



In situ incubation of iron(II)-bearing minerals and Fe(0) reveals insights into metabolic flexibility of chemolithotrophic bacteria in a nitrate polluted karst aquifer

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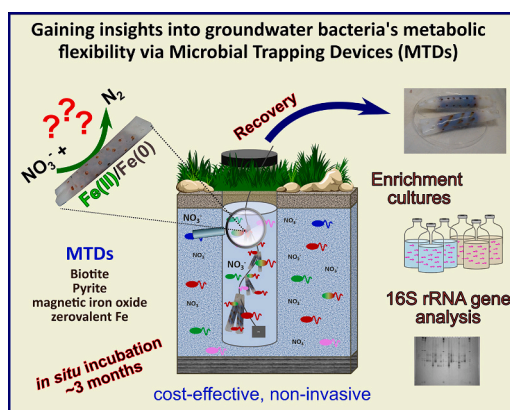
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HIGHLIGHTS

- Testing for the metabolic potential residing within the prevailing microbial communities of contaminated groundwater
- Microbial trapping device (MTD) – a non-invasive, cost-effective tool to enrich and locally stimulate the prevailing microbial community in situ
- MTDs enable enrichment and isolation of microorganisms from groundwater systems
- Comprehensive utilization of chemolithotrophic pathways, including anaerobic Fe(II) oxidation by the aquifer's microbial community

GRAPHICAL ABSTRACT



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ABSTRACT

Groundwater nitrate pollution is a major reason for deteriorating water quality and threatens human and animal health. Yet, mitigating groundwater contamination naturally is often complicated since most aquifers are limited in bioavailable carbon. Since metabolically flexible microbes might have advantages for survival, this study presents a detailed description and first results on our modification of the BacTrap® method, aiming to determine the prevailing microbial community's potential to utilize chemolithotrophic pathways. Our microbial trapping devices (MTDs) were amended with four different iron sources and incubated in seven groundwater monitoring wells for ~3 months to promote growth of nitrate-reducing Fe(II)-oxidizing bacteria (NRFeOxB) in a nitrate-contaminated karst aquifer. Phylogenetic analysis based on 16S rRNA gene sequences implies that the identity of the iron source influenced the microbial community's composition. In addition, high throughput amplicon sequencing revealed increased relative 16S rRNA gene abundances of OTUs affiliated to genera such as

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Thiobacillus, *Rhodobacter*, *Pseudomonas*, *Albidiferax*, and *Sideroxydans*. MTD-derived enrichments set up with Fe(II)/nitrate/acetate to isolate potential NRFeOx, were dominated by e.g., *Acidovorax* spp., *Paracoccus* spp. and *Propionivibrio* spp. MTDs are a cost-effective approach for investigating microorganisms in groundwater and our data not only solidifies the MTD's capacity to provide insights into the metabolic flexibility of the aquifer's microbial community, but also substantiates its metabolic potential for anaerobic Fe(II) oxidation.

1. Introduction

Pristine groundwater systems provide naturally-clean drinking water to approximately 50 % of the world's population (World Water Assessment Programme, 2012). Its protection and remediation is therefore an important subject in environmental research and ongoing political debates (Adelana, 2004; CEC, 1999; European Commission, 2018; World Health Organization, 2011). Groundwater ecosystems are predominantly threatened by anthropogenic activities, leading to high inputs of organic (Bombach et al., 2015; Schwarz et al., 2011) and inorganic (Almasri, 2007; Böhlke et al., 2006) contaminants such as pesticides, metals or nitrogen-bearing species. Particularly fractured and karstified aquifers are known for their high vulnerability towards anthropogenically induced contaminants (Einsiedl and Mayer, 2006).

Karstified aquifers are characterized by a complex network of fractures and fissures which result from natural corrosion of soluble rocks such as limestone and dolomite (Ford and Williams, 2007). The connectivity of these conduit networks leads to an increased heterogeneity and responsiveness towards hydraulic events, increasing the aquifers vulnerability especially towards mobile pollutants (Bakalowicz, 2005; Goldscheider, 2005). Particularly the epikarst and the vadose zone play a major role in the storage and the redirection of vertical infiltrating waters, resulting in the promotion of the formation of biological hotspots, which could enhance biologically induced pollutant reduction (Culver and Pipan, 2014; Jones, 2003; Lian et al., 2011; Pipan and Culver, 2007; Visser et al., 2021). Although groundwater systems are known to harbour diverse microbial communities (Nyyssönen et al., 2014), their ability to attenuate NO_3^- groundwater contamination naturally is often limited. Since most aquifers are oligotrophic environments, bioavailable organic substrates are scarce and low temperatures slow the activity of metabolic reactions additionally (e.g., Goldscheider et al., 2006; Jewell et al., 2016; Kumar et al., 2018). Microbial life in the subsurface is also strongly regulated by the availability of oxygen (O_2). Depending on aquifer type and hydrological conditions, O_2 levels can vary greatly from oxygen saturation to complete anoxia. As shown previously, chemolithotrophy may become a favourable metabolic lifestyle for microbes when both, O_2 and bioavailable carbon, become limiting (Hancock et al., 2005; Herrmann et al., 2015; Jewell et al., 2016; Sierra-Alvarez et al., 2007; Torrento et al., 2010). To gain a deeper understanding of microbial metabolic flexibility and substrate limitation in the subsurface it is necessary to identify dominant microbial key players, as well as to investigate the chemolithotrophic pathways utilized. Common methods applied to access these remote habitats involve groundwater pumping and filtration to collect biomass (Ben Maamar et al., 2015; Herrmann et al., 2017, 2015), as well as drilling (Ginige et al., 2013; Ino et al., 2016; Lazar et al., 2019). While drilling is not only cost-intensive but may also impact the sampling process directly, groundwater pumping will omit microorganisms growing in biofilms or attached to sediment/rock surfaces. To overcome these issues, we applied a modified version of the BacTrap® method (Bombach et al., 2015; Herbst et al., 2013), here called microbial trapping devices (MTDs), to locally stimulate/enrich microbes with certain metabolic pathways and allowing for isolation of microbes from a karstified and nitrate (NO_3^-) contaminated aquifer.

The karst aquifer investigated in this study is characterized by localized high NO_3^- concentrations (≥ 0.8 mM) and was demonstrated to harbour possible hot spots of denitrification within the recharge area, possibly promoting natural NO_3^- remediation due to chemolithotrophic

NO_3^- reduction pathways (Schwientek et al., 2013; Visser et al., 2021). Hydrochemical analysis performed prior to this study revealed that besides low concentrations of dissolved organic carbon (DOC 0.1 to 0.3 mM), dissolved ferrous iron (Fe(II)) has been detected (up to 0.4 mM) (Visser et al., 2021). The potential source of the dissolved Fe(II) detected within the groundwater could either be the Fe(II)-bearing limestones and dolostones, as well as the pyrite crystals (FeS_2) located within the limestone rock matrix or associated with fractures (Osenbrück et al., 2022). Therefore, the conditions prevailing in this ecosystem indeed support chemolithotrophic growth occurring on a microscale (e.g., niches around particles or anoxic hotspots), which might therefore directly facilitate and thus promote natural NO_3^- removal.

Although no genetic evidence for a direct autotrophic, and thus purely enzymatically mediated, process of nitrate-reducing Fe(II) oxidation (NRFeOx) in a pure laboratory culture has been provided so far (He et al., 2016; Klueglein and Kappler, 2013; Nordhoff et al., 2017; Price et al., 2018; Visser et al., 2022), indirect evidence for the existence of an autotrophic mechanism has been presented previously in field studies and various enrichment cultures (Huang et al., 2023, 2022; Jakus et al., 2021a; Laufer et al., 2016; Tian et al., 2020; Tominski et al., 2018). Moreover, microbes known to require an additional organic carbon source (e.g., acetate, succinate) to oxidize Fe(II), have so far been categorized as mixotrophic NRFeOx bacteria (Dopffel et al., 2022; Kappler et al., 2005; Klueglein et al., 2015; Muehe et al., 2009). Again, in most of these mixotrophic cultures, evidence that supports a direct enzymatic pathway, coupling nitrate reduction to Fe(II) oxidation, so far, is lacking (Bryce et al., 2018; Klueglein and Kappler, 2013; Price et al., 2018; Visser et al., 2022). Whether the observed oxidation of Fe(II) in these cultures is caused by an abiotic reaction of Fe(II) with denitrification intermediates (i.e., nitrite), by e.g., *c*-type cytochromes within the excreted extrapolymeric substances (EPS) (T. Liu et al., 2018), or indeed by an indirectly executed enzymatic mechanism (Dopffel et al., 2022), remains elusive. Hence, some bacterial cultures, which have been described before as mixotrophs, are now referred to as chemodenitrifiers (Kappler et al., 2021). Nevertheless, considering that only limited or no organic carbon substrate is required for mixotrophic/autotrophic NRFeOx, both processes represent possible pathways to not only ensure microbial survival but also to naturally decrease NO_3^- contamination in groundwater.

This study seeks to present a detailed design of a cost-effective and non-invasive method to locally stimulate microbes of interest at groundwater monitoring wells, as well as to report the first results obtained for a test campaign conducted in 2015/16. The campaign aimed to (i) investigate the microbial community composition attached to the MTDs after exposure to the groundwater microbial community for three to four months and to (ii) to determine and possibly enrich potential microbial key players with focus on autotrophic and mixotrophic bacteria capable to couple NO_3^- reduction to Fe(II) oxidation. The gained results from this test, as well as from subsequent publications (see Jakus et al., 2021a, 2021b), provide sufficient evidence for the methods functionality to provide not only novel model cultures to study NRFeOx in karstic aquifers, but also to gain valuable insights in the extent of their metabolic flexibility.

2. Material and methods

2.1. Study catchment

As described in detail in Visser et al. (2021), the karstified and fractured Upper Muschelkalk aquifer, is located in the “Oberes Gäu”, a landscape situated approximately 30 km southwest of Stuttgart within the Southwest German Triassic Scarp lands (Fig. 1A). Part of this regional aquifer underlies the catchment of the Ammer River, which originates from karstic springs close to the city of Herrenberg (48° 35′ 42.133″ N, 8° 52′ 1.767″ E) and enters the Neckar river after ~23 km at the city of Tübingen (48° 31′ 17.891″ N, 9° 3′ 27.521″ E) (Grathwohl et al., 2013; Liu et al., 2013; Ludwig et al., 2003). Here, we focused on the major (30 km²) western section of the aquifer, situated west of Herrenberg (Fig. 1B). The study site, and indeed the entire Ammer catchment, is dominated by agriculture (~ 67 %) with a small contribution of forested areas (~ 18 %) (Grathwohl et al., 2013; Y. Liu et al., 2018). With a population density of roughly 600 inhabitants/km², possible inputs from urban areas (15 %) also need to be considered (Grathwohl et al., 2013; Y. Liu et al., 2018). NO₃⁻ and other compounds are released into the karstic groundwater system mainly by intense agriculture. Other possible sources of N-contaminants include leakages from sewer systems from farms or urban areas, however, the majority of which are located downgradient of the monitoring wells used in this study. The study area is part of a large water protection zone, supplying >150,000 people with drinking water from the Upper Muschelkalk aquifer.

Geologically, the area is characterized by Triassic rock formations of the Upper Muschelkalk (mo) and the Lower (ku) and Middle Keuper (km). Fractured and partly karstified limestones and dolomites with a total thickness of 80 to 90 m constitute the Upper Muschelkalk (Villingner, 1982). Porous to cavernous dolomites and dolomitic marls form the base of the aquifer, which overlie the clayey subsidence residues of the evaporite-dominated formations of the Middle Muschelkalk (mm). Karstification of the Upper Muschelkalk (mo) resulted in the presence of numerous dolines (Fig. 1B), which provide pathways for rapid infiltration of water to greater depths. Overall, micritic limestones with a low to intermediate porosity (0.5 to 10 %) characterize the Upper Muschelkalk in the study area. Small pyrite crystals (FeS₂) with concentrations of up to 2 mass-% are present within the limestone matrix or in fractures. Fe (II)-bearing dolostones (saddle dolomites) are commonly found in the Muschelkalk formation (Osenbrück et al., 2022). The organic carbon content of the limestone is reported to be low, ranging from <0.06 mass-% (Visser et al., 2021) to 0.12 mass-% (Osenbrück et al., 2022).

Groundwater access is provided by two karstic springs (Ammer springs (AMQs)), six groundwater monitoring wells near Haslach (Has1-Has6), and seven groundwater monitoring wells near Sulz am Eck (Sul1–8). Groundwater levels within the monitoring wells of Haslach and Sulz am Eck ranged between 35 and 100 m below the surface since both sites are located at the plateau. The screening sections of the wells range from 8 to 10 m length, except for the deepest wells in Sulz, featuring a longer screen length of 20 m. Three monitoring wells in Halsach (Has2, 4, 5) and three monitoring wells in Sulz am Eck (Sul1, 3, 4), as well as the artesian well (ArtAlt) in the confined part of the aquifer, situated in the east of the town of Herrenberg, were used for MTD insertion and in situ incubation. Since the geochemical conditions within the artesian well ArtAlt were different compared to the other monitoring wells, ArtAlt was chosen for comparison.

2.2. Microbial Trapping Devices

The MTDs presented, as well as used in subsequent publications (see Jakus et al., 2021a, 2021b), were designed after Stelzer et al. (2006) and modified to meet the requirements for the respective study. Inert Teflon tubing (VWR, Ø 1.5 cm) was cut into seven cm long sections and perforated to produce a sieve-like structure with holes 0.3 cm in

diameter, ensuring water percolation (Fig. 2). To stimulate and enrich potential mixotrophic/autotrophic NRFeOx, autoclaved sections (121 °C, 20 min) were filled with a mix consisting of one defined Fe-mineral and cleaned (acid washed, muffled) quartz sand grains (Ø 0.75 to 3 mm; see Fig. B1A). The latter was added to mimic a heterogeneous and thus more natural system further promoting water percolation within the MTDs. MTDs were closed with ceramic wool plugs and Teflon cords. Four different Fe-bearing minerals were chosen as possible electron donor for NO₃⁻ reduction: Magnetic iron oxide (mio-) coated sand (Fe(II)/Fe(III), Ø ~3 mm, Fig. B1A), zero valent iron (Fe⁰, Ø 3 to 8 mm, ST37 steel spirals, ETH Zürich, Fig. B1C), pyrite (Fe(II), Ø ~ 3 mm, Peru, with quartz impurities, Fig. B1D), and Fe-rich biotite flakes (Fe(II), Ø ~ 2 to 8 mm, Arendal, Norway, Fig. B1E). For MTD addition, neither acid washing nor heat sterilization were applied to avoid mineral alteration. Only the mio-coated sand was synthesized under sterile and anoxic conditions (protocol provided in A1). Mineral characterization for the biotite and magnetic iron oxide coated sand was performed to deduce valence state of Fe and approximate content (see A2, Figs. B2, B3, Tables B1–2). In addition, MTDs filled with only small pieces of limestone rock were added as controls. The limestone rock was neither acid washed nor otherwise treated. Filled MTDs were attached to a stainless-steel wire and stored anoxically in a sterile 1 L Schott bottle until being deployed into the wells (Fig. 2).

MTDs were deployed in duplicates in groundwater monitoring wells within the proximity of the limestone quarries in late December 2015 and incubated in situ until the end of April 2016. In ArtAlt, MTDs were deployed in February 2016 and incubated until May 2016 (Table B3). Additional information on the detailed sampling procedure is provided in A3. After approximately four months for the six groundwater monitoring wells and three months for ArtAlt, the MTDs were recovered, brought back to the laboratory under anoxic conditions at 4 °C and processed as shown in Fig. 2. Dissolved oxygen (DO), pH, temperature (Tw) and specific electrical conductivity (SEC) were measured in the field using hand-held probes (WTW GmbH) inserted to a flow-through cell (Table 1).

2.3. Enrichment cultures

Based on a classical serum bottle-based incubation approach, 448 cultures (prime enrichments, PEs) were incubated using the internal material obtained from the MTDs to enrich nitrate-reducing Fe(II)-oxidizing bacteria (Fig. B4). Half of the PEs were amended with 2 mM Fe (II)Cl₂ and half of them with either biotite (0.5 g), pyrite (1 g), mio-coated sand (1.2 g) or Fe⁰ spirals (0.5 g). The latter was performed to provide additional surfaces for growth and to test for possible mineral-degrading capabilities. For all cultures, anoxic low phosphate medium (see protocol in A4) was used. Under anoxic conditions (glovebox, MBraun, 100 % N₂), MTDs were separated and the internal material (sand plus Fe-mineral mix) was added to the serum bottles as inoculum (~1 g/50 mL). Teflon tubing, cord and ceramic wool plugs were stored in 50 mL Falcon tubes at -80 °C until further processing.

All PEs were amended with either high (2 mM) or low (0.1 mM) NaNO₃ and grown at heterotrophic (acetate; high: 1 mM, low: 0.05 mM) or autotrophic (no additional carbon source) conditions (see Fig. B4). All PEs were incubated in the dark and at 25 °C. Growth was regularly verified either via testing a decrease in NO₃⁻ concentrations (NO₃⁻ test strips, MERCK) or visually by microscopic observation of viable cells in the enrichments by fluorescence microscopy (LIVE/DEAD staining; Leica Dm5500 B) (Laufer et al., 2017; Mauerofer et al., 2018). Transfer of the PEs (4 % inoculum) depended on the amount of viable cells observed and NO₃⁻ reduced (-75 %). The presence of Fe minerals did not seem to influence growth performance, hence, PE transfers, formerly grown in the presence of the respective minerals, were subsequently amended with 2 mM FeCl₂ instead. PEs/enrichments with low viable cell counts, no Fe(II) oxidation but strong NO₃⁻ reduction capability were henceforth grown in the absence of Fe(II) to test for heterotrophic

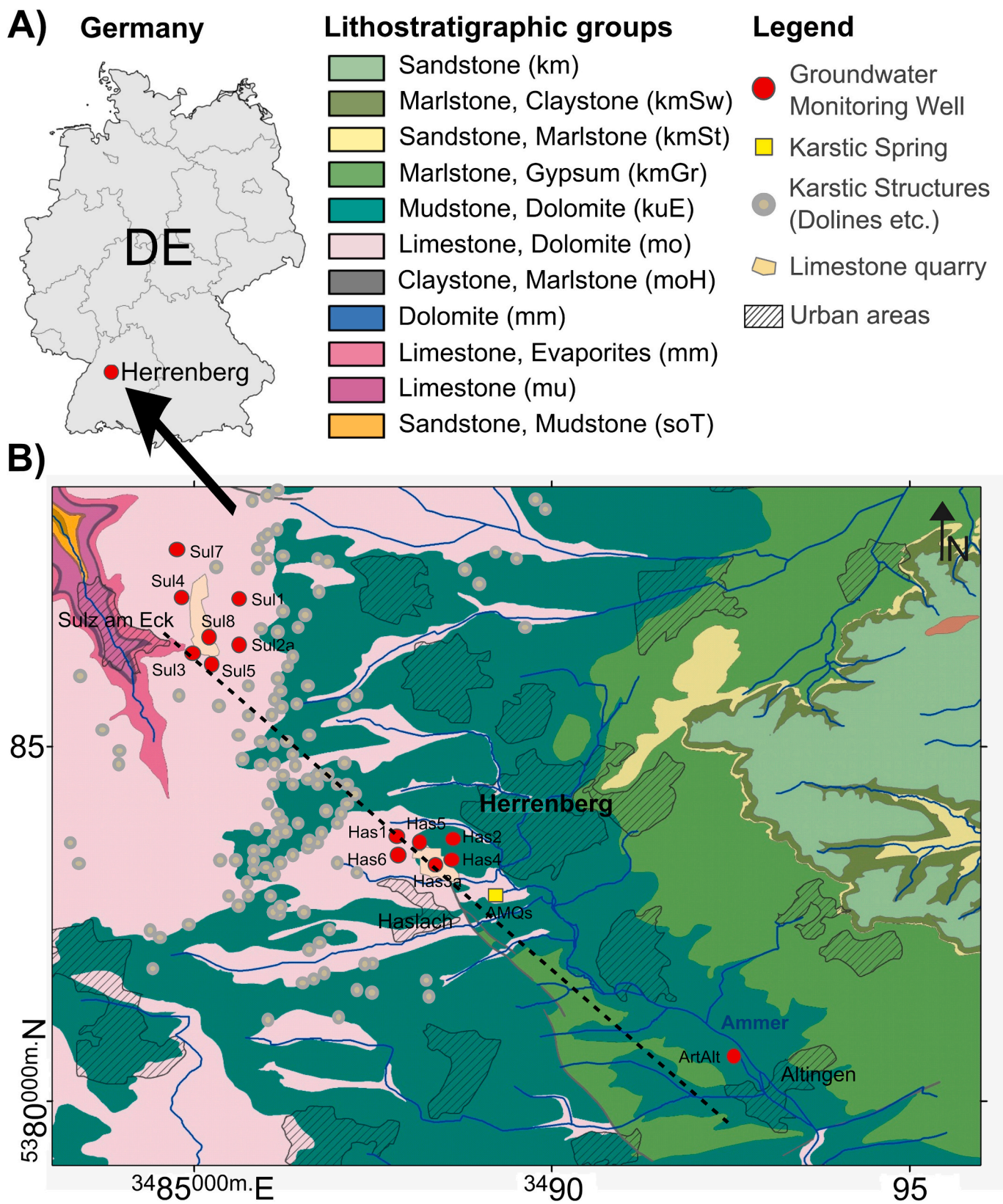


Fig. 1. Map visualizing the location of the city of Herrenberg in Germany (A); Lithostratigraphic map of the study catchment including Gauß-Krüger coordinates (B) - Red dots represent groundwater monitoring wells at the major study sites of Sulz am Eck (Sul), Haslach (Has), as well as the artesian well Altingen (ArtAlt). Dolines and other karstic structures are depicted as grey/beige dots. Yellow squares represent karstic springs. As indicated, Sulz am Eck and Haslach are both located in the vicinity of limestone quarries. See also Visser et al. (2021).

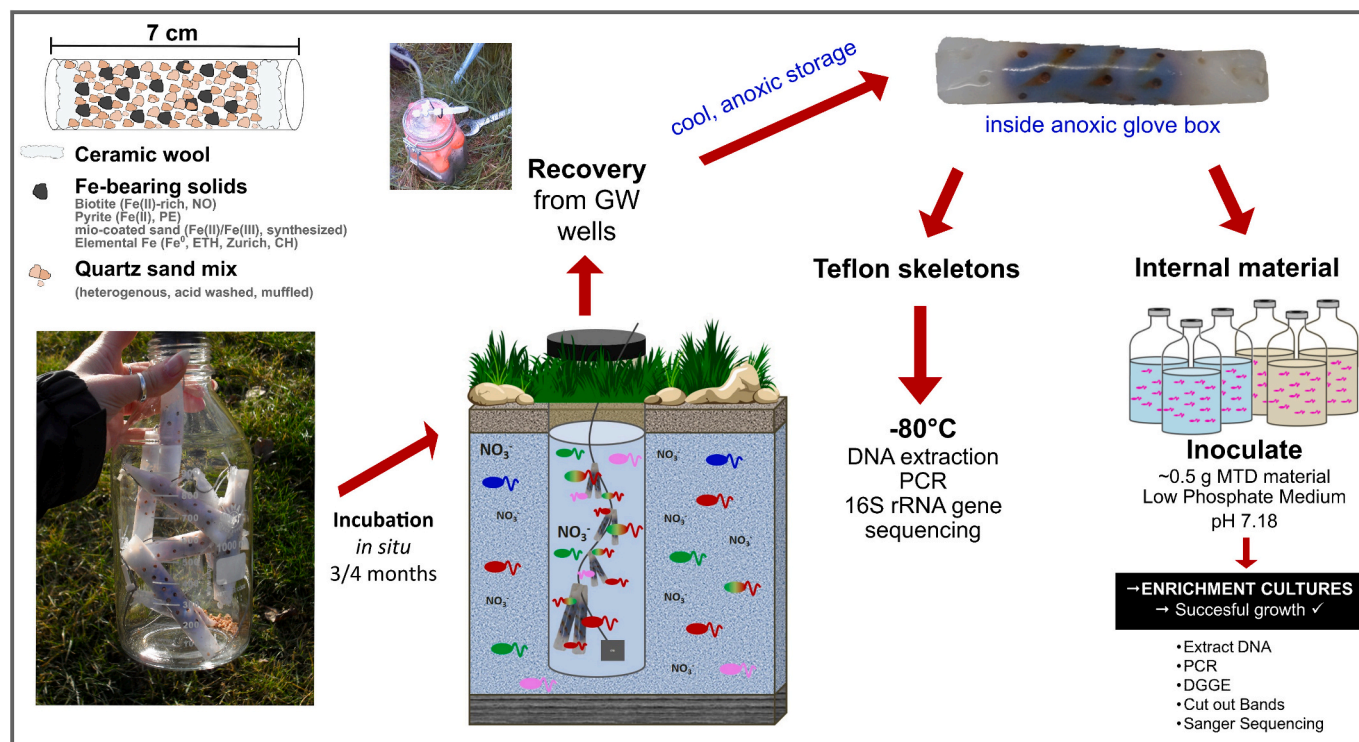


Fig. 2. Schematic illustration depicting microbial trapping device design and sample handling after recovery from the groundwater monitoring wells. First, a mix of Fe-bearing solids and heterogenous quartz sand grains was filled into seven centimetre long Teflon tubes to act as growth surface for the microbial community. Teflon tubes were perforated with ~ 40 holes (\varnothing 3 mm) to enable water percolation. MTDs were retrieved from monitoring wells and transported (anoxic, cooled) to the lab and opened within an anoxic glove box. The MTDs were then separated into Teflon skeleton and internal material, whereas the latter was further processed for an immediate set up of enrichment cultures (see A2).

Table 1

Major physicochemical parameters of the groundwater given as average \pm standard error. Temperature, O_2 and SEC were measured via continuous data loggers. At both sites, Sulz am Eck (Sul) and Haslach (Has), three wells were equipped with the MTDs, however, wells Sul2^a and Has4^b are excluded, since the probes were compromised.

Site/parameter	Sul	Has	ArtAlt*
Temperature [$^{\circ}$ C]	10.1 \pm 0.003	11.0 \pm 0.004	19.3
pH	7.3 \pm 0.1	7.3 \pm 0.1	7.4
SEC [μ S/cm]	635 \pm 1.6	708 \pm 2.7	899 \pm 41
O_2 [μ M]	212 \pm 0.06 ^a	180 \pm 0.16 ^b	<20
E_h [mV]	361 \pm 21	316 \pm 9	-42
NO_3^- [mM]	0.67 \pm 0.13	0.32 \pm 0.09	0.01
DOC [mM]	0.10 \pm 0.01	0.09 \pm 0.01	<0.08
Fe(II) [mM]	0.002–0.05	0.002–0.4	<0.002

Note: Hydrogeochemical parameters obtained in winter 2015/2016 are part of a large dataset previously published in Visser et al., Hydrogeology Journal (<https://doi.org/10.1007/s10040-021-02314-2>).

* Values for the artesian well at Altingen (ArtAlt) are single measurements.

denitrification. PEs/enrichments with no or very few viable cells and neither Fe(II) oxidation or NO_3^- reduction were not further transferred.

2.3.1. Metabolic flexibility testing of enrichment cultures by altering the carbon substrate

To test metabolic flexibility with regards to the carbon source, enrichments with a different microbial composition, based on their DGGE results (Table 2), that were also able to mixotrophically oxidize Fe(II) even after being transferred up to eight times, were chosen. Five cultures (ArtAlt_mio, Sul4_pyrite, Sul4_Fe0, Has2_pyrite, Has5_biotite; see Table 2) were cultivated in the presence of 4 mM Fe(II), 4 mM $NaNO_3$ and on 2 mM of an organic co-substrate (acetate, succinate, lactate, propionate, butyrate, glucose or ethanol). For each substrate, a control

cultivated in the absence of Fe(II) was used as base for comparison (see also Fig. B5). All batch cultures were grown on low phosphate medium (protocol A4). Incubation was performed at 28 $^{\circ}$ C and in the dark. Cultures were sampled at either four or seven different time points under anoxic conditions (MBraun glovebox, N_2 100%). Fe(II) and Fe(tot) concentrations in the liquid and the solid phase were quantified using a Ferrozine assay protocol revised for nitrite (NO_2^-)-containing samples (Klueglein and Kappler, 2013; Schaedler et al., 2018). NO_3^- and NO_2^- were quantified using continuous flow analysis (AA3 HR, SEAL Analytics) based on DIN 38405/ISO 13395. Organic acids were determined via HPLC (Shimadzu). Further details are provided in the Supplementary information (A4).

2.4. Molecular biology based methods

2.4.1. DNA extraction and DGGE

DNA was extracted from cultures successfully reducing NO_3^- and/or oxidizing Fe(II) even after the 7th/8th transfer, by using the MoBio PowerSoil Kit (PowerSoil DNA Isolation Kit, MoBio). For samples containing Fe(III) (oxyhydr)oxides, however, prior to bead beating, washing steps with an oxalate solution (28 g ammonium oxalate +15 g oxalic acid per litre; adjusted to pH 3), as well as tris-EDTA buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA), were applied (Nicomrat et al., 2006). Bacterial 16S rRNA genes were amplified using primers (GC-)341F and 907R and separated using denaturing gradient gel electrophoresis (DGGE) fingerprinting (Muyzer et al., 1995). From 13 enrichment cultures, the dominant bands were then excised (Fig. B6) and sent away for Sanger sequencing (GATC, Konstanz). NCBI BLASTn (Madden, 2013) as well as the SILVA web aligner (<https://www.arb-silva.de/aligner/>) SILVA ribosomal database (V 128) (Pruesse et al., 2012; Quast et al., 2012) were used to for taxonomic assignment of dominant taxa in the enrichments. Here we focus on the results of those enrichments that showed high Fe

Table 2

Overview of the BlastN results obtained from the sequences of the excised DGGE bands for Fe(II)-oxidizing and nitrate-reducing enrichment cultures. *E*-value for all equals zero. A summary of the blast search is provided in Supplementary material.

Batch culture ID (wellID_mineral)	Sequence ID	BlastN-accession # ^{a,b}	Closest relative	%Identity	BlastN-accession #
ArtAlt_mio ^a	<i>ArtAlt_mio</i>	SAMN36715043	<i>Acidovorax defluvii</i> strain C2-3	100	MT279968.1
Sul3_pyrite	<i>Sul3_Band1_pyrite</i>	SAMN36715044	<i>Acidovorax defluvii</i> strain HS1-68	100	MK396574.1
			<i>Acidovorax defluvii</i> strain C2-3	100	MT279968.1
Sul4_mio ^a	<i>Sul4_Band1_mio</i>	SAMN36715045	<i>Acidovorax defluvii</i> strain HS1-68	100	LR638215.1
			<i>Propionivibrio limicola</i>	100	MT000014.1
Sul4_pyrite	<i>Sul4_pyrite</i>	SAMN36715046	<i>Rhodocyclus</i> sp. HOD 5	100	AY691423.1
			<i>Propionivibrio militaris</i>	100	AY530552.1
			<i>Paracoccus yeii</i>	98.41	MW314786.1
			<i>Paracoccus</i> sp.	98.41	MG685697.1
Sul4_Fe0 ^a	<i>Sul4_Band1_Fe0</i>	SAMN36715050	<i>Azospira</i> sp. 109	100	AP021844.1
			<i>Sul4_Band2_Fe0</i>	SAMN36715051	<i>Azospira oryzae</i>
Has2_pyrite ^a	<i>Has2_pyrite</i>	SAMN36715049	<i>Propionivibrio limicola</i>	99.44	MT000014.1
			<i>Rhodocyclus</i> sp. HOD 5	99.44	AY691423.1
			<i>Propionivibrio militaris</i>	99.4	NR_125528.1
			<i>Acidovorax</i> sp.	100	MT255184.1
			<i>Acidovorax</i> sp. W20	100	KF560400.1
Has5_biotite ^a	<i>Has5_biotite</i>	SAMN36715047	Elbe River snow isolate Iso34	100	AF150707.1
			<i>Paracoccus</i> sp.	100	MN547319.1
			<i>Paracoccus yeii</i>	100	CP031078.1

^a Marks most potent Fe(II)-oxidizing enrichment cultures.

^b Uploaded on NCBI 26.07.23 under project PRJNA814479.

(II) oxidation capabilities. These sequences were also published at NCBI under the accession number PRJNA814479.

DNA was extracted from the Teflon skeletons and the ceramic wool using CTAB (Sigma Aldrich) and Phenol/Chloroform extraction according to Griffiths et al. (2000). DNA was successfully extracted from 28 skeletons of the 56 in situ incubated MTDs. Skeletons were separated into three sections (Fig. B7) and treated separately yielding a total amount of 80 samples, of which the DNA extracts were later pooled together depending on their origin and amended Fe-source. DNA yields were quantified by using NanoDrop (Thermo Scientific™) or Qubit (Thermo Scientific™). DNA extracts were stored at -20°C until further processing.

2.4.2. Phylogenetic analysis of the MTD skeleton biofilms

Phylogenetic analysis of microbes present on the Teflon skeletons was performed based on the partial 16S rRNA gene. To determine the local influence of each mineral amended on the microbial community, obtaining DNA from a full set (all four minerals) from at least one well was necessary. However, albeit biofilm formation upon MTD skeletons being observable for most MTDs recovered (for example see Fig. B8), PCR success was rather low. Sufficient DNA yields and PCR success on MTD skeletons from all four minerals were only achieved in well Sul1, thus only those were chosen for further analysis. The DNA extracts were prepared for amplicon library production and high throughput sequencing (Quantitative Genomics Facility, Basel, CH). A two-step PCR approach was applied to prepare the library. A first PCR of 25 cycles was performed using primers 515F—Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'), targeting the V4-V5 regions of the 16S rRNA gene (Parada et al., 2016). Sample indices and Illumina adaptors were added in a second PCR of 8 cycles. Purified indexed amplicons were finally pooled at equimolar concentration into one library and sequenced on an Illumina MiSeq using the 250 bp paired-end protocol (V2). Details of the sequence read treatment can be found elsewhere (Su et al., 2020; Weber et al., 2018). Briefly, quality control of the raw reads was carried out using *FastQC* (Andrews, 2010). Then, reads were merged into amplicons using *flash* (Magoč and Salzberg, 2011) and primer sites were trimmed using *cutadapt* (Martin, 2011). Trimmed and quality filtered (prinseq; Schmieder and Edwards, 2011) sequences were clustered into operational taxonomic units using *USEARCH* (Edgar, 2017, 2010), based on a 97 % sequence similarity threshold. We used *SINTAX* (Edgar, 2016) for taxonomic predictions based on the *SILVA* SSU database v128 (Quast et al., 2012). Subsequent

analysis of 16S rRNA gene sequence data was done in R (R Core Team, 2014) using the *phyloseq* package (McMurdie and Holmes, 2013). All sequences obtained are published at NCBI under the accession number PRJNA814479 and their visualisation as Krona charts is presented in the supplements.

3. Results

3.1. In situ incubation conditions at the study site

Major physical parameters such as temperature, specific electrical conductivity (SEC), pH and dissolved oxygen (DO) were measured during the incubation period by CTD probes and averages are given in Table 1 (Visser et al., 2021). CTD-based temperature measurements from Sul and Has ranged between 10.0 and 11.0 °C, except at Sul3 where a temperature of 12.0 °C was detected. The temperature in ArtAlt was measured via a hand-held probe and since the water column was standing at the time of insertion, water temperature detected here was highest with 19.3 °C. Overall, temperatures detected at Sul and Has are close to the mean annual temperature previously reported for the study area (Visser et al., 2021). In addition, average values for redox potential (Eh), ferrous iron (Fe(II)) and NO_3^- are also given in Table 1. The detected circumneutral to slightly alkaline pH falls within the range that has previously been observed in karstified areas (Ford and Williams, 2007; e.g. Pronk et al., 2006). Except for Sul3 ($1474 \pm 8.4 \mu\text{S}/\text{cm}$), SEC values for all three field sites fall within a similar range, with a slight elevation at ArtAlt, but still considered common for karstified areas (Ravbar et al., 2011). Reducing conditions and lowest DO values were detected only for ArtAlt, whereas at Sul and Has, averaged DO levels were relatively high. DO concentrations measured during in situ incubation by the oxygen probes showed fluctuations, generally ranged from ~ 100 to $\sim 260 \mu\text{M}$ at Sul and Has, respectively. Lowest DO concentrations were measured at Sul1 ($< 51 \mu\text{M}$) and Sul4 ($< 20 \mu\text{M}$) (Visser et al., 2021). The DO concentrations detected at Has4 ($\sim 100 \mu\text{M}$), however, were most likely compromised due to sediment contact of the probe near the bottom, since the probe had to be installed close to the bottom of the well to ensure permanent water coverage. DO measured in the standing water column at ArtAlt was below $20 \mu\text{M}$.

NO_3^- concentrations were highest in samples taken from Sul, followed by Has, and were lowest at ArtAlt ($< 10 \mu\text{M}$). Dissolved organic carbon (DOC) concentrations followed a similar trend, and were lowest at ArtAlt (Table 1). Fe(II) concentrations varied strongly between the

different monitoring wells and no Fe(II) was detected in ArtAlt. Our previously published hydrogeochemical facies analysis showed that the groundwater of the area can be classified as $\text{Ca}^{2+}/\text{Mg}^{2+}\text{-HCO}_3^-$ type waters (Visser et al., 2021), and thus is reflecting the dissolution of the calcite and dolomite minerals that form the matrix of the aquifer (see e. g. Einsiedl and Mayer, 2005). Groundwater in wells Sul3 and Sul5 showed elevated concentrations of Cl^- and particularly SO_4^{2-} , which probably is resulting from admixing waters exposed to the underlying evaporite layers of the Middle Muschelkalk (e.g. Blanchette et al., 2010; Warren, 2016). The continuously recorded changes in the hydraulic head levels during the in situ incubation (Visser et al., 2021) showed that wells Sul1, and Has2 in particular, were characterized by large fluctuations in the water table height during the first 2 months. The hydraulic head levels in wells Sul3 and Sul4, were much less variable although Sul4 showed strong short-term fluctuations resulting in reoccurring peaks in hydraulic head levels (Visser et al., 2021). Overall, 2015 was a hot and very dry year for SW Germany, leading to a lowering

in the groundwater table by approximately 2 m. The dry summer/autumn was followed by a dry winter 2015/16, resulting in less water available for groundwater recharge. This extreme situation even led to an “inactivation” of the ArtAlt, resulting in a standing water column in the well.

3.2. Molecular biological analysis of the MTD skeletons

DNA extracts, yielding 6 to 72.5 ng/ μL , were obtained from 22 out of 28 MTDs. DNA extraction failed for MTDs filled with limestone rock material only, which were intended as controls. PCRs of DNA acquired from MTDs containing all four minerals were only successful at well Sul1. Illumina sequencing was therefore only performed for samples of well Sul1. All samples, independent of the mineral amended, were ~ 99 % dominated by Bacteria and ≤ 1 % were affiliated with Archaea. Based on the relative 16S rRNA gene abundance of the 50 most abundant family OTUs (97 %), slight variations in the microbial community

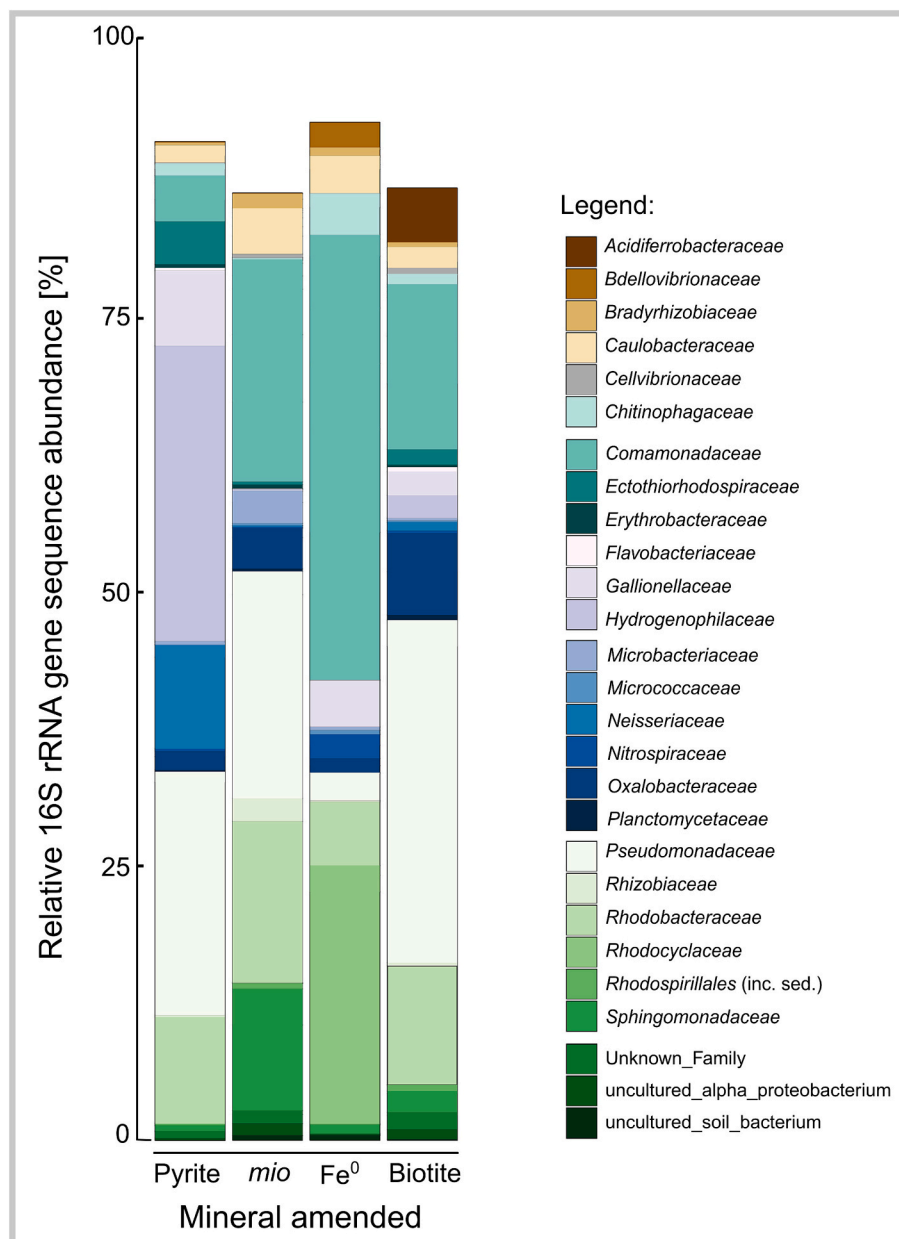


Fig. 3. Relative 16S rRNA gene abundance of the 50 most abundant family OTUs (97 %) in well Sul1. DNA was extracted from the MTD Teflon skeletons and analysed by Illumina MiSeq V2 sequencing; *mio* = magnetic iron oxide coated sand.

composition were observed depending on the mineral present within the MTD (Fig. 3). In all four Fe-mineral amendments, *Pseudomonadaceae*, *Comamonadaceae*, and *Rhodobacteraceae* were present, however, their relative abundance varied depending on the mineral added (Fig. 3). In the pyrite amended setup, *Hydrogenophilaceae*, *Neisseriaceae*, and *Gallionellaceae* had increased relative abundance compared to other amendments (Fig. 3). In the presence of mio-coated sand, families including *Sphingomonadaceae*, *Rhodobacteraceae*, and *Caulobacteraceae* showed increased relative abundance in comparison to the other setups. In situ incubations with zero valent iron (Fe^0) resulted in higher relative abundances of OTUs associated to the family *Rhodocyclaceae* and were dominated by *Comamonadaceae* (~30 %). In contrast to the other three minerals, biotite-containing MTDs were dominated by *Pseudomonadaceae* (up to 35 %) and to a lesser extent by *Comamonadaceae*. When compared to the other minerals, biotite stimulated families such as *Oxalobacteraceae* and *Acidiferrobacteraceae*, which were either present at much lower relative abundances or were not detected in the other setups.

At genus level, in the presence of pyrite, *Thiobacillus* (27 %) and *Pseudomonas* (~20 %) were dominant, followed by *Sideroxydans* (~7 %) and *Rhodobacter* (10 %) (Fig. 4). MTDs amended with mio-coated sand, however, led to an increase in the relative abundances of *Pseudomonas* (21 %), *Rhodobacter* (15 %), and *Sphingobium* (11 %). In addition, mio-coated sand also resulted in the detection of OTUs affiliated to the genus of *Albidiferax* (19 %, previously *Rhodiferax*) (Fig. 4). Fe^0 amended MTDs appeared to be more complex with regards to the diversity of the sample. Results obtained indicate that the sample was dominated by OTUs affiliated to the genera *Hydrogenophaga* (14 %), *Albidiferax* (~20 %) and *Dechloromonas* (22 %). In addition, genera such as *Sideroxydans* (4 %), *Rhodobacter* (6 %) and *Leptothrix* (~7 %) as well as *Sphingobium*, *Pseudomonas*, *Massilia* and *Caulobacter* (<10 %), were also present (Fig. 4). Biotite amended MTDs were dominated by OTUs affiliated with genera such as *Pseudomonas* (>30 %) followed by *Rhodobacter* (11 %), and to a lower extent (<10 %) by *Thiobacillus*, *Sphingobium*, *Sideroxydans* and *Massilia* (Fig. 4).

3.3. Enrichment culture growth and Sanger sequencing results

Of 448 initially inoculated PE cultures (see Fig. B4), only 123 showed viable cells (DEAD/LIVE staining) and NO_3^- reduction during/after an incubation of three to six weeks. As illustrated in Fig. 5, for PEs inoculated on the respective minerals but in the absence of FeCl_2 , no growth was observed. In PEs amended with 2 mM FeCl_2 and 2/0.1 mM nitrate, Fe(II) oxidation was observed in 33 cultures, of which 21 were grown in the presence of the organic co-substrate acetate and 12 in the absence of an additional carbon source (N_2/CO_2 headspace only, see Fig. 5). The ability to grow in the absence of an additional carbon source (autotrophically) and to successfully oxidize Fe(II) , however, was lost in all autotrophic enrichments after the 2nd or 3rd transfer. 90 PEs, that were amended with FeCl_2 and an organic acid, showed NO_3^- reduction but no obvious ability to oxidize Fe(II) . Furthermore, active NO_3^- reduction and moderate amounts of viable cells were found in 47 PEs although no additional carbon source was provided. The moderate viability of the cells observed in these cultures presumably resulted from organic remnants that were transferred with the inoculum. All 90 PEs were further transferred as cultures solely performing denitrification (i.e. in the absence of Fe(II) but in the presence of acetate). Transfers derived from the successfully grown PEs, that were later grown in the presence or absence of Fe(II) , were successfully cultivated for up to nine transfers.

After the 8th or 9th transfer, 13 enrichment cultures grown in the presence of Fe(II) , NO_3^- and acetate (4/4/2 mM) still showed strong Fe(II) -oxidizing capabilities and were therefore chosen for further molecular biological analysis. DNA was extracted from duplicate serum bottles and screened using DGGE (Fig. B6). For 7 of the 13 enrichments, apparent differences were observed between the number and positioning of the bands on the DGGE gel, hence bands from the 7 enrichments were excised and prepared for sequencing. Sanger sequencing results showed that the enrichment cultures obtained from the PE ArtAlt_mio contained bacteria that were 100 % similar to two *Acidovorax delafieldii* strains (Table 2). The same results were observed for enrichments derived from PE Sul3_pyrite and Has2_pyrite. Has5_biotite

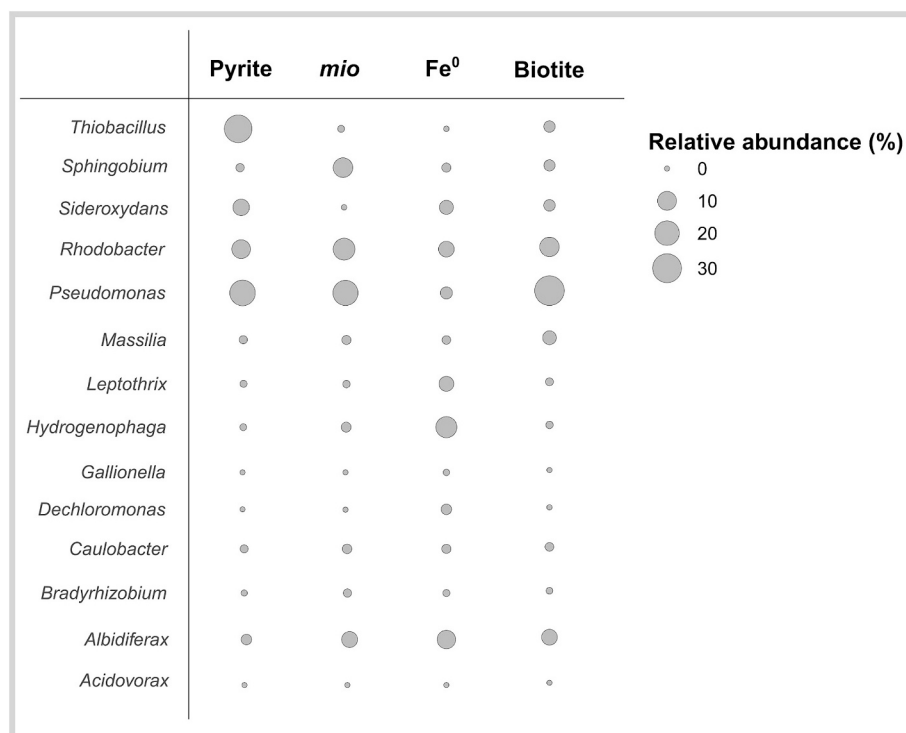


Fig. 4. Relative abundance of OTUs at the genus level in well Su11. DNA was extracted from MTD Teflon skeletons and analysed via Illumina MiSeq V2 sequencing; mio = magnetic iron oxide coated sand.

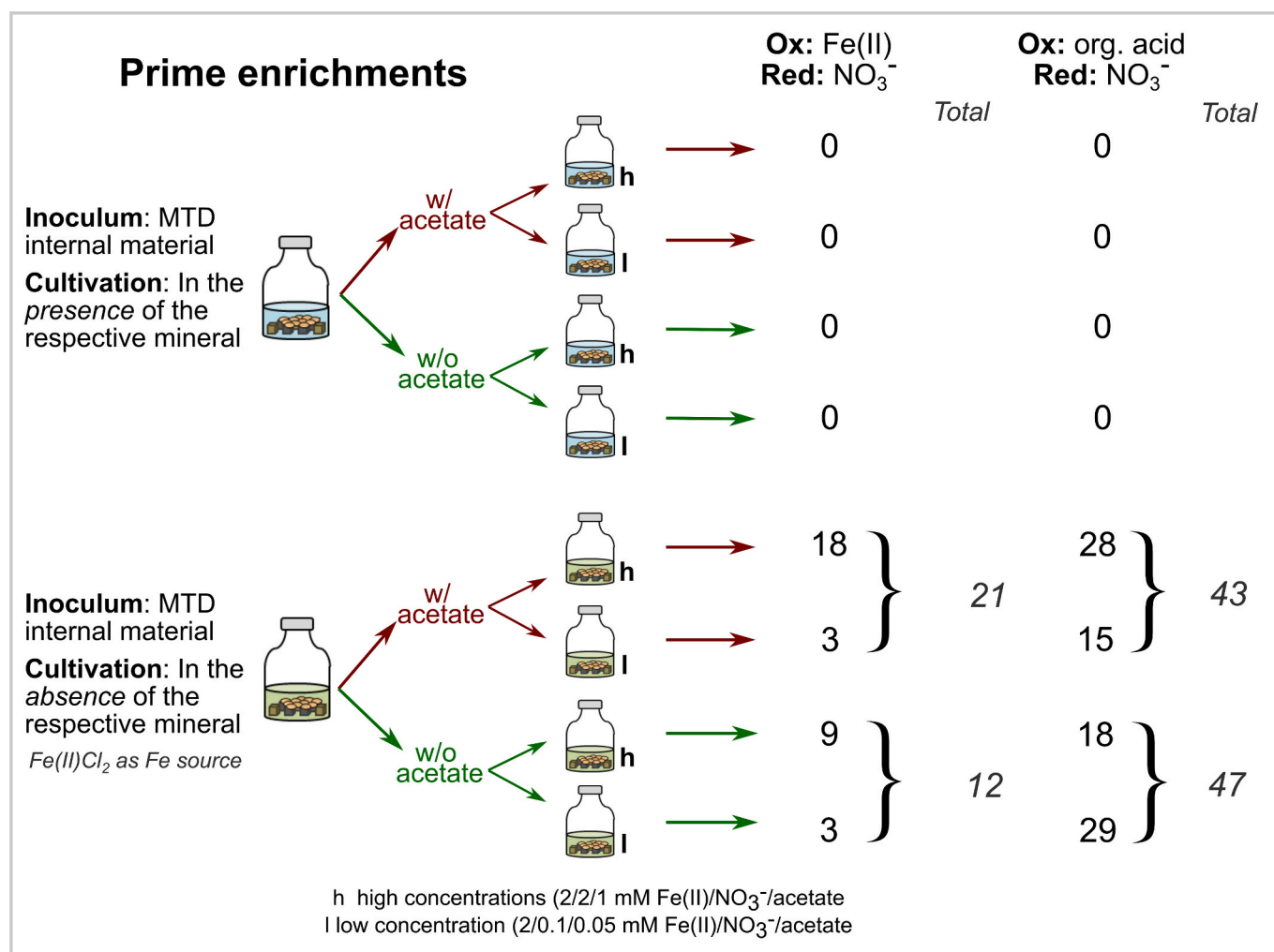


Fig. 5. Schematic illustration of the cultivation progress and success for the prime enrichment (PE) cultures. Minor to no growth was observed for batches inoculated on the respective mineral as major iron source. In contrast, successful growth was observed in the presence of $Fe(II)Cl_2$. $Fe(II)$ oxidation was observed in 21 cultures grown with acetate and 12 cultures without acetate. No $Fe(II)$ oxidation but nitrate reduction was observed in 90 prime enrichment cultures, of which 47 grew even if no acetate was amended initially.

derived enrichments contained bacteria affiliated to *Paracoccus* spp., similar to the Sul4_pyrite enrichments. Sul4_mio enrichments harboured relatives of two *Propionivibrio* strains and a *Rhodocyclus* sp. The Sul4_Fe0 enrichments had the highest diversity even after several transfers, harbouring sequenced 16S rRNA gene fragments that were similar to different *Azospira* strains, a *Propionivibrio* strain as well as a *Rhodocyclus* sp. strain (Table 2). Overall, enrichments obtained from ArtAlt and groundwater monitoring wells Has2 and Has5 were less diverse

compared to enrichments obtained from well Sul4. Furthermore, diversity appears not to be limited by the mineral originally amended via the MTD.

3.4. Metabolic flexibility experiments on the enrichment cultures

From the seven cultures shown in Table 2, only the enrichments yielding highest $Fe(II)$ oxidation potential were further investigated to

Table 3

Average amount of NO₃⁻ reduced per max. 250 h (in mM) within the five enrichment cultures under heterotrophic (H) and mixotrophic (M) growth conditions. In all cultures grown under mixotrophic conditions, $Fe(II)$ oxidation has been observed. Observed minor NO₃⁻ reduction (although $Fe(II)$ was oxidized and the organic acid fully consumed) is marked with -; no growth is marked with +.

Enrichment	ArtAlt		Has2_pyrite		Has5_biotite		Sul4_mio		Sul4_Fe0	
	H	M	H	M	H	M	H	M	H	M
Acetate	2.4	3.6	2.6	2.6	2.4	2.0	2.5	2.7	0.9 ⁺	2.5
Butyrate	3.2	3.9	4.1	3.4	2.3	3.6	3.8	4.0	1.3	3.6
EtOH	2.8	4.0	4.1	3.8	3.0	3.2	3.0	3.0	4.2	4.1
Glucose	4.1	4.0	3.5	2.8	0.4	3.0	3.7	2.7	4.1	0.09 ⁻
Lactate	4.4	3.2	1.2	1.2	2.9	1.4 ⁻	2.9	4.3	5.6	3.3
Propionate	4.1	3.6	3.3	4.7	3.2	1.7	3.4	2.6	3.4	3.7
Succinate	3.8	3.4	4.0	3.3	3.4	3.6	3.4	3.8	3.6	3.6

Heterotrophic setup: ~4/2 mM NO₃⁻/organic acid; mixotrophic setup: ~4/4/2 mM $Fe(II)$ /NO₃⁻/organic acid.

determine the impact of varying the organic co-substrate on Fe(II) oxidation (details for the cultures provided in Table B4). Most enrichments showed the ability to utilize the carbon co-substrates provided resulting in the successful reduction of NO_3^- and partially in the oxidation of Fe(II) (Table 3, Fig. B5). The ArtAlt_mio enrichment (100 % similarity *Acidovorax* spp.) reduced NO_3^- mixotrophically regardless of the organic acids supplied (Tables 3, B4; Fig. B9). Conversely, the enrichment originating from Has2_pyrite (100 % similarity to *Acidovorax* spp.), seemed to be inhibited to some extent by lactate but nevertheless oxidized Fe(II) in all treatments (Table 3). The enrichment culture from Has5_biotite (100 % 16S rRNA gene sequence similarity to *Paracoccus* spp.) showed slightly lower NO_3^- reduction levels and under heterotrophic conditions it seemed to be inhibited by glucose amendment (Tables 3, B4; Fig. B9). The highly diverse enrichment of Sul4_Fe0 (100 % similarity *Azospira* spp., *Rhodocyclus* sp., *Propionivibrio* spp.) generally led to high NO_3^- reduction, however, in some cases growth was drastically inhibited (heterotrophic on acetate and mixotrophic on glucose) (Tables 3, B4).

4. Discussion

4.1. Potential microbial processes based on in situ incubation conditions

Median NO_3^- concentrations (0.1 to 0.7 mM) in the investigated groundwater were commonly high (Table 2) and in some wells even exceeded the German threshold value of 50 mg NO_3^-/L (≈ 0.8 mM) (Visser et al., 2021). In contrast, for all examined wells, no ammonium (NH_4^+) has been detected (data not shown), potentially indicating ongoing nitrification (Visser et al., 2021). NO_3^- concentrations are, however, consistent with concentrations reported for catchments in which intense agricultural practices were identified as the dominant source of NO_3^- (Einsiedl and Mayer, 2006; Gurdak and Qi, 2012; Wild et al., 2018). Also, since all field sites are not only located in close proximity of intensively used farm- or forested land, but urbanized areas, other potential anthropogenic N sources might include leakage from sewer systems of isolated houses and workshops (Wakida and Lerner, 2005). However, concentrations of typically sewage-derived organic microcontaminants such as carbamazepine, clofibric acid or metoprolol, were below 1 ng/L (Visser et al., 2021), hence sources other than wastewater are likely to cause the N contamination observed (Heberer, 2002; Whelehan et al., 2010). Besides isotope analysis of NO_3^- and NO_2^- , and low concentrations of microcontaminants, hydraulic and chemical responses detected also suggest that the NO_3^- in the Ammer catchment is mainly originating from fertilizer (manure and/or artificial fertilizers) application (Visser et al., 2021).

Considering that karstified aquifers are commonly characterized by oxygen-rich waters (Benk et al., 2019; Ford and Williams, 2007; Kendall and Doctor, 2005), and since the dissolved oxygen (DO) concentrations were generally high (Visser et al., 2021), N turnover processes favouring oxic conditions should prevail in the water column of the groundwater monitoring wells. Furthermore, reducing conditions (based on E_h data) were only found at ArtAlt (Tables 1, B4), which also corresponded to very low or no NO_3^- concentrations. Since it is known that the expression of genes necessary to perform complete denitrification is inhibited by the presence of O_2 (Codispoti et al., 2001; Dalsgaard et al., 2014), nitrification appears to be the major process dominating the water column in the Sul and Has monitoring wells. Although previous findings demonstrated O_2 sensitivity of denitrifying enzymes (Kuypers et al., 2018; Schreiber et al., 2012; Takaya et al., 2003), several studies have shown that various microorganisms can successfully perform dissimilatory NO_3^- reduction to NO_2^- under hypoxic and even oxic conditions (Cojean et al., 2019; Roco et al., 2016; Zhou et al., 2019). Therefore, the large temporal changes of DO concentrations observed in some wells, which resulted in minimum DO values of $< \sim 51$ μM (Visser et al., 2021), could support ongoing albeit incomplete denitrification in either the water column or even in suboxic and anoxic niches located within the

vadose zone of the aquifer (Visser et al., 2021). This is also supported by previous studies, which provided evidence for ongoing denitrification not only within the vadose zone and/or the epikarst (Brahana et al., 2005; Kuniansky and Spangler, 2014; Panno et al., 2001), but also in oxic and anoxic monitoring wells of a limestone aquifer (Henson et al., 2017; Wegner et al., 2018). Although lower DO concentrations detected in Sul1 and other wells mainly indicate that, at least temporarily, parts of the water in the system are influenced by enhanced but not complete O_2 consumption, suboxic and anoxic niche formation, particularly on a microscale and thus within the MTDs, might actively promote denitrification. Therefore, even if complete denitrification was most likely hampered in the free water phase, microorganisms utilizing anaerobic respiration may still be active in anoxic microenvironments, i.e. inside of particles and biofilms (Bianchi et al., 2018; Schramm et al., 1999; Seifi and Fazaelpour, 2012).

Although the DOC concentrations were slightly higher than previously reported (0.04 to 0.17 mM) for oligotrophic groundwater systems (Goldscheider et al., 2006), values were still relatively low (< 0.3 mM, Table 1). Low DOC concentrations in the groundwater might also be a result of leachates originating from organic matter degradation in overlying soils entering the system via the epikarst and the vadose zone. Still, the bioavailability of carbon is limited and thus chemolithotrophic bacteria, which are known to be well adapted to these nutrient-poor systems (Goldscheider et al., 2006; Griebler and Lueders, 2009), most likely play a major role in N turnover processes. Ben Maamar et al. (2015) investigated a hard-rock aquifer microbial community showing that particularly in the recharge zone, putative denitrifiers were more abundant, whereas in ancient and deeper (> 100 m) groundwater, chemolithoautotrophic bacteria capable of Fe(II)/Fe(III) and S/ SO_4^- redox cycling were identified. In general, autotrophic chemolithotrophs are considered to be linked to deep habitats in subsurface environments (Griebler and Lueders, 2009). Although the wells investigated in this study were not that deep (36.5 to 92 m), our data implies the possible presence of chemolithotrophic microorganisms also in shallow wells. While the first test did not provide sustaining evidence for chemolithoautotrophic pathways in this system, the application of the MTD method by Jakus et al., resulted in the successful enrichment and isolation of lithoautotrophic NRFeOxB (Jakus et al., 2021a). Metagenomic analysis performed on these cultures revealed the presence of novel putative NRFeOx taxa, thus providing additional support for the proposed importance of chemolithoautotrophs in (karst) groundwater systems. This is also supported by previous studies, which investigated microbial communities in shallow limestone aquifers, as well as the water column of groundwater monitoring wells (Herrmann et al., 2017; Kumar et al., 2018; Wegner et al., 2018). Furthermore, Herrmann et al. (2017) investigated water from a pristine limestone aquifer, which was characterized by a highly diverse community harbouring a high potential for NO_3^- reduction. They concluded that, not O_2 availability, but the availability of suitable inorganic versus organic electron donors is determining the activity of denitrifying microbial communities within groundwater systems (Herrmann et al., 2017). Their findings support that denitrification, regardless of the degree of groundwater oxygenation, does occur on a microscale and hence support our hypothesis of ongoing denitrification within the MTDs.

4.2. Impact of mineral amendment on microbial community members

Phylogenetic analysis based on the partial 16S rRNA gene obtained from MTD skeletons showed a variation in the dominant OTUs according to the Fe mineral amended (Figs. 3 and 4). The impact of Fe(II) on the aquifers community has also been confirmed by a consecutive study using the MTD method (there called passive samplers) in the same aquifer, published by Jakus et al. (2021b). Our combined results provide strong evidence for the presence of microorganisms capable of utilizing a chemolithotrophic mode of metabolism such as e.g. H_2 , Fe(II) and S oxidation (Ben Maamar et al., 2015; Jewell et al., 2016; e.g., Torrente

et al., 2010). The enrichment of these microbes seems to not have been limited to the internal material, but was also found as biofilms attached to MTD Teflon skeletons. Furthermore, the chemolithotrophic bacteria present in this karstified aquifer may play a major role in the weathering of autochthonous Fe-bearing minerals and/or Fe corrosion. In particular, OTUs affiliated to genera known to contain Fe(II)-oxidizing bacteria such as *Thiobacillus*, *Sideroxydans*, *Leptothrix* and *Pseudomonas* (Cornelis and Dingemans, 2013; Di Capua et al., 2019; Emerson et al., 2013; Katsoyiannis and Zouboulis, 2004) have been identified. However, our results indicate that this ability to attack and utilize structurally bound Fe is not only mineral- but also genus-dependent, as follows:

Biotite is a phyllosilicate that is characterized by strong bonds between Si^{4+} (tetrahedral layer), $\text{Mg}^{2+}/\text{Fe}^{2+}$ (octahedral layer) and K^{+} forming the intermediate layer, resulting in a perfect cleavage in the (001) basal plane and thus between the T-O-T sheets (Brigatti and Davoli, 1990). As a result of this perfect cleavage, the mineral is highly anisotropic with regards to surface reactivity, meaning that the majority of biotite dissolution occurs at the reactive (hk0) edges and thus perpendicular to the basal (001) plane (Bray et al., 2015). Hence, the Fe (II) sitting within the octahedral layer is well protected and biotite dissolution, which also strongly depends on particle size and shape, is, particularly at a circumneutral pH, not easily achievable (Bray et al., 2015). Nevertheless, previous studies provided evidence that biotic dissolution by either local acidification or the release of organic ligands is indeed an important process for phyllosilicate weathering (e.g., Balland et al., 2010). Studies investigating microbially mediated biotite dissolution so far mainly identified members of the family *Gallionellaceae*, particularly the genus *Sideroxydans*, as being able to utilize the structurally bound Fe(II) in biotite (Aquilina et al., 2018; Jewell et al., 2016; Shelobolina et al., 2012). In our experiments, the highest number of sequence reads in the biotite amended MTDs belonged to OTUs associated to the genus *Pseudomonas* but other genera such as *Rhodobacter*, *Massilia*, *Sideroxydans*, *Albidiferax*, as well as *Sphingobium* and *Thiobacillus* were also present but at low abundance (<10 %). Considering that *Pseudomonas* spp. have mainly been reported to oxidize dissolved Fe(II) (Li et al., 2018; Su et al., 2015) and to promote smectite dissolution and thus Fe(III) solubilization via siderophore secretion (Ferret et al., 2014), their prevalence in the biotite-filled MTDs is noteworthy and possibly indicates the presence of an indirect Fe(II) mobilisation mechanism.

Pyrite (FeS_2), a highly crystalline mineral is considered to be even more stable than biotite and so far nitrate-dependent pyrite oxidation under laboratory conditions has been demonstrated for batch cultures of *Thiobacillus denitrificans* (Bosch et al., 2012; Torrento et al., 2010), as well as two enrichment cultures harbouring two *Rhizobiales* species (*Bradyrhizobium* sp. and *Mesorhizobium* sp.) and a *Ralstonia* species (Percak-Dennett et al., 2017). Yet, pyrite oxidation in *Thiobacillus denitrificans* cultures is probably linked to the oxidation of the mineral's reduced sulfur species, as opposed to Fe(II) (Yan et al., 2019). Nevertheless, in pyrite amended MTDs, OTUs affiliated to the genera *Thiobacillus* and *Pseudomonas* were dominant, followed by OTUs assigned to the genera of *Rhodobacter* and *Sideroxydans*. The subsequent studies performed by Jakus et al. (2021b, 2021c) demonstrated the potential relevance of NRFeOx bacteria for pyrite oxidation in this very aquifer. Furthermore, they also obtained bacterial cultures/isolates related to *Thiobacillus denitrificans* and/or *Sideroxydans* spp. (Jakus et al., 2021a, 2021b). The *Sideroxydans* genus has been suggested to break down minerals such as biotite (Shelobolina et al., 2012) and is also known to harbour NO_3^- -reducing and microaerophilic Fe(II)-oxidizing bacteria (Emerson et al., 2013; Huang et al., 2022; Jakus et al., 2021b). Thus, the occurrence of OTUs affiliated to this genus could additionally be linked to O_2 being present in the wells. Contrarily, the detection of OTUs assigned to *Rhodobacter* spp., particularly in biotite, mio and Fe^0 setups, is quite surprising. Members of the family of *Rhodobacteraceae* are not only known to harbour a variety of facultative anaerobic heterotrophs but also for Fe(II)-oxidizing phototrophic species (Hedrich et al., 2011),

however, photoferrotrophy can be excluded in these dark monitoring wells. Similar to *Thiobacillus* spp., chemolithotrophic sulfur, instead of (or in addition to) Fe(II), oxidation coupled to NO_3^- reduction might serve as a plausible explanation for the presence of *Rhodobacter* and *Thiobacillus* in pyrite amended MTDs, since sulfur oxidation by species belonging to the genera *Thiobacillus* and *Rhodobacter* have previously been reported (Ghosh and Dam, 2009; Yan et al., 2019). Furthermore, in mio-coated sand amended MTDs, Fe(III) reduction might play an important role. The mio was synthesized especially for the MTDs and product analysis revealed a Fe(II)/Fe(III) ratio of 0.36, indicating that the magnetite produced was partially oxidized (~32.8 %) to Fe(III) (Fig. S2; see also Usman et al., 2018). Hence, the product is defined by a magnetite-maghemite intermediate state (Usman et al., 2018). Therefore, the mio-coated sand does not only provide Fe(II) as electron donor, but also Fe(III), which can be used by certain bacteria as electron acceptor. Genera such as *Albidiferax* and *Pseudomonas* have been shown to reduce Fe(III) (Cornelis and Dingemans, 2013; Finneran et al., 2003; Ramana and Sasikala, 2009) coupled to the oxidation of H_2 (Lovley, 1997).

In addition, MTDs amended with zero valent iron (Fe^0) might have initiated even more reactions due to the high chemical reactivity of Fe^0 . Besides microbially induced corrosion, several studies have shown the advantages, but also disadvantages, of Fe^0 as possible bioremediation agent. For example, Fe^0 is known to promote microbially mediated corrosion, resulting in the production of Fe^{2+} and H_2 (Peng et al., 2015; Zhu and Getting, 2012). This release of electron donors has been demonstrated to locally increase the denitrification potential of the system (Peng et al., 2015; Zhu and Getting, 2012). Consequently, MTDs supplied with Fe^0 harboured, with regards to the genera identified via 16S rRNA gene sequence analysis, the highest diversity (Fig. 3). Genera containing putative Fe(II)-oxidizing and Fe(III)-reducing species, such as *Leptothrix* and *Albidiferax*, were identified in relatively high abundances. Furthermore, a corrosion related release of H_2 could explain the increased abundances of *Hydrogenophaga* spp., as well as *Dechloromonas* spp., which are known to couple H_2 oxidation to NO_3^- reduction (Chakraborty and Picardal, 2013; Sun et al., 2009; Yan et al., 2017; Zhang et al., 2002; Zhao et al., 2011). Interestingly, *Dechloromonas* spp. have not only been demonstrated to oxidize H_2 via perchlorate reduction, but also to couple NO_3^- reduction to the oxidation of dissolved Fe (II) (Chakraborty and Picardal, 2013; Zhang et al., 2002). Relative 16S rRNA gene sequence abundances of *Dechloromonas* spp. have also been identified in cultures enriched/isolated by Jakus et al. (2021b). Here, DNA extraction was only performed successfully from MTDs containing Fe-bearing minerals, but not from the limestone-filled controls. Hence, the hypothesis that microbial growth/activity is limited by the availability of a suitable (inorganic) electron donor appears to be true. Contrarily, Jakus et al. (2021a) deployed the MTDs filled with pyrite-bearing limestone (mother rock, mu) for four months and successfully enriched (similar culture conditions) a culture dominated by members of the *Gallionellaceae* family. However, whether the successful growth on the mother rock here was related to a longer in situ incubation, seasonal differences or simply the fact that the Artesian well was indeed active again during their campaign, remains elusive. Nevertheless, our combined results demonstrate that a localized in situ stimulation of certain microbial key players is indeed possible simply by increasing substrate availability. However, in contrast to previous studies investigating groundwater microbial communities (i.e. Herrmann et al., 2017), the communities present on the MTDs are less diverse and most certainly do not reflect the total groundwater microbial community.

4.3. Successful enrichment of Fe(II)-oxidizing and denitrifying bacteria under laboratory conditions

MTDs were tested as a tool for microbial enrichment and isolation of nitrate-reducing Fe(II)-oxidizing bacteria and classical denitrifiers. The gained results show that the internal material obtained from MTDs acted

as potent inoculum. Furthermore, the results evidently support the presence of facultative NRFeOxB, as well as the method's functionality with regards to stimulate their growth in situ but also ex situ, i.e., in laboratory enrichment cultures. Hence, facultative NRFeOxB might yet play a crucial role in oligotrophic systems. However, in contrast to Jakus et al. (2021c), the presence of obligate NRFeOxB and/or a chemolithoautotrophic pathway could not be confirmed. Since the ability to grow under autotrophic conditions was diminished and even lost after several transfers, the continuous cultivation of autotrophic NRFeOxB is, in fact, not trivial. Sanger sequencing of the most potent Fe(II)-oxidizing enrichment cultures (see Table 2, cultures marked with *), grown at mixotrophic conditions, supported a successfully performed bacterial enrichment. Furthermore, in enrichments of all three field sites, putative NRFeOxB such as members of the genus *Acidovorax* have been identified. Considering, however, that the medium used for the enrichment process is commonly applied for the cultivation of the mixotrophic *Acidovorax* sp. strain BoFeN1 (Kappler et al., 2005; Klueglein and Kappler, 2013; Muehe et al., 2009), might explain the apparent ability of *Acidovorax* spp. to outcompete other species in our enrichments. Furthermore, *Acidovorax* spp. (Comamonadaceae) are not only abundant in many freshwater systems but also have been investigated intensively with regards to Fe(II) oxidation (Bryce et al., 2018; Chakraborty et al., 2011; Dopffel et al., 2022; Kappler et al., 2010; Visser et al., 2022). The ability to perform NRFeOxB has been reported for several *Acidovorax* strains (Chakraborty et al., 2011; Hohmann et al., 2011; Kappler et al., 2005; Muehe et al., 2009; Pantke et al., 2012), however, genomic evidence supporting a direct enzymatically mediated Fe(II) oxidation in *Acidovorax* spp., is lacking (Price et al., 2018). Although temperature-dependent incubations suggest a partial enzymatic Fe(II) oxidation by *Acidovorax* sp. BoFeN1 (Dopffel et al., 2022), other studies analysing NRFeOxB in *Pseudogulbenkiania* sp. strain 2002 and in BoFeN1 based on e.g., isotope analysis do not support a direct link between enzymatic nitrate reduction and Fe(II) oxidation (Chen et al., 2020; Visser et al., 2022).

MTD, as well as enrichment Has5_biotite and Sul4_pyrite based results indicate that genera commonly attributed to "classical" denitrification pathways (e.g., *Paracoccus* spp.), conceivably harbour a greater metabolic flexibility than previously assumed (e.g. carbon source utilization flexibility shown in Table 3, Fig. B9, cultures Sul4_pyrite and Has5_biotite). Albeit, *Paracoccus* spp. are known organotrophic denitrifiers, certain *Paracoccus* strains are able to oxidize Fe(II) if grown under mixotrophic conditions (Klueglein et al., 2014; Muehe et al., 2009). Enrichments dominated by *Paracoccus yeei* strains, of which several are considered pathogenic and are associated with opportunistic human infections (Lasek et al., 2018), successfully performed Fe(II) oxidation (Tables 3, B2). Members of the genus *Paracoccus* are known to be highly adaptive and versatile (Lasek et al., 2018) and their ability to perform sulfide oxidation coupled to denitrification has been demonstrated before (Cardoso et al., 2006; Chen et al., 2010). The enrichment originating from PE Sul4_Fe⁰ showed the highest diversity, containing bacteria closely related to known Fe(II)-oxidizers including *Propionivibrio* spp., *Rhodocyclus* spp., and *Azospira* spp. Fe(II) oxidation under microoxic conditions, and in the presence of acetate, has already been observed in members of the genus *Rhodocyclus* have been reported to oxidize Fe(II) under microoxic conditions or in the presence of acetate (Sobolev and Roden, 2004). Mejia et al. (2016) investigated the impact of O₂ and NO₃⁻ on iron mineral transformations by performing NO₃⁻-spiked experiments of soil microbial communities. Their results showed not only Fe(II) oxidation in the presence of NO₃⁻ and glucose, but also revealed growth of *Propionivibrio* and *Rhodocyclus*. Species of the genera *Propionivibrio*, *Azospira* and *Rhodocyclus* are considered organotrophic NO₃⁻ reducers and have been shown to degrade organic contaminants (Byrne-Bailey and Coates, 2012; Chu and Wang, 2017; Thrash et al., 2010; Zhang et al., 2002; Zhao et al., 2011). Testing the metabolic flexibility of these cultures showed that most organic acids supplied were easily utilized (Tables 3, B2). Only glucose amendment decreased

Fe(II) oxidation abilities in all cultures although NO₃⁻ was fully consumed (Fig. B8, Table B2). According to previous studies, NO₃⁻ addition resulted in a decreased glucose turnover, suggesting denitrification is inhibited in the presence of glucose (Bowman and Focht, 1974; Chidthaisong and Conrad, 2000). Considering that Fe(II) oxidation would yield less energy compared to breaking down glucose and using the by-products, utilizing glycolysis might indeed be more favourable. For most anaerobic bacteria, glycolysis usually occurs by breaking down glucose to smaller molecules such as pyruvate during the glycolytic pathway, since pyruvate can be further used, after conversion to acetate or acetyl-CoA, in the TCA cycle. Pyruvate oxidation is commonly coupled to the reduction of an inorganic substrate such as NO₃⁻ (Fuhrer et al., 2005; Jurtschuk, 1996; Park et al., 2014), which would explain why NO₃⁻ was fully consumed in all pyruvate amended cultures. Furthermore, butyrate also decreased the Fe(II) oxidation ability in enrichments containing bacteria closely related to *Acidovorax* spp., *Paracoccus* spp., and in the diverse enrichment culture of PE Sul4_Fe⁰ (*Rhodocyclus* sp., *Azospira* sp., *Propionivibrio* sp.). Butyrate has been shown to be utilized in a fermentative pathway similar to glucose and also to partially inhibit NO₃⁻ reduction (Chen et al., 2017). This might also explain why most butyrate amended cultures showed only low Fe(II) oxidation capabilities. Furthermore, while the accumulation of NO₂⁻ (or NO_x species) has been suggested to play a major role in mixotrophically grown, putative NRFeOxB (Klueglein and Kappler, 2013; Visser et al., 2020), its absence observed in the glucose amended (and partially butyrate amended) enrichments could also serve as possible explanation why Fe(II) oxidation was hampered. In general, most enrichments were able to utilize all organic acids provided and the ability to oxidize Fe(II) was most likely linked to the production of reactive NO_x species produced during denitrification.

5. Conclusions and outlook

The modified MTDs filled with Fe(II)-bearing minerals and Fe⁰ stimulated the microbial community of a karstic aquifer in situ resulting in a local enrichment of different Fe-transforming bacteria, depending on the mineral amended. In addition, the internal material acted as a potent inoculum to enrich putative NRFeOxB under laboratory conditions. Our data shows that the enriched microorganisms generally thrive on organic substrates (heterotrophs and mixotrophs), but seem to be also characterized by a high metabolic flexibility, enabling them to utilize Fe(II) as additional energy and electron source. Considering not only the results obtained by sequencing the DNA obtained from the Teflon skeletons, but also the strong fluctuations in DO concentrations, the local microbial community appears to predominantly consist of facultative aerobes which might even dwell as organotrophs under oxic conditions. Although the insights gained by this study are very promising, slight adjustments applied in the discussed subsequent studies increased the enrichment success particularly with regards to the utilization of the mother rock and thus the isolation of an autotrophic culture. Hence, we advise to include detailed information about the aquifer of interest into the planning process. We suggest not only to increase the in situ incubation time, but also to enhance the overall enrichment methods by providing additional materials such as various carbon substrates, which do not impede with groundwater regulations and thus quality. Furthermore, applying MTDs as a physical remediation test tool by filling them with reactive Fe-bearing minerals and solids (i.e., magnetite, Fe⁰) to remove heavy metals or organic pollutants such as nitrobenzene from contaminated groundwaters, might also be worth testing. Our results provide valuable insights in the importance of metabolic flexibility, particularly in oligotrophic aquifers, showing that regardless of its in situ oxygen concentration or redox state, chemolithotrophic Fe(II)-oxidizing denitrifiers can be found in all aquifers.

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CRedit authorship contribution statement

Anna-Neva Visser: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joseph D. Martin:** Writing – review & editing, Visualization, Methodology, Investigation. **Karsten Osenbrück:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Hermann Rügner:** Writing – review & editing, Supervision, Resources, Project administration. **Peter Grathwohl:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Andreas Kappler:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.172062>.

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