (*p*-values < 0.0001, Table 2). Prokaryotic communities of biofilms obtained from station C were richer (ASV richness) and more diversified (Shannon and PD diversities) than biofilm communities collected in stations A and B (Figure 4). The average ASV richness of biofilm communities

developed in station C (1313 ASVs) was approximately 3.3 times and 1.3 times higher than average ASV richness measured in station B (400 ASVs) and station A (1003 ASVs), respectively. Similar patterns were obtained for the Shannon and PD indices, with the highest prokaryotic diversity measured in biofilms of station C and the lowest prokaryotic diversities reported from biofilms of station B.

Statistical analyses showed significant differences in prokaryotic richness and diversity amongst incubation periods (*p*-values < 0.0001, Table 2). However, significant interactions between the factors ‘station’ and ‘period’ were also detected (*p*-values < 0.0001, Table 2), indicating that the temporal dynamics of alpha diversity metrics varied amongst stations. More precisely, alpha diversity metrics showed higher temporal variabilities in station A than in stations B and C (Figure 4). In station A, fluctuations in alpha diversity values were observed during the course of the experiment with lowest richness and diversity indices for periods P1 and P3 and highest indices for periods P2 and P5. In stations B and C, alpha diversity values were more stable during the course of the experiment than in station A, although the Shannon index decreased during the course of the experiment in station B and the ASV richness and the PD index of biofilms decreased for the period P7 in station C (Figure 4).

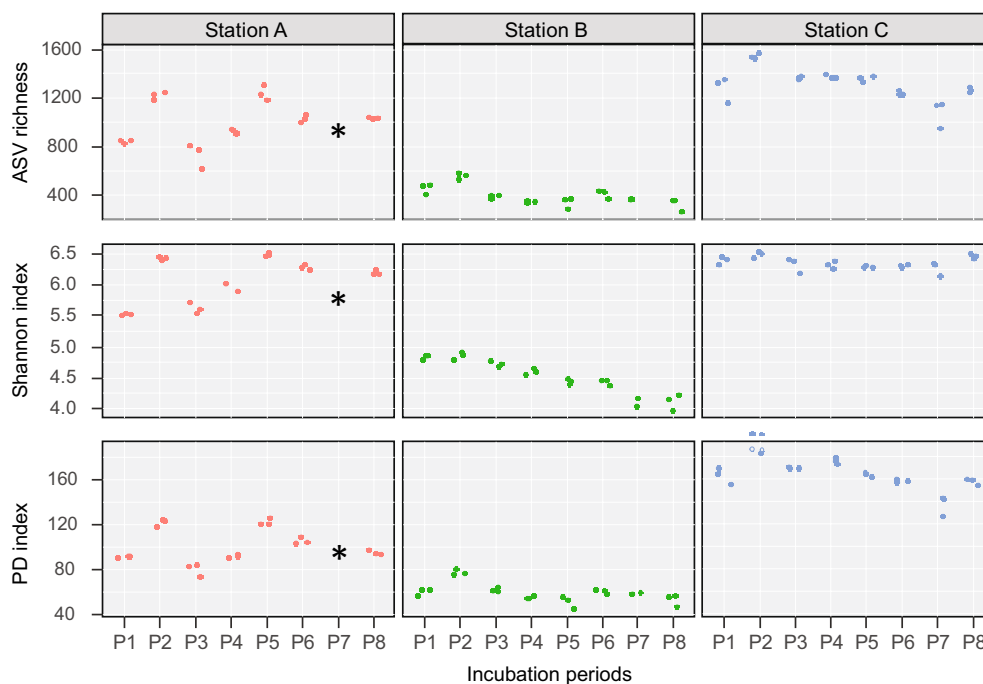


FIGURE 4 | Alpha diversity metrics (ASV richness, Shannon index and Faith Phylogenetic Diversity (PD) index) of the prokaryotic communities collected in the three stations for the 8 incubation periods. The * symbol indicates missing data.

3.5 | Correlations Between Environmental Factors (Meteorological and Chemical Variables), biofilm Biomass, Biofilm Activities, and Prokaryotic Community Richness and Diversity

The Spearman's correlation matrix (Figure 5) highlighted significant and positive correlations amongst nutrient variables (PO_4^{3-} , NO_3^- , and DOC) at the exception of NH_4^+ concentrations. The same correlation patterns were observed amongst biofilm variables (biofilm biomasses and activities), amongst alpha diversity metrics of prokaryotic communities, and between two meteorological variables (cumulative precipitation and number of rainfall events exceeding 20 mm for each period). Unlike our expectations, no significant correlations between nutrient and hydrological variables nor between nutrient and biofilm biomass/activities were detected. This statistical result was largely due to the fact that the highest concentrations of nutrients were measured in groundwater of station C (Figure 2) but the highest microbial biomass and activities were not observed for biofilms collected in this station (Figure 3). There was a probable inhibition of biofilm growth in this station. In contrast, the three prokaryotic alpha diversity metrics were significantly and positively correlated with the concentrations of PO_4^{3-} , NO_3^- , and DOC measured in groundwater.

3.6 | Difference in Prokaryotic Community Structure Amongst the Three Stations

The PCoA analysis represented 71.9% of the variability contained in the weighted Unifrac distance matrix (Figure 6). This graphical representation clearly illustrated the significant difference in ASV structure amongst the three stations

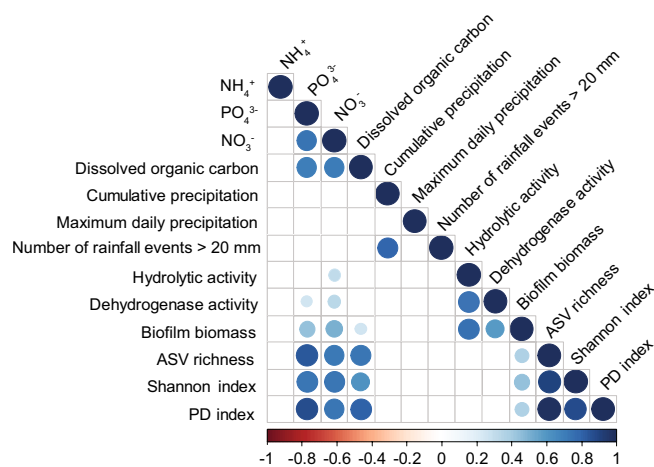


FIGURE 5 | Spearman correlation matrix for the studied variables, including environmental variables (NH_4^+ , PO_4^{3-} , NO_3^- , DOC, cumulative precipitation, maximum daily precipitation, and number of days with rainfall > 20 mm; see Table 1), biofilm variables (hydrolytic activity, dehydrogenase activity, and biomass) and alpha diversity metrics of prokaryotic communities (ASV Richness, Shannon index and PD index). The intensity of the correlations between two variables increases with size of the circles, with colours ranging from red (negative correlations) to blue (positive correlations). Only significant correlations ($p < 0.05$) are displayed in the figure.

(PERMANOVA, $F_{(2, 67)} = 61$, $p < 0.001$). The prokaryotic communities of biofilms recovered from clay beads incubated in stations A and B were quite comparable for the first incubation period (P1) but they largely differed for the other incubation periods (pairwise comparison of prokaryotic communities between stations A and B, $F_{(1, 43)} = 19$, $p < 0.001$). Prokaryotic communities of biofilms collected in station C were different

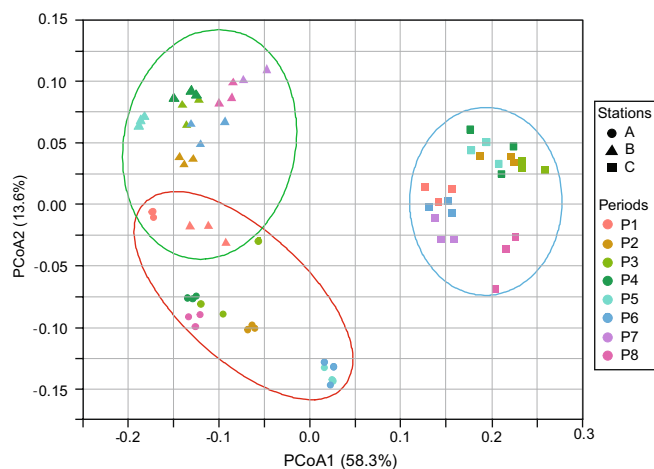


FIGURE 6 | PCoA ordination plot representing differences in prokaryotic community structure based on Weighted Unifrac distances. Point shapes represent the three stations and colours indicate incubation periods.

from those collected in stations A and B for all incubation periods (pairwise comparisons of prokaryotic communities between stations C and A, $F_{(1, 44)} = 67$, $p < 0.001$; and between stations C and B, $F_{(1, 46)} = 108$, $p < 0.001$). Whilst significant differences in prokaryotic community structure were observed amongst stations, no significant influence of the incubation period was detected on prokaryotic community structure (PERMANOVA: $F_{(7, 67)} = 1.1$, $p = 0.31$).

Significant differences in prokaryotic community structure amongst stations were observed at the kingdom level (Figure 7; see Supporting Information S7–S9 for the Krona html files). For example, detected Archaea represented only 1% of the relative abundance of the prokaryotic community in stations A and B, whilst archaea members constituted around 14% of the community of biofilms in station C (all percentages in this section are expressed as % of reads). The archaeal part of the community was mainly composed of the phyla Crenarchaeota (more than 80% of the archaeal community) and Nanoarchaeota (17% of the archaeal community recovered from station C, 2% from station A, and 1% from station B). For Bacteria, the most represented phylum in the three stations was Proteobacteria, which constituted more than 38% of the relative abundance of Bacteria in the three stations. This bacterial phylum was mainly composed of the Gammaproteobacteria (~65% of relative abundance) and Alphaproteobacteria (~35%) classes. The phylum Bacteroidota was also found in the three stations with relative abundances higher than 9%. Several bacterial phyla displayed higher relative abundances in the prokaryotic communities of stations A and C than in the community reported from station B: Verrucomicrobiota (7% versus 2%), Planctomycetota (more than 4% versus 2%), Firmicutes (more than 2% versus 0.002%), and Acidobacteriota (2% in station A, 5% in station C, and 0.7% in station B). Conversely, a higher relative abundance of members of the Actinobacteriota phylum was reported in biofilms of station B (9%) than in biofilms of the two other stations (1% in station A and 3% in station C).

Using the Indicator Value index (IndVal), 63 prokaryotic families out of 278 were detected as station-specific (i.e., with

IndVal indexes > 0.7 and p -value < 0.05) with 11, 9, and 43 families for stations A, B and C, respectively (Supporting Information S10). The station-specific prokaryotic families for station A were Moraxellaceae (2%), Burkholderiaceae (0.2%), and Xanthomonadaceae (2%), all belonging to the class Gammaproteobacteria (Figure 7). Belonging to other classes, the families Chitinophagaceae (3%) and Flavobacteriaceae (5%) were also found as characteristic taxa of station A. In Station B, the families Microbacteriaceae (7%), Sporichthyaceae (1%), and Micrococcaceae (1%) of the phylum Actinobacteriota were selected by IndVal (Figure 7). Other families specific of station B included Myxococcaceae (0.4%), Caulobacteraceae (6%) and Dongiaceae (2%). Several orders not assigned at the family level were also selected by IndVal for this station, notably FFCH16767 (0.2%), Blfdi19 (0.5%), and Lineage 4 (1%) of the phylum Elusimicrobiota. In Station C, several families of the archaeal order Woesarchaeales were identified as station-specific (Figure 7). Additionally, the families Nitrosopumilaceae (80%) and Nitrososphaeraceae (1%) of the phylum Crenarchaeota were detected as characteristic taxa of station C. IndVal also selected many bacterial families in which were specific to station C like Bacillaceae (0.4%), Silvanigrellaceae (0.3%), Peptostreptococcaceae (2%), Erysipelotrichaceae (0.4%), and Pirellulaceae (4%).

4 | Discussion

4.1 | Sources and Dynamics of Nutrients and Dissolved Organic Carbon Concentrations

The aim of this study was to examine the influence of nutrient and organic matter concentrations on microbial biofilms and prokaryotic community composition. As expected, nutrient and dissolved organic carbon (DOC) analyses confirmed that contrasting catchment areas led to differences in groundwater quality amongst the three stations. Station B, which has a watershed dominated by a forested area, was the least affected by nutrient and organic carbon supplies. Station A, which collected water from a large watershed, received diverse organic matter inputs (road runoff, wastewater, debris, etc.), leading to moderate nutrient and organic carbon supplies. Finally, station C, despite a reduced watershed, was directly influenced by farm effluents from the surface, leading to high inputs of nutrients and DOC.

We expected that the nutrient and organic matter supplies to the karstic system would vary with the seasonal variations in hydrology. Meteorological data allowed us to identify two main periods during the course of the experiment: a dry season extending from P3 to P6 (January to October 2023) and a rainy season from P7 to P8 (October 2023 to February 2024). These observations were coherent with the classical meteorological context encountered in this mountain environment: reduced precipitations are generally observed in summer and in winter (snow cover), whilst spring and autumn are classically characterised by rainy conditions (Najib Molaï 2000). Based on these meteorological data, nutrient and organic carbon concentrations were expected to vary with the hydrological conditions occurring during the 18 months of experiment. Some studies (Guan et al. 2023; Odabaşı et al. 2022) have reported that nitrate and ammonium concentrations were higher during low-flow periods

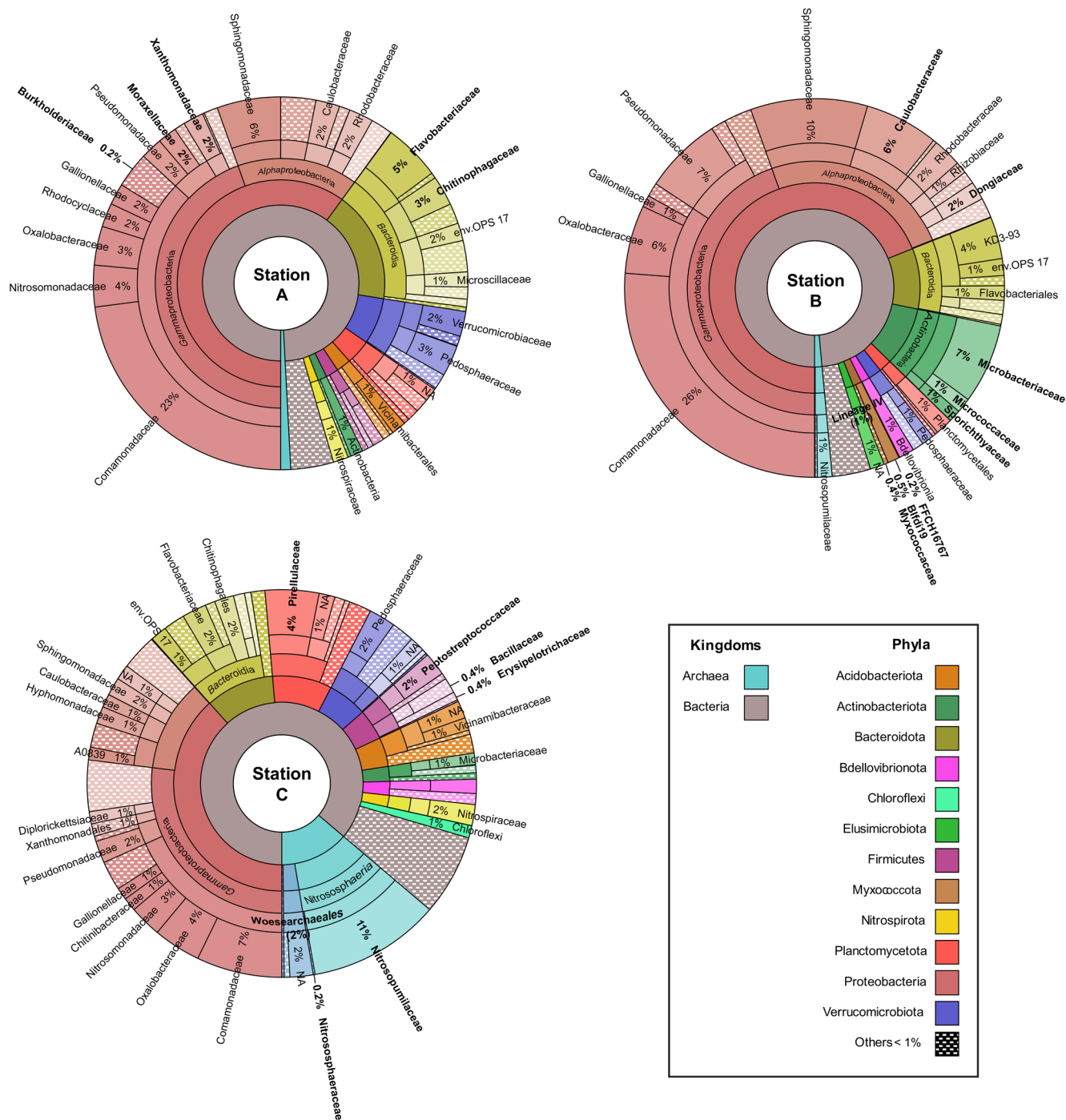


FIGURE 7 | Krona charts of prokaryotic community structures from station A, station B and station C. Taxa displayed in bold are the most promising taxa detected with the Indicator Value index (IndVal). All detected indicator taxa are listed in Supporting Information S10 and the Krona charts are available in interactive format in Supporting Information S7–S9.

than during rainy periods in karst systems. These fluctuations were explained by the dilution effect of nutrients during the rainy season. Such a mechanism could explain the annual dynamics of NO_3^- and PO_4^{3-} concentrations measured in station A during the present experiment. Nevertheless, this potential dilution mechanism was not observed in the two other stations (B and C). For example, the NO_3^- concentrations in station C were the lowest during the summer period (P5 and P6), invalidating the main influence of dilution on nutrient concentrations in this station. The different responses of nutrient concentrations

to meteorological conditions amongst stations were probably due to the large variability in catchment areas amongst these stations. The three studied stations have catchments with different areas and structural heterogeneities (from intricate grain arrangements at the pore scale to larger geologic structures and discontinuities at the meso- and macroscopic scales) that could explain their distinct responses to rainfall (Bakalowicz 2005). Thus, more specific analyses coupling meteorological data with measurements of water discharge rates and nutrient concentrations in the three stations would be necessary to decrypt the

hydrological mechanisms which could explain nutrient dynamics in each station. Due to this potential hydrological variability amongst stations, we did not detect a significant influence of meteorological conditions on nutrients and dissolved organic carbon on the whole dataset. Without any clear seasonal variation of trophic resources in groundwater, no consistent temporal variations during the experiments were measured on biofilm variables. In contrast, biofilm biomass, activities, and prokaryotic community structures were significantly different amongst stations.

4.2 | Links Between Trophic Conditions and Biofilm Characteristics

The marked differences in groundwater nutrient and DOC concentrations amongst stations allowed us to evaluate whether these distinct trophic conditions determine the biofilm development and the prokaryotic community structure developed on artificial substrates. Based on Lebon et al. (2021) and Voisin et al. (2020) we hypothesised that the station with the highest nutrient and dissolved organic carbon concentrations would support the highest microbial biomasses, microbial activities and prokaryotic diversity (H1). This hypothesis was not validated by the measures of biofilm biomass and activities performed in the three stations as these microbial variables did not exhibit the highest values in the station (station C) characterised by the highest concentrations of nutrients and DOC. Consequently, we did not observe the positive relationship between the concentration of dissolved organic carbon and biofilm variables which has been reported in sedimentary aquifers (Claret 1998; Mermillod-Blondin et al. 2013; Voisin et al. 2020; Williamson et al. 2012) or other karstic aquifers (Zhou et al. 2012). This lack of positive relationship observed in the present study might have several explanations. First, the availability of the dissolved organic matter for microorganisms could have been highly variable amongst stations and the fraction of biodegradable DOC could be very low in station C characterised by the highest DOC concentrations. Consequently, the biofilm growth and activity would be lower in station C than in station A because the availability of organic carbon sources for biofilms in groundwater was lower in station C than in station A. Second, despite high concentrations of nutrients and DOC in station C, the use of antibiotics associated with livestock farming on the catchment of this station could have produced biocidal effects on microbial communities (reduction of the growth and activity of microorganisms, Zielesny et al. 2006).

In contrast with biofilm biomass and activity measured on clay beads, alpha diversity metrics supported the hypothesis stating that the station with the highest nutrient and dissolved organic carbon concentrations would support the highest prokaryotic diversity of biofilms. More precisely, the number of ASVs (richness), the Shannon index, and the PD index were higher for biofilms collected in station C than for biofilms recovered from the two other stations. Moreover, these prokaryotic alpha diversity metrics were the lowest on biofilms developed in station B which was characterised by the lowest concentrations of nutrients and DOC. As observed in previous studies (Hofmann et al. 2020; Lebon et al. 2021; Voisin et al. 2020), the positive response of the prokaryotic diversity of biofilms to dissolved organic carbon

availability in groundwater was mainly due to the organic carbon limitation of most groundwater ecosystems. Due to this limitation, inputs of dissolved organic carbon by infiltration from catchment areas can increase the number of ecological niches by increasing the availability of more resources and gradients (Hubalek et al. 2016). For example, large inputs of organic matter could permit the maintenance of copiotrophic bacteria that were not adapted to live in the more oligotrophic areas of aquifers (e.g., Fierer et al. 2007; Lever et al. 2015).

It is worth noting that biofilms developed in station C were not the densest and the most active but they presented the highest prokaryotic diversity. This specificity could be associated with the presence of antibiotics which were reported to decrease the growth and activity of biofilms without affecting their diversity in the environment (Cycoń et al. 2019; Guan et al. 2023; Li et al. 2011). Indeed, several antibiotics used in livestock farming in the catchment of station C could have been excreted by animals and could have reached groundwater through runoff and percolation (Zhang et al. 2009). Antibiotic infiltration in this station could not have impacted bacterial diversity but might have promoted the presence of antibiotic-resistant taxa as observed for the genera *Flavobacterium* and *Pseudomonas* (Guan et al. 2023) and the classes Clostridia and Bacilli (Li et al. 2011) in groundwater. This hypothesis was partially supported in the present study as Clostridia and Bacilli families had higher relative abundances in the prokaryotic community of station C (2.7% and 2.1% for Clostridia and Bacilli, respectively) than in communities of stations A (1.5% and 0.5% for Clostridia and Bacilli, respectively) and B (>0.01% for both classes). In addition to changes in the prokaryotic community structure, antibiotic infiltration can lead to the development and selection of resistance genes and bacteria carrying these genes (Guan et al. 2023), potentially explaining the prokaryotic diversity measured in station C. To test this hypothesis, targeted analyses on the presence of antibiotics in groundwater samples should be conducted, along with metagenomic approaches to assess the presence of antibiotic resistance genes, as demonstrated in Smith et al. (2012).

In summary, assessment of alpha diversity through a 16S metabarcoding approach appeared as a pertinent proxy to detect organic matter supplies from the catchment. However, the potential presence of antibiotics prevented the use of more conventional metrics such as biofilm growth and activities. Consequently, the analysis of the forthcoming 16S metabarcoding results could be a promising solution for identifying taxonomic markers of various types of contamination, including the presence of biocides (as indicated above with the Clostridia and Bacilli families).

4.3 | Prokaryotic Community Structures of Biofilms and Candidate Taxa for Bioindication

We expected that the structures of prokaryotic communities would diverge amongst stations due to the selection of taxa which were able to develop and maintain under the specific groundwater chemistry of each station (H2). Although Alphaproteobacteria, Gammaproteobacteria, Firmicutes, Bacteroidetes, Acidobacteria, Actinobacteria, Verrucomicrobia, and Planctomycetes were common to the three stations as

classical classes found in groundwater environments (Kato et al. 2024; Tomczyk-Żak and Zielenkiewicz 2016), our hypothesis was validated because specific prokaryotic taxa were detected for each station. Interestingly, these station-specific prokaryotic taxa could be easily linked to the inputs of nutrients, organic carbon sources, and potential contaminants associated with catchment areas.

For instance, station A drains a watershed comprising residential and road areas, forests and meadows, leading to inputs of a wide variety of nutrients, organic carbon sources but also contaminants (wastewater, hydrocarbons, Wilhelm 2007) in the karstic aquifer. This station can receive high amounts of particulate organic matter (wood and leaf debris) and can be colonised by a dense invertebrate fauna (*Niphargus* sp., *Gammarus* sp., *Proasellus valdensis*, Dodelin and Dodelin 2003) following floods (see Supporting Information S11 for an example of organic matter accumulation after a flood). Under these conditions, the prokaryotic community obtained from station A was dominated by Gammaproteobacteria, a class of bacteria commonly found in subterranean environments which tends to proliferate in ecosystems affected by inputs of nutrients or pollutants due to surface water infiltration (Fillinger et al. 2021; Shabarova 2013). Several families within this class are classified as copiotrophic bacteria (Fierer et al. 2007) which have the ability to degrade a wide range of organic compounds (Farnleitner et al. 2005; Weng et al. 2022) and may be favoured in hydrocarbon-enriched environments (Hershey et al. 2018; Herzyk et al. 2017). For example, amongst the families of Gammaproteobacteria selected as indicators in station A, Xanthomonadaceae and Burkholderiaceae are known to be involved in the remediation of soils contaminated with hydrocarbons, phenol, pesticides and metals (Fillinger et al. 2021; Yavari-Bafghi et al. 2023). Members of the Xanthomonadaceae also contribute to the decomposition of organic matter, cellulose, and chitin (Jiang et al. 2025; Weng et al. 2022). It is also the case for other specific families of station A like Moraxellaceae, Flavobacteriaceae and Chitinophagaceae (Farnleitner et al. 2005; Fillinger et al. 2021; Glöckner et al. 2000; Kirchman 2002; Shabarova 2013; Zhao et al. 2023). Interestingly, the presence of high density of invertebrates in this station could easily explain the presence of families such as Chitinophagaceae and Xanthomonadaceae, which can degrade chitin, a major component of invertebrate exoskeletons (Fillinger et al. 2023).

Station B receives water from a small catchment located in a forested area and was characterised by oligotrophic conditions. According to these oligotrophic conditions, prokaryotic communities found at this station had a high relative abundance of Actinobacteria, a class frequently observed in environments with low organic matter concentrations (Arocha-Garza et al. 2017; Eisenlord and Zak 2010; Glöckner et al. 2000; Zhang et al. 2019). Other taxonomic groups associated with oligotrophy were also specific of station B like bacterial members of the family Caulobacteraceae (Henrici and Johnson 1935; Poindexter 1964; Seballos et al. 2020; Wilhelm 2018) found in aquifers, stagnant waters, soils and plant rhizosphere (Farnleitner et al. 2005; Henrici and Johnson 1935; Wilhelm 2018). Similarly, members of the phylum Myxococcota (orders FFCH16767 and Blfdi19, family Myxococcaceae) specifically abundant in station B are recognised for their ability to survive in nutrient-poor

environments (Villeneuve et al. 2022; Weng et al. 2022). In this station, some specific taxa were associated with the use and degradation of cellulose and complex carbon sources like the families Microbacteriaceae, Sporichthyaceae, Micrococcaceae (Shabarova 2013; Tamura 2014), the order Lineage IV (class Elusimicrobia) (Méheust et al. 2020) or the family Dongiaceae (Weng et al. 2022). This observation could be linked to the inputs of organic matter in groundwater of station B which collects water from a forested area.

Station C was strongly impacted by water infiltration from cattle farming. Groundwater chemistry indicated a strong enrichment in dissolved organic matter, nitrate and phosphate in this station in comparison with the two other stations. Associated with nitrogen contamination, the prokaryotic community of biofilms in station C was characterised by several taxa involved in the nitrogen cycle. More precisely, the relatively high abundance of archaeal taxa in this station was due the family Nitrosopumilaceae which is an ammonia oxidising archaea commonly found in environments rich in ammonium, nitrate, or other nitrogen compounds (Hatzenpichler 2012; Liu et al. 2023). Although this family was initially discovered in marine environments, its presence in aquifers has also been reported by Fillinger et al. (2021). It was also worth noting that nitrate concentration in groundwater varied strongly in station C (from 12.35 mg/L at T3 to 1.27 mg/L at T5, Figure 2) without having a significant influence on the proportion of Nitrosopumilaceae in the prokaryotic community of the biofilm. The proportion of Nitrosopumilaceae varied between 8.7% for P1 to 17.2% for P5 (Supporting Information S12) without any correlation with nitrate concentrations measured during periods (Pearson's correlation, $R^2 = 0.0076$ and $p > 0.838$). In addition, the proportions of Nitrosopumilaceae always remained more than 3-fold higher than the proportion of this family reported from the other stations during the course of the experiment (Supporting Information S12). These results suggest that the family Nitrosopumilaceae developed in biofilms could be a pertinent candidate to detect long term impacts of cattle farming on karstic aquifers. Within the bacterial community, the family Pirellulaceae was found as a specific taxon of station C. Like Nitrosopumilaceae, the selection of this family for station C might be also associated with nitrogen contamination as multiple Pirellulaceae members are nitrifying bacteria (Colombo et al. 2023). The phylum Firmicute containing copiotrophic bacteria (Cleveland et al. 2007; Francioli et al. 2016) was also specific of station C. Within this phylum, we detected the families Peptostreptococcaceae and Erysipelotrichaceae which are known to play on role in nitrogen metabolism in the digestive tracts of dairy cows (Castro-Ramos et al. 2022; Kanno et al. 2015). It was not surprising to find them in station C which receives water from grazing areas with bovine faeces (Wolinska et al. 2017). As indicated previously for the class Bacilli, Bacillaceae family was specific of station C most probably due to its high antibiotic resistance (Li et al. 2011) but also to its preference for fertilised sites in comparison with unfertilized sites (Wolinska et al. 2017). Analyses of prokaryotic communities also highlighted that the phylum Acidobacteriota was relatively more abundant in biofilms from station C than in biofilms from the two other stations. The presence of Acidobacteriota has already been reported in subsurface environments (Fillinger et al. 2021), but also in acidic environments such as peatlands

and alpine meadow soils impacted by nitrogen enrichment (Reji and Zhang 2022). It is therefore probable that the predominance of this phylum in station C resulted from soil acidification associated with the transformation of organically bound N from urine and dung to nitrate (e.g., Bouman et al. 1995; Cai et al. 2014). Overall, the multiple taxa specifically found at station C seemed to be promising indicators of cattle farming, presumably highlighting nitrogen and organic matter enrichment, antibiotic presence and soil acidification.

In summary, the structural differences in microbial communities observed amongst the three stations seemed tightly linked to the chemical groundwater conditions in the stations. Based on a statistical approach and literature in microbial ecology, station-specific taxa were selected as promising candidates for biological indication in karstic aquifers. Other candidate taxa were detected with IndVal but they were not highlighted in the present study because of the limited documentation on their ecology in the literature. However, a full list of these potential indicators is provided in Supporting Information S10.

5 | Conclusions

This study contributes to a better understanding of the interactions between groundwater chemical quality and microbial communities in karstic aquatic ecosystems. This knowledge opens a way to more precise management of groundwater resources through the integration of microbiological tools. The use of artificial substrates, such as clay beads, combined with 16S metabarcoding analyses could improve strategies for monitoring and managing karst aquifers by facilitating the identification of groundwater contaminations with nutrients and organic matter originating from the catchments. Bulk biofilm measurements of biomass and activities did not provide reliable information on nutrients and organic matter contaminations. In contrast, prokaryotic richness and diversity could be used as indicators of an enrichment of groundwater with nutrients and organic matter. The metabarcoding approach permitted identifying potential indicator taxa of groundwater quality for the studied stations, but this approach is still in its early stages. The widespread use of such microbial indicators in biomonitoring approaches for groundwater ecosystems needs to extend our experimental approach to other karstic environments for validation.

Author Contributions

Lina Fabre: investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **Clémentine Francois:** conceptualization, investigation, funding acquisition, methodology, supervision, data curation, formal analysis, writing – review and editing. **Félix Vallier:** investigation, writing – review and editing, resources, formal analysis. **Mailys Gauthier:** investigation, writing – review and editing, formal analysis, resources, data curation. **Emmanuel Malet:** investigation, writing – review and editing, methodology, resources. **Ludovic Guillard:** investigation, writing – review and editing, methodology, resources. **Yves Perrette:** conceptualization, investigation, funding acquisition, writing – review and editing, methodology, supervision. **Florian Mermillod-Blondin:** supervision, data curation, project administration, formal analysis, methodology, validation, writing – original draft, writing – review and editing, funding acquisition, investigation, conceptualization.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data and Supporting Information that support some findings of this study are openly available in Zenodo at <http://doi.org/10.5281/zenodo.17951662>. Sequence data are openly available in the NCBI repository at <https://www.ncbi.nlm.nih.gov/> under the BioProject name PRJNA1390062.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Supporting Information: S1.** Main characteristics of sediments collected in the three stations. **Supporting Information: S2.** (A) Meteorological data (daily precipitation) measured at the weather station of La Feclaz and daily discharge rates measured at the outlet of the Doria karstic system during the experiment and (B) correlation between total precipitation and average discharge rates for the experimental periods of 2 months (9 periods). **Supporting**

Information: S3. Correspondance between samples used in the article (with information for each sample on station, replicate number and period of incubation) and their names for sequenced samples deposited on NCBI database under the BioProject PRJNA1390062. **Supporting Information: S4.** PCoA ordination plot representing differences in prokaryotic community structure between biofilms developed on clay beads (circles) and negative controls (asterisks) based on Bray-Curtis distances before decontamination. The colour of the symbols was relative to the concentration of DNA recovered from each sample after extraction. **Supporting Information: S5.** Rarefaction curves for each sample indicating that 35,243 reads (black dotted vertical line) was selected to obtain an accurate assessment of ASV richness. One sample was suppressed from the analysis as its number of reads was too below (19,764reads, red dotted vertical line). See Supporting Information 3 for correspondence between the codes displayed in the figure and the sample information (station, period, replicate number) in the present study. **Supporting Information: S6.** Chemical and biofilm variables measure during the study. DOC=Dissolved Organic Carbon, PD=Phylogenetic Diversity. **Supporting Information: S7.** Interactive Krona chart of the biofilm community in station A. **Supporting Information: S8.** Interactive Krona chart of the biofilm community in station B. **Supporting Information: S9.** Interactive Krona chart of the biofilm community in station C. **Supporting Information: S10.** Main results obtained with Indval to select indicators at the Family level for each station. The taxa having significant *p*-values but also Indval values > 0.7 are indicated in bold. **Supporting Information: S11.** Picture from Doria cave close to station A done on 24/01/2024 after a flood. Black material corresponds to vegetal debris supplied to the cave during the flood event. **Supporting Information: S12.** Average relative proportions of the family Nitrosopumilaceae in the prokaryotic community for all sampling periods and the three stations.