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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 of 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 °C, 40 °C 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 °C 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 of 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, 1992; Griffioen and Appelo, 1993; Lerm et al. 2011, 2013) as well as impacts on the groundwater and sediment chemistry (Brielmann et al. 2009, 2011; Bonte et al. 2011a, b, 2013a, b, 2014; Jesußek 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 (Brielmann et al. 2009; Lerm et al. 2011; Brielmann et al. 2011; Bonte et al. 2013a). Bacterial diversity in groundwater was shown to increase with temperature (Brielmann et al. 2009). In addition, conditions may shift from iron-reducing to sulfate-reducing and methanogenic processes due to a temperature increase from 11 °C 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 (Brielmann et al. 2011; Jesußek et al. 2013a, b; Bonte et al. 2013a, b). In the studies of Jesußek 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 (Jesußek 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 Jesußek 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 modelling 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 modelling 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 °C, 25 °C, 40 °C, 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 cm 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^{-1} of oxygen, 0.50 mmol L^{-1} of nitrogen, 0.51 mmol L^{-1} of sulfate, 0.01 mmol L^{-1} of nitrate, 0.87 mmol L^{-1} of chloride, 0.50 mmol L^{-1} of silicon, 0.0001 mmol L^{-1} of iron, 0.0002 mmol L^{-1} of manganese, 0.36 mmol L^{-1} of magnesium, 3.16 mmol L^{-1} of calcium, 0.64 mmol L^{-1} of sodium, 0.08 mmol L^{-1} of potassium, 0.23 mmol L^{-1} of non-purgeable organic carbon (NPOC) and 5.91 mmol L^{-1} 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. Afterwards, three of the columns were heated up to 25 °C, 40 °C 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_{diss} was done by first stabilizing the samples with 65% HNO_3 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). Afterwards, the filters were stored at -20 °C until further processing.

DNA Extraction

DNA was extracted from filters using the FastDNA™ Spin Kit for Soil (MP Biomedicals) according to the manufactures' 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).

174 *Genetic fingerprinting (PCR-DGGE)*

175 Partial 16S rRNA genes from bacteria were amplified by polymerase chain reaction (PCR)
176 using the primer pair 341F-GC (according to Primer 3 in Muyzer et al. 1993) and 907R
177 (according to UNIV907-R in Amann et al. 1992) (94 °C for 2:45 min, followed by 40 cycles
178 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
179 min). PCR was performed in 50 µL reactions containing 5 µL of 10x buffer (Genecraft), 6 µL
180 of deoxynucleotides (dNTPs) (10 mM, Thermo Fisher), 3 µL of $MgCl_2$ (50 mM, Genecraft), 3
181 µL of forward primer (10 mM), 3 µL of reverse primer (10 mM), 0.4 µL of bovine serum
182 albumin (BSA) (20 mg/mL, Thermo Fisher), 0.3 µL of Taq polymerase (5 u/µL, Genecraft),
183 28.3 µL of RNA/DNA-free water (Thermo Fisher) and 1 µL of template DNA. Amplicons
184 were subsequently purified (Thermo Fisher GeneJET PCR Purification Kit). Denaturing
185 gradient gel electrophoresis (DGGE) was performed using equal concentrations of amplicons
186 and a gradient of 35 % to 65 % urea and 6 % to 9 % formamide. Since the number of samples
187 exceeded the number of lanes in the gel, two separate gel runs were performed. It has to be
188 considered that slight variations in the urea gradient between the gels might cause slight
189 differences in the gel runs.

190 The DGGE gels were run for 17 hours at 110 V and 60 °C. The most intensive gel bands were
191 excised and reamplified using the primer pair 341F/907R (94 °C for 1:30 min, followed by 30
192 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
193 10 min). Re-amplification of some bands failed. Successfully re-amplified PCR products were
194 purified using the Thermo Fisher GeneJET PCR Purification Kit. The concentrations of the
195 amplicons were measured fluorimetrically (BMG Labtech FLUOstar OPTIMA) by labeling
196 the DNA with Quant-iT PicoGreen (Invitrogen). The PCR products were sequenced by
197 GATC Biotech AG. Sequence homologies were checked using BLAST (Basic Local
198 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; 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 *dsr*A 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 µL of Power SYBR Green (Life Technologies), 0.5 µM of each primer, 10 µg BSA (Thermo Scientific) and 1 µL of template DNA. Nuclease-free water (Thermo Scientific) was added to a final volume of 20 µl. 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 *dsr*AB 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 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 *dsr*A 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 mmol L^{-1} in all columns, indicating a release of sulfate from the sediment. At 10 °C and 25 °C, the sulfate increase remained at 0.08 mmol L^{-1} with ongoing runtime. In contrast, temporary decreases in sulfate concentration by 0.02 mmol L^{-1} at 40 °C and 0.1 mmol L^{-1} at 70 °C were detected after 12 days and five days, respectively (Fig. 1). Afterwards, a release of sulfate of up to 0.06 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 mmol L^{-1} and 0.08 mmol L^{-1} after the equilibrium phase. In terms of the temperature elevation to 40 °C and 70 °C, the TOC increase was 0.22 mmol L^{-1} and 0.31 mmol L^{-1} after five days, respectively (Fig. 1). Afterwards, at 70 °C, the TOC increase resulted in 0.08 mmol L^{-1} on days 53 and 81, whereas at 40 °C the TOC increase resulted in 0.03 mmol L^{-1} on day 12 followed by a stronger increase to 0.14 mmol L^{-1} with ongoing runtime.

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

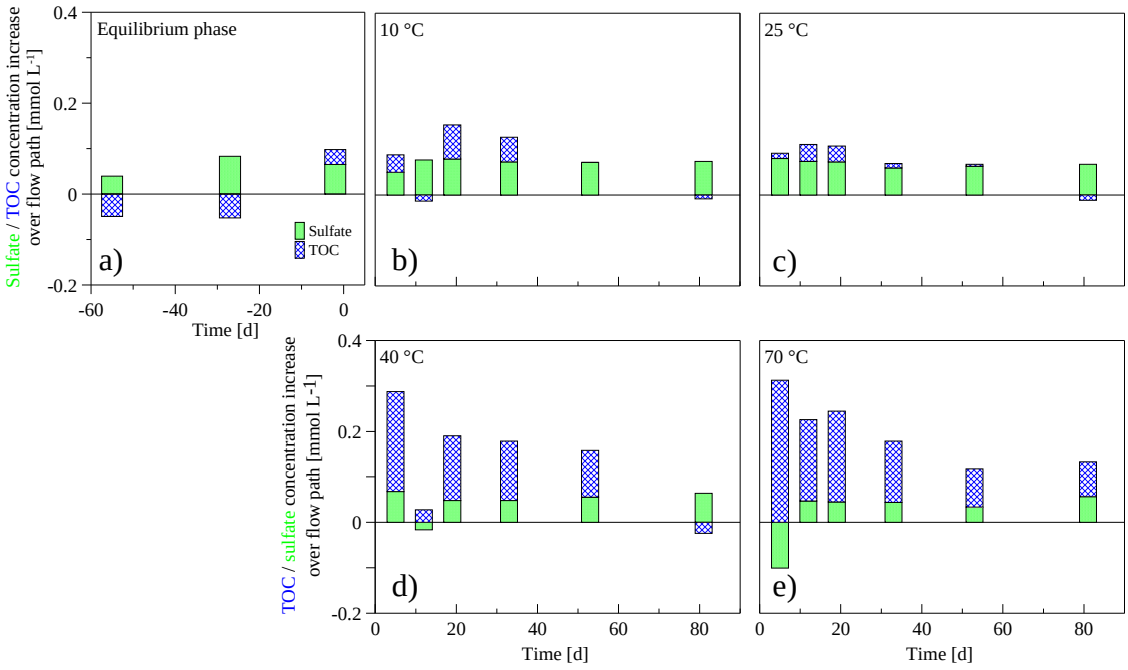

Fig. 1 Increase in TOC and sulfate concentrations over column flow path in the equilibrium phase (a) (average values of all columns) and at 10 °C (b), 25 °C (c), 40 °C (d) and 70 °C (e) during the heating phase.

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).

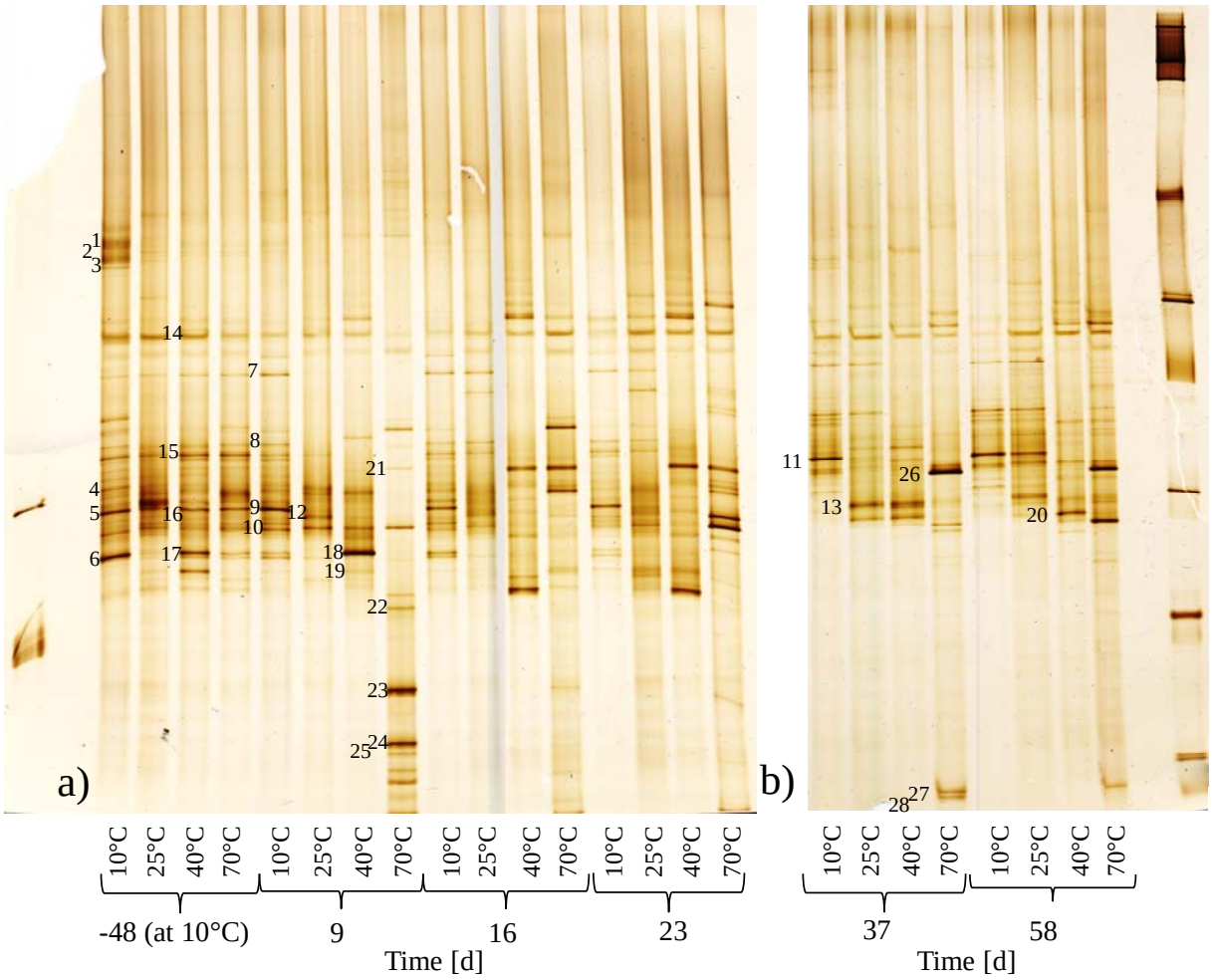

Fig. 2 DGGE profiles of bacterial 16S rRNA gene fragments. The first DGGE run was conducted for the days -48, 9, 16 and 23 (a) and the second DGGE run was done for the days 37 and 58 (b). Sequenced bands are labeled with numbers. The taxonomic affiliation is shown in Table 1.

Table 1 Sequencing of partial 16S rRNA genes retrieved from DGGE fingerprints. The taxonomic assignment was performed using NCBI BLAST. n.d., not determined.

Temperature	Band	Closest relative (Genbank accesion number)	Phylum	Genbank accesion number	Similarity
10 °C	1	Uncultured *Bacteroidetes* bacterium (AY948070.1)	*Bacteroidetes*	KU136344	97%
	2	Uncultured *Bacteroidetes* bacterium (AY948070.1)	*Bacteroidetes*	KU136345	97%
	3	Uncultured *Bacteroidetes* bacterium (AY948070.1)	*Bacteroidetes*	KU136346	93%
	4	*Polaromonas* sp. (JX950027.1)	*Proteobacteria*	KU136347	96%
	5	Uncultured *Gallionellaceae* bacterium (JQ177958.1)	*Proteobacteria*	KU136348	97%
	6	*Aquabacterium commune* (NR_024875.1)	*Proteobacteria*	KU136349	100%
	7	Uncultured *Nitrosomonadales* bacterium (EF562022.1)	*Proteobacteria*	KU136354	92%
		Uncultured *Rhodocyclaceae* bacterium (JQ278780.1)	*Proteobacteria*		92%
	8	Uncultured *Serratia* sp. (AY963526.1)	*Proteobacteria*	KU136355	97%
	9	Uncultured *Sideroxydans* sp. (JQ178033.1)	*Proteobacteria*	KU136356	97%
	10	*Simplicispira* sp. (KC113247.1)	*Proteobacteria*	KU136357	99%
	11	Uncultured *Gallionellaceae* bacterium (JQ177958.1)	*Proteobacteria*	KU136370	95%
25 °C	12	*Aquabacterium commune* (NR_024875.1)	*Proteobacteria*	KU136358	99%
	13	*Aquabacterium parvum* (NR_024874.1)	*Proteobacteria*	KU136369	100%
40 °C	14	Uncultured *Serratia* sp. (AY963526.1)	*Proteobacteria*	KU136350	94%
	15	Uncultured *Thiobacillus* sp. (JQ087157.1)	*Proteobacteria*	KU136351	98%
	16	*Aquabacterium commune* (NR_024875.1)	*Proteobacteria*	KU136352	100%
	17	*Rhodobacter* sp. (JX949604.1)	*Proteobacteria*	KU136353	99%
		Pseudorhodobacter sp. (JX949602.1)	*Proteobacteria*		99%
	18	*Aquabacterium commune* (NR_024875.1)	*Proteobacteria*	KU136359	99%
	19	*Caldicoprobacter algeriensis* (NR_117466.1)	*Firmicutes*	KU136360	97%
	20	*Aquabacterium commune* (NR_024875.1)	*Proteobacteria*	KU577262	99%
70 °C	21	Uncultured *Clostridia* bacterium (DQ208699.1)	*Firmicutes*	KU136361	91%
	22	*Desulfurispora thermophila* (NR_042969.1)	*Firmicutes*	KU136362	98%
	23	*Desulfotomaculum* sp. (EF494253.1)	*Firmicutes*	KU136363	100%
	24	Uncultured *Thermoanaerobacteriaceae* bacterium (AY684090.1)	*Firmicutes*	KU136364	92%
	25	*Hydrogenophaga* sp. (DQ854970.1)	*Proteobacteria*	KU136365	97%
	26	Uncultured *Hydrogenophaga* sp. (KC110482.1)	*Proteobacteria*	KU136366	100%
	27	*Thermoanaerobaculum aquaticum* (NR_109681.1)	*Acidobacteria*	KU136367	98%
	28	Uncultured candidate division OP10 bacterium (EF205572.1)	n.d.	KU136368	95%

The Sørenson-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 Sørenson-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 Sørenson-Dice coefficient showed decreasing similarities of the communities (0.29). The calculations of the Sørenson-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 Sørenson-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.

The band pattern at 70 °C showed differences to the other temperatures. In accordance, the Sørenson-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 Sørenson-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*.

Table 2 Comparison of bacterial community similarities in the equilibrium phase on day -48 and between different days by calculation of the Sørenson-Dice coefficient. Values give the similarity between one column or one day to another.

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

Quantification of bacterial, SRB and SOB abundances

The qPCR analyses of the bacterial 16S rRNA gene copy numbers as well as of *dsr*A and *Thiobacillus* 16S rRNA gene copy numbers showed changes over time and corresponding to the temperature increase (Fig.s 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 (Fig.s 3, 4). Particular at 70 °C, *dsr*A 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 *dsr*A 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 °C and 40 °C (Fig. 4). Co-existence of SRB and the SOB genus *Thiobacillus* occurred over experimental runtime at all temperatures (Fig. 5).

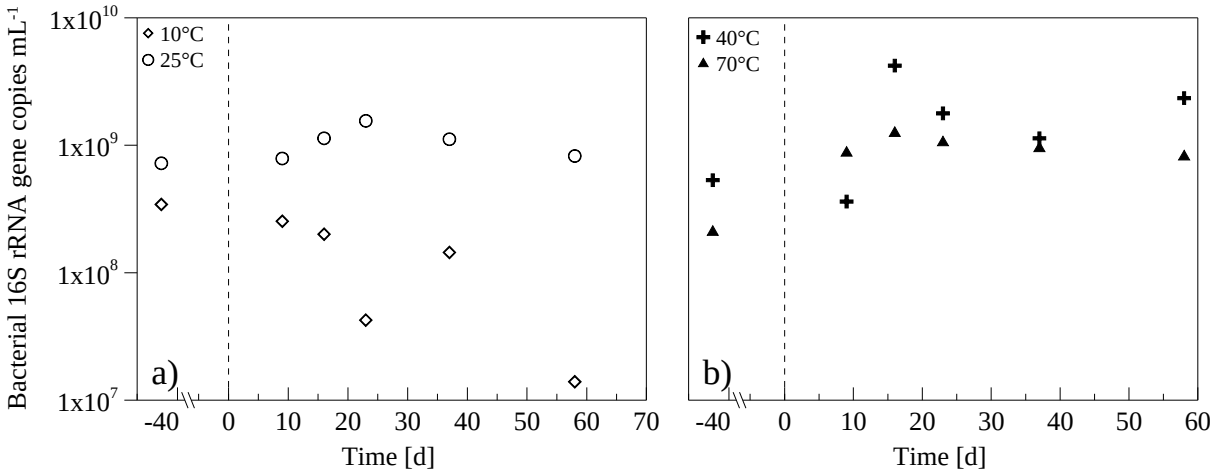

Fig. 3 16S rRNA gene copy numbers of *Bacteria* at 10 °C and 25 °C (a) as well as at 40 °C and 70 °C (b). The dashed line represents the start of heating.

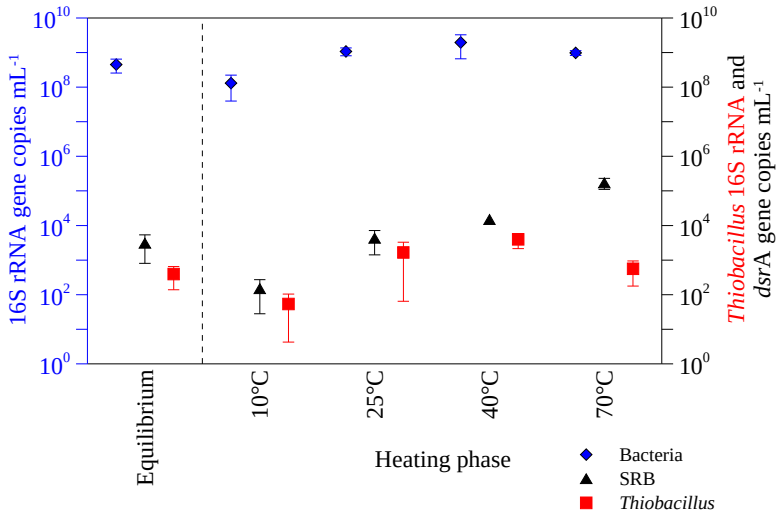

Fig. 4 16S rRNA gene copy numbers of *Bacteria*, SRB specific *dsr*A gene copy numbers and 16S rRNA gene copies of sulfur oxidizing bacterial genus *Thiobacillus* on day -48 in equilibrium phase (average values of all columns) and average values (n=5) in the heating phase over 58 days.

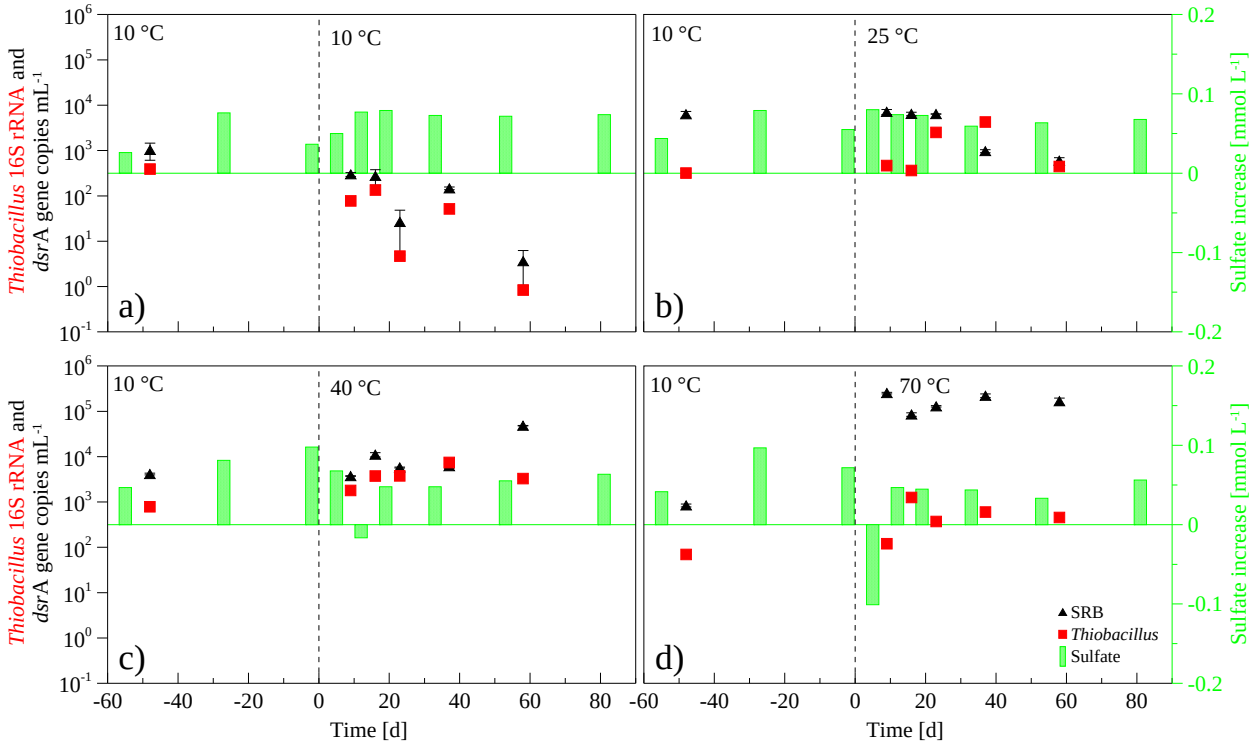

Fig. 5 Gene copy numbers of SRB and sulfur oxidizing bacterial genus *Thiobacillus* as well as increase in sulfate concentration over column runtime at 10 °C (a), 25 °C (b), 40 °C (c) and 70 °C (d). The dashed line represents the start of heating.

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 were conducted (Fig. 6). At 10 °C, on days 5-9 (t1) and 12-16 (t2) 16S rRNA gene copy numbers of bacteria, *dsr*A gene copies and *Thiobacillus* 16S rRNA gene copy numbers correlated (Fig. 6a). Moreover, on days 33-37 (t4) TOC concentrations solely 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 *dsr*A 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), *dsr*A gene copy numbers correlated to *Thiobacillus* 16S rRNA gene

copies. At 70 °C, a divergence of *dsr*A 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 *dsr*A gene copy numbers and *Thiobacillus* 16S rRNA gene copies.

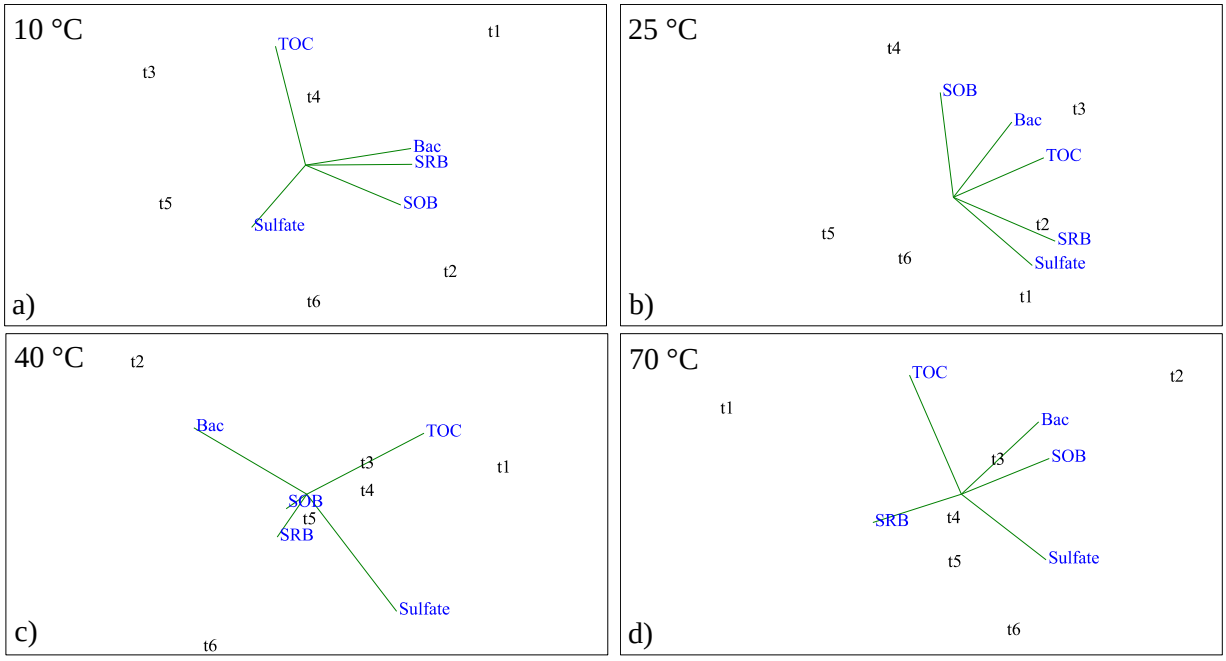

Fig. 6 Principle component analysis (PCA) correlating sulfate and TOC concentrations and gene copy numbers of bacteria, SRB and SOB with respect to the temperatures 10 °C (a), 25 °C (b), 40 °C (c) and 70 °C (d) during the heating phase (t1=days 5-9, t2=days 12-16, t3=days 19-23, t4=days 33-37, t5=days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

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.

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 °C to 45 °C also the lowest cell numbers were found in the 10 °C fluids (Brielmann et al. 2011).

In our study at 10 °C, sequences were affiliated to facultative anaerobic bacteria such as *Aquabacterium, 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 °C to 25 °C (Irgens et al. 1996; Margesin et al. 2012), whereas *Simplicispira* grows optimally between 10 °C and 40 °C (Lu et al. 2009). 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

403 was outcompeted by a community dominated by *Gallionellaceae*-like bacteria. Only one
404 species, *Gallionella ferruginea*, was described in the family *Gallionellaceae* to date (Hallbeck
405 and Pedersen 2014). Besides *Sideroxydans*, *Gallionella ferruginea* was also described as an
406 aerobic IOB. Thus, chemolithotrophic metabolism probably played a major role at 10 °C due
407 to limited availability of OM. Since the sediment contained approximately 1200 mg kg^{-1} of
408 pyrite (Dethlefsen, unpublished data) this might have been an iron source for the IOB. Pyrite
409 was probably metabolized to sulfate by sulfur oxidizing bacteria (SOB), as indicated by the
410 increased sulfate concentrations in the effluents. Besides representatives of the genus
411 *Sideroxydans*, which were likely involved in pyrite oxidation, qPCR also showed an
412 abundance of the SOB genus *Thiobacillus* at 10 °C with ongoing runtime. Members of this
413 genus were also shown to take part in pyrite oxidation (Hedrich et al. 2011).
414 Taken together, at the *in situ* aquifer temperature of 10 °C a succession in the microbial
415 community was apparent, first as a result after the setup of the column, then as succession by
416 competition of dominant genera for the available nutrients.
417
418 *Release of OM and microbial degradation after increase in temperature*
419 The heating of groundwater and sediment influenced the groundwater chemical composition
420 and the microbial community composition in the sediment columns. Genetic fingerprinting
421 and qPCR analyses showed that the impact on the aquifer microbial community composition
422 is stronger with higher temperature and goes along in phases with ongoing runtime.
423 After heating the column to 25 °C, changes in the bacterial community composition went
424 along with geochemical alterations in the fluids. Already nine days after the start of heating to
425 25 °C, a shift in bands indicated an adaption of the community to the higher temperature. An
426 *Aquabacterium*-like organism dominated the community. However, bacterial 16S rRNA gene
427 copies as well as the TOC concentration in the effluents remained constant. After 37 days,

428 additional bands appeared and the community was further dominated by an *Aquabacterium*-
429 like organism. An appearance of the genus *Aquabacterium* was also shown for other sediment
430 column experiments (Westphal et al. 2017). However, in their sediment column experiment,
431 acetate was constantly added and the oxygen was already depleted in the first centimeters of
432 the column flow path. Therefore, the *Aquabacterium* dominance was not as strong, because
433 oxygen as terminal electron acceptor was very limited. In our study at 25 °C, the bacterial 16S
434 rRNA gene copy numbers and number of SRB specific *dsr*A gene copies decreased with
435 ongoing runtime and reflected the change in the bacterial community composition.
436 Accordingly, Bonte et al. (2013a) also showed a significant change in the microbial
437 community composition and only slight changes in the dissolved organic carbon
438 concentration when increasing the temperature from 11 °C to 25 °C in a groundwater based
439 experimental setup. Obviously in our study, heating to 25 °C had an influence on the
440 microbial community and caused a shift in dominant genera even though the changes in the
441 community were only slight over a longer period of time. The increase of temperature by
442 15 K with respect to the natural aquifer temperature obviously enhanced the activity of e.g.
443 *Aquabacterium* and thus allowed for its dominance at this temperature. In contrast,
444 psychrophilic organisms such as *Polaromonas* were less adapted to the temperature increase
445 and were outcompeted.
446 A more complex pattern of changes was visible in the 40 °C experiment and the highest
447 bacterial 16S rRNA gene copy numbers were detected in the effluents of this column. This is
448 in accordance with the study of Westphal et al. (2017). Brielmann et al. (2011) also showed a
449 strong increase in microbial activity at a temperature of 40 °C compared to 10 °C and 20 °C,
450 however cell counts and bacterial diversity were not higher at 40 °C. The reason for this
451 might be the 10-fold higher flow rate and thus a shorter contact time of the fluid and the
452 sediment in the study of Brielmann 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 °C 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 *dsr*A 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 *dsr*A 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 °C 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 Jesußek 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 °C, 25 °C and 40 °C. Accordingly, in our study, a temporary sulfate reduction was detected simultaneously with an OM release after five 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 (Daumas et al. 1988; Kaksonen et al. 2007). At the same time, SRB specific *dsr*A 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 °C and 70 °C, an increase in SRB specific *dsr*A gene copies was detected over time, while in the 25 °C effluents *dsr*A 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 °C 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 °C 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 °C and 70 °C, which also led to reduced sulfate concentrations compared to the 10 °C and 25 °C columns. The finding of the temporary sulfate reduction at 40 °C 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 *dsr*A 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 *dsr*A 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

551 by several studies (Okabe et al. 2005; Baumgartner et al. 2006; Satoh et al. 2009; Handley et
552 al. 2013; Einsiedl et al. 2015; Westphal et al 2016). Thereby, SRB reduce sulfate to sulfide in
553 the anaerobic zones of e.g. biofilms and the SOB oxidize the reduced sulfur to sulfate or
554 sulfuric acid. The same processes have probably occurred in the sedimentary biofilms in this
555 study.
556 Sulfate and sulfur metabolizing microorganisms are known causers of corrosion e.g. due to
557 the production of hydrogen sulfide by SRB or sulfuric acid by SOB (Satoh et al. 2009;
558 Westphal et al. 2016). Moreover, SOB of the genus *Thiothrix* were shown to cause filter
559 clogging in topside facilities of a cold store (Lerm et al. 2011). Indeed, the study presented
560 here shows alterations in the abundance of SRB and SOB due to an increase in temperature.
561 However, formations of sulfuric acid or hydrogen sulfide were not detected. Exclusively at
562 70 °C, low hydrogen sulfide concentrations were detected by odor. The coexistence of sulfate
563 reducers and sulfur oxidizers in the sediments is a natural process. Hereby, harmful chemical
564 compounds such as sulfide or sulfuric acid resulting from environmental impacts such as a
565 temperature increase due to ATES are reduced by building closed sulfur cycling in aerobic
566 aquifers with temporal increases in OM availability and thus redox changes.
567
568 Taken together this study shows complex alterations in the bacterial community composition
569 due to temperature increases of up to 70 °C. The study was conducted to mimic conditions in
570 a shallow aerobic aquifer used as geothermal heat storage. The main focus was thereby on the
571 formation of harmful chemical compounds such as sulfide or sulfuric acid, which might lead
572 to corrosion or a local acidification of the groundwater. In particular the changes in redox
573 conditions due to the elevated temperatures were important. Exclusively at 70 °C, a change
574 from oxygen respiration to sulfate reduction was detected. However, this change was
575 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 co-existence 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 modelling 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 °C 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

601 laboratory-scale study can only reflect one specific set-up, more studies are necessary to
602 evaluate the impact of ATES on the environment.
603
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607
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