FEMS Microbiology Ecology, 97, 2021, fiab145

https://doi.org/10.1093/femsec/fiab145
Advance Access Publication Date: 1 November 2021
Research Article

RESEARCH ARTICLE

Presence of Fe(II) and nitrate shapes aquifer-originating communities leading to an autotrophic enrichment dominated by an Fe(II)-oxidizing *Gallionellaceae* sp.

Natalia Jakus[1,2], Nia Blackwell[2], Daniel Straub[2,3], Andreas Kappler[1,4] and Sara Kleindienst[2,*,†]

[1]Geomicrobiology, Center for Applied Geoscience, University of Tübingen, D-72076 Tübingen, Germany, [2]Microbial Ecology, Center for Applied Geoscience, University of Tübingen, D-72076 Tübingen, Germany, [3]Quantitative Biology Center (QBiC), University of Tübingen, D-72076 Tübingen, Germany and [4]Cluster of Excellence: EXC 2124: Controlling Microbes to Fight Infection, D-72076 Tübingen, Germany

*Corresponding author: Microbial Ecology, Center for Applied Geoscience, University of Tuebingen, Schnarrenbergstrasse 94–96, D-72076 Tübingen, Germany. Tel: +49-(0)7071-29-74701; E-mail: sara.kleindienst@uni-tuebingen.de

One sentence summary: Nitrate in organic-poor aquifers can be removed by autotrophic Fe(II)-oxidizing communities, dominated by microorganisms belonging to the family Gallionellaceae.

Editor: Tillmann Lueders

†Sara Kleindienst, https://orcid.org/0000-0001-8304-9149

ABSTRACT

Autotrophic nitrate reduction coupled to Fe(II) oxidation is an important nitrate removal process in anoxic aquifers. However, it remains unknown how changes of O_2 and carbon availability influence the community structure of nitrate-reducing Fe(II)-oxidizing (NRFeOx) microbial assemblages and what the genomic traits of these NRFeOx key players are. We compared three metabolically distinct denitrifying assemblages, supplemented with acetate, acetate/Fe(II) or Fe(II), enriched from an organic-poor, pyrite-rich aquifer. The presence of Fe(II) promoted the growth of denitrifying *Burkholderiaceae* spp. and an unclassified *Gallionellaceae* sp. This *Gallionellaceae* sp. was related to microaerophilic Fe(II) oxidizers; however, it did not grow under microoxic conditions. Furthermore, we explored a metagenome and 15 metagenome-assembled genomes from an aquifer-originating, autotrophic NRFeOx culture. The dominant *Gallionellaceae* sp. revealed the potential to oxidize Fe(II) (e.g. *cyc2*), fix CO_2 (e.g. *rbcL*) and perform near-complete denitrification leading to N_2O formation (e.g. *narGHJI*, *nirK/S* and *norBC*). In addition, *Curvibacter* spp., *Methyloversatilis* sp. and *Thermomonas* spp. were identified as novel putative NRFeOx taxa. Our findings provide first insights into the genetic traits of the so far only known autotrophic NRFeOx culture originating from an organic-poor aquifer, providing the genomic basis to study mechanisms of nitrate removal in organic-poor subsurface ecosystems.

Keywords: denitrification; autotrophic; NRFeOx; aquifer; metagenomics; *Gallionellaceae*

Received: 6 July 2021; **Accepted:** 29 October 2021

INTRODUCTION

Nitrate (NO_3^-) pollution in groundwater is one of the major worldwide environmental issues as many aquifers exhibit values exceeding the maximum regulatory concentration of nitrate in drinking water (50 mg L^{-1}) (European Union 2006; European Commission 2018; Kazakis *et al.* 2020). Only two biological pathways are known that lead to nitrate attenuation: dissimilatory nitrate reduction to ammonium (DNRA) and denitrification (Kuypers, Marchant and Kartal 2018). Denitrification is an anaerobic respiratory process in which dissolved nitrate is reduced to nitrite (NO_2^-) and further to gaseous nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2) via a series of enzymatic steps, mediated by a wide range of bacterial and archaeal taxa (Kuypers, Marchant and Kartal 2018). Denitrifying bacteria can be divided into three different groups: heterotrophs, autotrophs or mixotrophs, depending on whether they gain energy from the oxidation of organic compounds, inorganic compounds or both, respectively (Bryce *et al.* 2018). In many subsurface ecosystems including aquifers, organic carbon is limited and as such, autotrophic denitrifiers utilizing inorganic electron donors like Fe(II), reduced sulfur or H_2 were suggested to play important roles in nitrate removal (Postma *et al.* 1991; Pauwels *et al.* 1998; Schwientek *et al.* 2008; Jørgensen *et al.* 2009; Zhang *et al.* 2012; Visser *et al.* 2020). However, the influence of the availability of alternative electron acceptors (e.g. O_2) as well as electron donors (e.g. organic compounds) when compared with nitrate and Fe(II) on autotrophic nitrate-reducing Fe(II)-oxidizing (NRFeOx) groundwater assemblages remains poorly understood.

Most prominent candidates for NRFeOx microorganisms are Fe(II)-oxidizing bacteria (FeOB) belonging to the family *Gallionellaceae*, typically inhabiting microoxic pH-neutral environments, which were also reported to have the potential to reduce nitrogen species under anoxic conditions (He *et al.* 2016; Huang *et al.* 2021b). Metagenome-assembled genomes (MAGs) of *Gallionellaceae* spp. were shown to harbor several denitrification genes (e.g. *narG*, *nirK*, *nirS*, *norB* or *nosZ*), co-occurring with at least one of the candidate Fe(II)-oxidase genes encoding, for example, cytochrome *c* Cyc2 or a MtoAB complex (He *et al.* 2016; Huang *et al.* 2021a,b). Additionally, *Gallionellaceae* spp. were demonstrated to fix CO_2 (Tominski *et al.* 2018), utilizing the Calvin–Benson–Bassham (CBB) cycle encoded by *cbbL*, *cbbM* or *rbcS* genes (Huang *et al.* 2021a). Since several *Gallionellaceae* sp. MAGs in field studies and cultivation experiments were found to have incomplete gene sets encoding denitrification (Jewell *et al.* 2016; Bethencourt *et al.* 2020; Huang *et al.* 2021a,b), complete autotrophic denitrification coupled to Fe(II) oxidation was suggested to be achieved via metabolic handoffs between *Gallionellaceae* spp. and other community members rather than by *Gallionellaceae* sp. alone (He *et al.* 2016; Huang *et al.* 2021a,b). However, our knowledge about this process and the underlying complex interdependencies in organic-poor aquifers is limited.

To fill this gap, a novel autotrophic NRFeOx culture was recently obtained from a nitrate polluted, pyrite-rich (4.1 ± 1.4 mg [g rock]$^{-1}$) limestone aquifer, and it is to date the only autotrophic NRFeOx culture originating from an organic-poor environment (dissolved organic carbon <2.0 mg L^{-1}) (Jakus *et al.* 2021a). This culture is composed of a mixed community with diverse bacterial taxa, dominated by *Gallionellaceae* sp., and was demonstrated to oxidize 1–3 mM of dissolved Fe^{2+} or Fe(II) minerals such as siderite and pyrite under anoxic conditions using nitrate as an electron acceptor (Jakus *et al.* 2021b). Yet, the genomic traits that enable the uncultured *Gallionellaceae* sp. in the aquifer-originating enrichment culture to grow under anoxic conditions, the role of other microbial members and the mechanism(s) of Fe(II) oxidation remain unknown.

Our overarching hypothesis was that the availability of Fe(II) and nitrate would substantially influence the composition and capability of autotrophic NRFeOx assemblages originating from a pyrite-rich, organic-poor limestone aquifer. We therefore compared the microbial community composition of the autotrophic NRFeOx culture with parallel enrichment cultures grown under microoxic, heterotrophic or mixotrophic conditions, to test the response of the denitrifying microbial community to the change of electron acceptor (i.e. O_2) and the availability of organic carbon (i.e. acetate) in the absence and presence of Fe(II). We further investigated the metagenome of the autotrophic NRFeOx culture, retrieved 15 MAGs and analyzed the genetic traits encoded in the MAGs to predict the metabolism of the most abundant microorganisms growing in the enrichment culture including the *Gallionellaceae* sp. and to search for candidates of NRFeOx bacteria that may play a critical role in nitrate removal in aquifers limited in organic carbon.

MATERIALS AND METHODS

Enrichment and cultivation

The field site location and the conditions and the procedure of enrichment were the same for all three types of nitrate-reducing cultures (autotrophic, mixotrophic and heterotrophic) as described previously for the autotrophic NRFeOx enrichment, Culture AG (Altingen groundwater, named after the source of the culture; Jakus *et al.* 2021a). Briefly, denitrifying communities were enriched using passive samplers filled with crushed, pyrite-rich limestone particles and incubated in an anoxic (dissolved O_2 of 0.1 ± 0.1 mg L^{-1}) low-nitrate (1.5 ± 0.6 mg L^{-1}) monitoring well. After incubation, rock particles with attached microbial cells were retrieved and transferred to sterile serum bottles (58 mL) filled with 25 mL of anoxic bicarbonate-buffered (22 mM), modified low-phosphate freshwater medium (LPM) (Jakus *et al.* 2021a), adjusted to pH 7.1 and amended with 2 mM of $NaNO_3$. Next, 24 h prior to the experiment, either 2 mM of $FeCl_2$, 1 mM of acetate or 1 mM of acetate plus 2 mM of $FeCl_2$ was added to the medium for autotrophic, heterotrophic and mixotrophic cultivation conditions, respectively. For cultures growing under microaerophilic conditions, gradient tubes and zero-valent iron (ZVI) plates were used. Gradient tubes with opposing gradients of Fe^{2+} and O_2 were prepared following Emerson and Floyd (2005). Cultures in ZVI plates were established using Petri dishes (55 mm diameter) containing 8 mL of modified Wolfe's mineral medium (Emerson and Floyd 2005) and ~0.5 g of ZVI powder (200 mesh; metal basis; Alfa Aesar, Ward Hill, MA). ZVI plates were inoculated by adding 100 μL of inoculum from a gradient tube and incubated in an acrylic jar (Merck, Darmstadt, Germany) with a gas pack (BD GasPak EZ Campy, New York, USA) to form microoxic conditions (6–10% atmospheric O_2).

Experimental setup and DNA extraction for 16S rRNA gene and metagenome sequencing

In order to grow a sufficient amount of cells for the experiment, a preculture of autotrophic NRFeOx enrichment culture was grown in six serum bottles containing modified LPM (25 mL of medium each) prepared as described earlier, supplemented with 2 mM NO_3^- and 2 mM Fe(II). After 5 days of growth, the content of all bottles was pooled into one sterile anoxic serum bottle and used as an inoculum. Immediately after obtaining

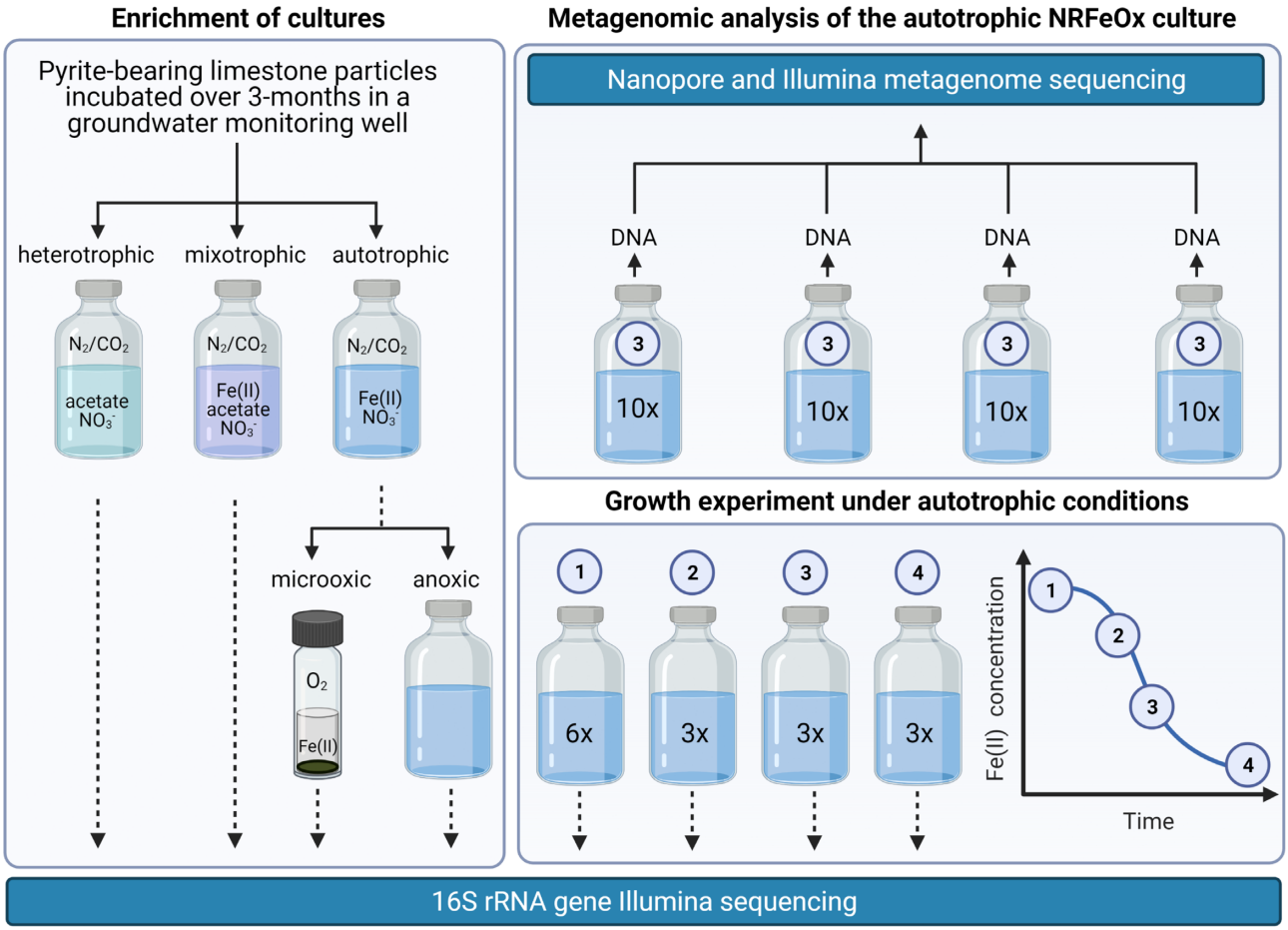

Figure 1. Schematic figure of the experimental setups. Different bottle colors represent three different setups containing growth medium used to enrich heterotrophic (pale blue), mixotrophic (purple) and autotrophic (blue) denitrifying communities using acetate and nitrate, Fe(II), acetate and nitrate, and Fe(II) and nitrate, respectively. The gray serum vial represents enrichment cultures grown under microoxic conditions in opposite gradients of oxygen (entering from the top into the solidified agar layer) and Fe^{2+} (released by dissolution of the black FeS from the bottom). Numbers on the bottles show the number of replicates used for DNA extractions followed by sequencing. Numbers in circles indicate the time point when samples were collected in relation to Fe(II) consumption. Dark blue boxes display sequencing method used. The figure was created with BioRender.com.

the preculture, a sample for 16S rRNA gene sequencing was collected (day 0) and 53 serum bottles, containing the same modified LPM, were inoculated with 10% (vol/vol) of the preculture ($\sim 2 \times 10^6$ cells mL^{-1}). To follow consumption of substrates, three inoculated bottles (biotic controls) were randomly selected and sampled daily. Additionally, abiotic controls (no cells, only modified LPM supplemented with substrates) were simultaneously run in triplicates. At days 2, 3 and 9 of the experiment, we collected DNA samples to follow changes in the relative abundance of the microbial community, by harvesting three bottles at each sampling point. After harvesting, all liquid together with microbial cells was centrifuged (20 min, 4000 rpm) to remove the supernatant. The biomass was frozen at –20°C and stored until DNA extraction. At day 3, we additionally collected a DNA sample for metagenome sequencing, for which, to have sufficient yield of DNA, 40 serum bottles were sacrificed by filtration using four (10 bottles per filter) 0.2-µm pore-size membrane filters (Isopore®, Millipore) (Fig. 1). Filters were subsequently placed in 2-mL centrifuge tubes and frozen at –20°C. All DNA samples collected in this study were extracted using the FastDNA SPIN Kit according to the manufacturer's instructions, and DNA extracts were then pooled for shotgun metagenome library construction.

Chemical analyses

Samples were taken daily in an anoxic glovebox (100% N_2) using a syringe with a needle through the butyl rubber stopper and centrifuged (14 000 × *g*, 10 min). For quantification of Fe(II) and Fe(III), a revised ferrozine protocol for nitrite-containing samples was used to eliminate the abiotic reaction of nitrite with Fe(II) during acidification as described in detail previously (Klueglein and Kappler 2013; Schaedler *et al.* 2018). Nitrate and nitrite were quantified following the DIN 38405/ISO 13395 standard quantification method using AA3 HR AutoAnalyzer System (Seal Analytical, Germany).

16S rRNA gene sequencing and analysis

DNA extracted from the cultures was used for amplification with universal primers (515F: GTGYCAGCMGCC-GCGGTAA [Parada, Needham and Fuhrman 2016] and 806R: GGACTACNVGGGT-W TCTAAT [Apprill *et al.* 2015]) fused to Illumina adapters. The PCR (Polymerase Chain Reaction), library preparation and sequencing are detailed in the Supporting Information. Quality control, reconstruction of 16S rRNA gene sequences and taxonomic annotation were performed with nf-core/ampliseq v1.1.0 (Ewels

et al. 2020; Straub et al. 2020) as described in detail in the Supporting Information.

Quantitative PCR

Total cell numbers of bacteria were estimated by quantitative PCR (qPCR) (Bio-Rad Laboratories GmbH, Munich, Germany) based on the amplification of bacterial 16S rRNA genes following a protocol described previously (Schaedler et al. 2018) and described in detail in the Supporting Information. Absolute abundances of 16S rRNA genes belonging to taxa present in the autotrophic NRFeOx culture were calculated by multiplication of 16S rRNA gene copy numbers and relative abundances of 16S rRNA gene amplicon sequences on species level.

Metagenome sequencing, assembly and annotation

Metagenome library preparation and shotgun Illumina sequencing were conducted by CeGaT, Tuebingen, Germany. Short- and long-read quality control, hybrid assembly, metagenome-assembled genome binning and taxonomic annotation were performed with nf-core/mag v1.0.0 (https://doi.org/10.5281/zenodo.3589528) (Krakau et al. 2021) as described in detail in the Supporting Information. The assembled metagenome and eight high-quality MAGs (≥89.9% completeness, <10% contamination) were uploaded to the Joint Genome Institute's Integrated Microbial Genome and Microbiome Expert Review (IMG/MER) pipeline for annotation, and subsequent analysis was performed using tools available through the pipeline IMGAP v5.0.19 (Chen et al. 2019). Putative Fe(II) oxidation genes were identified using the blast function in IMG (10^{-5} *E*-value cutoff). For the remaining seven MAGs with completeness between 89.9% and 56.1%, gene annotations were subset from the whole metagenome annotation. The genes of interest present in these MAGs were then identified by their KEGG Orthology numbers and analyzed manually. In addition, all 15 assembled MAGs were analyzed using the FeGenie tool (Garber et al. 2020) installed using Python 3.6 (Python Software Foundation; https://www.python.org/) to search for genes related to Fe cycling. A full list of the 15 MAG identifiers together with the completeness and the identifiers of the NCBI and IMG JGI platforms can be found in Table 1 and Table S1 (Supporting Information).

RESULTS AND DISCUSSION

Microbial community composition under autotrophic NRFeOx growth conditions

Using 16S rRNA gene sequencing of the V4 region (~250 bp) in combination with quantification of 16S rRNA gene copy numbers and substrate concentrations over time during a 10-day incubation of the autotrophic NRFeOx culture growing under anoxic conditions, we determined microbial community changes linked with nitrate and Fe(II) consumption (Fig. 2). Overall, the microbial community structure was stable as we did not observe any major changes over 10 days (Fig. 2A). Minor changes in the microbial community occurred between day 2 and day 3, where the largest increase in absolute 16S rRNA gene copy numbers occurred (Fig. 2A). After a total consumption of 0.49 ± 0.07 mM of NO_3^- and 1.22 ± 0.07 mM of Fe(II), the relative abundance of the unclassified *Gallionellaceae* sp. decreased from 61.8% (day 2) to 54% (day 3), while relative 16S rRNA gene sequence abundances of both *Dechloromonas* sp. and *Ferribacterium* sp. nearly doubled between these two time points (from 7.0% to 13.1% and from 2.1% to 5.2%, respectively). The overall increase in absolute 16S rRNA gene copies over time from 3.08×10^5 (day 0) to 1.36×10^6 (day 10) suggests the ability of this culture (or at least some of its members) to fix inorganic carbon for biomass production coupled to Fe(II) oxidation. This is in agreement with our previous study, where the culture was shown to produce more cells when incubated in medium containing nitrate and 1–3 mM of Fe(II) vs nitrate alone (no Fe(II)) (Jakus et al. 2021a). Interestingly, our growth experiment results presented here point to a slight delay between substrate consumption and cell growth at the beginning of the experiment. The largest increase in the 16S rRNA gene copy numbers between day 2 and day 3 did not correlate with the largest decrease in Fe(II) and NO_3^- concentrations between day 0 and day 2 (Fig. 2), which may indicate that uptake of substrates took place ahead of CO_2 fixation and subsequent cell production. Alternatively, the discrepancy between cell growth and consumption of substrates could result from oxidation of either electron donors carried over from the inoculum, or MQ water-derived organics as discussed previously (Jakus et al. 2021a).

Oxygen as potential electron acceptor for autotrophic growth of Fe(II) oxidizers

To examine the capability of the autotrophic NRFeOx culture to perform microaerophilic Fe(II) oxidation that may occur in the aquifer due to the presence of microoxic niches (Visser et al. 2020), the culture was transferred to gradient tubes and plates containing FeS and zero valent iron (ZVI), respectively. With this approach, we intended to test the community's response to O_2 and to promote growth and ultimately isolate the uncultured *Gallionellaceae* sp., as these bacteria are typically known to be microaerophilic Fe(II) oxidizers and some of the cultured members of this family were isolated using one of the applied techniques (Hallbeck, Ståhl and Pedersen 1993; Emerson and Moyer 1997). However, none of these *Gallionellaceae* sp. isolates were reported to perform Fe(II) oxidation under anoxic conditions, i.e. coupled to (partial) denitrification. After inoculation into a gradient tube, our culture was observed to oxidize Fe(II), evidenced by the appearance of a bacterial layer above the bottom FeS-containing layer. In contrast, after inoculating ZVI plates, we did not observe an increase in cell numbers under the microscope and no notable difference in Fe(II) oxidation was observed compared with the abiotic control; therefore, further transfers were only done in gradient tubes. After three continuous transfers under microoxic autotrophic conditions, the very diverse bacterial community initially growing in the autotrophic, anoxic culture was reduced to four taxa: the unclassified *Burkholderiaceae* sp. (56.2%), *Rhizobium* sp. (36.5%), *Bradyrhizobium* sp. (5.2%) and *Paracoccus* sp. (1.8%) (Fig. 3). Surprisingly, the uncultured *Gallionellaceae* sp. was not identified among the sampled microbial community, suggesting its adaptation to strictly anoxic conditions or its dependence on other community members that could not thrive under microoxic conditions.

Organic carbon as substrate for the aquifer-originating enrichment cultures

To evaluate the impact of inorganic versus organic electron donors that could both serve for microbial growth at the field site (though organotrophic denitrification was suggested to play

Table 1. Overview of taxonomic classification, completeness and contamination of MAGs, contig numbers, contig and MAG sizes, GC content together with number of ORFs (Open Reading Frames) and estimated coverage of MAGs retrieved in this study from an autotrophic NRFeOx culture. A complete table with NCBI and IMG identifiers is provided as Table S1 (Supporting Information).

MAG ID	Taxonomy classification (last common ancestor of a majority of genes)	Top BLASTN hit to 16S rRNA gene amplicon sequences	Completeness (%)	Contamination (%)	Number of contigs	Largest contig (Mbp)	Genome size (Mbp)	GC (%)	Number of ORFs in MAG	Estimated coverage
AG16	*Rhodobacteraceae* sp. QY30		97.3	0	2	2.5	3.6	65.0	3484	63.2
AG20	Unclassified *Betaproteobacteria* sp.	*Thermomonas* sp.	95.9	0.7	12	1.0	3.8	65.0	3421	37.8
AG27	*Thermomonas fusca*		95.3	0	6	1.3	3.0	68.4	2722	31.4
AGun1	Unclassified *Bradyrhizobiaceae* sp.	*Bradyrhizobium* sp.	93.2	0	1	4.1	4.1	65.0	3828	64.7
AG31	*Methylotenera* sp. G11		93.2	0	53	0.2	2.5	45.4	2434	4.6
AG5	*Geothrix fermentans*	*Geothrix fermentans*	89.9	0.7	2	2.0	3.3	67.1	3081	27.2
AG29	*Curvibacter delicatus*		89.9	1.4	6	1.6	3.8	63.3	3586	229.5
AG11	Unclassified *Nitrosomonadales*	*Gallionellaceae* sp.	89.9	0	9	0.8	2.2	58.0	2038	3617.9
AG26	*Acidovorax* sp.		84.5	0.7	63	0.2	3.6	63.9	3349	631.9
AG17	Unclassified *Burkholderiales*		83.8	0	42	0.3	3.0	64.5	2710	13.6
AG1	*Curvibacter* sp. PAE-UM		70.9	0.7	82	0.2	3.0	67.0	3106	11.4
AG28	Unclassified *Betaproteobacteria*		68.2	0.7	17	0.5	2.9	62.1	2737	199.9
AG14	*Thiobacillus denitrificans*		66.2	1.4	14	0.7	2.5	63.5	2496	10.0
AG22	*Burkholderiales* sp.		56.8	1.4	322	0.1	4.1	69.6	4331	3.7
AG18	*Rhizobium* sp.	*Rhizobium* sp.	56.1	0.7	455	0.1	4.4	64.1	4963	3.4

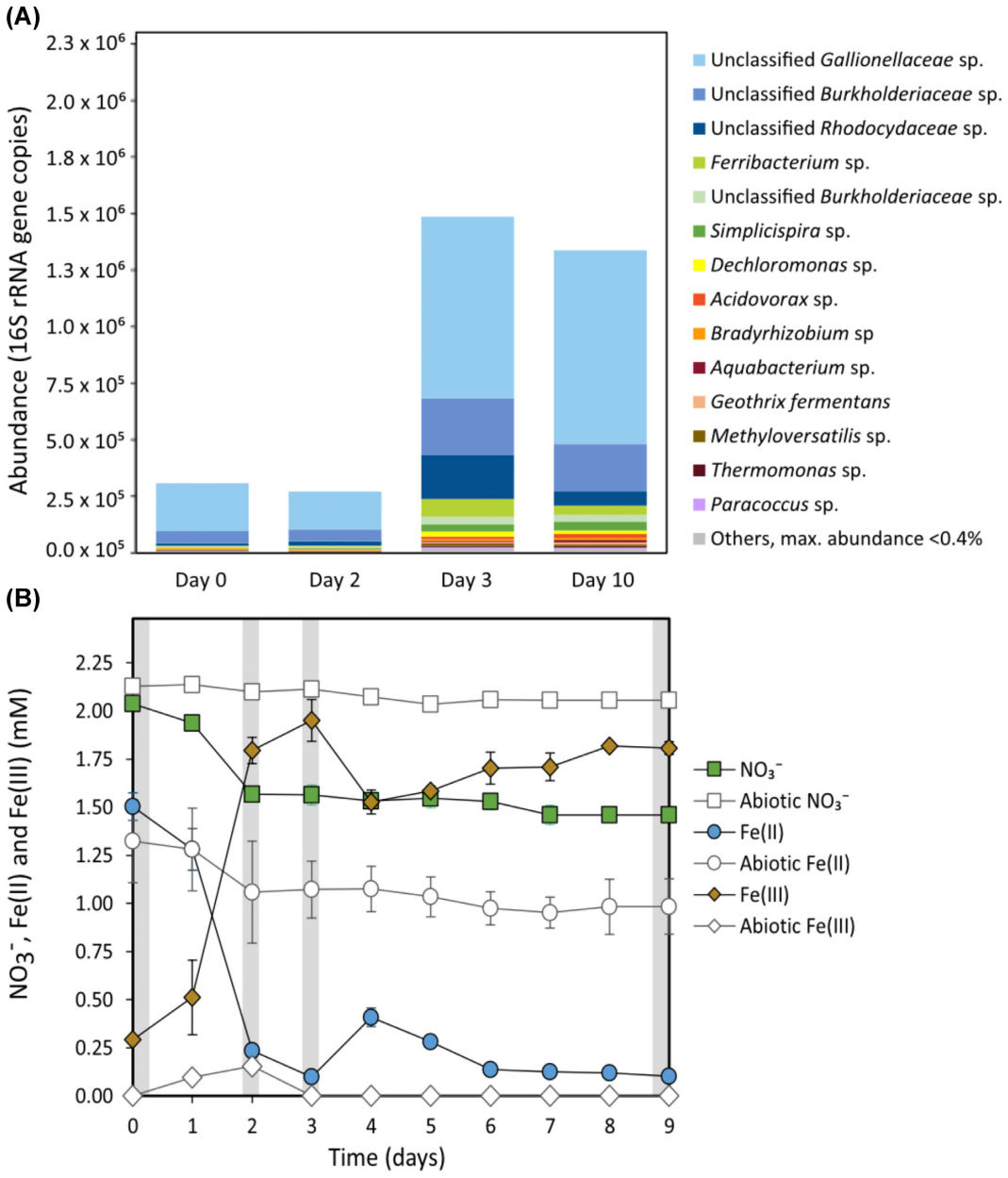

Figure 2. Changes in microbial community composition based on 16S rRNA gene amplicon sequencing **(A)** and concentrations of NO_3^- (green), total Fe(II) (blue) and total Fe(III) (brown) **(B)** in a 10-day incubation experiment using the autotrophic nitrate-reducing Fe(II)-oxidizing enrichment culture. All data points are mean values of samples collected from three biological replicate bottles; error bars represent standard deviations.

a rather minor role when compared with other metabolic pathways at this field site; Visser *et al.* 2020), the community composition of heterotrophic denitrifying cultures supplemented with organic carbon (i.e. acetate), simultaneously enriched from the same field site, was compared with the autotrophic NRFeOx culture growing under anoxic conditions (Fig. 3). The 16S rRNA gene sequencing revealed that the heterotrophic denitrifying culture grown on acetate was dominated by known nitrate-reducing bacteria belonging to the genus *Thauera* (Etchebehere and Tiedje 2005; Wang *et al.* 2017), yielding 84.8% relative sequence abundance. Interestingly, the second most dominant (4.6% relative 16S rRNA gene sequence abundance) bacterial taxon in the heterotrophic denitrifying culture shares 100% 16S rRNA gene sequence identity (based on the Amplicon Sequence Variant (ASV) region) with the predatory bacterium *Bdellovibrio bacteriovorus*, probably preying on cells living in this culture.

Effect of Fe(II) on the community structure of denitrifiers

We further analyzed the composition of the culture supplemented with both acetate and Fe(II) (mimicking fluxes of organic carbon compounds in iron-rich fractures in the aquifer). Surprisingly, despite being supplied with two different electron donors (i.e. acetate and Fe(II) at the same time), this culture had the least complex, i.e. the least diverse microbial community composition (Shannon's diversity indices for heterotrophic and mixotrophic cultures were 1.03 and 0.23, respectively). This

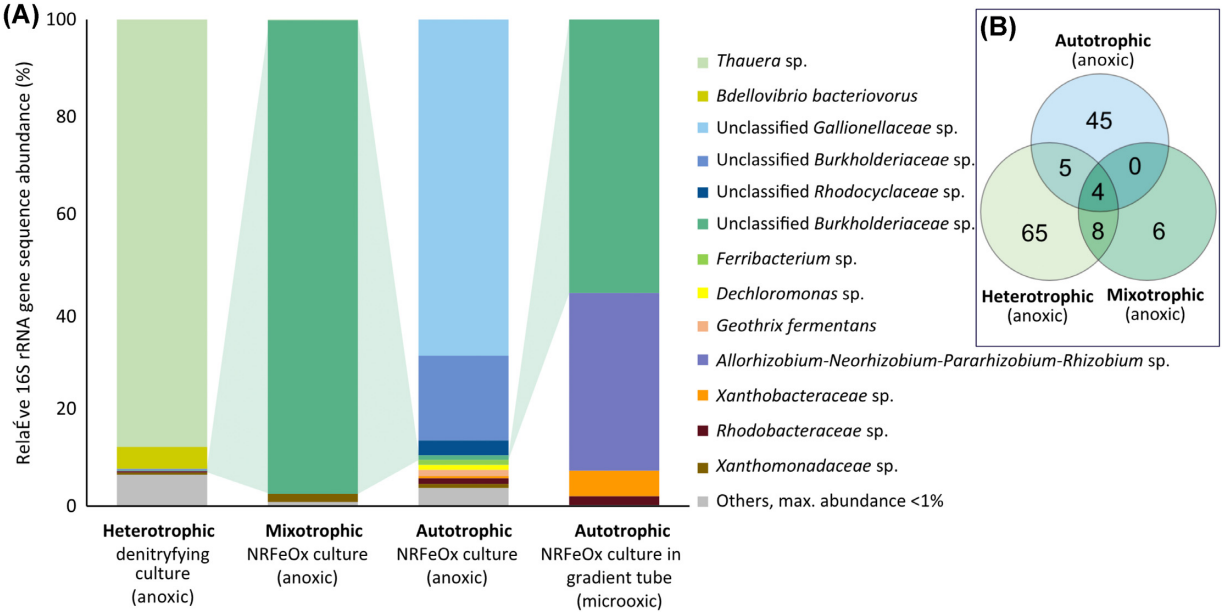

Figure 3. Microbial community composition based on 16S rRNA gene amplicon sequencing of three enrichment cultures **(A)** incubated under heterotrophic denitrifying, mixotrophic nitrate-reducing iron(II) oxidizing (NRFeOx) and autotrophic NRFeOx (anoxic) conditions using nitrate-containing freshwater medium supplemented with either 1 mM acetate, 1 mM acetate and 2 mM Fe(II), or 2 mM Fe(II), respectively. The fourth bar chart represents the community composition of the autotrophic NRFeOx enrichment culture growing under microoxic conditions in a gradient tube with FeS as Fe(II) source and air (with O_2) in the headspace. Venn diagram **(B)** depicts the shared and distinct ASVs (Supporting Information) of the three metabolically different enrichment cultures. Autotrophic culture stands here for the enrichment NRFeOx culture grown under anoxic conditions.

suggests that the addition of Fe(II) acted as a stressor, strongly inhibiting the growth and probably activity of most of the heterotrophic and autotrophic denitrifiers. The culture grown under mixotrophic conditions was dominated by the unclassified *Burkholderiaceae* sp., which accounted for up to 97.3% relative 16S rRNA gene sequence abundance. In general, *Burkholderiaceae* spp. were present in all cultures ranging from 1.4% (heterotrophic denitrifying culture) to 97.5% (mixotrophic denitrifying culture) of relative abundance, represented by a wide range of genera, including those that were previously found to directly and/or indirectly, i.e. via chemodenitrification (Bryce *et al.* 2018), catalyze Fe(II) oxidation such as *Acidovorax* spp. (Muehe *et al.* 2009; Carlson *et al.* 2013; Chakraborty and Picardal 2013) and *Curvibacter* spp. (Gülay *et al.* 2018). Our observation based on the microbial community changes among the different treatments leading to the enrichment of *Burkholderiaceae* spp. in Fe(II)-containing media further confirmed that these microbes play an important role in Fe cycling, especially in environments where redox conditions and supply of nutrients are changing.

A comparison of the microbial communities of all three cultures revealed that the unclassified *Burkholderiaceae* sp., together with three other taxa identified as *Thiobacillus denitrificans*, a *Thermomonas* sp. and another less abundant unclassified *Gallionellaceae* sp., was identified in all three cultures, being able to survive under heterotrophic, mixotrophic or autotrophic conditions (Fig. 3). Interestingly, besides these four ASVs, there was no further overlap detected for the microbial community composition in autotrophic and mixotrophic cultures (see the Supporting Information).

Recovery of MAGs of the autotrophic NRFeOx community

Since autotrophic communities are hypothesized to play a key role of nitrate removal at the field site (Visser *et al.* 2020), the metabolic potential of the autotrophic NRFeOx community was furthermore assessed using metagenome sequencing, assembly and genome binning methods and yielded in the recovery of 15 MAGs with a minimum of 56.1% estimated completeness and a maximum of 1.4% estimated contamination (Table 1). Most (10 out of 15) MAGs were assigned to the class Betaproteobacteria as expected from 16S rRNA gene amplicon sequencing that showed *Betaproteobacteria* spp. to be the most dominant class (96.3%, based on estimated coverage). The MAG with the highest estimated coverage (AG 11) was identified as unclassified member of the order Nitrosomonadales (Table 1). Please note that in the Genome Taxonomy Database (GTDB) taxa formerly known as Betaproteobacteria, are now classified as *Burkholderiales* spp., an order of Gammaproteobacteria. Details on taxonomic classification of MAGs can be found in Table S2 (Supporting Information). The 16S rRNA gene sequence retrieved from this MAG was compared with 16S rRNA gene amplicon sequences and showed 100% identity to the most dominant *Gallionellaceae* sp. identified in our cultivation experiments. The MAG was then compared with other genomes of *Gallionellaceae* spp. using average nucleotide identity and average amino acid identity analyses, confirming that the MAG belongs to the family Gallionellaceae (Figs S1 and S3, Supporting Information). Therefore, the *Nitrosomondales* sp. MAG (AG11) will be further referred to as the unclassified *Gallionellaceae* sp. MAG (AG11). In addition, four other MAGs were identified using pairwise comparison of MAG-recovered 16S rRNA gene sequences and 16S rRNA gene amplicon sequencing data obtained from the culture (Table 1).

Nitrate reduction by the autotrophic NRFeOx community

Among all of the genes associated with nitrogen cycling, genes involved in nitrate reduction were the most abundant genes identified in the metagenome (Fig. 4). The first step of denitrifi-

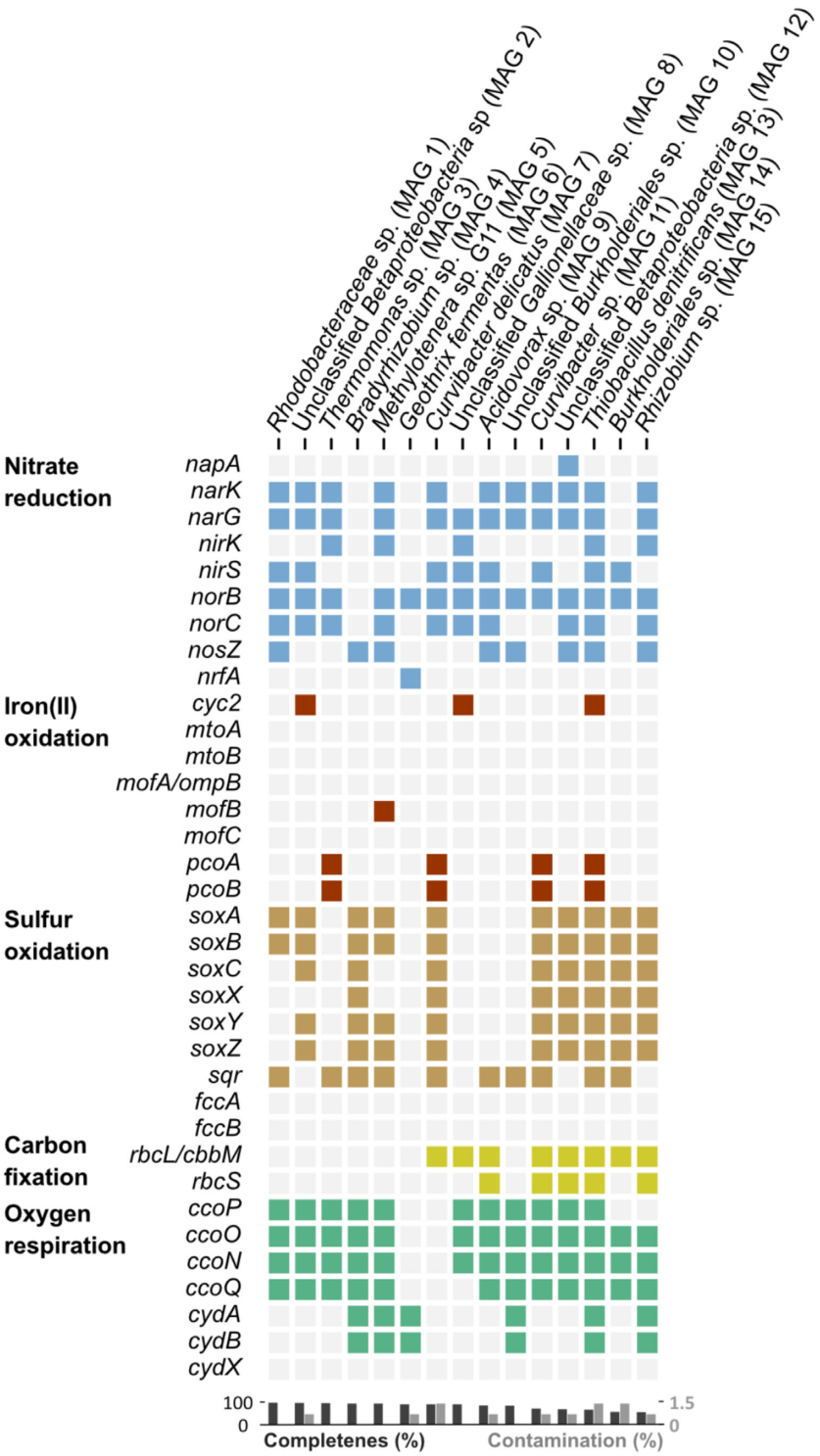

Figure 4. Summary of key genes involved in putative energy generation pathways. Colored squares indicate presence of genes involved in nitrate reduction (blue), Fe(II) oxidation (red), sulfur oxidation (brown), inorganic carbon fixation (bright green) and oxygen respiration (dark green). MAGs are ordered according to the genome completeness, starting from the most complete (97.3%) to the least complete (56.1%) MAG. Bar plot underneath the table shows completeness (0–100%, dark gray) and contamination (0–1.5%, bright gray) of each individual MAG.

cation and DNRA is performed by the periplasmic or membrane-bound nitrate reductases encoded by *napAB* or *narKGHJI* genes, respectively, that were detected in 12 of the 15 MAGs (Fig. 4). Only one copy of *nrf*, a functional gene specific for DNRA, was identified in the whole metagenome, while genes encoding nitrite reductases (*nirK* and *nirS*) and/or nitric oxide reductase (*norBC*), and/or nitrous oxide reductase (*nosZ*) were annotated in all 15 MAGs. In general, all analyzed MAGs harbored at least one of the functional genes involved in the denitrification pathway, but only five MAGs revealed all the genes necessary to complete

the entire process, starting from reduction of NO_3^- to N_2 formation. MAGs encoding genes for the complete pathway were identified as an unclassified *Rhodobacteraceae* sp., *Methylotenera* sp., *Acidovorax* sp., *Thiobacillus denitrificans* and a *Rhizobium* sp., all previously described to be members of heterotrophic and/or autotrophic denitrifying communities (Bellini *et al.* 2013; Mustakhimov *et al.* 2013; Chu and Wang 2017; Li *et al.* 2017; Kumar *et al.* 2018). In contrast, the *Gallionellaceae* sp. MAG (AG11) was found to harbor all genes required to reduce NO_3^-, NO_2^- and transform toxic NO to N_2O, but the last step was missing due to the lack of the *nosZ* gene in the genome. However, the lack of *nosZ* in this MAG might be attributed to the relatively low completeness (89.9%). Alternatively, the organism may simply not possess the full set of enzymes and rely on other microbial community members to complete denitrification as suggested previously for *Gallionellaceae* spp. from cultures KS and BP (He *et al.* 2016; Huang *et al.* 2021a,b). The autotrophic NRFeOx culture presented in this study is expected to also produce N_2 resulting from denitrification based on gas measurements (Jakus *et al.* 2021a) and on the previously experimentally determined stoichiometric ratio of $nitrate_{reduced}/Fe(II)_{oxidized}$, which was 0.28 ± 0.1, approximating the expected value of 0.2 for complete denitrification. Therefore, the microbial community members likely have to cooperate to complete the process via a series of metabolic handoffs.

Iron oxidation by the autotrophic NRFeOx community

The mechanisms for autotrophic Fe(II) oxidation in neutrophilic Fe(II)-oxidizing bacteria remain largely unknown and are hypothesized to be facilitated by extracellular electron transfer (Ilbert and Bonnefoy 2013; He *et al.* 2017). Using the FeGenie software (Garber *et al.* 2020), we searched for putative genes involved in Fe(II) oxidation (i.e. *cyc1*, *cyc2*, *foxABC*, *foxEYZ*, *sulfocyanin*, *pioABC* and *mtoAB*) that resulted in the detection of the genes in 3 out of all 15 MAGs: the *Gallionellaceae* sp. (AG11), *Thiobacillus denitrificans* (AG14) and unclassified *Betaproteobacteria* sp. (AG20). All three MAGs possessed homologs of the gene encoding an outer membrane cytochrome *c*, *cyc2* (Figs 4 and 5A). However, it was previously found that Fe(II) oxidation in *Thiobacillus denitrificans* is not catalyzed by any *c*-type cytochrome (Beller *et al.* 2013), and we therefore conclude that the identified homolog might rather be a heme-containing protein that may participate in a yet-to-be defined electron transfer pathway rather than Fe(II) oxidation. For further discussion, we thus consider only the dominant *Gallionellaceae* sp. and unclassified *Betaproteobacteria* MAGs to contain the gene potentially involved in energy generation via Fe(II) oxidation. The *cyc2* homolog recovered from the unclassified *Gallionellaceae* sp. grouped phylogenetically with the putative *cyc2* of other known neutrophilic Fe(II) oxidizers (Fig. 5A), indicating that this microbe may also use Cyc2 to facilitate Fe(II) oxidation. A homolog of the *cyc2* gene identified in the MAG AG20 was found to be more phylogenetically similar to cytochromes present in *Dechloromonas* sp. and *Methylotenera* sp. genomes and other microbes not commonly classified as typical Fe(II) oxidizers (Fig. 5A). The 16S rRNA gene retrieved from this MAG is incomplete (954 bp), and we therefore performed additional comparative genomic analyses (multilocus sequence analysis, see Fig. S3 [Supporting Information]) that revealed that the unclassified *Betaproteobacteria* MAG (AG20) containing a *cyc2* gene might be affiliated with so far unclassified *Methyloversatilis* spp. Interestingly, in previously published studies, a methylotrophic nitrate-reducing *Methyloversatilis* sp. was found to grow in the presence of Fe(II), but no potential Fe(II) oxidase was identified. Further, using annotations of the MAGs and metagenome produced by the IMG/MER pipeline, we searched for putative Fe(II) oxidation genes and detected the presence of homologs of three more putative oxidases: a homolog of a gene encoding MofB in the MAG of *Methylotenera* sp. (AG31), and PcoAB in MAGs of both *Curvibacter* sp. (AG29) and *Thiobacillus denitrificans* (AG14) (see the Supporting Information). Since we were unable to retrieve a 16S rRNA gene sequence of the *Curvibacter* sp. MAG (AG29), we compared 16S rRNA gene amplicon sequence-based relative abundances of ASVs in the autotrophic NRFeOx culture with estimated coverages of the MAGs (Table 1), revealing that the *Curvibacter* sp. MAG (AG29) most likely represents the unclassified *Burkholderiaceae* sp. (accounting for 3% relative abundance). The same ASV was additionally identified in the culture growing in the gradient tube and in the culture growing under mixotrophic conditions (Fig. 3), which further supports the hypothesis that this taxon has the ability to oxidize Fe(II). Although *Curvibacter* spp. were recently recognized as FeOB (Gülay *et al.* 2018a), the genes encoding proteins involved in this process have not yet been identified; in addition, the electron transfer pathway has not yet been proposed. Therefore, we suggest that Fe(II)-oxidizing *Curvibacter* spp., closely related to the organism identified in this study, are likely to use the putative Fe(II) oxidase PcoAB to promote Fe(II) oxidation.

Sulfur oxidation by the autotrophic NRFeOx community

Pyrite present in the aquifer is a potential source of both Fe(II) and reduced sulfur. Since the complete oxidation of sulfur involves multiple electron transfer steps, we searched for genes involved in the first and the last oxidation step to identify potential microbial key players in this process. Ten out of 15 most complete MAGs contained sulfide:quinone oxidoreductase (*sqr*) genes providing the potential for oxidation of sulfide (S^{2-}) to zero-valent sulfur (S^0) (Berben *et al.* 2019). This oxidation step can also be mediated by the flavocytochrome *c* sulfide dehydrogenase (*fccAB*) genes (Berben *et al.* 2019) that were not detected in the metagenome (Table S1, Supporting Information). Genes needed for thiosulfate ($S_2O_3^{2-}$) oxidation (*sox* cluster) leading to sulfate (SO_4^{2-}) formation were present in most of the MAGs, including those identified as *Thiobacillus denitrificans* (AG14), *Methylotenera* sp. (AG31) and both *Curvibacter* spp. (AG29 and AG1). Similarly, homologs of sulfur-oxidizing *sox* genes were recovered in all of the MAGs in our study, which cover a diverse range of community members, except for the *Chitinophagaceae* sp. The genome of the dominant *Gallionellaceae* sp. (AG11) assembled in our study possessed neither *sqr* nor *sox* genes, and is most probably unable to participate in steps initiating and completing the sulfur oxidation pathway. This implies that the most dominant *Gallionellaceae* sp. of our study is likely better adapted for growth with Fe(II) than reduced S-compounds, even though the ability to grow on these compounds would give it a competitive advantage during pyrite oxidation after all available Fe(II) is oxidized.

Autotrophic carbon fixation by the NRFeOx community

Even though the NRFeOx culture enriched from the aquifer is able to continuously grow under autotrophic conditions, with CO_2 being the sole carbon source (Jakus *et al.* 2021a), only eight MAGs were found to contain the ribulose-1,5-bisphosphate carboxylase/oxygenase gene, encoding the key enzyme of the CBB cycle, used for CO_2 fixation. In total, 19 copies of this gene

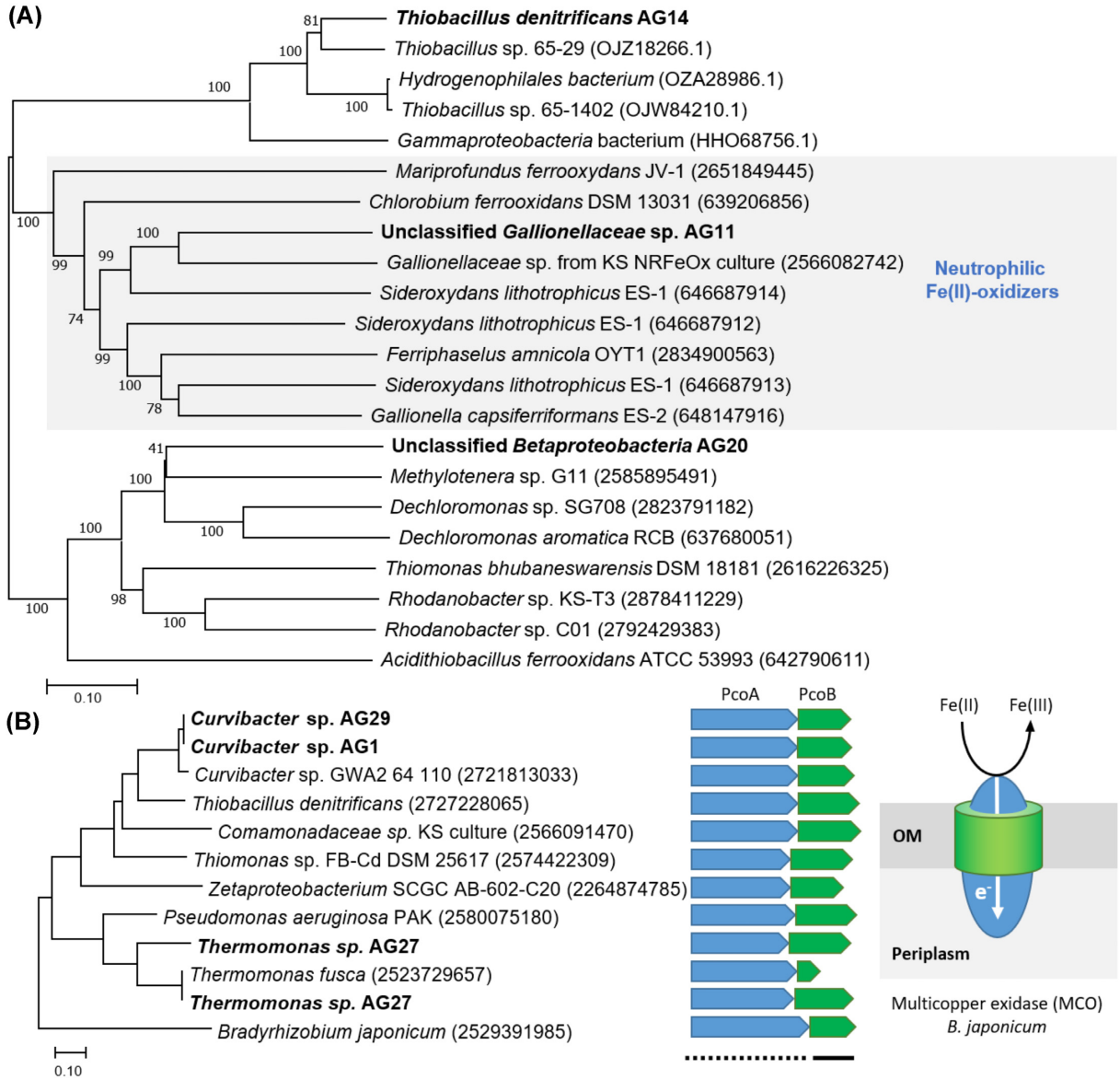

Figure 5. Phylogenetic trees of putative Fe(II)-oxidase homologs: Cyc2 **(A)** and PcoAB **(B)** identified from MAGs recovered in this study (bold font). Sequences of *Thiobacillus denitrificans* sp. (AG14), unclassified *Gallionellaceae* sp. (AG11) and unclassified *Betaproteobacteria* sp. (AG20) are shown in relation to other homologs of Cyc2, including protein sequences of commonly known neutrophilic Fe(II) oxidizers (gray background). *Curvibacter* spp. (AG29 and AG1) and *Thermomonas* sp. (AG27) are shown in relation to homologous sequences of PcoAB. Predicted cellular locations of encoded PcoAB are shown by different line types under the genes, with dashed lines and thick lines indicating periplasm and outer membrane, respectively. The trees were produced by neighbor joining in the MEGAX software suite using the *p*-distance model and pairwise gap deletion. Bootstrap values are the percentage of 1000 trials in which a given node was present. Numbers in brackets are IMG gene ID accession numbers. The scale bar corresponds to 0.1 nucleotide substitution per site.

were annotated in the whole metagenome but seven of them were excluded from further analysis due to short sequence length (<150 bp; Table S3, Supporting Information). Four copies of the gene belonging to the MAGs of the *Thiobacillus denitrificans* (AG14), *Acidovorax* sp. (AG26), *Rhizobium* sp. (AG18), as well as an unbinned sequence, were identified as *rbcS* genes. The *rbcS* gene encodes RuBisCO Form I (Fig. S4, Supporting Information), a key protein participating in CO_2 fixation particularly in environments with elevated levels of O_2 (Badger and Bek 2008). The unbinned sequence of the *rbcS* gene showed the highest similarity to *rbcS* found in the genome of *Acidovorax* sp. SD340 (WP_055394172.1, 100% query cover, 100% identity). The other gene encoding RuBisCO, *rbcL/cbbM* (Form II; Fig. S4, Supporting Information), was identified in the MAGs of *Gallionellaceae* sp. (AG11), unclassified *Betaproteobacteria* sp. (AG28), *Curvibacter delicatus* (AG29) and two more sequences that were not affiliated to any genome bins but had the highest similarity to *rbcL/cbbM* sequences identified in *Dechloromonas aromatica* (WP_011289362.1, 100% query cover, 96.51% and 91.72% identity). Additionally, the *Thiobacillus denitrificans* MAG (AG17) was found to also possess genes encoding Form II RuBisCO alongside the Form I, which was expected for this organism based on a previous study (English *et al.* 1992). We also found that two MAGs contained genes encoding RuBisCO Form IV (Fig. S4, Supporting Information) that is unable to catalyze CO_2 fixation because of key substitutions of many essential active-site

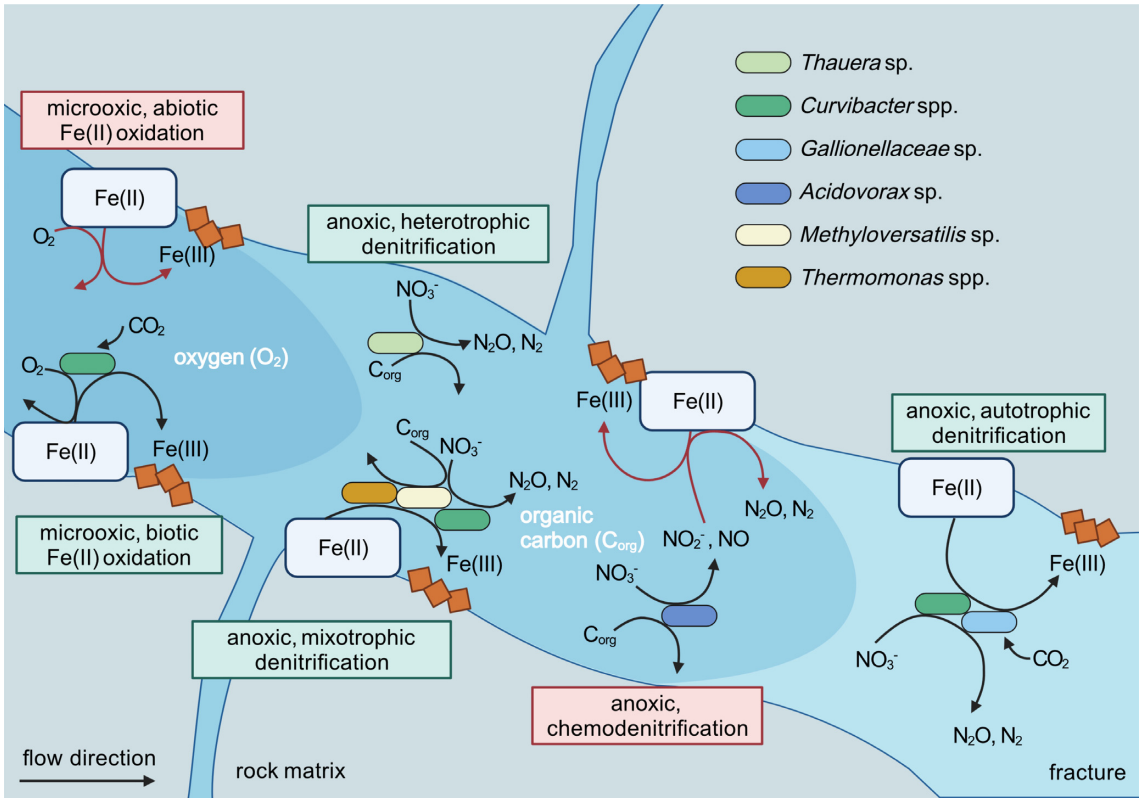

Figure 6. A conceptual model of biogeochemical reactions and microbial key players controlling the fate of Fe(II) and NO_3^- in response to fluxes of O_2 and bioavailable organic carbon (C_{org}) along the groundwater flow through fractures in the studied aquifer. First, oxygen entering the aquifer reacts either abiotically with Fe(II) phases or is oxidized by microaerophilic FeOB (e.g. *Curvibacter* spp.), leading to precipitation of Fe(III) oxyhydroxides. Once all O_2 is exhausted, NO_3^- reduction is coupled to C_{org} oxidation via heterotrophic denitrification (e.g. by *Thauera* sp.), and mixotrophic denitrification (with Fe(II) as a co-substrate, e.g. by *Thermomonas* spp., *Methyloversatilis* sp. and *Curvibacter* spp.) commences, likely leading to the formation of N_2O or N_2. Additionally, heterotrophic denitrification leading to the formation of reactive N-species (NO_2^-, NO) (e.g. by *Acidovorax* sp.) may trigger abiotic oxidation of Fe(II) and, as a consequence, further reduce the reactive N-species (chemodenitrification). Finally, after all bioavailable C_{org} is consumed, NO_3^- can be reduced via Fe(II) oxidation by autotrophic denitrifiers (e.g. *Gallionellaceae* sp., *Curvibacter* spp.). Note that white rectangles represent all forms of Fe(II) available for microbes in the studied aquifer, including pyrite, Fe(II)-bearing carbonates and aqueous Fe^{2+}. Red arrows and boxes indicate abiotic reactions.

residues, but it was found to play a role in sulfur metabolism (thiosulfate oxidation) (Hanson and Tabita 2001, 2003; Tabita *et al.* 2008). Additionally, using the IMG JGI platform, we surveyed the metagenome for the presence of key genes involved in CO_2 fixation pathways alternative to the CBB cycle. The search resulted in the identification of 3-methylfumaryl-CoA hydratase homologs, belonging to the 3-hydroxypropanoate bicycle (Berg *et al.* 2010), and a 4-hydroxybutanoyl-CoA dehydratase homolog, involved in the 3-hydroxypropanoate, 4-hydroxybutyrate and dicarboxylate pathways (Berg *et al.* 2010). However, none of the other key genes involved in these pathways were found and, therefore, we hypothesize that the identified genes might be involved in other pathways than CO_2 fixation. A detailed list of the investigated CO_2 fixation pathways and genes can be found in Tables S5 and S6 (Supporting Information).

Potential oxygen reduction by the autotrophic NRFeOx community

Conventional biological denitrification requires hypoxic conditions with dissolved oxygen concentrations lower than 0.2 mg L^{-1} (Seitzinger *et al.* 2006). However, in the studied aquifer, structural discontinuities such as bedding planes or fractures produce preferential flow paths, which can transport oxygen (Osenbrück *et al.* 2021). Microbes living in such environments are often adapted to take an advantage of local redox changes and respire oxygen when it is present at low concentrations. Genes encoding for the *cbb3*-type cytochrome *c* (*ccoGPONQ*) were identified in all 15 MAGs with the exception of *Curvibacter delicatus* (AG29) and *Geothrix fermentans* (AG5). The lack of the genes encoding *cbb3*-type cytochrome *c* in the *Curvibacter delicatus* MAG is particularly surprising and might be linked to the MAG incompleteness (89.9% completeness) since this taxon was found to dominate the community growing under microoxic conditions. The majority of the MAGs, including the *Gallionellaceae* sp. (AG11), were also missing gene *cydABX* coding for another terminal oxidase: the cytochrome *bd* oxidase. As expected from our culture originating from an anoxic aquifer, none of the genomes was found to possess *aa3*-type cytochrome oxidases, which is a gene involved in oxygen reduction and therefore would require elevated oxygen levels (Castelle *et al.* 2008).

Candidates of NRFeOx bacteria

The unclassified *Gallionellaceae* sp. dominating the autotrophic NRFeOx culture most likely is a truly autotrophic NRFeOx organism, performing Fe(II) oxidation via Cyc2 coupled to near-complete denitrification. Other potential autotrophic NRFeOx candidates identified in this study are *Curvibacter* spp. and *Thiobacillus denitrificans*, previously reported to be able to couple

nitrate reduction to Fe(II) and pyrite oxidation (Bosch and Meckenstock 2012; Bosch *et al*. 2012). However, *Thiobacillus denitrificans* has never been shown to maintain Fe(II) oxidation over several transfers without organic carbon addition (Bryce *et al*. 2018) and therefore its ability to continuously perform autotrophic NRFeOx remains questionable. Furthermore, we classified *Methyloversatilis* sp. and *Thermomonas* sp. as additional candidates for NRFeOx bacteria, since these organisms possessed all genes required to facilitate almost complete denitrification and genes putatively involved in Fe(II) oxidation. Neither of them were found to harbor genes required for carbon fixation. The lack of these genes in the *Methyloversatilis* sp. MAG can be associated with the MAG incompleteness, since genomes of other bacteria related to this genus (e.g. *Methyloversatilis* sp. RAC08) possess genes involved in carbon fixation. This further indicates that the *Methyloversatilis* sp., enriched in the culture supplemented with nitrate and Fe(II) (only), is most probably a facultative mixotrophic NRFeOx organism, able to survive under autotrophic conditions. Additionally, the MAG of the *Thermomonas* sp. revealed denitrification genes and *pcoAB* but was lacking genes involved in carbon fixation pathways. Bacteria related to *Thermomonas* spp. are typically heterotrophs (Mergaert, Cnockaert and Swings 2003), and therefore PcoAB might be used facultatively for ATP production under limitation of organic carbon. Alternatively, the identified homolog of the putative Fe(II) oxidase may facilitate a different process than Fe(II) oxidation and we currently do not have enough evidence to consider this bacterium a potential NRFeOx candidate. Interestingly, the MAG of the second most abundant species in the autotrophic NRFeOx culture, *Acidovorax* sp. (16.4%), was found to have a complete set of genes to reduce NO_3^- to N_2 together with genes encoding CO_2 fixation but none of the putative Fe(II) oxidation genes was present. The lack of the enzymatic component of Fe(II) oxidation was previously reported in a draft genome of the NRFeOx *Acidovorax* sp. BoFeN1 (Price *et al*. 2018). Fe(II) oxidation catalyzed by this taxon was therefore interpreted to be a result of chemodenitrification (Klueglein and Kappler 2013; Bryce *et al*. 2018). Thus, the *Acidovorax* sp. present in the NRFeOx culture is therefore most likely a 'chemodenitrifier', catalyzing nitrate reduction (coupled to organic carbon consumption) and indirectly contributing to Fe(II) oxidation, although an enzymatic contribution to Fe(II) oxidation cannot be ruled out.

Fe(II) and organic carbon availability changes the composition of subsurface denitrifying communities

The availability of Fe(II) selects for a subset of denitrifying microbial assemblages that in the presence of organic substrates are able to tolerate, resist or transform Fe(II) by chemodenitrification or by enzymatic oxidation, to gain energy from simultaneous utilization of Fe(II) and organic compounds (mixotrophy). Alternatively, when the readily degradable organic carbon is limited (which often is the case in deeper aquifers; Griebler and Lueders 2009), microbes are able to perform denitrification coupled to Fe(II) oxidation, and fixation of CO_2 for energy and biomass production (autotrophy) may become predominant. Here, we found that only four taxa were shared between the different enrichment cultures taking an advantage of metabolic adaptation to exploit various electron donors and acceptors, which indicates that the supply of Fe(II) and in/organic carbon shapes very specialized and distinct assemblages. Among these organisms, the most metabolically flexible were bacteria related to *Burkholderiaceae* sp. and identified as *Curvibacter delicatus*, dominating mixotrophic communities and the culture subjected to microoxic conditions. This flexibility is especially advantageous in an aquifer, where fluxes of recent groundwater transported along the horizontal, karstified bedding planes or fractures may carry oxygen, nitrate and organic compounds and mix with anoxic, electron donor-depleted groundwater (Hofmann *et al*. 2020; Osenbrück *et al*. 2021) (Fig. 6).

The culture represents one of a few known existing autotrophic NRFeOx cultures that are continuously grown for a span of several years without the addition of organic carbon, and fulfilling most of the criteria for truly NRFeOx cultures defined by Bryce *et al*. (2018). Additionally, the capability of the CO_2 uptake was now confirmed by the presence of genes encoding RuBisCO in 8 out of 15 recovered MAGs. The culture contains several so far uncultured taxa and potential candidates for autotrophic NRFeOx bacteria. The physiology of these taxa remains rather speculative, and thus, our experiments provide novel insights into the potential lifestyles of uncultivated organisms relevant for nitrate-contaminated aquifer sites. Our findings have implications for assessing the denitrification potential of microbial communities thriving in subsurface ecosystems like many aquifers, where the host rock contains Fe(II) minerals, which can serve as electron donor, supporting chemolithotrophic microbial metabolisms and nitrate removal even under organic carbon limitation.

ACKNOWLEDGMENTS

We would like to thank Stefanie Becker, Caroline Schlaiss and Jonah Schooss for the maintenance of cultures and help with DNA extractions. AK acknowledges infrastructural support by the DFG under Germany's Excellence Strategy, Cluster of Excellence [EXC2124, project ID 390838134].

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in GenBank/Sequence Read Archive at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA678222, and can be accessed with PRJNA678222.

AUTHOR CONTRIBUTIONS

NJ, NB and SK designed the study. NJ and DS analyzed the data. NJ, NB and SK interpreted the data and NJ wrote the manuscript with the help of NB, DS, SK and AK. All authors commented and approved the final manuscript.

FUNDING

This work was funded by the Institutional Strategy of the University of Tübingen (German Research Foundation; Deutsche Forschungsgemeinschaft [DFG], Zukunftskonzept [ZUK] 63) and by the Collaborative Research Center 1253 CAMPOS (Project 5: Fractured Aquifers), funded by the German Research Foundation (DFG grant agreement 1253/1 2017). SK is supported by an Emmy Noether fellowship (DFG grant 326028733).

Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Apprill A, McNally S, Parsons R *et al.* Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol* 2015;**75**:129–37.

Badger MR, Bek EJ. Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. *J Exp Bot* 2008;**59**:1525–41.

Beller HR, Zhou P, Legler TC *et al.* Genome-enabled studies of anaerobic, nitrate-dependent oxidation in the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Front Microbiol* 2013;**4**:1–16.

Bellini MI, Gutiérrez L, Tarlera S *et al.* Isolation and functional analysis of denitrifiers in an aquifer with high potential for denitrification. *Syst Appl Microbiol* 2013;**36**:505–16.

Berben T, Overmars L, Sorokin DY *et al.* Diversity and distribution of sulfur oxidation-related genes in *Thioalkalivibrio*, a genus of chemolithoautotrophic and haloalkaliphilic sulfur-oxidizing bacteria. *Front Microbiol* 2019;**10**:1–15.

Berg IA, Kockelkorn D, Ramos-Vera WH *et al.* Autotrophic carbon fixation in archaea. *Nat Rev Microbiol* 2010;**8**:447–60.

Bethencourt L, Bochet O, Farasin J *et al.* Genome reconstruction reveals distinct assemblages of Gallionellaceae in surface and subsurface redox transition zones. *FEMS Microbiol Ecol* 2020;**96**:1–13.

Bosch J, Lee KY, Jordan G *et al.* Anaerobic, nitrate-dependent oxidation of pyrite nanoparticles by *Thiobacillus denitrificans*. *Environ Sci Technol* 2012;**46**:2095–101.

Bosch J, Meckenstock RU. Rates and potential mechanism of anaerobic nitrate-dependent microbial pyrite oxidation. *Biochem Soc Trans* 2012;**40**:1280–3.

Bryce C, Blackwell N, Schmidt C *et al.* Microbial anaerobic Fe(II) oxidation: ecology, mechanisms and environmental implications. *Environ Microbiol* 2018;**20**:3462–83.

Carlson HK, Clark IC, Blazewicz SJ *et al.* Fe(II) oxidation is an innate capability of nitrate-reducing bacteria that involves abiotic and biotic reactions. *J Bacteriol* 2013;**195**:3260–8.

Castelle C, Guiral M, Malarte G *et al.* A new iron-oxidizing/O_2-reducing supercomplex spanning both inner and outer membranes, isolated from the extreme acidophile *Acidithiobacillus ferrooxidans*. *J Biol Chem* 2008;**283**:25803–11.

Chakraborty A, Picardal F. Induction of nitrate-dependent Fe(II) oxidation by Fe(II) in *Dechloromonas* sp. strain UWNR4 and *Acidovorax* sp. strain 2AN. *Appl Environ Microbiol* 2013;**79**:748–52.

Chen IMA, Chu K, Palaniappan K *et al.* IMG/M v.5.0: an integrated data management and comparative analysis system for microbial genomes and microbiomes. *Nucleic Acids Res* 2019;**47**:D666–77.

Chu L, Wang J. Denitrification of groundwater using a biodegradable polymer as a carbon source: long-term performance and microbial diversity. *RSC Adv* 2017;**7**:53454–62.

Emerson D, Floyd MM. Enrichment and isolation of iron-oxidizing bacteria at neutral pH. *Methods Enzymol* 2005;**397**:112–23.

Emerson D, Moyer C. Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl Environ Microbiol* 1997;**63**:4784–92.

English RS, Williams CA, Lorbach SC *et al.* Two forms of ribulose-l,5-bisphosphate carboxylase/oxygenase from *Thiobacillus denitrificans*. *FEMS Microbiol Lett* 1992;**73**:111–9.

Etchebehere C, Tiedje J. Presence of two different active nirS nitrite reductase genes in a denitrifying *Thauera* sp. from a high-nitrate-removal-rate reactor. *Appl Environ Microbiol* 2005;**71**:5642–5.

European Commission. Report from the commission to the council and the European Parliament on the implementation of Council Directive 91/676/EEC. *Eur Comm* 2018:14.

European Union. Directive 2006/118/EC of the European Parliament and of the council of 12 December 2006 on the protection of groundwater against pollution and deterioration. *Off J Eur Union 2006*;**19**:19–31.

Ewels PA, Peltzer A, Fillinger S *et al.* The nf-core framework for community-curated bioinformatics pipelines. *Nat Biotechnol* 2020;**38**:271.

Garber AI, Nealson KH, Okamoto A *et al.* FeGenie: a comprehensive tool for the identification of iron genes and iron gene neighborhoods in genome and metagenome assemblies. *Front Microbiol* 2020;**11**:1–23.

Griebler C, Lueders T. Microbial biodiversity in groundwater ecosystems. *Freshw Biol* 2009;**54**:649–77.

Gülay A, Çekiç Y, Musovic S *et al.* Diversity of iron oxidizers in groundwater-fed rapid sand filters: evidence of Fe(II)-dependent growth by *Curvibacter* and *Undibacterium* spp. *Front Microbiol* 2018;**9**:1–14.

Hallbeck L, Ståhl F, Pedersen K. Phylogeny and phenotypic characterization of the stalk-forming and iron-oxidizing bacterium Gallionella ferruginea. *J Gen Microbiol* 1993;**139**:1531–5.

Hanson TE, Tabita FR. A ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO)-like protein from *Chlorobium tepidum* that is involved with sulfur metabolism and the response to oxidative stress. *Proc Natl Acad Sci USA* 2001;**98**:4397–402.

Hanson TE, Tabita FR. Insights into the stress response and sulfur metabolism revealed by proteome analysis of a *Chlorobium tepidum* mutant lacking the Rubisco-like protein. *Photosynth Res* 2003;**78**:231–48.

He S, Barco RA, Emerson D *et al.* Comparative genomic analysis of neutrophilic iron(II) oxidizer genomes for candidate genes in extracellular electron transfer. *Front Microbiol* 2017;**8**:1–17.

He S, Tominski C, Kappler A *et al.* Metagenomic analyses of the autotrophic Fe(II)-oxidizing, nitrate-reducing enrichment culture KS. *Appl Environ Microbiol* 2016;**82**:2656–68.

Hofmann R, Uhl J, Hertkorn N *et al.* Linkage between dissolved organic matter transformation, bacterial carbon production, and diversity in a shallow oligotrophic aquifer: results from flow-through sediment microcosm experiments. *Front Microbiol* 2020;**11**:2425.

Huang Y-M, Straub D, Blackwell N *et al.* Meta-omics reveal Gallionellaceae and Rhodanobacter as interdependent key players for Fe(II) oxidation and nitrate reduction in the autotrophic enrichment culture KS. *Appl Environ Microbiol* 2021a;**87**:e0049621.

Huang Y-M, Straub D, Kappler A *et al.* A novel enrichment culture highlights core features of microbial networks contributing to autotrophic Fe(II) oxidation coupled to nitrate reduction. *Microb Physiol* 2021b;**1–16**:000517083.

Ilbert M, Bonnefoy V. Insight into the evolution of the iron oxidation pathways. *Biochim Biophys Acta* 2013;**1827**:161–75.

Jakus N, Blackwell N, Osenbrück K *et al*. Nitrate removal by a novel lithoautotrophic nitrate-reducing iron(II)-oxidizing culture enriched from a pyrite-rich limestone aquifer. *Appl Environ Microbiol* 2021a;**87**:e0046021.

Jakus N, Mellage A, Hoeschen C *et al*. Anaerobic neutrophilic pyrite oxidation by a chemolithoautotrophic nitrate-reducing iron(II)-oxidizing culture enriched from a fractured aquifer. *Environ Sci Technol* 2021b;**55**:9876–84.

Jewell TNM, Karaoz U, Brodie EL *et al*. Metatranscriptomic evidence of pervasive and diverse chemolithoautotrophy relevant to C, S, N and Fe cycling in a shallow alluvial aquifer. *ISME J* 2016;**10**:2106–17.

Jørgensen CJ, Jacobsen OS, Elberling B *et al*. Microbial oxidation of pyrite coupled to nitrate reduction in anoxic groundwater sediment. *Environ Sci Technol* 2009;**43**:4851–7.

Kazakis N, Matiatos I, Ntona MM *et al*. Origin, implications and management strategies for nitrate pollution in surface and ground waters of Anthemountas basin based on a δ^{15}N-NO_3- and δ^{18}O-NO_3- isotope approach. *Sci Total Environ* 2020;**724**:138211.

Klueglein N, Kappler A. Abiotic oxidation of Fe(II) by reactive nitrogen species in cultures of the nitrate-reducing Fe(II) oxidizer *Acidovorax* sp. BoFeN1: questioning the existence of enzymatic Fe(II) oxidation. *Geobiology* 2013;**11**:180–90.

Krakau S, Straub D, Gourlé H *et al*. nf-core/mag: a best-practice pipeline for metagenome hybrid assembly and binning. *bioRxiv* 2021. DOI: 10.1101/2021.08.29.458094.

Kumar S, Herrmann M, Blohm A *et al*. Thiosulfate- and hydrogen-driven autotrophic denitrification by a microbial consortium enriched from groundwater of an oligotrophic limestone aquifer. *FEMS Microbiol Ecol* 2018;**94**:1–13.

Kuypers MMM, Marchant HK, Kartal B. The microbial nitrogen-cycling network. *Nat Rev Microbiol* 2018;**16**:263–76.

Li J, Hao H, Cheng G *et al*. Enhancing nitrate removal from freshwater pond by regulating carbon/nitrogen ratio. *Front Microbiol* 2017;**8**:1–9.

Mergaert J, Cnockaert MC, Swings J. *Thermomonas fusca* sp. nov. and *Thermomonas brevis* sp. nov., two mesophilic species isolated from a denitrification reactor with poly(E-caprolactone) plastic granules as fixed bed, and emended description of the genus *Thermomonas*. *Int J Syst Evol Microbiol* 2003;**53**:1961–6.

Muehe EM, Gerhardt S, Schink B *et al*. Ecophysiology and the energetic benefit of mixotrophic Fe(II) oxidation by various strains of nitrate-reducing bacteria. *FEMS Microbiol Ecol* 2009;**70**:335–43.

Mustakhimov I, Kalyuzhnaya MG, Lidstrom ME *et al*. Insights into denitrification in *Methylotenera mobilis* from denitrification pathway and methanol metabolism mutants. *J Bacteriol* 2013;**195**:2207–11.

Osenbrück K, Blendinger E, Leven C *et al*. Nitrate reduction potential of a fractured Middle Triassic carbonate aquifer in southwest Germany. *Submitted* 2021.

Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016;**18**:1403–14.

Pauwels H, Kloppmann W, Foucher JC *et al*. Field tracer test for denitrification in a pyrite-bearing schist aquifer. *Appl Geochem* 1998;**13**:767–78.

Postma D, Boesen C, Kristiansen H *et al*. Nitrate reduction in an unconfined sandy aquifer: water chemistry, reduction processes, and geochemical modeling. *Water Resour Res* 1991;**27**:2027–45.

Price A, Macey MC, Miot J *et al*. Draft genome sequences of the nitrate-dependent iron-oxidizing proteobacteria *Acidovorax* sp. strain BoFeN1 and *Paracoccus pantotrophus* strain KS1. *Microbiol Resour Annouc* 2018;**5**:17–8.

Schaedler F, Lockwood C, Lueder U *et al*. Microbially mediated coupling of Fe and N cycles by nitrate- reducing Fe(II)-oxidizing bacteria in littoral freshwater sediments. 2018;**84**:1–14.

Schwientek M, Einsiedl F, Stichler W *et al*. Evidence for denitrification regulated by pyrite oxidation in a heterogeneous porous groundwater system. *Chem Geol* 2008;**255**:60–7.

Seitzinger S, Harrison JA, Böhlke JK *et al*. Denitrification across landscapes and waterscapes: a synthesis. *Ecol Appl* 2006;**16**:2064–90.

Straub D, Blackwell N, Langarica-Fuentes A *et al*. Interpretations of environmental microbial community studies are biased by the selected 16S rRNA (gene) amplicon sequencing pipeline. *Front Microbiol* 2020;**11**:1–18.

Tabita FR, Satagopan S, Hanson TE *et al*. Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. *J Exp Bot* 2008;**59**:1515–24.

Tominski C, Lösekann-Behrens T, Ruecker A *et al*. Insights into carbon metabolism provided by fluorescence in situ hybridization-secondary ion mass spectrometry imaging of an autotrophic, nitrate-reducing, Fe(II)-oxidizing enrichment culture. *Appl Environ Microbiol* 2018;**84**:1–19.

Visser AN, Lehmann MF, Rügner H *et al*. Fate of nitrate during groundwater recharge in a fractured karst aquifer in Southwest Germany. *Hydrogeol J* 2020;**29**:1153–71.

Wang Y, Tian H, Huang F *et al*. Time-resolved analysis of a denitrifying bacterial community revealed a core microbiome responsible for the anaerobic degradation of quinoline. *Sci Rep* 2017;**7**:1–11.

Zhang Y-C, Slomp CP, Broers HP *et al*. Isotopic and microbiological signatures of pyrite-driven denitrification in a sandy aquifer. *Chem Geol* 2012;**300–301**:123–32.