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Research Article

RESEARCH ARTICLE

Thiosulfate- and hydrogen-driven autotrophic denitrification by a microbial consortium enriched from groundwater of an oligotrophic limestone aquifer

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One sentence summary: Initial electron acceptor/electron donor ratios of nitrate and thiosulfate strongly affected complete denitrification of a groundwater enrichment culture, dominated by *Thiobacillus* sp., *Sulfuritalea hydrogenivorans*, *Sulfuricella denitrificans* and *Hydrogenophaga* sp.

ABSTRACT

Despite its potentially high relevance for nitrate removal in freshwater environments limited in organic carbon, chemolithoautotrophic denitrification has rarely been studied in oligotrophic groundwater. Using thiosulfate and H_2 as electron donors, we established a chemolithoautotrophic enrichment culture from groundwater of a carbonate-rock aquifer to get more insight into the metabolic repertoire, substrate turnover, and transcriptional activity of subsurface denitrifying consortia. The enriched consortium was dominated by representatives of the genus *Thiobacillus* along with denitrifiers related to *Sulfuritalea hydrogenivorans*, *Sulfuricella denitrificans*, *Dechloromonas* sp. and *Hydrogenophaga* sp., representing the consortium's capacity to use multiple inorganic electron donors. Microcosm experiments coupled with Raman gas spectroscopy demonstrated complete denitrification driven by reduced sulfur compounds and hydrogen without formation of N_2O. The initial nitrate/thiosulfate ratio had a strong effect on *nosZ* transcriptional activity and on N_2 formation, suggesting similar patterns of the regulation of gene expression as in heterotrophic denitrifiers. Sequence analysis targeting *nirS* and *nosZ* transcripts identified *Thiobacillus denitrificans*-related organisms as the dominant active *nirS*-type denitrifiers in the consortium. An additional assessment of the *nirS*-type denitrifier community in the groundwater clearly confirmed the potential for sulfur- and hydrogen-dependent chemolithoautotrophic denitrification as important metabolic feature widely spread among subsurface denitrifiers at the Hainich Critical Zone Exploratory.

Keywords: Raman gas spectroscopy; chemolithoautotrophy; *nirS*; *nosZ*; groundwater

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INTRODUCTION

Increasing reactive nitrogen can negatively impact terrestrial and aquatic ecosystems worldwide, leading to eutrophication cascades and health hazards (Fields 2004; Rivett *et al.* 2008). Underlying subsurface structures, especially karst aquifers that are the major source of drinking water for approximately 20%–25% of the global population, are under continuous threat from nitrate contamination (Ford and Williams 2007; Stevanoivić 2015). Groundwater denitrifying microbial populations are known to have a profound effect on the fate of these solutes, by reducing nitrate to N_2 gas, with their diversity and contribution to nitrogen loss being affected by the availability of different electron donors (Hiscock, Lloyd and Lerner 1991; Korom 1992; Seitzinger *et al.* 2006; Højberg *et al.* 2017).

Pristine aquifers are often limited in organic carbon, restricting the overall contribution of the heterotrophic denitrifying microbial community to groundwater nitrogen loss (Alföldi 1988; Ghiorse and Wilson 1988). In contrast, autotrophic denitrifying bacteria can prevail in these organic carbon-limited ecosystems, utilizing inorganic electron donors such as reduced sulfur derived from iron sulfide minerals and H_2 as energy source for CO_2-fixation (Chapelle 1993; Nelson and Hagen 1995; Ottley, Davison and Edmunds 1997; Lau *et al.* 2016). In addition, recent metatranscriptomics and metaproteomics analyses demonstrated a high chemolithoautotrophic potential in aquifers linked to C, S, N and Fe cycling (Jewell *et al.* 2016, 2017; Starke *et al.* 2017). Most of the chemolithotrophic denitrifying organisms isolated and described from freshwater environments such as lakes and aquifers belong to the *Proteobacteria*, such as the genera *Thiobacillus, Sulfuricella, Sulfuritalea, Sulfurimonas, Dechloromonas, Hydrogenophaga* and *Acinetobacter* (Smith, Ceazan and Brooks 1994; Alfreider, Schirmer and Vogt 2003, 2009, 2012; Aburto *et al.* 2009; Kellermann and Griebler 2009; Kojima and Fukui 2010, 2011; 2012; Han and Perner 2015; Zeng *et al.* 2016). Although the presence of chemolithoautotrophic denitrifiers in oligotrophic subsurface environments has been well documented, their capacity to utilize reduced sulfur compounds and H_2 on the community level has rarely been demonstrated.

Among inorganic electron donors for nitrate reduction that may typically occur in subsurface environments, thiosulfate is a both readily available and a non-toxic sulfur compound, while hydrogen-driven nitrate removal has relatively faster kinetics, and the presence of both electron donors may synergistically support the effective reduction of nitrate to N_2 gas (Ergas and Reuss 2001; Lee and Rittmann 2002; Cardoso *et al.* 2006; Zhu and Getting 2012). From a stoichiometric point of view, complete reduction of 1 mol of nitrate to N_2 requires 0. 625 mol thiosulfate (1) or 2.5 mol H_2 (2) (Mateju *et al.* 1992).

$$8\,NO_3^- + 5\,S_2O_3^{2-} + H_2O \rightarrow 4\,N_2 + 10\,SO_4^{2-} + 2\,H^+ \quad (1)$$

$$2\,NO_3^- + 5\,H_2 \rightarrow N_2 + 4H_2O + 2OH^- \quad (2)$$

Groundwater of the Hainich Critical Zone Exploratory (CZE) (Thuringia, Germany) has previously been reported to harbor a high genetic potential for chemolithoautotrophic denitrification linked to the oxidation of reduced sulfur compounds (Herrmann *et al.* 2015, 2017; Kumar *et al.* 2017). Moreover, carbon isotope signatures of dissolved inorganic carbon supported a strong contribution of chemolithoautotrophy to the build-up of groundwater microbial biomass (Nowak *et al.* 2017; Schwab *et al.* 2017). Previous studies also revealed that groundwater denitrification activity proceeds at rates as low as 0.7 nmol N_2 L^{-1} d^{-1} (Kumar *et al.* 2017), providing limited resolution when studying substrate turnover and activity of key players at the *in situ* scale. Consequently, to follow production and consumption of intermediates of denitrification and transcriptional activity of key players in more detail, we enriched a consortium of chemolithoautotrophic denitrifiers from groundwater of the Hainich CZE, using thiosulfate and H_2 in combination as electron donors. We carried out experimental incubations under simulated groundwater conditions to test the consortium's capacity to perform complete denitrification, followed via ^{15}N-based Raman gas spectroscopy, and to identify key players in autotrophic denitrification based on *nirS* and *nosZ* gene transcripts along with a 16S rRNA gene-targeted assessment of community structure. We hypothesized (i) that the ratio of electron acceptor to electron donor is crucial for completing the last steps of chemolithoautotrophic denitrification and that these expected effects are reflected (ii) on the level of *nirS* and *nosZ* transcriptional activity and (iii) by changes in the community structure of the enriched consortium during the experimental incubations. Furthermore, we traced back the relevance of the key players of our enrichment culture to the denitrifying community in groundwater of the Hainich CZE.

MATERIALS AND METHODS

Study site and groundwater sampling

The limestone aquifer assemblages of the Hainich CZE in northwestern Thuringia, Central Germany allow access to groundwater for long-term monitoring in the framework of the Collaborative Research Centre AquaDiva (Küsel *et al.* 2016). The Hainich CZE covers 29 km^2 of a hill slope subcatchment featured with different land uses (managed forest, agriculture and pasture). Groundwater wells access two superimposed aquifer assemblages (Hainich transect upper aquifer assemblage = HTU and Hainich lower aquifer assemblage = HTL) at five different locations (H1–H5) with sampling depth ranging from 5 to 90 m. Well construction, hydrogeology and groundwater sampling have been described in more detail in Küsel *et al.* (2016) and Kohlhepp *et al.* (2017). Groundwater from eight wells along the groundwater observation transect was used to set up enrichment cultures of chemolithoautotrophic denitrifiers. In brief, groundwater samples were collected in sterile 1 L glass bottles filled to the top, transported to the laboratory at <4°C and used within 3 h as inoculum. To enrich preferentially attached denitrifier communities, crushed rock material that had been exposed in groundwater wells for approximately 6 months to allow microbial colonization (Küsel *et al.* 2016; Herrmann *et al.* 2017) was used as inoculum. Upon retrieval, thimbles were immediately placed in a sterile glass bottle with anoxic atmosphere, transferred to the laboratory at ca. 4°C and processed inside an anoxic chamber within a few hours after sampling. For additional molecular assessment of the groundwater denitrifier community, groundwater samples were obtained from wells H43 (12 m) and H53 (50 m) of HTU and from wells H41 (48 m) and H51 (88 m) of HTL during five sampling campaigns from July 2014 to August 2015. Groundwater samples were collected in sterile glass bottles, transported at <4°C, and immediately filtered through 0.2 μm polyethersulfone (PES) membrane filters (Supor, Pall Corporation, Dreieich, Germany) with 5 to 6 L of water collected on one filter. The filters were then transferred to sterile 2 mL tubes and frozen on dry ice, followed by storage at

80°C until nucleic acid extraction. pH, groundwater temperature and total organic carbon (TOC) concentration at the time of sampling in all locations showed little variation at values of approximately pH 7.2, 12°C and, < 1.8 mg L^{-1} TOC, respectively. While wells H43 and H53 of HTU usually showed anoxic to suboxic conditions (oxygen < 0.3 μmol L^{-1}) and mean nitrate concentrations of 1 μmol L^{-1} (H43) and 26 μmol L^{-1} (H53), the groundwater of wells H41 and H51 was oxic (oxygen concentration 162 and 88 μmol L^{-1}, respectively) with higher nitrate concentrations (143 and 142 μmol L^{-1}). All wells exhibited equally low concentrations of reduced sulfur compounds (sulfide and thiosulfate < 0.5 μmol L^{-1}) but differed regarding the concentrations of sulfate with the highest values in well H51 (2937 μmol L^{-1}) and the lowest values in well H43 (373 μmol L^{-1}). The basic hydrochemical characteristics of the sampling sites have been described by Kohlhepp *et al.* (2017) and Kumar *et al.* (2017).

Enrichment set up and incubation conditions

Enrichment cultures of chemolithoautotrophic denitrifiers obtained from either groundwater or exposed crushed rock material were set up using two cultivation strategies. The first approach utilized a traditional liquid medium in serum bottles, while the second approach was based on a modified agar shake dilution technique replacing agar with Gelrite (0.5%) in Hungate tubes (Miller and Wolin 1974; Pfennig and Trüper 1981). The mineral medium used for the enrichment culture contained 0.94 mmol NH_4Cl, 0.08 mmol $MnCl_2$, 0.04 mmol $FeCl_3.6H_2O$, along with 10 mL of Cl-solution (1.0% $MgCl_2$ + 0.5% $CaCl_2$), 10 mL of a 0.4% KH_2PO_4 solution per liter and $CuCl_2$ solution to a final concentration of 10 nmol (part A) and was purged with 90% N_2–10% CO_2 using an MCQ gas blender (GB-103) for 15 min (modified medium after Kojima and Fukui 2010). After autoclaving, part B was added consisting of filter-sterilized (0.2 μm), anoxic solutions of $NaNO_3$ (0.01 mmol L^{-1}), $Na_2S_2O_3^{2-}$ (0.01 mmol L^{-1}), and $NaHCO_3$ (10 mmol L^{-1}), followed by the addition of vitamin solution [DSMZ 461 Mineral medium (Nagel and Andreesen): http://www.dsmz.de] (5 mL L^{-1}) and trace element solution [DSMZ 461 Mineral medium (Nagel and Andreesen): http://www.dsmz.de] (1 mL L^{-1}). pH of this 'nitrate thiosulfate carbonate medium' (NTC) was 7.3 and the headspace was 80% N_2–10% CO_2–10% H_2. 60 mL of anoxic NTC medium in 120 mL butyl septum-aluminium crimped serum vials or 20 mL of anoxic semi-solid NTC medium in 30 mL butyl septum-aluminium crimped Hungate tubes were inoculated with 5 mL or 0.5 mL groundwater, respectively. Incubations were performed in the dark at 15°C with shaking at 50 rpm for liquid cultures. Subcultures were made after five (liquid cultures) and three months (gelrite tubes). After the first transfer of fresh inoculum and colonies to fresh NTC medium, enrichment cultures were incubated for 30 days in similar conditions and were further sub-cultured every month in a fresh medium with gradually increased concentrations of thiosulfate and nitrate up to 2 and 5 mmol L^{-1} respectively. Throughout the whole enrichment procedure including the initial enrichment phase, subsamples were taken at different time points to monitor nitrate and sulfate concentrations as substrates and products of nitrate-dependent thiosulfate oxidation, followed by the addition of additional electron donor and acceptor, if necessary.

Microcosm experiments, ion chromatography and Raman-based gas analysis

Out of the 50 parallel cultivation approaches, four enrichment cultures (E1, E2, E3 and E4) showed denitrification under chemolithoautotrophic conditions. Based on the taxonomic identity of the community members after the 35th transfer, enrichment culture E1 was selected for microcosm experiments to study denitrification activity in more detail. Two subsequent microcosm experiments were set up in order to follow the consortium's response to two different electron acceptor/electron donor ratios: microcosm experiment M_I with a nitrate/thiosulfate ratio of 2.5 (electron acceptor in excess) and microcosm experiment M_II with a nitrate/thiosulfate ratio of 1 (supportive of complete denitrification according to the stoichiometry of the reaction). During these incubation experiments, we traced turnover reactions of electron donors (thiosulfate and H_2), and electron acceptor (nitrate) (M_I), and gaseous intermediates and end products using Raman gas spectroscopy (Keiner *et al.* 2014) (M_II). A pre-culture of enrichment culture E1 (35th transfer) was setup in triplicate 120 mL serum vials with butyl rubber stoppers and aluminium crimps. The medium composition was the same as used for the enrichment culture, except that part A was made under argon gas atmosphere. For part B of the medium, filter-sterilized $Na^{15}NO_3$ (98 atom% of ^{15}N) was used, corresponding to a final concentration of 5 mmol L^{-1} $^{15}NO_3^-$, and supplemented with $Na_2S_2O_3$ (2 mmol L^{-1}) and $NaHCO_3$ (10 mmol L^{-1}). 40 mL of $^{15}NO_3^-$-spiked NTC medium was filled in each serum vial under argon atmosphere supplemented with a gas mixture of 4% H_2 and 5% CO_2. Cells were pre-incubated with $^{15}NO_3^-$ for 10 days, centrifuged at 4000 x g for 6 min at 15°C in sterile 50 mL centrifuge tubes, and washed two times with anoxic carbonate mineral medium (original medium without nitrate and thiosulfate) to minimize the carryover of nutrients. The cell pellets obtained from this pre-incubated culture were then used to inoculate the denitrification microcosm experiments in anoxic ^{15}N-NTC medium. Microcosms were maintained as batch assays at 15 °C and 50 rpm for an incubation period of 14 and 19 days during microcosm experiment M_I and M_II, respectively. The pH of the microcosms remained in the range of 7.5±0.2, while the pressure was in the range of 1.2–1.4 bar in all serum bottles throughout the incubation period. During microcosm experiment M_I, one control and three inoculated cultures were destructively sampled every day from day 0 to day 11 and additionally at day 14. The entire content of each vial was transferred to a sterile 50 mL centrifuge tube, followed by centrifugation at 20 000 x g for 20 min at 4°C. Cell pellets were immediately frozen on dry ice and stored at −80°C until nucleic acid extractions were performed. The supernatant was filtered through a 0.22 μm pore size filter and processed immediately for tracking the concentrations of dissolved inorganic nitrogen species (NO_3^-, NO_2^- and NH_4^+) and sulfur species ($S_2O_3^{2-}$ and SO_4^{2-}). Measurements for NO_3^-, NO_2^- and SO_4^{2-} were performed on an ion chromatography system DX-500 (Thermo Fisher Scientific GmbH, Dreieich, Germany), while $S_2O_3^{2-}$ and NH_4^+ were quantified on an ion chromatography system ICS-5000 (Thermo Fisher Scientific GmbH, Dreieich, Germany). The second microcosm experiment (M_II) was carried out with a focus on following gaseous intermediates and end products using Raman gas spectroscopy. The 39th transfer of enrichment E1 was used as inoculum. To realize a nitrate/thiosulfate ratio of 1, the concentration of thiosulfate was increased

to 5 mmol L^{-1} and the volume of supplemented growth medium in each serum vial was increased to 60 mL. One control and three inoculated cultures were destructively sampled after 0, 7, 10, 12, 14, 17 and 19 days of incubation. The headspace was analysed for $^{15}N_2$, $^{15}N_2O$, CO_2, H_2, $^{14}N_2$ and O_2 using Raman gas spectroscopy. Generation of bacterial cell pellets for later molecular analysis was carried out as during microcosm experiment M_I.

Cavity-enhanced Raman gas spectroscopy

Analysis of $^{15}N_2$, $^{15}N_2O$, CO_2, H_2, $^{14}N_2$ and O_2 in the headspace was carried out using a specially designed Raman gas sensor based on cavity enhanced Raman spectroscopy, which allows simultaneous measurements of these gases down to concentrations of approximately 100 ppm (parts per million) with subsecond time resolution (Frosch *et al.* 2013; Jochum *et al.* 2015a, 2015b, 2017) (Fig. S1, Supporting Information). Calibration spectra were generated by flushing the device with the pure gases CO_2, $^{15}N_2$, $^{15}N_2O$, $^{14}N_2$ and O_2 at fixed pressure and temperature. For H_2, a gas mixture of 5% H_2 in N_2 was used. The peaks belonging to H_2 were scaled up to 100% and the nitrogen peak was removed to generate the calibration spectrum. The concentrations of the gases were calculated *via* a least squares fit using the whole set of calibrated reference spectra and the measured spectrum from 500 to 3000 cm^{-1}, and by monitoring temperature, pressure and laser intensity with the help of additional sensors (Keiner *et al.* 2014). All analysed gases have a distinct spectral signature in the Raman spectrum and could be quantified without cross-sensitivity. More details on the measurement procedure are provided as Supplementary Material (Supporting Information).

Nucleic acids extractions and reverse-transcription PCR

Extraction of DNA from the initial enrichment cultures (E1, E2, E3 and E4: 35th transfer) was performed by centrifugation of 60 mL cell suspension at 20 000 x g at 4°C for 20 min and further processing using the Bacterial DNA extraction kit (GenElute Bacterial Genomic DNA kit; NA2120–1KT, Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer instructions.

Cell pellets collected from both microcosm experiments (M_I, M_II) were subjected to simultaneous DNA and RNA extraction using the All prep bacterial DNA/RNA/Protein kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Bead-beating was performed using a Bio 101 FastPrep FP120 instrument (Thermo Electron Corporation, Milford, MA, USA) for three times 20 s at a speed setting of 4.5 m/s. RNA extracts were treated with Ambion TURBO DNA-free (ThermoFisher Scientific, Darmstadt, Germany), followed by reverse-transcription PCR using ArrayScript reverse transcriptase (ThermoFisher Scientific, Darmstadt, Germany) following the manufacturer's instructions along with a control which was set up with RT-PCR grade water instead of reverse transcriptase to test for carry-over of non-digested DNA. Genomic DNA from groundwater was extracted from PES membrane filters using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA), following the manufacturer's instructions.

Clone library construction and phylogenetic analysis

Four clone libraries targeting bacterial 16S rRNA genes were generated to assess the community composition of the four enrichment cultures (35th transfer). PCR products were generated using the universal bacterial primers 8F and 907R (Lane *et al.* 1985; Felske *et al.* 1997). *nirS*- and *nosZ*- transcript-based clone libraries were prepared using cDNA generated from samples collected during incubation experiment M_I. PCRs were carried out with previously published primer combinations, cd3aF/R3cd and nosZ-F-1181/nosZ-R-1880, respectively, for *nirS,* and *nosZ* genes encoding cytochrome cd1-type nitrite reductase and nitrous oxide reductase, using cycling conditions as described in Rich *et al.* (2003), Throbäck *et al.* (2004) and Kandeler *et al.* (2006). PCR reactions were performed in triplicates using Hotstart Mastermix (Qiagen, Hilden, Germany) PCR products were purified using the NucleoSpin extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Purified PCR products were ligated into pGEM T-Easy cloning vector (Promega, Madison, Wisconsin, USA), and transformation was performed using chemically competent *Escherichia coli* (JM109) in accordance with the manufacturer's protocols. PCR products from positive clones were sequenced with M13 primers at Macrogen (Amsterdam, The Netherlands).

Quantification of genes and transcripts

To quantify the abundances of *nirS*, *nosZ* genes and transcripts along with 16S rRNA genes during the microcosm experiments, qPCR was performed on a Mx3000P qPCR cycler (Agilent Technologies, Waldbronn, Germany) using Maxima SYBR Green Mastermix (Thermo Fisher Scientific, Darmstadt, Germany). Bacterial 16S rRNA genes were quantified using the primer combination Bac8Fmod/Bac338Rabc (Loy *et al.* 2002; Nercessian *et al.* 2005) with cycling conditions as described elsewhere (Herrmann, Hädrich and Küsel 2012). *nirS* and *nosZ* genes were quantified using the same primer combinations as used for the cloning approach, following the thermal cycling conditions given in Rich *et al.* (2003) and Throbäck *et al.* (2004). Standard curves were produced based on a serial dilution of plasmids containing inserts of the respective target genes obtained in this study or in previous work (Kumar *et al.* 2017). Standard curves were linear from 5×10^1 to 5×10^8 copies per reaction with $R^2 > 0.99$ and qPCR efficiencies ranging from 89.7% to 106.2%. 16S rRNA gene abundances were used to estimate cell numbers by correcting for multiple 16S rRNA gene operons per cell. Based on 16S rRNA gene operon numbers provided at the rrnDB-website (Stoddard *et al.* 2014) (https://rrndb.umms.med.umich.edu/) for the closest available relatives of the 20 most abundant OTUs in the enriched consortium, covering 85%–90% of the enriched community, these correction factors were 2.2 for microcosm experiment M_I and 2.28 for microcosm experiment M_II.

Illumina sequencing of 16S rRNA and nirS genes

To assess changes in the bacterial community structure of the enriched consortium during the two microcosm experiments, we used 16S rRNA gene-targeted Illumina MiSeq amplicon sequencing for four time points (M_I: day 0 and day 14; M_II: day 0 and day 17). 16S rRNA gene PCR products were generated using the primer combination Bakt_341F/Bakt_805R (Herlemann *et al.* 2011), covering the V3–V5 region of the bacterial 16S rRNA gene and HotStar Taq Mastermix (Qiagen, Hilden, Germany). PCR products were purified using NucleoSpin Gel & PCR Clean-Up Kit (Macherey-Nagel, Düren, Germany). Amplicon libraries were prepared using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Frankfurt, Germany) and purified using AMPure XP Beads (Beckman Coulter, Krefeld, Germany)

according to the manufacturer's instructions. Sequencing was performed on an Illumina MiSeq platform using v3 chemistry (Illumina, Eindhoven, The Netherlands).

In addition, we assessed the community composition of *nirS*-type denitrifiers in groundwater samples by *nirS*-targeted MiSeq Illumina amplicon sequencing for wells H41, H43, H51 and H53 during five sampling campaigns (July 2014 and January, March, June and August 2015). Because of low *nirK* gene abundances in the groundwater (Kumar *et al.* 2017), a detailed analysis of denitrifier community composition was focused on *nirS*-type denitrifiers only. Amplicons of *nirS* genes were generated from genomic DNA using the primer combination cd3aF/R3cd (Throbäck *et al.* 2004; Kandeler *et al.* 2006) and HotStarTaq Mastermix (Qiagen, Hilden, Germany) as described elsewhere (Kumar *et al.* 2017), followed by PCR product purification using NucleoSpin Gel and PCR Cleanup kit (Macherey-Nagel, Düren, Germany). Generation of barcoded amplicons and amplicon sequencing using the Illumina MiSeq platform was performed by LGC Genomics (Berlin, Germany) as described previously (Kumar *et al.* 2017). Sequence analysis of *nirS* amplicons was performed using Mothur (Schloss *et al.* 2009, v.1.36.1), with few modifications necessary to adjust the pipeline for the analysis of protein-encoding genes as previously described (Kumar *et al.* 2017), integrating BioEdit (Hall 1999) and the ARB package (Ludwig *et al.* 2004). Sequences were assigned to OTUs based on a 0.18 distance cutoff on the nucleic acid level (Palmer, Biasi and Horn 2012). Closest relatives were determined using nucleotide Basic Local Alignment Search Tool (blastn) of one representative sequence per OTU against the Nucleotide collection (nr/nt) database at the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences obtained in this study have been submitted to Genbank for the clone library data (accession numbers MH085257-MH085433 and MH155692-MH155863) and to the ENA archive for the data obtained by MiSeq Illumina amplicon sequencing (project PRJEB25618, sample accession numbers ERS2303714-ERS2303737, run accession numbers ERR2405446-ERR2405469).

RESULTS

Comparison of enriched consortia across the different enrichment approaches

The four enrichment cultures that showed chemolithoautotrophic denitrification (E1, E2, E3 and E4), obtained from groundwater or exposed rock material, varied in their community composition (35th transfer). For enrichment culture E1 (liquid medium with groundwater from well H53 as inoculum), 59% of the sequences were affiliated with the genus *Thiobacillus* (*T. thiophilus, T. thioparus* and *T. denitrificans*), followed by sequences related to *Hydrogenophaga* (21%), *Sulfuritalea hydrogenivorans* (7%), *Ferribacterium limneticum* (7%), *Dechloromonas aromatica* (4%) and *Sulfuricella denitrificans* (2%) (Fig. 1; Fig. S2A, Supporting Information). In enrichment culture E2 obtained from exposed rock material (well H53), even 95% of the clone sequences were affiliated with the genus *Thiobacillus*, (e.g. *Thiobacillus* sp. K6.2, *T. denitrificans* and *T. thioparus*), while the other 5% were affiliated with the genus *Pseudomonas* (*P. panacis* and *P.* sp. LS197) (Fig. S2B, Supporting Information). In contrast, the enrichment cultures obtained from modified agar shake dilutions (gelrite) were each dominated by two presumably heterotrophic members that is bacteria related to *Pseudomonas extremaustralis* strain 14-3 and *P. veronii* strain CIP 104 663 in enrichment culture E3, and

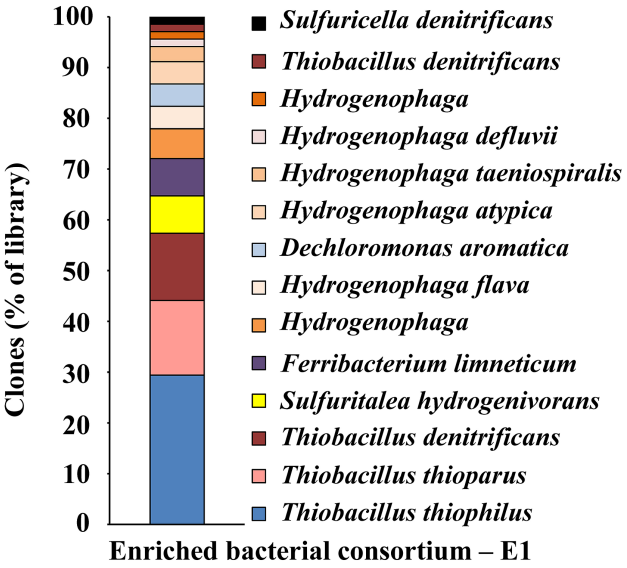

Figure 1. Community composition of the chemolithotrophic denitrifying enrichment culture E1 after the 35th transfer based on 16S rRNA gene sequences. Sequence identities with cultured representatives ranged from 93% to 99%. Analysis based on 67 sequenced clones.

related to *Simplicispira psychrophila* strain CA 1 and *Thermomonas fusca* strain R-10 289 in enrichment culture E4 (Figs S2C and D, Supporitng Information). Enrichment culture E1 showed the highest enrichment of chemolithoautotrophs and the largest number of representatives observed in previous molecular surveys of the groundwater (Herrmann *et al.* 2017; Kumar *et al.* 2017) and was chosen for further microcosm experiments.

Microcosm experiment M_I: turnover of nitrogen and sulfur compounds by the enriched consortium E1

Using a nitrate/thiosulfate ratio of 2.5, up to 93% of the nitrate was readily reduced on the expense of thiosulfate and H_2 oxidation by the end of the 14 days incubation period. The initially gradual depletion of nitrate up to day 8 was followed by a rapid decrease in the nitrate concentration until the end of the incubation (Fig. 2A). Accumulation of nitrite was observed but never exceeded 912 μmol NO_2^- L^{-1}. Thiosulfate was consumed completely after ten days, resulting in the accumulation of 4495 ± 60 μmol SO_4^{2-} L^{-1}. Ammonium concentrations showed negligible variations, averaging 2600 ± 200 μmol NH_4^+ L^{-1}. In the non-inoculated controls, nitrite was not detected nor were any changes observed in ammonium, thiosulfate and nitrate concentrations (data not shown). The initial rates of nitrate reduction and sulfate production were calculated as 220 μmol L^{-1} d^{-1} and 292 μmol L^{-1} d^{-1} respectively, based on the near-linear change of concentrations between days 1 and 7. The total bacterial population increased by about two orders of magnitude with an increase from 2.2×10^9 bacterial 16S rRNA genes L^{-1} culture to a maximum of 7.8×10^{11} genes L^{-1} on day 10 (Fig. 2), corresponding to an increase from 1×10^9 to 3.6×10^{11} cells L^{-1} (Fig. S3, Supporting Information), and exhibited an estimated doubling time of ca. 28 h. Estimated per cell activities of nitrate reduction and sulfate production calculated for the interval days 6 to 7 were 0.42 ± 0.35 fmol $cell^{-1}$ h^{-1} and 0.59 ± 0.36 fmol $cell^{-1}$ h^{-1}, respectively (Table S1, Supporting Information). Gene abundances for *nirS* and *nosZ* ranged from

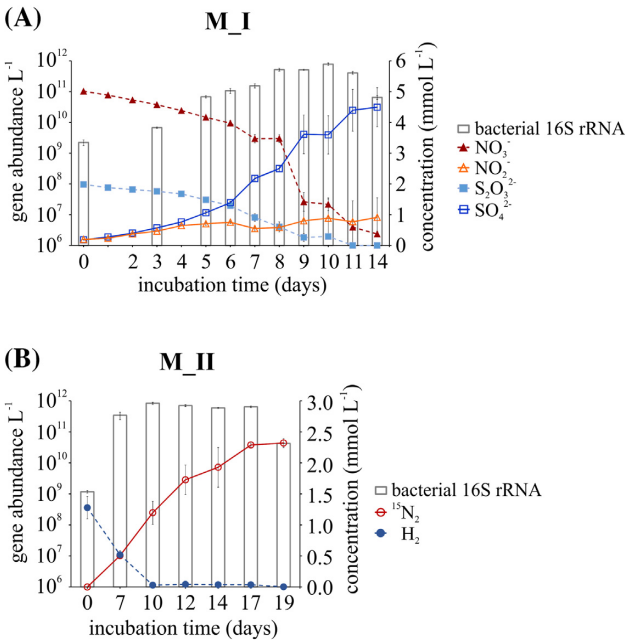

Figure 2. Substrate turnover and growth followed by bacterial 16S rRNA gene abundances of the enrichment culture E1 in two microcosm experiments M_I (**A**) and M_II (**B**). All data points for concentrations of gases and ions are mean values of samples from three replicate culture bottles. Bacterial 16S rRNA gene abundance data are mean values of nine replicates (DNA extracts from three replicate culture bottles, for each DNA extract, three technical replicates were performed in the qPCR assay); error bars are SEMs.

1.4 × 10^8 to 4.0 × 10^{11} and 2.0 × 10^8 to 1.6 × 10^{11} genes L^{-1} culture, respectively (Fig 3A and B). *nirS* transcript/gene ratios showed a 415-fold increase from days 5 to 10 with ratios increasing from 0.0003 to 0.1163, corresponding to 8.8 × 10^6 *nirS* transcripts L^{-1} culture at day 5 to a maximum of 1.3 × 10^{10} *nirS* transcripts L^{-1} at day 10, respectively (Figs 3A and 4A). In contrast, *nosZ* transcript/gene ratios were highest on day 0 (0.2887) and remained on a low level throughout microcosm experiment M_I (Figs 3B and 4A).

Microcosm experiment M_II: complete denitrification and H_2 utilization followed by cavity enhanced Raman spectroscopy

With a nitrate/thiosulfate ratio of 1 (each 5 mmol L^{-1}) and 4% H_2 (corresponding to 1.7 mmol L^{-1}), nearly complete (98%) consumption of the initially 5 mmol L^{-1} ^{15}N-NO_3^- was observed with subsequent accumulation of ^{15}N-N_2 to 2.4 mmol L^{-1} at day 19 of the microcosm experiment M_II (Fig. 2B). The concentration of H_2 decreased to 519 μmol L^{-1} after seven days, and H_2 was completely consumed by the end of the incubation. The rates of N_2 production and H_2 consumption under denitrifying conditions were 243 μmol L^{-1} d^{-1} and 22 μmol L^{-1} d^{-1}, respectively, calculated based on the near-linear change in concentrations between day 7 and day 12 of the experiment for $^{15}N_2$ and between day 0 and day 10 for H_2. Nitrous oxide was not detected nor were significant changes in the CO_2 concentrations detected during the incubation period. We did not observe any formation of $^{15}N_2$ or $^{15}N_2O$ in the non-inoculated controls. Growth dynamics were similar to microcosm experiment M_I with an increase of bacterial 16S rRNA gene abundances from 1.2 × 10^9 to 8.4 × 10^{11} genes L^{-1} culture on

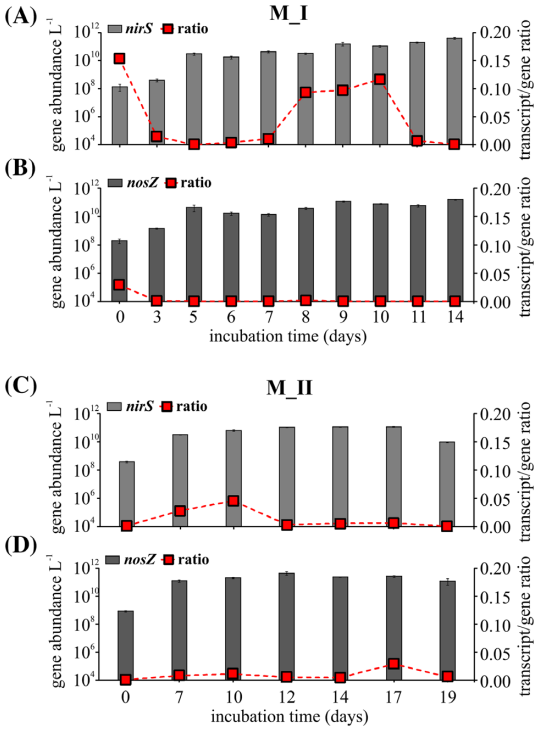

Figure 3. Abundance and transcript/gene ratios of *nirS* and *nosZ* genes. (**A**) *nirS*, (**B**) *nosZ* based analysis for microcosm experiment M_I; (**C**) *nirS*, (**D**) *nosZ* for microcosm experiment M_II. Please note that the x-axis in (**A**) and (**B**) covers 14 days while the x-axis in (**C**) and (**D**) covers 19 days. All data points for gene abundances are mean values of nine replicates (DNA extracts from three replicate culture bottles; for each DNA extract, three technical replicates were performed in the qPCR assay); error bars are SEMs.

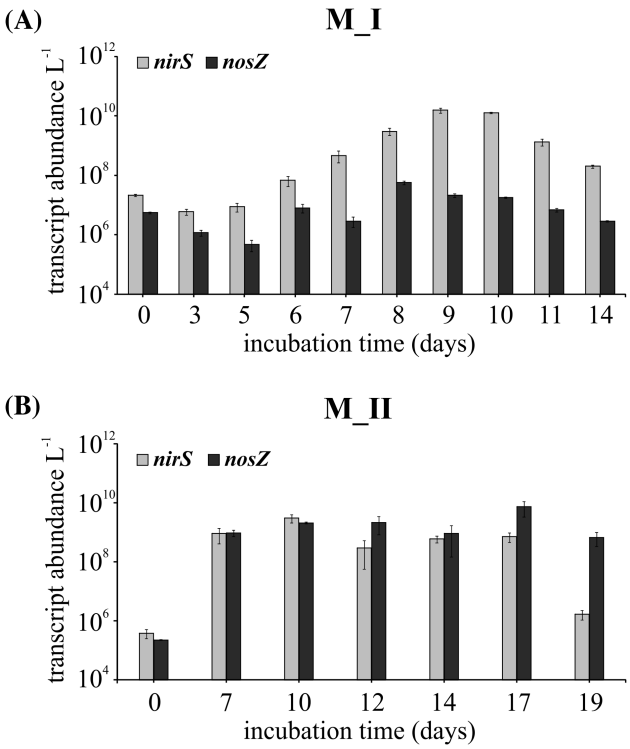

Figure 4. Transcript abundances of *nirS* and *nosZ* over time during the two microcosm experiments, M_I (**A**) and M_II (**B**). Please note that the x-axis in (**A**) covers 14 days while the x-axis in (**B**) covers 19 days. All data points are mean values of nine replicates (DNA extracts from three replicate culture bottles; for each DNA extract, three technical replicates were performed in the qPCR assay); error bars are SEMs.

day 10 in M_II (Fig. 2B), corresponding to an increase from 5.2×10^8 to 3.7×10^{11} cells L^{-1} (Fig. S3, Supporting Information). Estimated per cell activities of N_2 production and H_2 consumption calculated for the interval day 10 to day 12 were 0.06 ± 0.004 fmol cell^{-1} h^{-1} and 0.03 ± 0.02 fmol cell^{-1} h^{-1}, respectively (Table S1, Supporting Information). *nirS* and *nosZ* gene abundances were in a similar range as 16S rRNA gene abundances with 3.9×10^8 to 1.2×10^{11} and 8.2×10^8 to 4.6×10^{11} genes L^{-1} (Fig 3C and D). Transcriptional activity of *nirS* during microcosm experiment M_II showed a 46-fold increase from the beginning to day 10 (*nirS* transcript/gene ratio ranging from 0.001 at day 0 to 0.045 at day 10, corresponding to 3.84×10^5 transcripts L^{-1} to 3.02×10^9 transcripts L^{-1}; Figs 3C and 4B). Transcriptional activity of *nosZ* revealed a gradual increase by 104-fold from 0.0003 (day 0) to 0.02854 (day 17) corresponding to 2.3×10^5 transcripts L^{-1} and 7.3×10^9 transcripts L^{-1}, respectively (Figs 3D and 4B).

Shifts in community composition during the microcosm experiments

For both microcosm experiments, 52% of the community was initially dominated by members of the *Betaproteobacteria*. This fraction increased to 87% and 71% at day 14 and day 17 of M_I and M_II, respectively (Fig. S4, Supporting Information). The second most abundant class was *Alphaproteobacteria*, which decreased from 21% to 10% during the first microcosm experiment and from 28% to 18% during the second experiment, respectively. *Bacteroidetes* changed from 23% to 1% and 7% to 9% during microcosm assays M_I and M_II, respectively. At a more detailed taxonomic resolution, nearly 60% of the sequences represented sulfur oxidizing bacteria of the genus *Thiobacillus* (*T. thioparus* and *T. denitrificans*) (Fig. 5). During microcosm experiment M_I, fractions of *T. thioparus*-related sequences decreased from 37% to 9%. On the contrary, the relative abundance remained nearly the same (43% to 45%) between the corresponding time points during experiment M_II. Sequence fractions related to *T. denitrificans* showed an increase in both microcosm experiments (2% to 41% during M_I and 2% to 12% during M_II). *Rhizobium selenitireducens* -related sequence reads decreased from 18% to 7% during M_I, and from 16% to 12% during M_II. Read fractions of sequences related to *Lutibacter maritimus* decreased from 21% to 1% during M_1 but showed a moderate increase from 2% to 7% during M_II. (Fig. 5). *Hydrogenophaga* sp. related sequences showed an increase from 7% to 16% during M_1 but contributed only a minor fraction during M_II (3% to 0%). *Ferribacterium limneticum* like sequences showed an increase in both experiments with an increase from 1% to 11% and 1% to 6% in M_1 and M_II, respectively.

Thiobacillus denitrificans-related organisms as key transcriptionally active denitrifiers in the consortium

Analysis of metabolic activity exhibited by the denitrifying consortium was complemented with the identification of the dominant transcriptionally active denitrifiers at days 3 and 9 of the first microcosm experiment (M_I), targeting *nirS* and *nosZ* transcripts. *Thiobacillus denitrificans*-related sequences accounted for 65% of the *nirS*-transcripts in the early stage of the incubation but for only 24% on day 9 (Fig. 6). *Sulfuricella denitrificans*, *Sulfuritalea hydrogenivorans*, *D. aromatica* and *Hydrogenophaga sp*. were also represented on the *nirS* transcript level with an increase of their fractions from days 3 to 9. Clone library analysis targeting *nosZ* gene transcripts provided further evidence of *T. denitrificans* as dominant transcriptionally active member in the denitrifier community at both day 3 and day 9 (90% and 91% of all sequenced *nosZ* transcripts, respectively).

Hydrogen and sulfur oxidizers form a high fraction of the groundwater denitrifier community

To trace back the key players of the enriched denitrifying consortium to the source of isolation, groundwater of the Hainich CZE, we assessed the *nirS*-type denitrifier community structure in suboxic well H53 in addition to three other wells representing oxic (H41, H51) and anoxic conditions (H43). Furthermore, including previously published data (August 2014; Kumar *et al*. 2017), we followed community fluctuations over six sampling events between July 2014 and August 2015 with a total of 1822,947 *nirS* sequence reads obtained in these wells (Table S2, Supporting Information). In the groundwater of well H53, *Sulfurifustis variabilis*-related sequences (with 84% sequence identity) constituted more than half (56±6%) of the sequence reads. Remaining reads were associated with *Sulfuricella denitrificans* (with 82% sequence identity) (30±8%) and *Sulfuritalea hydrogenivorans* (with 90% sequence identity) (8±5%), pointing to a high fraction of chemolithoautotrophic denitrifiers (Fig. 7). Similarly, at well H43 of the upper anoxic aquifer assemblage and well H51 of the lower oxic aquifer assemblage, most of the *nirS*-type denitrifier community was constituted by putative chemolithoautotrophic or mixotrophic organisms such as *Sulfuritalea hydrogenivorans* (50±20%) and *Rubrivivax gelatinosus* (38±20%) in H43 and *Sulfurifustis variabilis* (41±16%), *Sulfuritalea hydrogenivorans* (13±8%) and *Sulfuricella denitrificans* (14±3%) in H51. In contrast, at well H41, presumably heterotrophic denitrifiers were present at higher fractions with 25±15% of the sequence reads affiliated with *Pseudomonas* sp., followed by denitrifiers related to *Sulfurifustis variabilis* (22±11%) and *Sulfuricella denitrificans* (11±3%). Interestingly, the community structure of groundwater *nirS*-type denitrifying bacteria showed less fluctuation over time in well H53 compared to the other wells (Fig. 7).

DISCUSSION

Here, we used a chemolithoautotrophic denitrifying enrichment culture as a model system to follow substrate turnover and transcriptional activity of groundwater denitrifiers utilizing reduced sulfur compounds and H_2. We selected thiosulfate as electron donor that is usually involved in oxidation, disproportionation and reduction pathways (Jørgensen 1990), keeping its concentration in the groundwater of the Hainich CZE below 0.5 μmol L^{-1}. Similarly, H_2 as another suitable electron donor may be subject to rapid turnover *in situ*. Concentrations of H_2 in the unsaturated zone of the Hainich CZE always remained below the detection limit of 100 ppm (data not shown) and only nanomolar concentrations have been observed in groundwater in other studies (Alter and Steiof 2005; Flynn *et al*. 2013). Molecular H_2 is an important product of fermenters (Laanbroek and Veldkamp 1982) and can also occur as a byproduct of the nitrogenase reaction by nitrogen-fixing microbes (Dixon 1978), members of which have been ubiquitously found in the Hainich aquifers and could provide H_2 to chemolithoautotrophic denitrifiers (Lazar *et al*. 2017; Nowak *et al*. 2017; Starke *et al*. 2017). Both thiosulfate and H_2 might be simultaneously available to chemolithoautotrophic denitrifiers in the groundwater while total concentrations and

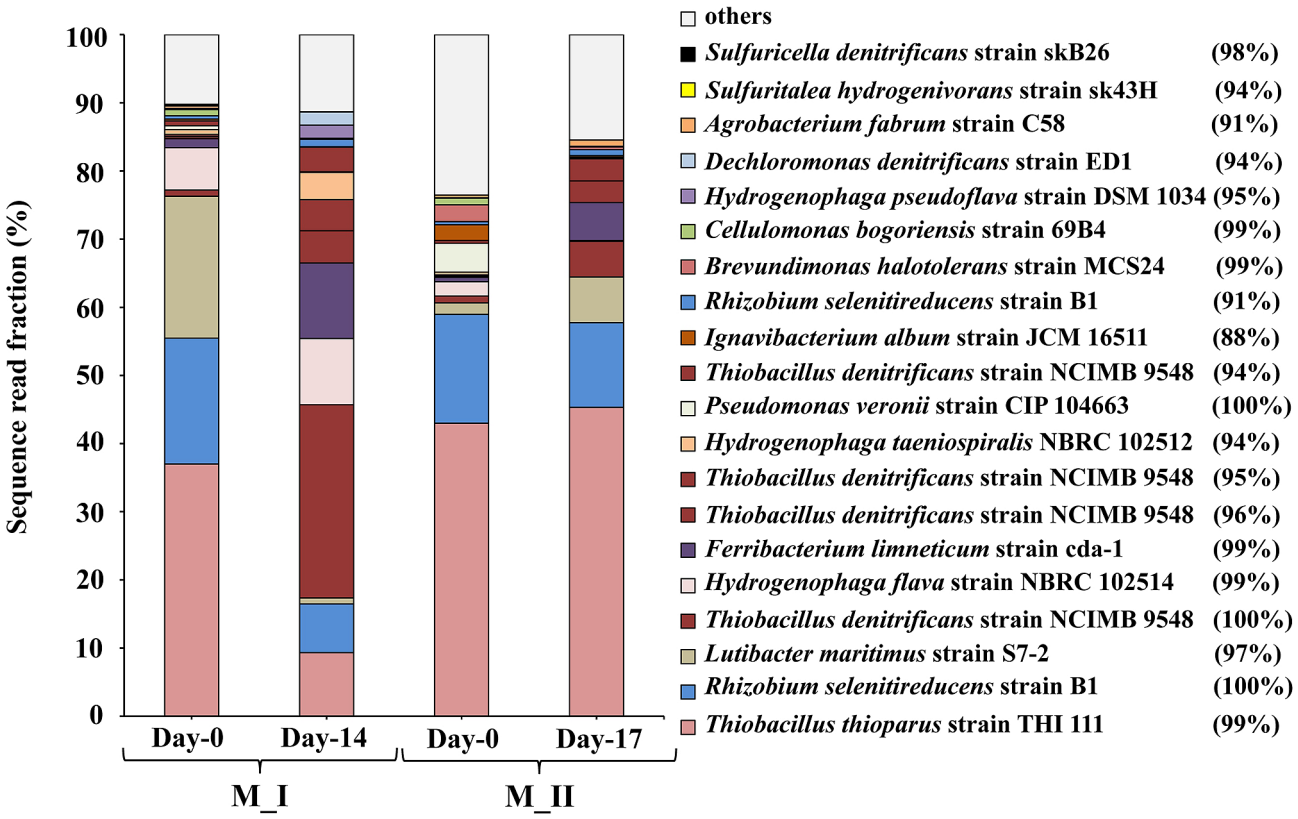

Figure 5. Changes of bacterial community structure of the denitrifying consortium E1 during two microcosm experiments M_I and M_II. Nitrate/thiosulfate ratios were 2.5 in M_I and 1 in M_II. Data are results of MiSeq amplicon sequencing targeting bacterial 16S rRNA genes. PCR products from three biological replicates were pooled for sequencing. Numbers in parentheses indicate sequence identities with the most closely related cultured representatives.

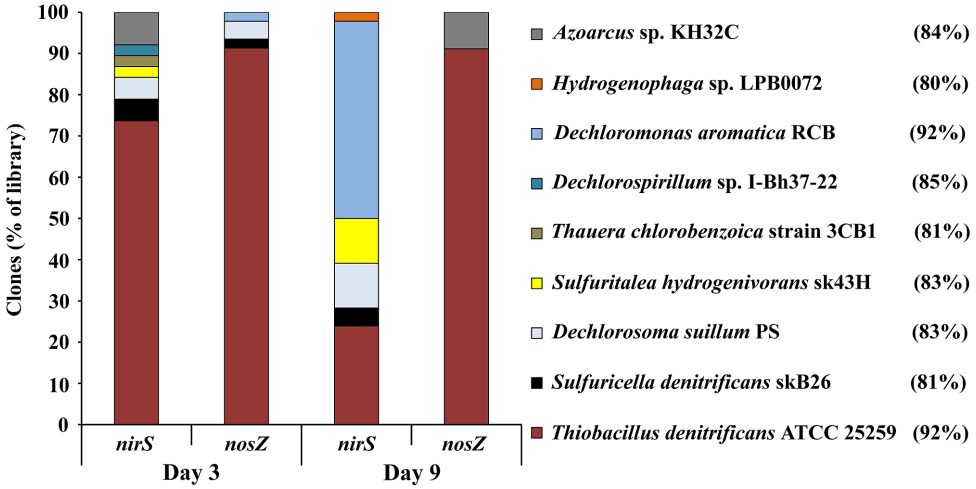

Figure 6. Transcriptionally active denitrifying bacteria based on *nirS* and *nosZ* transcripts on days 3 and 9 of microcosm experiment M_I. Numbers in parentheses indicate sequence identities with most closely related cultured representatives.

the ratio between the two compounds are likely to fluctuate over time. Hence, microorganisms that are able to switch between these two electron donors could have a competitive advantage in oligotrophic groundwater. Consequently, in this study we chose enrichment conditions which especially also support growth of these organisms.

Cultivation using liquid cultures resulted in the most successful enrichment of chemolithoautotrophic denitrifiers from both groundwater and microbiota attached to exposed rock material, while the modified agar shake dilution technique yielded enrichments of presumably heterotrophic organisms with only poor representation in the groundwater denitrifier communities. High fractions of denitrifiers related to the genera *Thiobacillus*, *Sulfuricella*, *Sulfuritalea*, or *Hydrogenophaga* along with low abundance or absence of heterotrophs identified enrichment E1 derived from groundwater of suboxic well H53 as the best approximation of the metabolic strategies

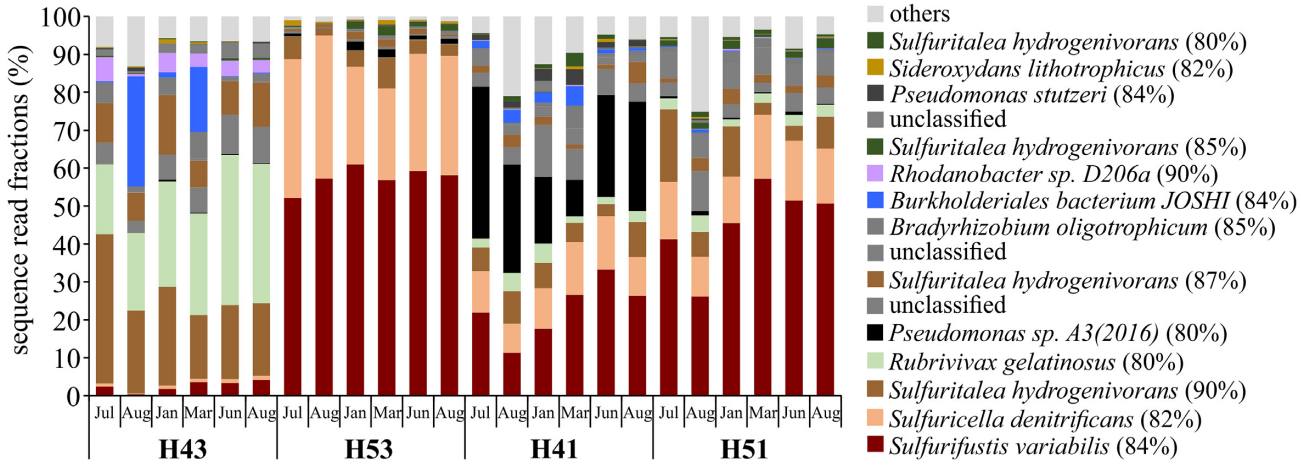

Figure 7. Taxonomic affiliation of the *nirS*-type denitrifying bacterial communities in the groundwater of four wells across the two aquifer assemblages at the Hainich CZE (well H43: anoxic conditions; well H53: suboxic conditions; wells H41, H51: oxic conditions). Analysis based on Illumina MiSeq amplicon sequencing of *nirS* genes in July, August 2014 and January, March, June, and August 2015. Numbers in parentheses indicate sequence identities with most closely related cultured representatives. Sequences showing less than 80% *nirS*-sequence identity with cultured denitrifiers are referred to as 'unclassified'. *nirS* sequence data from August 2014 were integrated from Kumar *et al.* (2017).

represented by the denitrifying communities in the groundwater of the Hainich CZE (Herrmann *et al.* 2017; Kumar *et al.* 2017). Chemolithoautotrophic *Thiobacillus* lineages utilizing reduced sulfur compounds, which dominated the enriched consortium, have been described and also isolated from other aquifer samples (Katayama and Kuraishi 1978; Vlasceanu, Popa and Kinkle 1997; Kellermann and Griebler 2009; Zhang *et al.* 2017). Complete denitrification along with the capability of autotrophic CO_2-fixation or growth with reduced sulfur compounds and H_2 have also previously been reported for *Sulfuricella denitrificans* and *Sulfuritalea hydrogenivorans* (Kojima and Fukui 2010, 2011; Watanabe, Kojima and Fukui 2014), which accounted for about 7% of our enrichment. About 20% of the enriched microorganisms were potential H_2 utilizers. A mixotrophic and facultative anaerobic lifestyle was represented by *Hydrogenophaga* sp. RAC07 for which H_2 and thiosulfate oxidation has so far only been postulated based on genomic information (Fixen *et al.* 2016). Similarly, *D. aromatica* RCB, a facultative chemolithotroph, is known to possess the capability of oxidation of H_2, H_2S and Fe (II) along with anaerobic aromatic degradation coupled to nitrate reduction (Coates *et al.* 2001; Chakraborty *et al.* 2005; Shrout *et al.* 2005). The fact that enrichment of chemolithoautotrophic denitrifiers only succeeded with groundwater or exposed rock material from suboxic well H53 as inoculum agrees well with observations that showed highest abundances of *nirS*-type denitrifiers at this site (Kumar *et al.* 2017). *nirS* gene abundances approximated 2.3×10^7 per liter for well H53 but ranged from 2.4×10^4 to 7.6×10^6 per Liter for the other wells when groundwater was used for inoculum in this study. Consequently, both molecular data and the enrichment approach confirmed the high potential for chemolithoautotrophic denitrification at well H53 of the Hainich CZE.

Both thiosulfate and H_2 were oxidized by the enrichment culture E1 under chemolithotrophic denitrifying conditions in the two microcosm experiments. In agreement with our hypothesis, nitrite accumulated under electron donor-limited conditions, whereas almost complete denitrification to N_2 with very low nitrite accumulation and formation of sulfate up to 8 mmol L^{-1} was achieved at a nitrate/thiosulfate molar ratio of 1. Lack of formation of nitrous oxide (N_2O) or ammonium clearly indicated that the consortium was able to mediate complete denitrification and no dissimilatory nitrate reduction to ammonium (Cardoso *et al.* 2006; Harrold *et al.* 2016). The estimated doubling time of 28 h was in the lower range of doubling times reported for sulfur and H_2 oxidizing chemolithoautotrophs (1–15 days; Kodama and Watanabe 2003) and represented about ten times faster growth compared to rates reported for communities in aquifers (Mailloux and Fuller 2003). The slow but steady increase in gene abundances of 16S rRNA, *nirS* and *nosZ* over time coincided with increasing sulfate production and H_2 consumption coupled with N_2 production, indicating that the built-up of biomass occurred at the expense of the oxidation of the inorganic electron donors. Maximum abundances of *nirS* genes exceeded abundances in groundwater of the Hainich CZE by four orders of magnitude (Herrmann *et al.* 2017; Kumar *et al.* 2017). Rates of nitrate reduction of 220 μmol L^{-1} d^{-1} in the first microcosm experiment were even five orders of magnitude higher than denitrification activity measured earlier in groundwater samples (Kumar *et al.* 2017).

In line with our hypothesis, analysis of transcriptional activity of *nirS* and *nosZ* genes showed that during the first microcosm experiment at a nitrate/thiosulfate ratio of 2.5, only expression of *nirS* genes appeared to be induced, most likely as a result of rapid nitrate reduction to nitrite, while during the second microcosm experiment, at a nitrate/thiosulfate ratio of 1, transcriptional activity of *nirS* and *nosZ* reached comparable levels. Limitation of electron donors in the first microcosm experiment resulted in incomplete denitrification and an accumulation of nitrite. Similar to previous gene expression studies with heterotrophic denitrifiers (Härtig & Zumft 1999; Spiro 2012), nitrite probably triggered transcription of *nirS* in the chemolithoautotrophic denitrifiers but was not converted to sufficient amounts of NO to induce transcription of *nosZ* genes. Low levels of *nosZ* transcriptional activity probably resulted in only low levels of nitrous oxide reductase responsible for the last step of complete denitrification. An optimized nitrate/thiosulfate ratio of 1 applied in the second microcosm experiment most likely supported more efficient nitrite reduction to NO, which was followed by an upregulation of *nosZ* expression, allowing complete denitrification at higher nitrous oxide reductase activity similar to other studies (Manconi, Carucci and Lens

2007). Along with nitrite accumulation, a nitrate/thiosulfate ratio of 2.5 also resulted in more pronounced community shifts during the incubation experiment, with the most obvious being a strong increase of *Thiobacillus denitrificans* along with a strong decrease of *Thiobacillus thioparus*, while the latter remained the most dominant community member throughout microcosm experiment M_II at a nitrate/thiosulfate ratio of 1. Interestingly, early depletion of the main electron donor thiosulfate in microcosm experiment M_I obviously favored denitrifiers capable of using alternative electron donors such as H_2 (*Hydrogenophaga* sp., *Dechloromonas* sp.) or ferrous iron (*Ferribacterium* sp.). A shift from *Thiobacillus* sp. to H_2 utilizers as key active denitrifiers during microcosm experiment M_I was also clearly reflected on the level of *nirS* transcripts, where the proportion of sequences related to *Hydrogenophaga* sp., *Dechloromonas* sp. and *Sulfuritalea hydrogenivorans* increased from less than 10% on day 3 to about 60% on day 9.

Molecular support of ongoing H_2 oxidation during microcosm assay M_I could be verified by measured H_2 consumption during microcosm experiment M_II. We cannot rule out that other transcriptionally active groups may have been overlooked due to bias associated with the *nirS* primer sets used in this study or the limited amount of data from the cloning approach. *Thiobacillus thioparus*-related denitrifiers were highly abundant on the 16S rRNA gene level but harbor a copper-dependent instead of a cytochrome-cd1-type nitrite reductase and thus could not be detected by the *nirS* approach. Similarly, primer mismatches may have prevented the detection of *T. thioparus*-related *nosZ* transcripts.

Based on our analysis of denitrification intermediates and products as well as the molecular assessment of 16S rRNA and functional genes, we could demonstrate the community-level capacity of groundwater-originated sulfur and H_2 oxidizing denitrifiers to simultaneously use multiple electron donors and their community shifts in response to different electron acceptor/electron donor ratios. Assessment of the *nirS*-type denitrifier community in the groundwater across four wells and six time points clearly confirmed that chemolithoautotrophic denitrification driven by reduced sulfur compounds and H_2 was an important metabolic feature widely spread among the groundwater denitrifiers, especially in anoxic groundwater of wells H43 and H53 of the upper aquifer assemblage, and agreed with observations in other aquifers (Kellermann *et al.* 2012; Zeng *et al.* 2016). Under oxic conditions in the groundwater of the lower aquifer, we observed higher relative abundances of denitrifiers related to *Pseudomonas* sp. A3, which might be linked to the capability of several *Pseudomonas* sp. to perform aerobic denitrification (Kong *et al.* 2006; Xu *et al.* 2017). Supply of fresh surface-derived organic carbon may be one of the key factors driving denitrifier community composition across the two aquifer assemblages. Previous investigations suggested faster water infiltration along with surface input signals of freshly introduced organic carbon for wells H41, H51 and H43 compared to well H53 (Kohlhepp *et al.* 2017; Nowak *et al.* 2017), which might result in conditions supportive of a fluctuating mixotropic and heterotrophic denitrifier community in wells H41, H51 and H43. In contrast, DIC natural isotope abundances indicated a stronger internal C cycling with a higher contribution of autotrophy for groundwater well H53 (Nowak *et al.* 2017). These findings agree well with the observed stable community structure of presumably mostly autotrophic denitrifiers at H53. Interestingly, *Sulfurifustis variabilis*-related denitrifiers that were highly abundant in the groundwater *nirS*-type denitrifier communities were not present in the enriched consortium. Given the fact that *Sulfurifustis variabilis* has been reported to utilize thiosulfate in a concentration range from 10 mM to 20 mM (Kojima, Shinohara and Fukui 2015), it remains unclear whether competition with *Thiobacillus* species played a major role in the absence of *Sulfurifustis* after 35 transfers. As oxidation of thiosulfate was previously not observed under anoxic conditions in the presence of nitrate (Kojima, Shinohara and Fukui 2015), *Sulfurifustis* might also not be able to perform chemolithoautotrophic denitrification with thiosulfate as electron donor or might not be able to denitrify at all. However, given the low sequence identity on *nirS*-level (84%), it is questionable whether the findings by Kojima, Shinohara and Fukui (2015) can directly be transferred to the *Sulfurifustis*-related bacteria we detected in oligotrophic groundwater, and the reasons for their absence from the enrichment culture remain speculative.

Overall, the denitrifying capacity of the enrichment reflected the common metabolic potential of large fractions of the denitrifier community in oligotrophic limestone aquifers. Shifts in the enrichment's community structure and active denitrifiers during the short experimental incubation period indicated the denitrifier community's ability to quickly adapt to changing environmental conditions such as sporadic inputs of nitrate or depletion of one of multiple possible electron donors. Given a sufficient supply of suitable inorganic electron donors, chemolithoautotrophic denitrification could provide an effective mechanism of nitrate removal in karstic aquifers or other groundwater environments where heterotrophic denitrification is limited.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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