

# Effects of thermal energy storage on shallow aerobic aquifer systems: temporary increase in abundance and activity of sulfate-reducing and sulfur-oxidizing bacteria

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**Abstract** Aquifer thermal energy storage may result in increases in the groundwater temperature up to 70 °C and more. This may lead to geochemical and microbiological alterations in the aquifer. To study the temperature effects on the indigenous microbial community composition, sediment column experiments at four different temperatures were carried out and the effluents were characterized geochemically and microbiologically. After an equilibrium phase at groundwater temperature of 10 °C for 136 days, one column was kept at 10 °C as a reference and the others were heated to 25, 40 and 70 °C. Genetic fingerprinting and quantitative PCR revealed a change in the bacterial community composition and abundance due to the temperature increase. While at 25 °C only slight changes in geochemical composition and gene copy numbers for bacteria were observed, increasing concentrations of total organic carbon in the 40 °C column were followed by a strong increase in bacterial abundance. Thermophilic bacteria became dominant at 70 °C. Temporary sulfate reduction took place at 40 and 70 °C and this correlated with an increased abundance of sulfate-reducing bacteria

(SRB). Furthermore, a coexistence of SRB and sulfur-oxidizing bacteria (SOB) at all temperatures indicated an interaction of these physiological groups in the sediments. The results show that increased temperatures led to significant shifts in the microbial community composition due to the altered availability of electron donors and acceptors. The interplay of SRB and SOB in sedimentary biofilms facilitated closed sulfur cycling and diminished harmful sulfur species.

**Keywords** Shallow aquifer thermal energy storage · Sediment column experiment · Temperature elevation · Microbial community composition shift · Sulfate-reducing bacteria · Sulfur-oxidizing bacteria

## Introduction

The use of underground thermal energy storage (UTES) is important for the transmission from a fossil to a renewable energy supply and for the reduction in carbon dioxide emissions (Saner et al. 2010; Blum et al. 2010). UTES provides heating and cooling for buildings or other facilities and thus substitutes the burning of coal, gas or oil. Several UTES techniques are used nowadays such as aquifer thermal energy storage (ATES) or borehole thermal energy storage (BTES). Beside the use of deep aquifers for the storage of thermal energy, also shallow aquifers in a depth below 400 m are used.

However, the environmental aspects of a temperature elevation in the subsurface due to heat addition are still rarely investigated. These influences include corrosion, clogging and scaling in geothermal power plants (Holm et al. 1987; Brons et al. 1991; Brons 1992; Griffioen and Appelo 1993; Lerm et al. 2011, 2013) as well as impacts on the groundwater and sediment chemistry (Briellmann et al.

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2009, 2011; Bonte et al. 2011a, b, 2013a, b, 2014; Jesušek et al. 2013a; Saito et al. 2016). Since groundwater is a resource of drinking water and habitat for a high number of organisms, assessment and planning concepts are necessary to evaluate the sustainability of the approach (Bauer et al. 2013; Hähnlein et al. 2013). Particularly, the impact on the aquifer-inhabiting microorganisms is insufficiently studied. Since a variety of microorganisms is present in the shallow underground, increased temperatures in the aquifer system can result in a shift of the microbial community composition and activity (Briellmann et al. 2009, 2011; Lerm et al. 2011; Bonte et al. 2013a). Bacterial diversity in groundwater was shown to increase with temperature (Briellmann et al. 2009). In addition, conditions may shift from iron-reducing to sulfate-reducing and methanogenic processes due to a temperature increase from 11 to 25 °C and thermophilic microorganisms were shown to establish at temperatures above 45 °C (Bonte et al. 2013a). Moreover, Lerm et al. (2013) showed that cooling fluids of an ATES system from above 68 °C to about 46 °C resulted in large differences in bacterial diversity and abundance.

Sediment column experiments were used to investigate changes in geochemical groundwater composition as well as microbial community structure and abundance due to increased temperatures in ATES systems (Briellmann et al. 2011; Jesušek et al. 2013a, b; Bonte et al. 2013a, b). In the studies of Jesušek et al. (2013a, b), the highest release of organic matter (OM) was detected for a temperature of 70 °C. For the same column experiment, Westphal et al. (2017) showed that the addition of acetate led to increased abundances of sulfate-reducing bacteria (SRB), which corresponded with a higher activity particular at 40 °C, and furthermore enhanced methanogenic activity at 25 °C. Most temperature studies assume anaerobic aquifer sediments (Bonte et al. 2013a, b, 2014) or a fast consumption of oxygen resulting in anaerobic conditions (Jesušek et al. 2013a, b). However, shallow aquifers may contain sufficient oxygen leading to another microbial community composition with a majority of microorganisms using oxygen as the terminal electron acceptor. The dominance of facultative and obligate aerobic microorganisms in oxygen-rich aquifers was demonstrated in several in situ studies (Balkwill and Ghiorse 1985; Griebler and Lueders 2009; Herrmann et al. 2015). Furthermore, Lerm et al. (2011) and Westphal et al. (2016) detected atmospheric oxygen ingress due to leaking equipment and a concurrent alteration in the microbial community composition in ATES systems during plant downtime.

A spatial proximity of aerobic and anaerobic microorganisms can be found in biofilms in the aquifer sediments. Increased temperatures might lead to a complete transmission from aerobic to anaerobic conditions and, thus, a concurrent change in the redox regime resulting in

emissions of, e.g., methane or hydrogen sulfide. An oxygen depletion and subsequent change to reductive conditions due to a temperature increase of up to 70 °C was already shown in the studies of Jesušek et al. (2013a, b) and Westphal et al. (2017). These changes in redox regimes were accompanied by increased sulfate reduction rates, methane formation and microbial activities. Bacteria taking part in the sedimentary sulfur cycle are of special interest in ATES systems. Increased temperatures might lead to enhanced activities of SRB and/or sulfur-oxidizing bacteria (SOB). SRB have been shown to enforce the damage of geothermal plant components due to the enhancement of corrosion processes (Lerm et al. 2013; Westphal et al. 2016). Furthermore, SOB such as *Thiobacillus* can induce corrosion or an acidification of the ambient groundwater by the production of sulfuric acid (Satoh et al. 2009). Biofilm formation of SOB was also shown to cause filter clogging in a shallow aquifer cold store (Lerm et al. 2011).

For the development of concepts to estimate the impact of geothermal heat storages in a specific area and for modeling the influences and creating guidelines for several regions, analyses concerning different chemical sediment and groundwater compositions are required (Kabuth et al. 2017). Microbiological data for most study sites are still rare or not available (Bauer et al. 2015). Since in situ investigations by trial and error are not of public interest and also cost intensive, experimental studies need to provide the data for modeling concepts.

This study presents temperature-induced changes in the bacterial community composition and abundance as well as geochemical parameters such as concentrations of TOC and terminal electron acceptors in the effluents of four columns filled with natural aquifer sediment from Odderade in Northern Germany. Genetic fingerprinting and quantitative PCR (qPCR) were used to study the structure and abundance of the bacterial community composition. To simulate a temperature increase in a shallow aquifer due to geothermal heat storage, those sediment columns were operated at 10, 25, 40 and 70 °C. The study encompasses the time before heating, directly after start of temperature increase and over a three months period at elevated temperatures. The focus of the investigations was on the abundance of SRB and SOB since their activity might be of environmental importance regarding the chemical groundwater composition.

## Materials and methods

### Experimental setup

Four high-density polyethylene (HDPE) columns with a length of 110 and 10 cm diameter were filled with

Pleistocene sand from Odderade in Northern Germany. The total organic carbon (TOC) content of the sediment was 0.185 wt% (Dethlefsen, unpublished data). The sediments were flowed through by tap water from the bottom to the top. The tap water contained approximately 0.16 mmol L<sup>-1</sup> of oxygen, 0.50 mmol L<sup>-1</sup> of nitrogen, 0.51 mmol L<sup>-1</sup> of sulfate, 0.01 mmol L<sup>-1</sup> of nitrate, 0.87 mmol L<sup>-1</sup> of chloride, 0.50 mmol L<sup>-1</sup> of silicon, 0.0001 mmol L<sup>-1</sup> of iron, 0.0002 mmol L<sup>-1</sup> of manganese, 0.36 mmol L<sup>-1</sup> of magnesium, 3.16 mmol L<sup>-1</sup> of calcium, 0.64 mmol L<sup>-1</sup> of sodium, 0.08 mmol L<sup>-1</sup> of potassium, 0.23 mmol L<sup>-1</sup> of non-purgeable organic carbon (NPOC) and 5.91 mmol L<sup>-1</sup> of total inorganic carbon (TIC). In the equilibrium phase, the columns were kept at 10 °C in the refrigerator for approximately 136 days to achieve a geochemical steady state. Afterward, three of the columns were heated up to 25, 40 and 70 °C by wrapping them with heating tapes and 1.9-cm-thick insulating material (AF-19MM/E; Armacell), while one column remained at 10 °C in the fridge as a reference. A more detailed description of the experimental setup is given in Lueders et al. (2016). In the following, the start of heating is termed as day 0, thus all data of days numbered with a “minus” were in the equilibrium phase at 10 °C.

### Geochemical analyses

For the geochemical sampling, three-way valves were attached to the columns. From the in- and outflow, 60 mL of fluid were taken to calculate increases or decreases in concentrations over the flow path. The NPOC concentrations were analyzed using a TOC/TN analyzer multi N/C 2000 (Analytik Jena). Assuming that the purgeable amount of TOC is negligible, the NPOC is termed as TOC in the following context. The nitrate and sulfate concentrations were measured by ion chromatography (IC 881, Metrohm). Oxygen concentration was measured using an oxygen probe (WTW oxy); however, changes in oxygen concentrations over the column flow path were too low for calculating reliable values. Accordingly, the geochemical data did not indicate a significant permeation of oxygen through the HDPE columns. The measurement of Fe<sub>diss</sub> was done by first stabilizing the samples with 65% HNO<sub>3</sub> sp. and then they were analyzed in an inductively coupled plasma optical emission spectrometer (Vista AX; Varian).

### Sampling and sample preparation for molecular biological analyses

Once during the equilibrium phase (48 days before heating start) and five times within 58 days during the heating phase (days 9, 16, 23, 37, 58 after heating start), approximately 1000 mL of fluid were collected at the outflow of

each column. The fluids were kept at 4 °C until vacuum filtration using a 0.2-µm-pore-size cellulose acetate membrane (Sartorius). Afterward, the filters were stored at -20 °C until further processing.

### DNA Extraction

DNA was extracted from filters using the FastDNA<sup>TM</sup> Spin Kit for Soil (MP Biomedicals) according to the manufacturer's protocol including an additional 10-min shaking step after addition of lysis buffer. Half a filter was used for DNA extraction. The concentration of the extracted DNA was determined fluorimetrically (FLUOstar OPTIMA, BMG Labtech) by labeling the DNA with Quant-iT PicoGreen (Invitrogen).

### Genetic fingerprinting (PCR-DGGE)

Partial 16S rRNA genes from bacteria were amplified by polymerase chain reaction (PCR) using the primer pair 341F-GC (according to Primer 3 in Muyzer et al. 1993) and 907R (according to UNIV907-R in Amann et al. 1992) (94 °C for 2:45 min, followed by 40 cycles of 94 °C for 0:45 min, 56 °C for 0:45 min and 72 °C for 0:50 min, followed by 72 °C for 30 min). PCR was performed in 50-µL reactions containing 5 µL of 10× buffer (Genecraft), 6 µL of deoxynucleotides (dNTPs) (10 mM, Thermo Fisher), 3 µL of MgCl<sub>2</sub> (50 mM, Genecraft), 3 µL of forward primer (10 mM), 3 µL of reverse primer (10 mM), 0.4 µL of bovine serum albumin (BSA) (20 mg/mL, Thermo Fisher), 0.3 µL of Taq polymerase (5 u/µL, Genecraft), 28.3 µL of RNA/DNA-free water (Thermo Fisher) and 1 µL of template DNA. Amplicons were subsequently purified (Thermo Fisher GeneJET PCR Purification Kit). Denaturing gradient gel electrophoresis (DGGE) was performed using equal concentrations of amplicons and a gradient of 35–65% urea and 6–9% formamide. Since the number of samples exceeded the number of lanes in the gel, two separate gel runs were performed. It has to be considered that slight variations in the urea gradient between the gels might cause slight differences in the gel runs.

The DGGE gels were run for 17 h at 110 V and 60 °C. The most intensive gel bands were excised and re-amplified using the primer pair 341F/907R (94 °C for 1:30 min, followed by 30 cycles of 94 °C for 0:30 min, 56 °C for 0:30 min, 72 °C for 0:30 min, followed by 72 °C for 10 min). Re-amplification of some bands failed. Successfully re-amplified PCR products were purified using the Thermo Fisher GeneJET PCR Purification Kit. The concentrations of the amplicons were measured fluorimetrically (BMG Labtech FLUOstar OPTIMA) by labeling the DNA with Quant-iT PicoGreen (Invitrogen). The PCR

products were sequenced by GATC Biotech AG. Sequence homologies were checked using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990). The comparative similarities of the communities in the equilibrium phase 48 days before start of heating (−48) and on days 9, 16 and 23 in the heating phase were calculated according to the Sørensen–Dice coefficient (Dice 1945; Sørensen 1948). This coefficient gives a value between 0 and 1. The closer the value is to 1, the more the communities have in common. DGGE gel analyses and band matching with a distance height of 0.5% were conducted using the PyElph software (Pavel and Vasile 2012). The similarities ( $CC$ ) were calculated using the formula  $CC = 2C/(S1 + S2)$  [ $C$ : number of bands the two communities have in common;  $S1$ : number of bands in community 1; and  $S2$ : number of bands in community 2]. Since the comparability of the Sørensen–Dice coefficient of two separately run gels is difficult, the values were only calculated for one gel.

### Quantitative PCR

Gene copies per ml of fluid were determined using a SYBR green approach with the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Primer sets used in the qPCR approach were 331F/797R for the total bacterial 16S rRNA, dsr1F/dsr500R for amplifying the SRB-specific *dsrA* operon as well as TT-F/TT-R for the *Thiobacillus*-specific 16S rRNA gene fragment (Nadkarni et al. 2002; Wagner et al. 1998; Wilms et al. 2007; Sercu et al. 2007). All analyses were performed in duplicates. Each PCR reaction contained 10  $\mu$ L of Power SYBR Green (Life Technologies), 0.5  $\mu$ M of each primer, 10  $\mu$ g BSA (Thermo Scientific) and 1  $\mu$ L of template DNA. Nuclease-free water (Thermo Scientific) was added to a final volume of 20  $\mu$ L. The thermal cycling included an initial denaturation step for 10 min at 95 °C followed by 40 cycles of amplification with 0:15 min at 95 °C, 0:20 min at respective primer-specific temperatures [57 °C (Bacteria), 58 °C (SRB) and 62 °C (*Thiobacillus*)] and 0:20 min (*Thiobacillus*) or 0:30 min (Bacteria, SRB) at 72 °C. After each run, a melting curve was recorded to discriminate between specific amplicons and unspecific fluorescence signals. For the determination of gene copy numbers per mL of fluid, the 16S rRNA gene of *Escherichia coli* strain JM109, the *dsrAB* gene of *Desulfotomaculum geothermicum* (DSMZ 3669) and the partial 16S rRNA of *Thiobacillus thioparus* were cloned using the pGEM-T Cloning Kit (Promega). Plasmid dilutions from  $10^{-1}$  to  $10^{-8}$  plasmids  $\text{mL}^{-1}$  served as template for performing qPCR standard curves. The efficiency of amplification

amounted to 83% for the bacterial 16S rRNA gene fragment, 94% for the *Thiobacillus* 16S rRNA gene fragment and 100% for the *dsrA* gene fragment with an  $R^2$  of 0.99 each.

### Statistical analyses

Univariate and multivariate statistical data analyses were done using the freeware Past version 3.12 (Hammer et al. 2001). Significant differences in gene copy numbers of bacteria, SRB and *Thiobacillus* were calculated according to a one-way analysis of variance (ANOVA) and Kruskal–Wallis test for equal medians. Moreover, principle component analysis (PCA) was conducted for determination of relation between geochemical data and microbiological data.

## Results

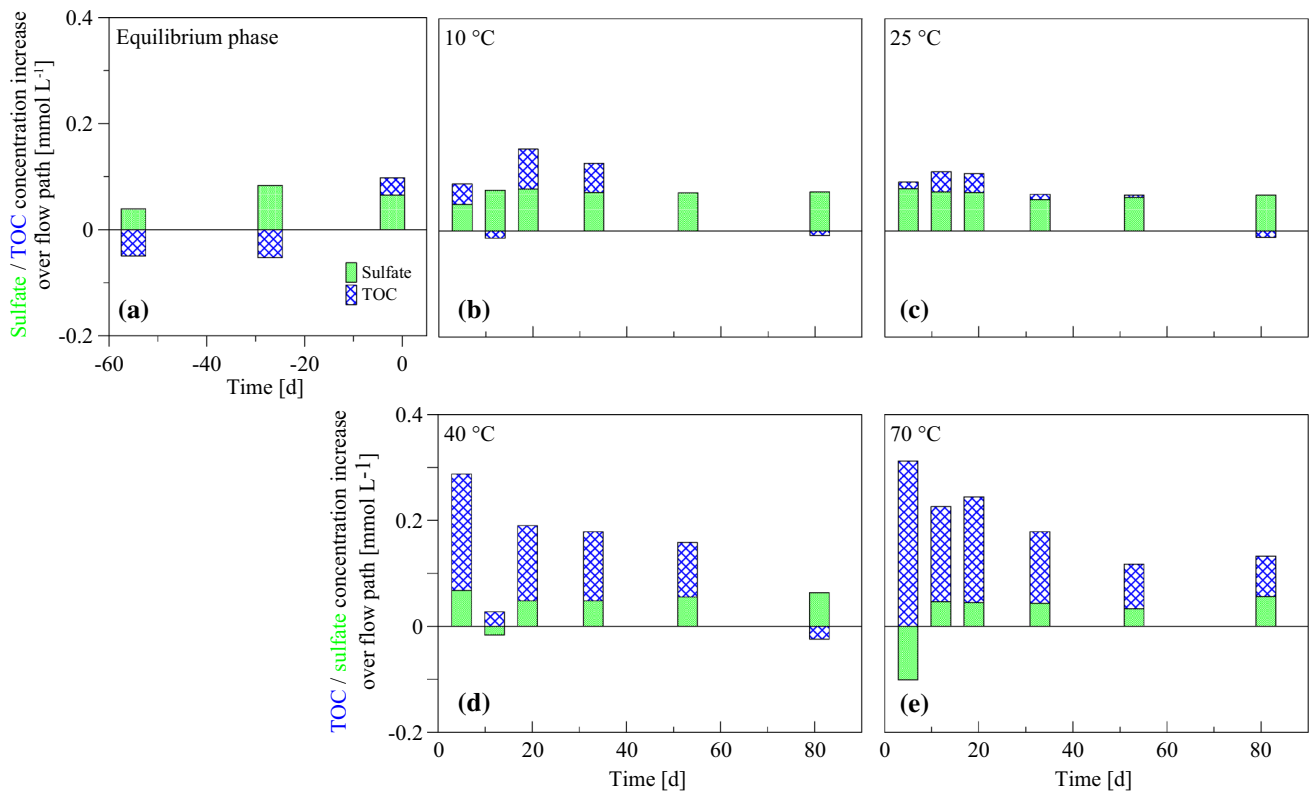
### Chemical characterization of fluids over time

The differences between out- and inflow of sulfate and TOC over time and due to the temperature increase are shown in Fig. 1.

During the equilibrium phase, sulfate concentrations increased over the column flow path by 0.09  $\text{mmol L}^{-1}$  in all columns, indicating a release of sulfate from the sediment. At 10 and 25 °C, the sulfate increase remained at 0.08  $\text{mmol L}^{-1}$  with ongoing runtime. In contrast, temporary decreases in sulfate concentration by 0.02  $\text{mmol L}^{-1}$  at 40 °C and 0.1  $\text{mmol L}^{-1}$  at 70 °C were detected after 12 days and five days, respectively (Fig. 1). Afterward, a release of sulfate of up to 0.06  $\text{mmol L}^{-1}$  occurred.

Increased TOC concentrations over the column flow path indicated a release of OM in the columns. In the reference column as well as in the 25 °C column, only low amounts of OM were mobilized resulting in TOC increases between 0.03  $\text{mmol L}^{-1}$  and 0.08  $\text{mmol L}^{-1}$  after the equilibrium phase. In terms of the temperature elevation to 40 and 70 °C, the TOC increase was 0.22 and 0.31  $\text{mmol L}^{-1}$  after five days, respectively (Fig. 1). Afterward, at 70 °C, the TOC increase resulted in 0.08  $\text{mmol L}^{-1}$  on days 53 and 81, whereas at 40 °C the TOC increase resulted in 0.03  $\text{mmol L}^{-1}$  on day 12 followed by a stronger increase to 0.14  $\text{mmol L}^{-1}$  with ongoing runtime.

In all columns, no decreases in nitrate concentrations and ferrous iron concentrations were observed (data not shown).



**Fig. 1** Increase in TOC and sulfate concentrations over column flow path in the equilibrium phase (a) (average values of all columns) and at 10 °C (b), 25 °C (c), 40 °C (d) and 70 °C (e) during the heating phase

**Determination of bacterial diversity at increased temperatures**

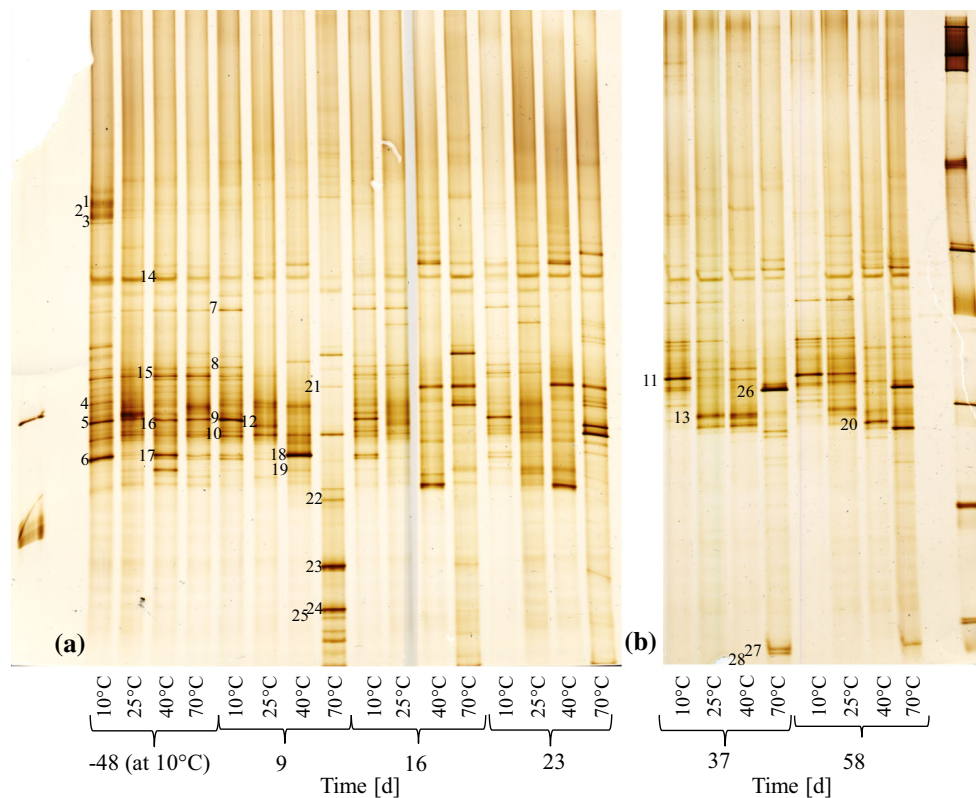
The genetic fingerprinting showed differences in the band patterns for all column effluents in the equilibrium phase and further alterations due to the temperature increase and experimental runtime (Fig. 2). The Sørensen–Dice coefficients for the comparison of the similarities of the four columns in the equilibrium phase showed differences in the bacterial communities with values between 0.38 and 0.48 (Table 1). Although the 10 °C column was kept as a reference, alterations in the genetic fingerprinting were observed. Sequences affiliated to a bacterium of the phylum *Bacteroidetes* (Bands 1, 2, 3), the genus *Polaromonas* (Band 4), a bacterium of the family *Gallionellaceae* (Band 5) and the genus *Aquabacterium* (Band 6) were found in the equilibrium phase (Table 1). A Sørensen–Dice coefficient of 0.34 indicated a significant dissimilarity of the microbial community on day nine compared to the band pattern of the equilibrium phase (Table 2). Additionally, a bacterium affiliated to the order *Nitrosomonadales* (Band 7) became more dominant in the effluents as well as two organisms affiliated to the genera *Sideroxydans* (Band 9) and *Simplicispira* (Band 10). These bands were further detected on the days 16 and 23. After 37 days, one band became more intensive while the other bands weakened.

This sequence was affiliated to a bacterium of the family *Gallionellaceae* (Band 11).

In the 25 °C effluents, the genetic fingerprinting also showed alterations over the runtime. Comparing the band patterns of the equilibrium phase and after nine days, the Sørensen–Dice coefficient showed decreasing similarities of the communities (0.29). The calculations of the Sørensen–Dice coefficients with ongoing runtime showed alterations between the community similarities from day nine to 16 (0.33) and 16 to 23 (0.21). After 37 days, one band, which sequence was affiliated to the genus *Aquabacterium* (Band 13), became most intensive.

At 40 °C, the band pattern after the start of heating also differed from the band pattern of the equilibrium phase. In the equilibrium phase, sequences affiliated to the genera *Serratia* (Band 14), *Thiobacillus* (Band 15), *Aquabacterium* (Band 16) and *Rhodobacter* (Band 17) were found. The Sørensen–Dice coefficient showed decreasing similarities from the initial phase to day nine (0.38) and from day nine to day 16 (0.25). One particular band assigned to the genus *Aquabacterium* (Band 18) became most intensive after the start of heating. The band pattern changed again resulting in two most intensive bands on day 16. After 37 days, the genetic fingerprinting showed further alterations. On day 58, one band, which was assigned to the genus *Aquabacterium* (Band 20), was most intensive.





**Fig. 2** DGGE profiles of bacterial 16S rRNA gene fragments. The first DGGE run was conducted for the days –48, 9, 16 and 23 (a) and the second DGGE run was done for the days 37 and 58 (b).

The band pattern at 70 °C showed differences to the other temperatures. In accordance, the Sørensen–Dice coefficient showed highly dissimilar community compositions (0.26). Organisms assigned to the class *Clostridia* (Band 21), the genera *Desulfurispora* (Band 22), *Desulfotomaculum* (Band 23) and *Hydrogenophaga* (Band 25) as well as to the family *Thermoanaerobacteriaceae* (Band 24) were detected. Bands 23, 24 and 25, which ran further in the gel, became weaker after 16 days. Accordingly, the Sørensen–Dice coefficient showed another change in the community similarities from day nine to day 16 as well as from day 16 to 23 (each 0.26). Correspondingly, after 23 days new intensive bands were detected. Sequences affiliated to *Hydrogenophaga* (Band 26), *Thermoanaerobaculum* (Band 27) and an uncultured bacterium (Band 28) were found after 37 days. Particularly the band affiliated to *Hydrogenophaga* became most dominant. On day 58, another band, whose microorganism was not identified, became co-dominant to the band affiliated to *Hydrogenophaga*.

#### Quantification of bacterial, SRB and SOB abundances

The qPCR analyses of the bacterial 16S rRNA gene copy numbers as well as of *dsrA* and *Thiobacillus* 16S rRNA gene copy numbers showed changes over time and

Sequenced bands are labeled with *numbers*. The taxonomic affiliation is shown in Table 1

corresponding to the temperature increase (Figs. 3, 4, 5). The highest bacterial 16S rRNA gene copy numbers were detected in the effluents of the 40 °C column, whereas the 10 °C effluents showed the lowest gene copies with a decreasing trend (Figs. 3, 4). Particularly at 70 °C, *dsrA* gene copy numbers increased by a factor of 300 directly after the start of heating (Fig. 5). The effluents of this column showed the highest *dsrA* gene copies over time (Fig. 4). 16S rRNA gene copies of the SOB genus *Thiobacillus* were detected at all temperatures with the highest numbers at 25 and 40 °C (Fig. 4). Coexistence of SRB and the SOB genus *Thiobacillus* occurred over experimental runtime at all temperatures (Fig. 5).

#### Statistical analyses according to geochemical data and abundances of microorganisms

A principle component analysis (PCA) with respect to the concentrations of sulfate and TOC as well as gene copy numbers of bacteria, SRB and *Thiobacillus* (SOB) depending on the time was conducted (Fig. 6). At 10 °C, on days 5–9 (t1) and 12–16 (t2) 16S rRNA gene copy numbers of bacteria, *dsrA* gene copies and *Thiobacillus* 16S rRNA gene copy numbers correlated (Fig. 6a). Moreover, on days 33–37 (t4) TOC concentrations solely

**Table 1** Sequencing of partial 16S rRNA genes retrieved from DGGE fingerprints

Temperature (°C)	Band	Closest relative (Genbank accession number)	Phylum	Genbank accession number	Similarity (%)
10	1	Uncultured <i>Bacteroidetes</i> bacterium (AY948070.1)	<i>Bacteroidetes</i>	KU136344	97
	2	Uncultured <i>Bacteroidetes</i> bacterium (AY948070.1)	<i>Bacteroidetes</i>	KU136345	97
	3	Uncultured <i>Bacteroidetes</i> bacterium (AY948070.1)	<i>Bacteroidetes</i>	KU136346	93
	4	<i>Polaromonas</i> sp. (JX950027.1)	<i>Proteobacteria</i>	KU136347	96
	5	Uncultured <i>Gallionellaceae</i> bacterium (JQ177958.1)	<i>Proteobacteria</i>	KU136348	97
	6	<i>Aquabacterium commune</i> (NR_024875.1)	<i>Proteobacteria</i>	KU136349	100
	7	Uncultured <i>Nitrosomonadales</i> bacterium (EF562022.1)	<i>Proteobacteria</i>	KU136354	92
		Uncultured <i>Rhodocyclaceae</i> bacterium (JQ278780.1)	<i>Proteobacteria</i>		92
	8	Uncultured <i>Serratia</i> sp. (AY963526.1)	<i>Proteobacteria</i>	KU136355	97
	9	Uncultured <i>Sideroxydans</i> sp. (JQ178033.1)	<i>Proteobacteria</i>	KU136356	97
	10	<i>Simplicispira</i> sp. (KC113247.1)	<i>Proteobacteria</i>	KU136357	99
25	11	Uncultured <i>Gallionellaceae</i> bacterium (JQ177958.1)	<i>Proteobacteria</i>	KU136370	95
	12	<i>Aquabacterium commune</i> (NR_024875.1)	<i>Proteobacteria</i>	KU136358	99
40	13	<i>Aquabacterium parvum</i> (NR_024874.1)	<i>Proteobacteria</i>	KU136369	100
	14	Uncultured <i>Serratia</i> sp. (AY963526.1)	<i>Proteobacteria</i>	KU136350	94
	15	Uncultured <i>Thiobacillus</i> sp. (JQ087157.1)	<i>Proteobacteria</i>	KU136351	98
	16	<i>Aquabacterium commune</i> (NR_024875.1)	<i>Proteobacteria</i>	KU136352	100
	17	<i>Rhodobacter</i> sp. (JX949604.1)	<i>Proteobacteria</i>	KU136353	99
		<i>Pseudorhodobacter</i> sp. (JX949602.1)	<i>Proteobacteria</i>		99
	18	<i>Aquabacterium commune</i> (NR_024875.1)	<i>Proteobacteria</i>	KU136359	99
	19	<i>Caldicoprobacter algeriensis</i> (NR_117466.1)	<i>Firmicutes</i>	KU136360	97
	20	<i>Aquabacterium commune</i> (NR_024875.1)	<i>Proteobacteria</i>	KU577262	99
	70	21	Uncultured <i>Clostridia</i> bacterium (DQ208699.1)	<i>Firmicutes</i>	KU136361
22		<i>Desulfurispora thermophila</i> (NR_042969.1)	<i>Firmicutes</i>	KU136362	98
23		<i>Desulfotomaculum</i> sp. (EF494253.1)	<i>Firmicutes</i>	KU136363	100
24		Uncultured <i>Thermoanaerobacteriaceae</i> bacterium (AY684090.1)	<i>Firmicutes</i>	KU136364	92
25		<i>Hydrogenophaga</i> sp. (DQ854970.1)	<i>Proteobacteria</i>	KU136365	97
26		Uncultured <i>Hydrogenophaga</i> sp. (KC110482.1)	<i>Proteobacteria</i>	KU136366	100
27		<i>Thermoanaerobaculum aquaticum</i> (NR_109681.1)	<i>Acidobacteria</i>	KU136367	98
28		Uncultured candidate division OP10 bacterium (EF205572.1)	n.d.	KU136368	95

The taxonomic assignment was performed using NCBI BLAST. *n.d.* not determined

branched. At 25 °C, a correlation of bacterial and *Thiobacillus* 16S rRNA gene copies as well as TOC concentrations was detected on days 19–23 (t3) (Fig. 6b). On days 12–16 (t2), the sulfate concentration corresponded to SRB-specific *dsrA* gene copy numbers. At 40 °C, bacterial 16S rRNA gene copies showed a divergence on days 12–16 (t2) to the other parameters (Fig. 6c). On days 53–58 (t5), *dsrA* gene copy numbers correlated to *Thiobacillus* 16S rRNA gene copies. At 70 °C, a divergence of *dsrA* gene copy numbers was detected on days 33–37 (t4) (Fig. 6d). Moreover, a correlation of bacterial and *Thiobacillus* 16S rRNA gene copy numbers was found on days 12–16 (t2).

Statistical analyses regarding differences in bacterial, SRB and *Thiobacillus* gene copy numbers according to

the temperature showed significant values for all parameters. Calculated *p* values for one-way ANOVA and Kruskal–Wallis test for equal medians were *p* < 0.01 each for bacterial 16S rRNA gene copies, SRB-specific *dsrA* gene copy numbers and *Thiobacillus* 16S rRNA gene copies.

## Discussion

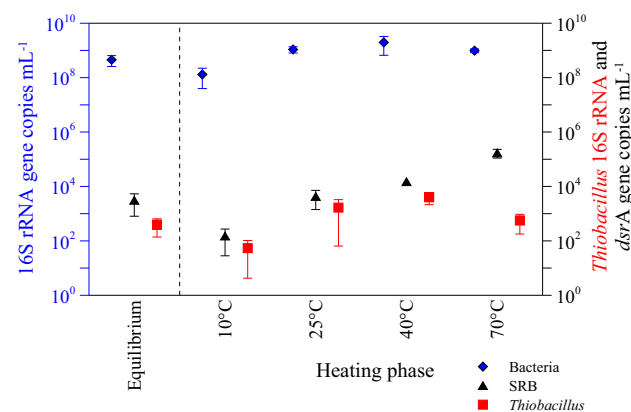
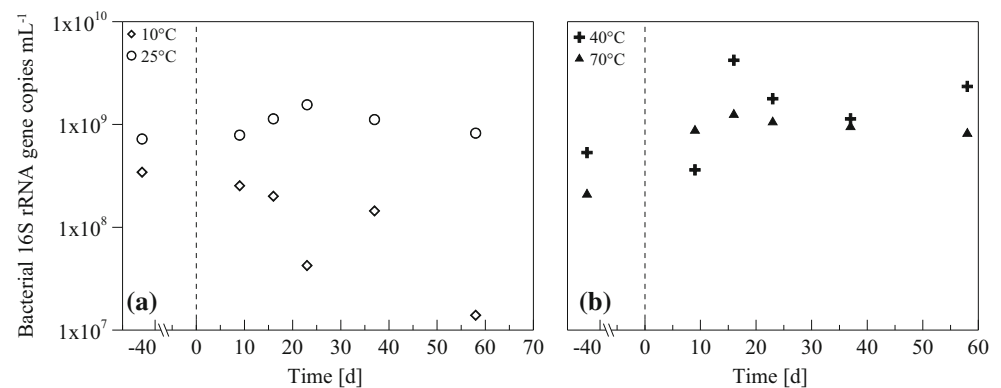
This study shows that increasing temperatures induce a change in the bacterial community composition and trigger complex interactions of different physiological microbial groups in natural aquifer sediments.

**Table 2** Comparison of bacterial community similarities in the equilibrium phase on day -48 and between different days by calculation of the Sørensen-Dice coefficient

Columns	Similarity coefficient in equilibrium phase at 10 °C on day -48		
10 versus 25 °C	0.38		
10 versus 40 °C	0.42		
10 versus 70 °C	0.42		
25 versus 40 °C	0.48		
25 versus 70 °C	0.48		
40 versus 70 °C	0.47		
Temperature (°C)	Similarity coefficient according to runtime		
	Day -48 to 9	Day 9 to 16	Day 16 to 23
10	0.34	0.41	0.48
25	0.29	0.33	0.21
40	0.38	0.25	0.24
70	0.26	0.26	0.26

Values give the similarity between one column or one day to another

**Fig. 3** 16S rRNA gene copy numbers of *Bacteria* at 10 and 25 °C (a) as well as at 40 and 70 °C (b). The dashed line represents the start of heating



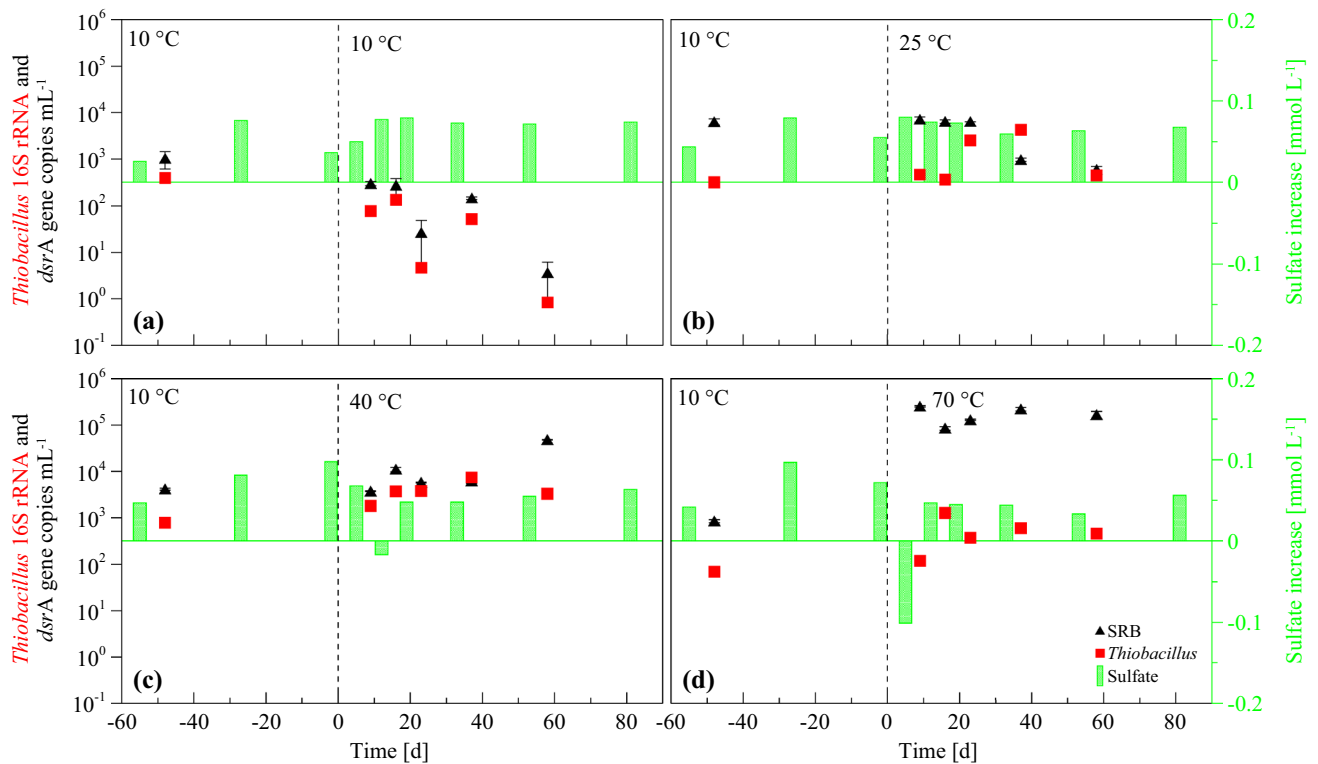
**Fig. 4** 16S rRNA gene copy numbers of *Bacteria*, SRB-specific *dsrA* gene copy numbers and 16S rRNA gene copies of sulfur-oxidizing bacterial genus *Thiobacillus* on day -48 in equilibrium phase (average values of all columns) and average values ( $n = 5$ ) in the heating phase over 58 days

### Release of OM and microbial degradation under in situ temperature

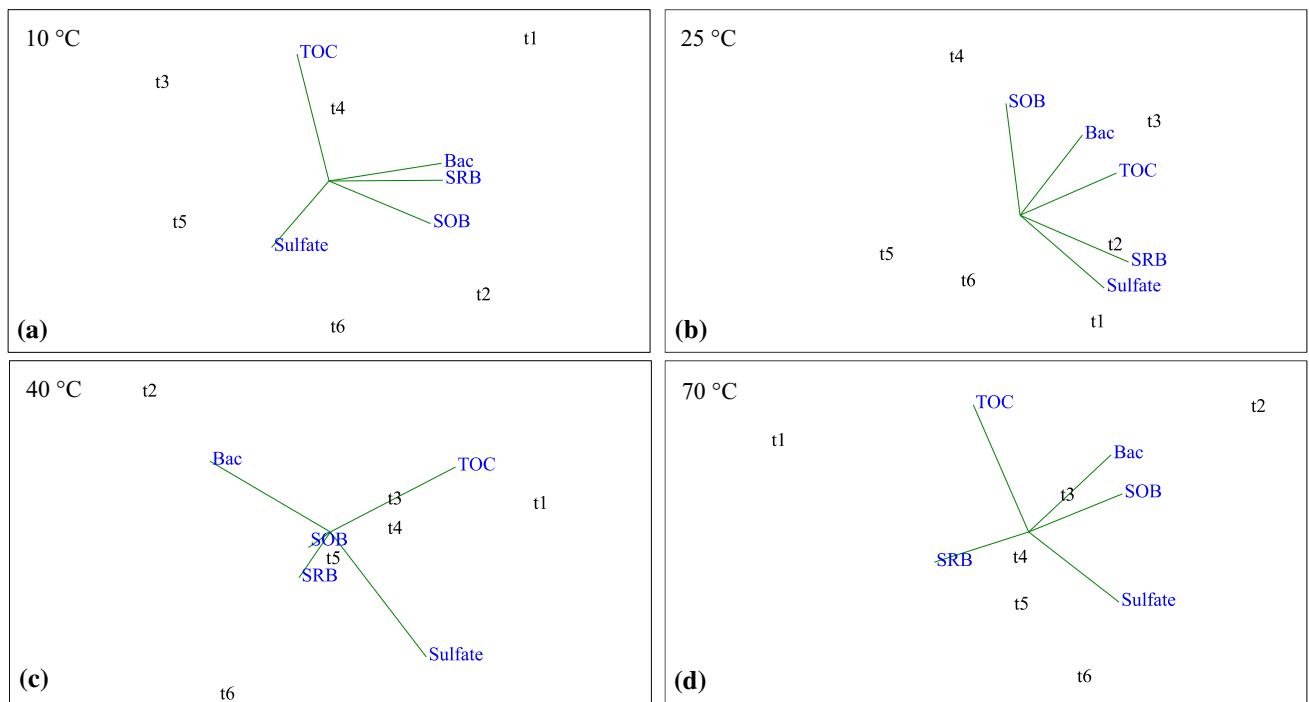
In the 10 °C column, which was run as a reference to the natural aquifer temperature, no equilibrium was reached after an initial phase of 136 days. Moreover, until the end of the experiment there were changes in chemical profiles and bacterial communities at 10 °C. During this, decreased OM release went along with decreasing bacterial 16S rRNA gene copy numbers. After a certain time, heterotrophic bacteria obviously became limited in available OM and the bacterial abundance decreased. In accordance to these results, in another study with sediment column experiments heated from 10 to 45 °C also the lowest cell numbers were found in the 10 °C fluids (Briellmann et al. 2011).

In our study at 10 °C, sequences were affiliated to facultative anaerobic bacteria such as *Aquabacterium*,





**Fig. 5** Gene copy numbers of SRB and sulfur-oxidizing bacterial genus *Thiobacillus* as well as increase in sulfate concentration over column runtime at 10 °C (a), 25 °C (b), 40 °C (c) and 70 °C (d). The dashed line represents the start of heating



**Fig. 6** Principle component analysis (PCA) correlating sulfate and TOC concentrations and gene copy numbers of bacteria, SRB and SOB with respect to the temperatures 10 °C (a), 25 °C (b), 40 °C (c) and 70 °C (d) during the heating phase (t1 = days 5–9, t2 = days 12–16, t3 = days 19–23, t4 = days 33–37, t5 = days 53–58, t6 = day 81)

*Polaromonas*, *Simplicispira*, *Serratia* and *Sideroxydans*. *Aquabacterium* is commonly found in biofilms of drinking water systems (Kalmbach et al. 1999). *Polaromonas* is a psychrophilic microorganism whose species have a growth range from 0 to 25 °C (Irgens et al. 1996; Margesin et al. 2012), whereas *Simplicispira* grows optimally between 10 and 40 °C (Lu et al. 2007). Some species of the genus *Serratia* are psychrotolerant and can grow at 5 °C (Grimont and Grimont 2006). Although *Sideroxydans* species are characterized as aerobic iron-oxidizing bacteria (IOB) (Beckwith et al. 2015), no changes in the iron concentration were detected in the effluents. Most probably, the iron(II) precipitated in the column sediment. The bacterial community composition changed with ongoing runtime. Obviously after 37 days, the diverse community of bacteria detected in the beginning was outcompeted by a community dominated by *Gallionellaceae*-like bacteria. Only one species, *Gallionella ferruginea*, was described in the family *Gallionellaceae* to date (Hallbeck and Pedersen 2014). Besides *Sideroxydans*, *G. ferruginea* was also described as an aerobic IOB. Thus, chemolithotrophic metabolism probably played a major role at 10 °C due to limited availability of OM. Since the sediment contained approximately 1200 mg kg<sup>-1</sup> of pyrite (Dethlefsen, unpublished data) this might have been an iron source for the IOB. Pyrite was probably metabolized to sulfate by sulfur-oxidizing bacteria (SOB), as indicated by the increased sulfate concentrations in the effluents. Besides representatives of the genus *Sideroxydans*, which were likely involved in pyrite oxidation, qPCR also showed an abundance of the SOB genus *Thiobacillus* at 10 °C with ongoing runtime. Members of this genus were also shown to take part in pyrite oxidation (Hedrich et al. 2011).

Taken together, at the in situ aquifer temperature of 10 °C a succession in the microbial community was apparent, first as a result after the setup of the column, then as succession by competition of dominant genera for the available nutrients.

### Release of OM and microbial degradation after increase in temperature

The heating of groundwater and sediment influenced the groundwater chemical composition and the microbial community composition in the sediment columns. Genetic fingerprinting and qPCR analyses showed that the impact on the aquifer microbial community composition is stronger with higher temperature and goes along in phases with ongoing runtime.

After heating the column to 25 °C, changes in the bacterial community composition went along with geochemical alterations in the fluids. Already nine days after the start of heating to 25 °C, a shift in bands indicated an adaptation

of the community to the higher temperature. An *Aquabacterium*-like organism dominated the community. However, bacterial 16S rRNA gene copies as well as the TOC concentration in the effluents remained constant. After 37 days, additional bands appeared and the community was further dominated by an *Aquabacterium*-like organism. An appearance of the genus *Aquabacterium* was also shown for other sediment column experiments (Westphal et al. 2017). However, in their sediment column experiment, acetate was constantly added and the oxygen was already depleted in the first centimeters of the column flow path. Therefore, the *Aquabacterium* dominance was not as strong, because oxygen as terminal electron acceptor was very limited. In our study at 25 °C, the bacterial 16S rRNA gene copy numbers and number of SRB-specific *dsrA* gene copies decreased with ongoing runtime and reflected the change in the bacterial community composition. Accordingly, Bonte et al. (2013a) also showed a significant change in the microbial community composition and only slight changes in the dissolved organic carbon concentration when increasing the temperature from 11 to 25 °C in a groundwater-based experimental setup. Obviously in our study, heating to 25 °C had an influence on the microbial community and caused a shift in dominant genera even though the changes in the community were only slight over a longer period of time. The increase in temperature by 15 K with respect to the natural aquifer temperature obviously enhanced the activity of, e.g., *Aquabacterium* and thus allowed for its dominance at this temperature. In contrast, psychrophilic organisms such as *Polaromonas* were less adapted to the temperature increase and were outcompeted.

A more complex pattern of changes was visible in the 40 °C experiment, and the highest bacterial 16S rRNA gene copy numbers were detected in the effluents of this column. This is in accordance with the study of Westphal et al. (2017). Briemann et al. (2011) also showed a strong increase in microbial activity at a temperature of 40 °C compared to 10 and 20 °C; however, cell counts and bacterial diversity were not higher at 40 °C. The reason for this might be the tenfold higher flow rate and thus a shorter contact time of the fluid and the sediment in the study of Briemann et al. (2011). In our study in the equilibrium phase, facultative anaerobic bacteria such as *Serratia*, *Thiobacillus*, *Aquabacterium* and *Rhodobacter* dominated. After the start of heating to 40 °C, the OM release increased, while the bacterial 16S rRNA gene copies remained on the same level until day nine in comparison to the equilibrium phase. Higher concentrations of dissolved organic carbon in sediment columns heated up to 40 °C in comparison to 10 and 25 °C were also found by Bonte et al. (2013a). Accordingly, Brons et al. (1991) showed an increased mobilization of organic carbon compounds at a

temperature higher than 45 °C in aerobic aquifer sediments. The following lower TOC concentration at the column outflow corresponded to a strong increase in the bacterial gene copy numbers and a small peak in SRB-specific *dsrA* gene copies after 16 days. The release of OM after the start of heating to 40 °C stimulated the bacterial metabolic rates and growth. Correspondingly, a higher sulfate reduction rate was detected on day 12 indicating enhanced anaerobic metabolic activity. It is likely that the increase in TOC availability triggered the anaerobic microorganisms in biofilms in the sediment. This is also shown by the increased *dsrA* gene copy numbers. Moreover, the PCA showed a divergence of the bacterial 16S rRNA gene copy numbers at this time, which also points out the strong increase in abundance. Also the fingerprinting results showed a change in the bacterial community between day nine and day 16 resulting in three most intensive bands and thus three dominating bacteria. Increased release of OM was detected after 19 days, whereas the bacterial gene copy numbers decreased from days 23 to 37 and the diversity diminished. This could be the result of an enhanced biofilm release from the sediment, which probably strongly developed during the previous activity increase. Band pattern changed again on day 58 showing a succession with lower diversity and one strong band, which was affiliated to an *Aquabacterium*-like organism. As already mentioned earlier, species of the genus *Aquabacterium* are very common in biofilms from drinking water systems (Kalmbach et al. 1999). The dominance of this organism on day 58 suggests that *Aquabacterium* outcompeted other organisms with ongoing experimental time.

Due to heating the column to 70 °C, the band patterns of the effluents differed significantly from the patterns at the other temperatures. Bands ran further in the gel indicating organisms with higher guanine–cytosine (GC) contents for DNA stabilization at high temperatures. Accordingly, sequences affiliated to the family *Thermoanaerobacteriaceae* and the genus *Thermoanaerobaculum*, both thermophilic bacterial divisions with high GC contents in the DNA, were detected exclusively at 70 °C. Although a temperature range from 50 to 65 °C with an optimum of 60 °C was described for the genus *Thermoanaerobaculum* (Losey et al. 2013), this study indicates that growth in this complex system also appears at 70 °C. A clear change of the microbial community structure with an appearance of thermophilic organisms was also shown in the study of Bonte et al. (2013a) for a temperature of 60 °C. In comparison to the other temperatures, the 70 °C effluents showed the strongest release of OM. Accordingly, the highest increase in the total organic carbon concentration was also shown in the study of Jesušek et al. (2013a) for sediment columns heated up to 70 °C. In their study, the

strongest release of OM at 70 °C was accompanied by a rudimentary sulfate reduction, whereas the sulfate concentrations did not decrease at 10, 25 and 40 °C. Accordingly, in our study, a temporary sulfate reduction was detected simultaneously with an OM release after 5 days at 70 °C. Correspondingly, the genetic fingerprint showed an appearance of relatives of the obligate anaerobic SRB genera *Desulfotomaculum* and *Desulfurispora*. Both genera were shown to grow under thermophilic conditions (Dumas et al. 1988; Kaksonen et al. 2007). At the same time, SRB-specific *dsrA* gene copy numbers were the highest. The detection of representatives of the genus *Desulfotomaculum* in particular at 70 °C is in accordance with the study of Bonte et al. (2013a), in which organisms related to *Desulfotomaculum* became enriched at a temperature of 60 °C. Westphal et al. (2017) also detected the genera *Desulfotomaculum* and *Desulfurispora* by applying universal genetic fingerprinting for bacteria in effluents of natural aquifer sediment column experiments at 70 °C. It is likely that organic matter was degraded to volatile fatty acids by thermophilic fermentative bacteria such as *Thermoanaerobaculum* (Losey et al. 2013) or bacteria from the class *Clostridia*, which stimulated the growth of SRB at 70 °C. This is in accordance with the study of Hubert et al. (2009), which showed the co-appearance of SRB and fermentative bacteria at a temperature of 50 °C. Most probably, biofilm formation in the sediments created anaerobic micro-niches which enabled these microbes to grow in an aerobic environment. After 37 days, in addition to a representative of *Thermoanaerobaculum*, a *Hydrogenophaga*-like organism became dominant. This aerobic chemoorganotrophic microorganism is capable of hydrogen oxidation (Willems et al. 1989) that might have been produced by fermentation, and thus it likely had an advantage over other microorganisms. On day 58, co-dominance of the aerobic *Hydrogenophaga*-like and *Aquabacterium*-like organisms appeared. The appearance of relatives of the genus *Aquabacterium* at 70 °C is in accordance with the study of Lerm et al. (2013), which also detected an *Aquabacterium*-like organism in an ATEs system at a temperature of 67 °C.

### Coexistence of SRB and SOB in sedimentary biofilms

It was shown that compared to the genetic fingerprinting, in which only the dominant microorganisms are detected, the specific analyses by qPCR showed a coexistence of the SOB genus *Thiobacillus* and SRB at all temperatures. At the reference temperature (10 °C), PCA showed a close correlation of bacterial, SRB and SOB gene copy numbers. At 40 and 70 °C, an increase in SRB-specific *dsrA* gene copies was detected over time, while in the 25 °C effluents

*dsrA* gene copy numbers decreased one order of magnitude compared to the equilibrium phase. At the same time in all three columns, the gene copy numbers of *Thiobacillus* increased at elevated temperatures.

The sulfate release was less at 40 and 70 °C in comparison to the equilibrium phase and to the 10 °C column. In accordance, the bands affiliated to bacteria taking part in pyrite oxidation such as *Sideroxydans* weakened. Lueders et al. (2016) showed a stronger gas phase formation in the 40 and 70 °C heated columns due to a lower solubility at higher temperatures. This likely led to a reduced retention time of the fluids in the columns, to local zones without oxygen and thus to less pyrite oxidation and better conditions for anaerobic bacteria such as SRB. Correspondingly, the sulfate reduction rate and abundance of SRB increased at 40 and 70 °C, which also led to reduced sulfate concentrations compared to the 10 and 25 °C columns. The finding of the temporary sulfate reduction at 40 and 70 °C is in agreement with the study of Bonte et al. (2013a), which also showed the highest sulfate reduction at these temperatures. At 70 °C, an increased OM release went along with an enhanced SRB-specific *dsrA* gene copy number while sulfate decreased. Apparently, the SRB activity was triggered by the temperature and probably by OM release and subsequent degradation resulting in sulfate reduction. However, although the gene copies of SRB remained in the same order of magnitude, geochemical data showed no decrease in sulfate concentration. At the same time, gene copy numbers of *Thiobacillus* increased. Also in the 40 °C column, the constant sulfate release went along with an increased *Thiobacillus* abundance. Presumably, the sulfur compounds reduced in the former period were oxidized by the SOB leading to an enhanced availability of sulfate as terminal electron acceptor for SRB. Thus, due to the sulfur cycling and pyrite oxidation, the sulfate concentrations resulted in constant values, although *dsrA* gene copies were still detected in high numbers and SRB were probably active. The correlation of SRB and SOB at 40 °C was also shown by the PCA. The coexistence and interaction of SRB and SOB in aerobic–anaerobic zones was already shown by several studies (Okabe et al. 2005; Baumgartner et al. 2006; Satoh et al. 2009; Handley et al. 2013; Einsiedl et al. 2015; Westphal et al. 2016). Thereby, SRB reduce sulfate to sulfide in the anaerobic zones of, e.g., biofilms and the SOB oxidize the reduced sulfur to sulfate or sulfuric acid. The same processes have probably occurred in the sedimentary biofilms in this study.

Sulfate- and sulfur-metabolizing microorganisms are known causers of corrosion, e.g., due to the production of hydrogen sulfide by SRB or sulfuric acid by SOB (Satoh et al. 2009; Westphal et al. 2016). Moreover, SOB of the genus *Thiothrix* were shown to cause filter clogging in topside facilities of a cold store (Lerm et al. 2011). Indeed,

the study presented here shows alterations in the abundance of SRB and SOB due to an increase in temperature. However, formations of sulfuric acid or hydrogen sulfide were not detected. Exclusively at 70 °C, low hydrogen sulfide concentrations were detected by odor. The coexistence of sulfate reducers and sulfur oxidizers in the sediments is a natural process. Hereby, harmful chemical compounds such as sulfide or sulfuric acid resulting from environmental impacts such as a temperature increase due to ATEs are reduced by building closed sulfur cycling in aerobic aquifers with temporal increases in OM availability and thus redox changes.

Taken together this study shows complex alterations in the bacterial community composition due to temperature increases of up to 70 °C. The study was conducted to mimic conditions in a shallow aerobic aquifer used as geothermal heat storage. The main focus was thereby on the formation of harmful chemical compounds such as sulfide or sulfuric acid, which might lead to corrosion or a local acidification of the groundwater. In particular, the changes in redox conditions due to the elevated temperatures were important. Exclusively at 70 °C, a change from oxygen respiration to sulfate reduction was detected. However, this change was temporary and over the long term the dominant metabolic pathway remained oxygen respiration. Although SRB were abundant in high numbers, sulfide formation was not detected and harmful sulfide species did not occur most probably because of the coexistence of sulfur-oxidizing bacteria. For ATEs systems being performed under aerobic aquifer conditions, this study indicates indeed alterations in the bacterial community with increased temperatures, but natural mechanisms in the sediments might overcome the critical changes without causing negative environmental impacts. However, it has to be considered that in this study anaerobic sediment was flushed with aerobic groundwater, which is not the common procedure. Moreover, the severity of the environmental impact due to ATEs depends on the sediment type and the nutrient supply by the water flowing through. Thus, more laboratory scale and in situ studies are necessary to evaluate possible impacts for different aquifer systems and to provide data for modeling concepts.

## Conclusions

The simulation of an ATEs-induced temperature increase in a shallow aquifer indicates long-term alterations in the microbial community composition and abundance due to higher temperatures. The succession was marked by an adaption of dominant genera to temperature and nutrient conditions. At 40 °C, the bacterial abundance and degradation of OM indicate the most efficient biomass



formation. Furthermore, under high temperatures of 40 and 70 °C a temporal shift in redox processes may occur. However, an interplay of SRB and SOB was shown for all temperatures leading to a closed S-cycling. The study indicates that within anaerobic aquifers, in which oxygen-containing groundwater is introduced, the main metabolic processes remain the same with increased temperatures and oxygen depletion does not occur necessarily, which would lead to the formation of harmful sulfuric species. Furthermore, it was shown that molecular biological analyses are a sensitive complementary approach to a geochemical monitoring of the geotechnical-used subsurface. Since every laboratory-scale study can only reflect one specific setup, more studies are necessary to evaluate the impact of ATEs on the environment.

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