Anaerobic oxidation of methane by sulfate in hypersaline groundwater of the Dead Sea aquifer

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ABSTRACT

Geochemical and microbial evidence points to anaerobic oxidation of methane (AOM) likely coupled with bacterial sulfate reduction in the hypersaline groundwater of the Dead Sea (DS) alluvial aquifer. Groundwater was sampled from nine boreholes drilled along the Arugot alluvial fan next to the DS. The groundwater samples were highly saline (up to 6300 mM chlorine), anoxic, and contained methane. A mass balance calculation demonstrates that the very low $\delta^{13}$C DIC in this groundwater is due to anaerobic methane oxidation. Sulfate depletion coincident with isotope enrichment of sulfur and oxygen isotopes in the sulfate suggests that sulfate reduction is associated with this AOM. DNA extraction and 16S amplicon sequencing were used to explore the microbial community present and were found to be microbial composition indicative of bacterial sulfate reducers associated with anaerobic methanotrophic archaea (ANME) driving AOM. The net sulfate reduction seems to be primarily controlled by the salinity and the available methane and is substantially lower as salinity increases (2.5 mM sulfate removal at 3000 mM chlorine but only 0.5 mM sulfate removal at 6300 mM chlorine). Low overall sulfur isotope fractionation observed ($\Delta^{34}S_{\text{org}} = 17 \pm 3.5\%$) hints at high rates of sulfate reduction, as has been previously suggested for sulfate reduction coupled with methane oxidation. The new results demonstrate the presence of sulfate-driven AOM in terrestrial hypersaline systems and expand our understanding of how microbial life is sustained under the challenging conditions of an extremely hypersaline environment.

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INTRODUCTION

During anaerobic organic matter oxidation, microbial respiration is coupled with the reduction in series of electron acceptors that provide decreasing free energy yields. The largest energy yield is associated with nitrate reduction (denitrification), then manganese and iron reduction, followed by sulfate reduction, and finally fermentation of organic matter or the reduction of CO$_2$ to methane via methanogenesis (Froelich et al., 1979). Due to the high concentration of sulfate in the ocean, dissimilatory bacterial sulfate reduction (Eq. 1) is responsible for the majority of organic matter oxidation in marine sediments (Kasten & Jørgensen, 2000).

$$\text{SO}_4^{2-} + 2(\text{CH}_2\text{O}) \rightarrow \text{H}_2\text{S} + 2\text{HCO}_3^-$$ (1)

When methane is available as the electron donor, microorganisms can oxidize it (methanotrophy), and in some cases, sulfate is reduced primarily by coupling with anaerobic oxidation of methane (AOM) (e.g., Nießner et al., 1998; Boetius et al., 2000; Aharon, 2000; Sivan et al., 2007), as shown in Eq. 2 (Hochler et al., 1994):

$$\text{CH}_4 + \text{SO}_4^{2-} \rightarrow \text{HS}^- + \text{HCO}_3^- + \text{H}_2\text{O}$$ (2)

Sulfate-driven AOM consumes almost all the upward fluxes of methane in marine sediments and prevents its release to the atmosphere. This process typically involves
microbial consortia of archaea and bacteria affiliated with methanosarcina-type methanogens and sulfate-reducing bacteria, respectively (Boetius et al., 2000). A common view is that anaerobic methanotrophic archaea (ANME) oxidize the methane while the bacterial partner uses the resulting reducing equivalents to reduce sulfate (Thauer & Shima, 2008; Basen et al., 2011). Recently, however, AOM mediated solely by archaea was reported (Milucka et al., 2012); whether this process is widespread in the natural environment is unknown. Sulfate-driven AOM has also been reported in a variety of terrestrial habitats, including terrestrial mud volcanoes (e.g., Alain et al., 2006), landfills (Grossman et al., 2002), in the anoxic waters of a eutrophic freshwater lake (Eller et al., 2005), and in anoxic coastal freshwater and brackish sediments (Segarra et al., 2013). ANME sequences have also been reported from diverse soils, aquifers, and oilfield production waters, although their linkage to sulfate-driven AOM in such habitats is unclear (Knittel & Boetius, 2009).

Anaerobic oxidation of methane seems uniquely suited to extreme natural environments: AOM has been observed in temperatures of up to 75 °C in the hydrothermal sediments of the Guaymas Basin (Holler et al., 2011), in CO₂-rich seep sediments with an in situ pH as low as 4 (Inagaki et al., 2006a), in alkaline fluids of carbonate chimneys with pH values of 9–11, and in diffuse vent fluids at temperatures up to 70 °C (Brazelton et al., 2006). However, documentation of AOM in hypersaline environments is limited. Thermodynamically, sulfate-driven AOM is not expected in salt-stressed environments because of the extremely low-energy yield and the high energy needed for the osmotic adaptation to the surrounding medium (Oren, 2011). Still, sulfate-driven AOM has been observed in hypersaline marine cold seep sediments (Orcutt et al., 2005; Lloyd et al., 2006; Maignien et al., 2013). However, the occurrence of this process in continental hypersaline environments has not been reported. Here, we provide the first time geochemical evidence of AOM in the Dead Sea (DS) aquifer, which expands our understanding of how microbial life is sustained under challenging conditions of an extreme hypersaline environment.

Traditional organoclastic bacterial sulfate reduction (not associated with methane) is often observed in hypersaline terrestrial systems (e.g., Canfield & Des Marais, 1991; Brander et al., 2001; Porter et al., 2007; Van der Wielen & Heij, 2007; Murray et al., 2012; Roychoudhury et al., 2013). Evidence for bacterial sulfate reduction has also been observed in the highly sulfate-depleted calcium chloride brines of the DS. Comparison of the isotopic composition of sulfate and sulfide from the lower anoxic water layer during the DS meromictic stage, before the 1978 overturn (Steinhorn, 1985), pointed to bacterial sulfate reduction as the source of the sulfide because this microbially mediated process selects for ³²S over ³⁴S (Nissenbaum & Kaplan, 1976). Sulfur isotopes and ammonium concentrations in two groups of brine along the western coast of the DS also indicate oxidation of the organic matter through bacterial sulfate reduction (Gavrili et al., 2001).

To date, no ANMEs have been identified in the DS environment; however, the potential for methanogenesis and AOM has been demonstrated. Microbial radiotracer studies of methanogenesis in sediments from the DS showed that methane is produced from methanol in sediment slurries (Marvin Di Pasquale et al., 1999). In a recent study (Avrahamov et al., 2010), methane was found in most hypersaline groundwaters, and from mass balance calculations of the carbon isotopic composition (δ¹³C) of the dissolved inorganic carbon (DIC), it was hypothesized that methanotrophy played an important role in this groundwater system.

The isotopic composition of sulfate and DIC in groundwaters provides evidence for biogeochemical processes involving methane and sulfate due to the significant isotopic fractionations associated with their biological transformations. Depending on the extent of reaction, AOM generates highly ¹²C-depleted DIC leaving ¹²C-enriched residual methane. This fingerprinting is the result of biological fractionation during methane oxidation (ε = 0–10‰) (Whiticar et al., 1986; Whiticar, 1999). An average ε value of 13 ± 9‰ was presented by Alperin & Hoehler (2009) and the initial highly depleted δ¹³C value of the methane itself, which is about 50‰ to -100‰ (Vienna Pee Dee Belemnite—VPDB) (Alperin et al., 1988; Martens et al., 1999). During bacterial sulfate reduction, a decrease of dissolved sulfate concentration coupled with isotopic enrichment of both ³⁴S and ¹⁸O in the dissolved sulfate (δ³⁴S₂O₄ and δ¹⁸O₂O₄) is expected, because sulfate-reducing bacteria discriminate against the heavy isotope (e.g., Mizutani & Rafter, 1973; Fritz et al., 1989).

This study presents geochemical and microbial evidence for sulfate-driven AOM in the aquatic, hypersaline groundwater system along the DS shore. This system includes the fresh-saline groundwater interface (FSI) from which the extremely saline samples in the alluvial fan of Wadi Arugot were taken (Fig. 1). We determined the sulfur and oxygen isotope fractionations and the apparent net sulfate reduction rate and explored the factors controlling the extent of sulfur isotope fractionation in this extreme environment.

**Study site**

The DS is a hypersaline terminal lake located in the deepest part of the DS Transform Fault. The salinity of the lake is ~345 g TDS/l (6400 mM Cl⁻). It is currently supersaturated with respect to gypsum (Reznik et al., 2011) and halite (Reznik et al., 2009), and its brine inorganically precipitates aragonite (Barkan et al., 2001) and halite (Gavrili et al., 1997). The DS brine has a calcium chloride
composition (Na/Cl<1 and Ca/ (SO$_4^{2-}$+HCO$_3^-$)>1), which is characterized by a low sulfate concentration relative to its salinity (Table 1). It was postulated that the DS brines are the result of seawater infiltration from the Mediterranean Sea in the Neogene (Neev & Emery, 1967; Zak, 1967), which underwent high evaporation and several stages of rock–water interaction (Starinsky, 1974).

The DS surface level has been dropping during the past 50 years due to human activity, with rates reaching more than 1 m year$^{-1}$ in the last several years (Anati & Shasha, 1989; Lensky et al., 2005; Yechieli et al., 2010). This drop in the DS water level has further concentrated the salts and increased the salinity, causing massive precipitation of halite from the water column to the lake bottom, and to the development of sinkholes in the surrounding aquifer. The DS aquifer is an ideal site to investigate sulfate-driven AOM in a hypersaline environment, because of the high ionic strength of the DS water (9 mol KgH$_2$O$^{-1}$), the relatively low sulfate concentrations (~4 mm in the DS) that may therefore show large isotopic shifts, the anoxic conditions of most of the hypersaline groundwater, and the presence of methane (Avrahamov et al., 2010).

The hydrological system in the Dead Sea aquifer

Two main aquifers exist in the western part of the DS rift: the limestone and dolomite layers of the Judea Group of Upper Cretaceous age, and the Quaternary alluvial aquifer (Fig. 2) (Arad & Michaeli, 1967; Yechieli et al., 1995).

This study focuses on the FSI in the alluvial aquifer with the DS, which consists of clastic deltaic (gravel, sand, and clay) and lacustrine (clay, aragonite, gypsum, and salt) sediments of the Lisan and Zeelim formations. Alternations between gravel and clay create several subaquifers that differ in their groundwater level, temperature, and chemical composition (Yechieli, 2006). The alluvial aquifer is generally separated from the Cretaceous aquifer by the western marginal faults of the DS rift, which sets the Cretaceous limestone rocks of the Judea Group against the Quaternary alluvial rocks. Due to the low precipitation and high evaporation rates in the DS area, only a small amount of water penetrates directly to the alluvial aquifer from floods. The main freshwater source of the alluvial aquifer is the Cretaceous aquifer from which water flows through the noted fault zone. The Cretaceous aquifer freshwater emerges in several springs along the western faults of the DS rift, such as the Ein Gedi spring.

The groundwater flow in the DS alluvial aquifer is presented in a schematic hydrogeological cross section (Fig. 2), showing that the groundwater in the upper phreatic aquifer has two opposite paths. Due to the hydraulic gradient in the region, the general groundwater flow is from the highlands from the west to the east. On the other hand, beneath the FSI zone, there is saline water circulation from the DS inland despite the fact that the water level in the DS is decreasing (Kiro et al., 2008; Avrahamov et al., 2010). In the FSI zone itself, the hypersaline groundwater mixes with the relatively fresh groundwater, generating continuous seaward outflow of saline water. In general, there are three main water bodies in the DS aquifer (Lewenberg, 2005; Kiro, 2007): (i) fresh groundwater in the west, at the foot of the Judean desert mountain (TDS>1 g L$^{-1}$); (ii) groundwater with chemical characteristics similar to that of the DS in the east (hereafter called DS circulating groundwater); and (iii) unique brines which are different from the DS brine in their chemical composition (Na/Cl, Mg/K, and Ca/Mg) and salinity in the west (hereafter called western brine).

In the first stage of groundwater mixing, the fresh groundwater flows eastward toward the western brine, causing dilution of the latter. Later, the western brine flows toward the lake and mixes with the DS water. It seems thus that at the Arugot alluvial fan, there is no direct flow of freshwater above the brines toward the DS (Kiro, 2007). The boundary between DS circulating water and the two other water bodies (western brine and fresh water) forms the FSI. The brine in the lower subaquifer below the upper aquifer is somewhat different. This research focuses on the anoxic part of the FSI zone in the upper aquifer. This is the more saline part of the FSI (>1600 mm Cl$^-$), where the two end members of the hypersaline groundwaters are the western brine and the DS water.

**METHODS**

**Sampling and field procedure**

Water samples were collected from nine boreholes drilled along the Arugot alluvial fan next to the DS (Fig. 1). Groundwater samples were collected with a submersible
Table 1 Chemical and isotopic compositions of the DS and groundwater and their group affiliation; Dead Sea water (DS), freshwater (FW), western brine (WB), lower subaquifer (LA), and mixed water of the DS and the western brine (BD). Gypsum saturation state ($\Omega_{\text{gy}}$) was calculated using the PhreeqC code.

| Sample | Water group | Date m.d.y | pH | Na$^+$ | Ca$^{2+}$ | Mg$^{2+}$ | Cl$^-$ | SO$_4^{2-}$ | Fe$^{3+}$ | DIC | DO | H$_2$S | CH$_4$ | $\delta^{13}$C$_{\text{DIC}}$ | $\delta^{13}$C$_{\text{CH}_4}$ | $\delta^{18}$O$_{\text{H}_2}O$ | $\delta^{34}$S$_{\text{SO}_4}$ | $\Omega_{\text{gy}}$ |
|--------|-------------|------------|----|--------|-----------|-----------|-------|-----------|--------|-----|-----|------|------|-----------------|-----------------|------------------|-----------------|----------------|
| DSW*   | DS          | 1983       | –   | 1662   | 444       | 1814      | 6290  | 4.55      | –      | –   | –   | –    | –    | –               | –               | –                | –               | 1.3             |
| DSW†   | DS          | 1983       | –   | 1435   | 449       | 1872      | 6346  | 4.07      | 2      | –   | –   | –    | –    | –               | –               | –                | –               | 1.4             |
| EG20/55† | DS         | 11.26.7    | 5.6 | 1605   | 422       | 1849      | 6377  | 2.2       | 2      | 0.98| 0.8 | –    | –    | –               | –               | 13.65            | 2               | 13.5            |
| EG20/55‡ | DS         | 11.26.7    | 5.6 | 1571   | 400       | 1761      | 6172  | 2.46      | 22     | 2.30| 0.5 | –    | –    | –               | –               | –                | –               | 17.0            |
| EG24/10 | DS          | 2.1.12     | 5.9 | 1319   | 436       | 1894      | 6284  | 2.87      | 2      | 0.77| 0.1 | –    | 0    | 12.6            | 15.8            | –                | –               | 0.6             |
| EG24/19 | DS          | 2.1.12     | 5.8 | 1333   | 456       | 1965      | 6319  | 2.52      | 3      | 0.77| 0.2 | –    | 0    | 11.76           | 14.1            | –                | –               | 0.7             |
| EG16/11† | DS         | 3.7 | 5.3 | 1393   | 408       | 1829      | 5965  | –         | –      | –   | –   | –    | –    | –               | –               | –                | –               | 10.2            |
| EG22/19.7| WB         | 1.31.12    | 6.4 | 428    | 98        | 278       | 1170  | 30.97     | 7      | 6.96| 0.1 | 0    | 126 | 14.6            | 15.8            | –                | –               | 11.8            |
| EG22/22 | BD          | 1.31.12    | 5.6 | 1041   | 203       | 719       | 2976  | 17.14     | 16     | 3.06| 0.1 | 0    | <7  | 67              | 17.9            | 19.9             | –               | 43.4            |
| EG22/25 ‡ | BD        | 11.26.7    | 5.7 | 1687   | 373       | 1548      | 5642  | –         | 1.8    | –   | –   | –    | 270 | –               | –               | –                | –               | 14.1            |
| EG22/25† | BD         | 6.13.10    | –   | 1427   | 324       | 1229      | 4758  | 6.99      | 0.3    | –   | –   | –    | –    | 19.3            | 22.4            | –                | –               | 1.0             |
| EG22/29 | BD          | 1.31.12    | 5.8 | 1501   | 315       | 1253      | 4755  | 6.46      | 0.12   | 21 | 1.05| 0.2 | <7  | 63              | 18.1            | 22.5             | –               | –4.2            |
| EG15/19† | BD         | 11.7 | 5.9 | 1724   | 375       | 1853      | 5829  | –         | –      | –   | –   | –    | 660 | –               | –               | –                | –               | –15.4           |
| EG26/22 | BD          | 2.1.12     | 6.1 | 1343   | 262       | 998       | 4013  | 9.78      | 0.03   | 2   | 2.34| 0.4 | <7  | 59              | 18.0            | 21.4             | –               | –36.4           |
| EG23/6.32 | DS        | 13.6.11    | 5.9 | 1635   | 386       | 1489      | 5528  | 4.72      | –      | 2   | 2.34| 0.1 | <7  | 0               | 16.4            | 19.4             | –               | –13.9           |
| EG19/50 | LA          | 2.1.12     | 6.2 | 529    | 122       | 331      | 1501  | 7.41      | 0.05   | 0   | 4.09| 0.09| 0   | 559             | 70              | 13.7             | 17.7            | –8.5            |
| EG19/58 | LA          | 2.1.12     | 7.5 | 940    | 223       | 657      | 2776  | 8.89      | 0.06   | 1   | 10.5| 0.2 | 0   | 1941            | 82              | 17.5             | 23.9            | –11.6           |
| EG25/4.5 | BD         | 13.6.11    | 6.4 | 1301   | 304       | 1108     | 4259  | 10.02     | 2      | 2.94| 0.14| <7  | 45  | 16.3            | 18.8            | –13.5            | –43.5           |
| Ein Arugot | FW      | 5.12.07   | 2.53 | 1.33 | 1.1 | 2.83 | 0.49 | – | 3.53 | – | – | – | – | – | – | – | – | – | – | – |

*Gavrieli (1997). †SO$_4$ concentration from Reznik et al. (2009). ‡Avrahamov et al. (2010). §Major elements and sulfate concentration from Kiro, unpublished data.
pump after three volumes of well water were removed. The density was measured in the field using a Paar digital DMA-55 density meter. Conductivity, pH, and temperature were also measured in the field using a portable multiparameters instrument (Multi 3500i, WTW, Weilheim, Germany). Sulfide was precipitated in the field as ZnS by adding 1–2 mL 1 M Zn-acetate to 1-L sampling bottles prior to sampling. Due to the high ionic strength of the DS, dissolved oxygen was measured in the field using a modified Winkler titration method (APHA, American Public Health Association, American Water Work association & Pollution Control Federation, 1985; Nishi & Ben-Yaakov, 1990). Samples for major ion concentrations were collected in plastic bottles. Subsamples for total alkalinity (TA), DIC, and carbon isotope analyses were immediately filtered through 0.45-µm filters and transferred into 20-mL pre-poisoned syringes (HgCl2 powder) to terminate bacterial activity. For methane analyses, 5 mL subsamples were transferred immediately to a vacutainer for headspace measurements. For the isotopic analyses of sulfur and oxygen of sulfates, sulfate was precipitated in the field from the groundwater as BaSO4, using a saturated barium chloride solution. The precision of the analysis of all the major elements was ±2%, except for sulfate, which is described below. Total alkalinity was measured by titration, with 0.01 N HCl as a titrant (Metrohm model 785, Herisau, Switzerland). The analytical precision from duplicates was 0.03 meq L⁻¹. Sulfate concentrations were measured using a Dionex DX500 high-pressure liquid chromatography (HPLC) with an external error of 0.4–4.2% between duplicates for samples (1σ). About 0.2 mL of each groundwater sample was transferred into a He-flushed vial containing H3PO4 for the headspace measurements of δ¹³CDIC by conventional isotopic ratio mass spectrometer (IRMS, DeltaV Advantage; Thermo, Waltham, MA, USA) with a precision of ±0.1‰. DIC concentration was also calculated from the IRMS results according to the peak height and a calibration curve (by standard samples prepared from NaHCO₃ with known DIC concentration) with an error of ±0.2 mm.

Methane concentrations were measured by a gas chromatograph (GC, Thermo) equipped with a Shin Carbon-packed column with precision of ±2 μm. The δ¹³CCH₄ values were measured via the IRMS equipped with a PreCon interface after oxidation to CO₂. The precision of the measurements was ±0.5‰ and the results are reported vs. the PDB standard. Total sulfide concentration was measured by titration with 5 mM thiosulfate, with an estimated error of 14 μm. Ferrous iron was fixed immediately using a ferrozine solution, and the absorbance at 562 nm was measured on a spectrophotometer (Stookey, 1970); concentrations were determined by comparison with a standard curve, and the error was less than 7 μm. The sulfur and oxygen isotope composition of the sulfates was analyzed at the University of Cambridge. Sulfate was precipitated as barite and pyrolyzed at 1450 °C in a temperature conversion element analyzer (TC/EA), and the resulting carbon monoxide (CO) was measured by continuous-flow GS-IRMS (Delta V Plus) for its δ¹⁸OSO₄. For the δ¹⁸OSO₄ analysis, the barite was combusted at 1030 °C in a flash element analyzer (EA), and the resulting sulfur dioxide (SO₂) was measured by continuous-flow GS-IRMS (Thermo, Delta V Plus). Samples for δ¹⁸OSO₄ were run in replicate, and the standard deviation of these replicate analyses was used as the external reported error (~0.5 ‰). The δ¹⁸OSO₄ values are reported vs. VSMOW and corrected for two barite standards of known δ¹⁸OSO₄ that

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were run at the beginning and end of each set of samples (NBS 127 $\delta^{18}$O$_{SO_4} = 8.6^{\circ}/_{oo}$ and EM barite $\delta^{18}$O$_{SO_4} = 15^{\circ}/_{oo}$). $\delta^{34}$S$_{SO_4}$ results are reported vs. the Canyon Diablo Troilite (VCDT), and the error was determined using the standard deviation of the standard (NBS 127) at the beginning and the end of each run ($\sim 0.4^{\circ}/_{oo}$). The $\delta^{34}$S$_{SO_4}$ was also corrected to two standards of known sulfur isotope composition, NBS 127 ($20.3^{\circ}/_{oo}$) and EM barite ($12^{\circ}/_{oo}$).

DNA extraction

Groundwater samples for DNA sequence analysis were extracted via phenol–chloroform DNA extraction (Sambrook & Russell, 2001). DNA extraction was repeated four times on each filter and pooled prior to purification. Samples were purified using the Genomic DNA Clean and Concentrator Kit (Zymo Research Co., Irvine, CA, USA).

16S amplicon sequencing and analysis

16S rRNA gene hypervariable region V4-V5 from the bacterial and archaeal communities was sequenced on an Illumina MiSeq sequencer (Illumina Inc. San Diego, CA, USA) at the Josephine Paul Bay Center, Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts, USA. Sample preparation, including primers used, PCR conditions, and cycling conditions are described at length on the MBL Web site (http://vamps.mbl.edu/resources/primers.php). Sequence QC and analyses were performed through the Visualization and Analysis of Microbial Population Structure (VAMPS) Web site (http://vamps.mbl.edu/index.php) and as described by Huse et al. (2007) and Sogin et al. (2006). Briefly, sequences of low quality, including sequences less than 50 bp in length, those with ambiguous taxonomic classifications, and those that did not include a forward primer sequence, were removed. Sequences were then screened for the presence of chimeric sequences using UCHIME (Edgar et al., 2011). Taxonomic designations were made using GAST (Global Alignment for Sequence Taxonomy) as described by Sogin et al. (2006). In this process, sequences are BLAST (Altschul et al., 1990) searched against the SILVA SSU database (Quast et al., 2013) and MUSCLE aligned (Edgar, 2004) to the top 100 BLAST hits. Consensus taxonomy is then determined and applied to each sequence. Sequences were assigned to operational taxonomic units (OTUs) at a 3% dissimilarity cutoff in QIIME 1.7.0 (Caporaso et al., 2010) using UCLUST (Edgar, 2010).

QIIME was also used to perform OTU rarefaction and richness analyses. All sequences are available via the VAMPS webpage http://vamps.mbl.edu/ listed as SBJ_BME_Av4v5 and SBJ_BME_Av4v5. The sequences have been submitted to the NCBI short-read sequence archive.

RESULTS

Full chemical and isotope data are presented in Table 1. Based on a plot of Na$^+$ vs. Cl$^-$, the collected water samples were mixtures of the fresh groundwater (represented by the fresh spring water) and DS brines (best represented by the 1980s DS water, Fig. 3A). However, as the sulfate concentration of the DS brine is low, another end member can be distinguished—the western brine, as presented in Fig. 3B and described hereafter. The continuous drop in the DS level since the 1970s has been accompanied by a change in the chemical composition of the DS. Due to halite precipitation and mixing with end brines released from the DS potash industries, the Na/Cl ratio in the DS has decreased from 0.30 to 0.21, while Mg/K ratio increased from 9 to 9.8 (Gavrieli, 1997; Reznik et al., 2009). As the chloride concentration in the DS is significantly higher than the sodium concentration (6300 mM vs. 1400 mM, respectively), during halite precipitation, sodium concentration decreases, whereas chloride concentration essentially remains constant. The behavior of these ions (sodium and chloride) in the aquifer, which is under-saturated with respect to halite, is expected to be conservative.

Fig. 3 (A) Na$^+$ and (B) SO$_4^{2-}$ vs. Cl$^-$ concentrations. (A) points to simple mixing between the two end members; the DS of the 80s and the freshwater spring (DS data from Gavrieli, 1997; Reznik et al., 2009). The error is smaller than the symbols. (B) shows a nonlinear relationship between SO$_4^{2-}$ and Cl$^-$ in groundwater samples between the two end members, the DS water and the western brine. The theoretical mixing line is exhibited by a solid line. The dashed line is the predicted SO$_4^{2-}$ concentration at saturation with gypsum ($\Omega_{\text{gyp}} = 1$) calculated from Phreeqc (Parkhurst & Appelo, 2007). Most of the groundwater samples fall below the two lines, suggesting partial removal of SO$_4^{2-}$ by BSR.
On the other hand, the carbon system, which was studied mainly through measurements of DIC, δ13C\textsubscript{DIC}, and alkalinity, exhibits non-conservative behavior (Avrahamov et al., 2010).

**Dissolved oxygen (DO)**

Theoretical calculations of oxygen solubility in hypersaline solutions such as the DS brine are difficult due to the uncertainty in the formulation of the interactions of the electrolytes at high ionic strength (Nishri & Ben-Yaakov, 1990). Dissolved oxygen concentration in the DS, measured using the modified Winkler titration method, was 43 ± 2 μM, which is comparable to previous measurements (45 μM, Nishri & Ben-Yaakov, 1990). The dissolved oxygen concentrations of the groundwater in the alluvial aquifer varied from saturation to below detection. In general, most of the low salinity groundwater (<1600 mM Cl\textsuperscript{–}) was anoxic, whereas the hypersaline groundwater was characterized by low (<5 μM) to below detection levels of oxygen.

**Dissolved sulfate and sulfide**

The dissolved sulfate concentration of the DS at the time of the study was 4.1 mM. Even at this low concentration (15% of seawater concentrations), the DS is oversaturated with respect to gypsum (Reznik et al., 2009). The DS water percolating into the alluvial aquifer was further depleted in sulfate relative to the DS water, with sulfate concentrations being in the range of 2.5–2.9 mM. The highest sulfate concentration, 31 mM, was found in the sample of lowest chloride concentration within the group of the higher salinity (Fig. 3B). In general, the sulfate concentration of this group of groundwater in the FSI zone decreases with increasing salinity and exhibits depletion from both the expected mixing values between the DS and the western brine (represented by borehole EG22/19.7 values, Table 1), as well as from the predicted sulfate concentration at saturation with respect to gypsum (Ω\textsubscript{gy} = 1, Fig. 3B). Exceptions to this were the samples from the lower subaquiifer that are distinguished by their ion ratios and salinity and therefore were defined as a separate water body (Fig. 3B).

The expected sulfate concentration of the groundwater samples in the FSI zone, prior to any geochemical reaction, was calculated by the mixing fraction of the DS end member (f_DS) according to the following equation:

$$f_{DS} = \frac{Cl_{sample} - Cl_{ wb}}{Cl_{DS} - Cl_{ wb}}$$

where Cl\textsubscript{sample} is the chlorine concentration in the measured sample, and Cl\textsubscript{wb} and Cl\textsubscript{DS} are the chlorine concentrations in the two saline end members the western brine (wb) and the DS, respectively. Due to halite precipitation in some of the groundwater samples, the mixing fractions for them (EG22/22 and EG22/25.3) were calculated using the magnesium concentration instead of chloride concentration. The calculated sulfate concentrations in the groundwater were then compared with the expected sulfate concentration at gypsum saturation (Ω\textsubscript{gy} = 1). The latter were calculated using the PhreeqC code (Parkhurst & Appelo, 2007) and its Pitzer database for high ionic strength solutions. The code 'precipitates' gypsum from the oversaturated brine until the brine attains saturation with respect to gypsum.

The aqueous geochemistry of the groundwater samples from the lower subaquiifer indicates that these samples have some unique brine component, in addition to the western brine. As there are not enough data on the lower subaquiifer, the pre-reaction sulfate and δ34S\textsubscript{SO4} values cannot be calculated, and these samples were excluded from the isotopic mass balance calculations.

Most of the groundwater samples contained low concentrations of sulfide (Table 1), but the low resolution of the sulfide measurements did not permit identification of a trend. However, sulfide concentrations in the western brine and in the lower subaquiifer samples differed significantly, being about 130 and 1940 μM, respectively.

**Methane**

Methane was found in most of the hypersaline groundwater samples at concentrations of up to 82 μM. The δ13C\textsubscript{CH4} ranged between −36 and −44‰ (Table 1).

**Fe\textsuperscript{2+}**

The concentrations of ferrous iron in some groundwater samples were measured and show depletion upon entrance to the aquifer (1.87 nM in the DS compared with 0.33 nM in groundwater 5 m from the DS) and then an increase to 19.9 nM at a distance of 80 m into the aquifer (Table 1).

**Sulfur and oxygen isotope compositions in sulfate**

The isotopic composition of sulfate in the groundwater ranged from 11.7‰ to 18.1‰ for the δ18O\textsubscript{SO4} (‰ VSMOW) and from 14.1‰ to 23.9‰ for δ34S\textsubscript{SO4} (‰ VCDT). Most of the groundwater samples were isotopically heavier than those of the modern DS (δ34S\textsubscript{SO4} = 15.9‰ and δ18O\textsubscript{SO4} = 13.7‰). An inverse correlation was observed between the sulfur and oxygen isotopic composition of sulfate and the sulfate concentration for most of the samples (Fig. 4), which suggests bacterial sulfate reduction, whereby the remaining sulfate progressively becomes enriched in the heavy sulfur and oxygen isotope as sulfate reduction proceeds. Two of the groundwater
samples deviate from the general trend. These samples were found very near the DS shoreline and have a similar chemical composition to that of the DS and thus probably represent intrusion of the DS brine into the coastal aquifer with only minor reaction (as discussed below).

**Microbial results**

To examine the microbial community potentially involved in AOM and bacterial sulfate reduction in the DS aquifer, 16s rRNA gene amplicons were sequenced from the archaecal and bacterial communities present at EG 19-50 m and EG 20-55 m (Table 2, Fig. 5). Between 22 226 and 44 449 unique sequences were obtained in the four libraries with Good’s coverage estimate over 99% in each library (Table 2). Bacterial libraries at both sites were more diverse than the archaecal counterparts, with more than 800 OTUs in both bacterial libraries and 309 or fewer in both archaecal libraries (Table 2). Shannon index values from the libraries agree with the OTU results (Table 2).

**DISCUSSION**

**DIC and δ¹³CDIC evidence for methane oxidation**

It has previously been suggested that methane oxidation occurs in the DS alluvial aquifer (Avrahamov et al., 2010), based on methane concentration and DIC–carbon isotope mass balance. This mass balance showed that the low δ¹³CDIC in the circulating DS groundwater and in the FSI zone, which only differ slightly in their DIC concentrations, was likely due to AOM. Here, we augment these data with oxygen concentration measurements, additional measurements of methane and sulfate concentrations, and stable isotopes of the sulfate in the anoxic aquifer. These measurements support the conclusion that AOM occurs in the DS alluvial aquifer and that it takes place at significant rates.

Mass balance calculations were performed to understand the evolution of isotopic signatures in the DS reservoir. Using these calculations, we explored four scenarios (Table 3). The ultimate objective was to track a parcel of DS water with known DIC concentration and elucidate the main processes affecting the DIC and δ¹³CDIC values when the DS water enters the aquifer. The mass balance calculation assumes that the initial DIC concentration and δ¹³CDIC value of the hypersaline groundwater were similar to that of the current DS (i.e., 0.98 mM and 2.8‰, respectively). Limited dilution of the DS groundwater by the freshwater coming from the west that flows on top of the circulating DS water does exist, but it has no significant effect on the DIC and δ¹³CDIC of the DS groundwater as the DS fraction is overwhelming. (according to mixing fraction calculation, the mixing fraction of the DS end member is 0.97–0.99.) The excess in DIC in the groundwater is then attributed to either organic matter oxidation (δ¹³COM ~ –25‰) or methane oxidation (δ¹³CH4 ~ –40‰), assuming no carbon isotope fractionation in the oxidation process. The four examples in Table 3 suggest that methane oxidation is the main source of the isotopically depleted DIC in two of them. The first two samples (EG15/19 and EG16/11 sites) have DIC lower than that of the DS with significantly lighter δ¹³CDIC, implying that
some of the carbon of the DS solution was lost while penetrating into the aquifer while another source was added. These two samples with the missing DIC emphasize the low $\delta^{13}C$ of the substrate involved, as precipitation of carbonate minerals is expected to enrich the residual DIC with the heavy isotopes ($+2.5\%$, Mook, 1980). However, in the last two samples (EG20/55 and EG22/25 sites), if the excess DIC was derived solely from AOM, the $\delta^{13}C_{\text{DIC}}$ would have been lighter than that measured. Thus, it seems that the carbon in these samples originated from a combination of both methane and organic matter oxidation.

The source of the methane could be a deep thermogenic one, as suggested for the hydrocarbon gases in the DS rift
SO₄ and δ³⁴S

Sulfate concentrations along the groundwater flow path reflect complex and multiple processes. Although the DS salinity is mainly preserved in the circulating DS groundwater, the sulfate concentration is lower in this hypersaline groundwater compared with the DS water (2.9 mM vs. 4 mM, respectively). As the DS water is oversaturated with respect to gypsum (Ωgyp = 1.4), it is reasonable to assume that gypsum precipitation accounts for some of these decreases. Maximum sulfate removal through precipitation can be determined from the difference in concentration between the expected sulfate due to mixing and sulfate concentration at saturation. Indeed, 5 m inland from the shore, the saturation state for gypsum (Ωgyp) has decreased to between 0.9 and 1.0. As the DS groundwater continues circulating, the Ωgyp drops to 0.6 (80 m from the lake, EG20/55 m), suggesting an additional sink for sulfate or calcium. The additional sink can be determined from the difference between the sulfate concentrations at saturation and the measured concentration. The δ³⁴SøSO₄ measured in the aqueous sulfate at EG20/55 m is 21.9‰, which is isotopically heavier (³⁴S rich) compared with the original DS brine (15‰), suggesting that ³⁴S was removed from the circulating sulfate. While not definitive, this is very suggestive of microbially mediated sulfate reduction occurring in the aquifer and the subsequent removal of the sulfide as FeS phases.

In the FSI zone (1200–6200 Cl⁻ mM), the sulfate concentrations are higher (up to 31 mM) compared with the circulated DS groundwater, and sulfate concentrations decrease with increasing salinity (Fig. 2B). Due to the changes in the lake’s water level, these data suggest that the extra sulfate source is gypsum dissolution by subsaturated groundwater within the alluvial aquifer that precipitated from the DS in the past, when lake level was higher (e.g., Neve & Hall, 1979). It should be noted that then at the Holocene period, δ³⁴SøSO₄ value was close to the present DS value (Gavrieli et al., 2001). The Ωgyp of the groundwater in the FSI zone ranges from 0.7 to 1.0; the δ³⁴SøSO₄ (18.8–21.4‰) is also enriched in ³⁴S, suggesting bacterial sulfate reduction.

Pre-reaction sulfate concentration

To estimate the extent of bacterial sulfate reduction and evaluate its sulfur isotope fractionation factor, the initial sulfate concentration must be known and the water sources must be traced. According to the end members described in the hydrological background, most of the groundwater in the FSI zone is a mixture of the western brine (represented by EG22/19.7) and the DS water in the east. Therefore, the expected mixing value prior to geochemical reactions can be calculated, as previously demonstrated. The two end member water bodies are supersaturated or saturated with respect to gypsum (Ωgyp = 1.4 and 1.0, for the DS and the western brine, respectively), but the mixtures measured from the boreholes are not saturated (Ωgyp<1, Table 1). Although gypsum has low precipitation kinetics in the DS brine, precipitation will be promoted when crystallization seeds are available (Reznik et al., 2009), as expected within the alluvial aquifer, and thus, it is reasonable to assume that gypsum precipitation proceeds before biochemical reaction. According to this, the first depletion in sulfate (Fig. 2B) from the expected mixing values at saturation state would be due to gypsum precipitation, while the second depletion would be due to bacterial sulfate reduction. The sulfate concentrations at saturation (Ωgyp = 1) were calculated with the PhreeqC code (Parkhurst & Appelo, 2007), using the Pitzer database for high ionic strength solutions. Precipitation of gypsum during the mixing process explains some of the changes in the sulfate concentrations of the DS circulating groundwater but not the isotopic variations, as sulfur isotope fractionation between dissolved sulfate and the precipitated gypsum is negligible (Worden et al., 1997). As

### Table 3 Estimation of the DIC source (organic matter oxidation (OM) or methane oxidation) in DS circulating groundwater

| Sample | Measured DIC (mM) | Initial DIC OM (mM) | Surplus OM oxidation (‰) | Initial DIC CH₄ oxidation (‰) | Expected δ¹³C DIC following OM oxidation (‰) | Expected δ¹³C DIC following CH₄ oxidation (‰) | Measured δ¹³C DIC (‰) |
|--------|------------------|---------------------|--------------------------|-----------------------------|---------------------------------------------|---------------------------------------------|---------------------|
| EG15/19* | 0.60             | 0.98                | –0.38                    | 2.8                         | –                                           | –                                           | –15.4              |
| EG16/11* | 0.57             | 0.98                | –0.41                    | 2.8                         | –                                           | –                                           | –10.2              |
| EG20/55* | 1.87             | 0.98                | 0.89                     | 2.8                         | –10.4                                       | –17.6                                       | –17.0              |
| EG22/25* | 1.80             | 0.98                | 0.82                     | 2.8                         | –9.9                                        | –16.7                                       | –14.1              |

*After Avrahamov et al. (2010).
discussed above, the mixed water at the FSI zone maintains higher sulfate concentrations due to gypsum dissolution in the Holocene alluvial aquifer, where this gypsum has a $\delta^{34}S_{SO_4}$ value close to that of the present DS (Gavrieli et al., 2001). Confirmation of this gypsum dissolution hypothesis can also be found in the oxygen isotope composition of the sulfate; oxygen isotope fractionation between sulfate and gypsum is around $+3.6^\circ$ (Lu et al., 2001). This is reflected in the heavier $\delta^{18}O_{SO_4}$ of the western brine end member ($\delta^{18}O_{SO_4} = 18.1^\circ$), where sulfate concentrations are higher due to gypsum dissolution compared with $\delta^{18}O_{SO_4}$ of the DS $-13.6^\circ$. Therefore, it is reasonable to assume that the main process responsible for this sulfate depletion and sulfur isotopic variations is bacterial sulfate reduction and subsequent sulfide precipitation. Given the above, the predicted sulfate concentrations at $S_{GS} = 1$ were compared with the measured value to estimate the amount of sulfate that was removed by bacterial sulfate reduction (Fig. 3B).

**Apparent net BSR and the environmental conditions**

Estimating sulfate reduction rates under aquifer conditions is not trivial. Processes such as advection, dispersion, and adsorption can lower sulfate concentration. However, a rough estimation of apparent net sulfate reduction can be achieved by subtracting measured sulfate values from the initial sulfate concentration. Figure 6 shows the sulfate, sulfur, and oxygen isotope compositions as a function of the apparent BSR reaction progress (pre-reaction value minus the measured value). Both the $\delta^{34}S_{SO_4}$ and $\delta^{18}O_{SO_4}$ show an increase with the progressive sulfate reduction, as expected from the heavy-isotope enrichment in the residual sulfate.

It is generally accepted that sulfate concentrations do not modify sulfate reduction rates unless the concentration is less than 1 mM, or even 200 $\mu$M (Martens & Berner, 1977; Jørgensen, 1981; Boudreau & Westrich, 1984). Most of the groundwater samples considered here contain more than 1 mM sulfate, indicating that sulfate concentration is not a primary controlling factor for sulfur isotope fractionation during sulfate reduction.

Besides sulfate concentrations, the observed differences in the apparent bacterial sulfate reduction between the samples may be attributed to temperature, pressure, and the amount of reactive organic carbon in the sediment and the extent to which it can be metabolized (Boudreau & Westrich, 1984 and references therein). In this study, all the samples are from the shallow aquifer, so temperature and hydrostatic pressure effects are irrelevant.

Previous studies have shown that the amount and reactivity of the organic carbon in sediments are the two most important factors controlling the rate of bacterial sulfate reduction (Goldhaber & Kaplan, 1975; Berner, 1978; Jørgensen, 1981; Westrich, 1983). Of these two variables, the reactivity of the organic carbon is considered to be more important (Westrich, 1983). In this case, the organic carbon available for bacterial sulfate reduction is mostly methane. While it is difficult to oxidize methane, under certain situations, methane can be more available to the sulfate-reducing bacteria than organic matter even though thermodynamically sulfate reduction coupled with methane is less favorable (Sivan et al., 2011 and references therein). The low concentrations of methane suggest that it may limit bacterial sulfate reduction in the groundwater, as indicated by the observed relationship between methane concentration and the apparent net rate bacterial sulfate reduction (Fig. 7A). The increase in the methane concentrations with the flow direction supports an increased net rate of sulfate reduction. We therefore suggest that bacterial sulfate reduction mainly occurs via AOM, although we cannot rule out organoclastic sulfate reduction entirely.

Figure 7B shows an inverse correlation between salinity and AOM ($R^2 = 0.78$). The salinity effect is not clear as there is also a correlation between methane and salinity. However, other studies have shown that hypersaline environments inhibit AOM (Maignien et al., 2013; Joye et al., 2009). In brine sediments of Mercator Marine mud volcanoes, AOM seems to be partially inhibited by hypersaline conditions. 

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conditions (Maignien et al., 2013). AOM appears to be inhibited at high salinity while bacterial sulfate reduction is not inhibited. However, no significant difference in the enrichment factor, $^{34}\delta_e$, in hypersaline environments has been demonstrated (Habicht & Canfield, 1997).

**Sulfur and oxygen isotope fractionations**

The observed inverse relationship between dissolved sulfate and $^{34}\delta_{SO_4}$ values (Fig. 4A) in the hypersaline groundwater fits a Rayleigh distillation of $^{32}\delta$ during sulfate reduction and may be a function of the methane concentrations or other limiting parameters (e.g., dissolved organic carbon concentration). The fractionation factors ($\alpha$) of sulfur during sulfate reduction can be derived from the Rayleigh equation for a closed system, based on measurements of the isotope ratios and sulfate concentrations (Broecker & Oversby, 1971):

$$R_t/R_0 = (N_t/N_0)^{(\alpha-1)}$$  \hspace{1cm} (4)

where $R_t$ and $R_0$ are the measured isotope ratios of sulfate at time $t$ and at the initiation of sulfate reduction, respectively; $\alpha$ is the fractionation factor for S; ($N_t/N_0$) is the fraction of sulfate ($f$) remaining in the groundwater at any given time. Equation (4) can be rewritten as follows (Aharon, 1985):

$$\Delta \delta = \delta_t - \delta_0 = 10^\delta (x - 1) \ln(f)$$  \hspace{1cm} (5)

where $\delta_t$ = $^{34}\delta_{SO_4}$ is the value of the residual sulfate fraction and $\delta_0$ = $^{34}\delta_{SO_4}$ is the value of the initial sulfate before microbial sulfate reduction commenced (according to mixing and chemical equilibrium with gypsum). $10^\delta (x-1)$ is also known as the enrichment factor ($\varepsilon$) in permil, which is the preference of $^{32}\delta$ over $^{34}\delta$ during bacterial sulfate reduction in the given natural conditions.

Several assumptions related to the origin of the groundwater can be used to constrain a closed system. The trend of increasing $^{34}\delta_{SO_4}$ values in the DS circulating groundwater along the flow path inland, while preserving the salinity in this groundwater, suggests a more advanced reduction stage along the Rayleigh distillation path, provided that the closed systems were maintained and that the initial isotopic compositions were similar. However, in the FSI zone, the two end members of the mixed brines have the same isotopic value ($15.9_{\%o}$ in the DS and $15.8_{\%o}$ in the western brine). In general, in a closed system, sulfides accumulate. However, it is possible that much of the produced sulfide escaped from the systems through degassing (as hinted at by the low pH of most of the groundwater samples compared to that in the two end members—Table 1) or by its precipitation as metal sulfide. We measured a decrease in ferrous iron concentrations that suggests that metal sulfides may be precipitated along the flow path.

In a plot of $\Delta \delta$ vs. $\ln(f)$, the slope represents the enrichment factor $\varepsilon$. In practice, the $\ln(f)$ member is actually the natural log of the apparent net bacterial sulfate reduction as calculated above and as presented in Fig. 4. The enrichment factor of sulfur ($^{34}\delta_e$) for the groundwater samples was found to be $17 \pm 3.5_{\%o}$ (Fig. 8). Sulfur isotope

**Fig. 7** Methane (A) and Cl$^-$ (B) concentrations vs. calculated apparent net BSR. The good correlation between the methane and salinity to the apparent net BSR suggests that methane and salinity might limit BSR coupled with AOM.

**Fig. 8** Sulfur isotope enrichment relative to the pre-reaction value vs. the residual sulfate fraction left in the groundwater after bacterial sulfate reduction (eq. 5). The sulfur isotope enrichment factor ($^{34}\delta_e$) is the slope of this relationship.

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fractionation during bacterial sulfate reduction is a function of various environmental factors. The $\delta^{34}S$ value calculated here is lower than the upper limit of sulfate isotope fractionations found to be between 0 and 72‰ in the natural environment (Wortmann et al., 2001; Brunner & Bernasconi, 2005; Canfield, 2001; Sim et al., 2011). Small sulfur isotope fractionation (17 ± 3.5‰) may occur under conditions of limited sulfate supply and/or high sulfate reduction rates (e.g., Canfield et al., 2006; Stam et al., 2011; Aharon & Fu, 2000). Given that the sulfate concentrations are high (as discussed above), we suggest that the small sulfur isotope fractionation may result from a high rate of bacterial sulfate reduction, which involves less re-oxidation of sulfur intermediates (Rees, 1973), as has also been concluded from both experimental data and theoretical work (e.g., Harrison & Thode, 1958; Kaplan & Rittenberg, 1964; Rees, 1973; Canfield et al., 2006; Brunner & Bernasconi, 2005; Sim et al., 2011). To better understand the effect of the substrate and salinity on the fractionation factor, Table 4 presents sulfur isotope fractionation data from different locations classified by their reaction type (organoclastic sulfate reduction vs. sulfate reduction by AOM) and salinity conditions (hypersaline or seawater). As several factors affect bacterial sulfate reduction, a reliable comparison between different sites is difficult and should be taken with caution.

Bacterial sulfate reduction has been documented in hypersaline water of organic rich sediments in Solar Lake in Egypt with a salinity range similar to that of the DS aquifer. In that case, the reported $\delta^{34}S$ was -20‰ (Habicht & Canfield, 1997). In cold seeps in the Gulf of Mexico, the sulfur isotope fractionation factor for bacterial sulfate reduction was 18‰ (Aharon & Fu, 2000) compared with 8.6‰ for bacterial sulfate reduction coupled with AOM at the same site.

The low $\delta^{34}S$ of the AOM in hypersaline environments compares with the $\delta^{34}S$ of bacterial sulfate reduction that may be controlled by the electron donor. As the molecule of methane is smaller and considerably simpler than that of refractory crude oil, some micro-organisms prefer methane over more complex organic matter (Aharon & Fu, 2000), even though thermodynamically it is less energetically favorable as an electron donor (Devol & Ahmed, 1981; Aharon & Fu, 2000). However, higher sulfate reduction rates at the methane-rich site may result from the higher methane availability, making it favorable over the less abundant organic matter. One of these two reasons could explain the current low $\delta^{34}S$ compared with Gavrieli et al.’s (2001) results, which calculated a higher sulfur isotope fractionation factor (30‰).

Besides sulfur isotopes, the oxygen isotopic composition of the residual sulfate contributes valuable information for understanding patterns and regulation of bacterial sulfate reduction (e.g., Fritz et al., 1989; Böttcher et al., 1998; Brunner et al., 2005; Wortmann et al., 2007; Turchyn et al., 2006; Antler et al., 2013). The partitioning of oxygen isotopes in the residual sulfate during bacterial sulfate reduction remains controversial. In the recent literature, two different mechanisms are discussed. The first hypothesis suggests the dominance of a kinetic enrichment of the heavy oxygen isotope in the residual sulfate over the preferential reduction of $^{16}$O-bearing sulfate (e.g., Mizutani & Rafter, 1969; Aharon & Fu, 2003; Mandernack et al., 2003). On the other hand, the second hypothesis favors the idea of an isotopic equilibration of the oxygen in the sulfate with ambient water. However, the timescale for abiotic oxygen isotope exchange between water and sulfate is very slow (20–60 million years; Lloyd, 1968; Chiba & Sakai, 1985), leading to the conclusion that oxygen isotope equilibrium is achieved via sulfur compounds generated intracellularly as intermediates during bacterial sulfate reduction (e.g., Mizutani & Rafter, 1973; Fritz et al., 1989; Brunner et al., 2005; Wortmann et al., 2007). According to this, while the sulfur isotope composition

### Table 4: Sulfur isotope fractionations by reaction type and salinity conditions in this study and previous studies

| Location                        | Sediment Type                        | Isotope Fractionation | References |
|---------------------------------|--------------------------------------|-----------------------|------------|
| Sulfate reduction coupled with anaerobic organic matter oxidation |                                      |                       |            |
| Hypersaline condition          |                                      |                       |            |
| Solar Lake, Egypt               | Microbial mats                       | 20 (1)                |            |
| Mono Lake, California           | Basaltic sediment                    | 5–21, mean value 12 (2) |            |
| Flow-through reactor            | Brackish estuarine sediments, 20 °C  | Mean value 17 (3)     |            |
| Sea water salinity              |                                      |                       |            |
| Gulf of Mexico                  | Oil seeps                            | 18 (4)                |            |
| Sulfate reduction coupled with anaerobic methane oxidation |                                      |                       |            |
| Seawater salinity               |                                      |                       |            |
| Gulf of Mexico                  | Gas seeps                            | 8.6 (4)               |            |
| Hypersaline condition          |                                      | 17 ± 3.5 (5)          |            |
| DS basin                        | Alluvial sediment                    |                       |            |

(1) Habicht & Canfield, 1997; (2) Stam et al. (2010); (3) Stam et al. (2011); (4) Aharon & Fu, 2000; (5) this study.
would increase during bacterial sulfate reduction, the oxygen value would be expected to be concave toward the equilibrium value. However, both isotopes respond to changes in the intermediate steps—fluxes and isotope fractionation (Brunner et al., 2005, 2012; Antler et al., 2013). Therefore, a plot of δ18O3SO4 vs. δ34S3SO4 can be useful for understanding the dynamics of the intracellular reactions during bacterial sulfate reduction (Brunner et al., 2005, 2012; Antler et al., 2013).

Figure 9 shows a linear correlation between δ18O3SO4 and δ34S3SO4 in the DS aquifer (R2 = 0.86). The moderate slope (0.76) indicates that the sulfur isotopes increase more rapidly relative to the oxygen isotopes. Figure 9 also demonstrates the expected isotopic ‘plateau’ for the oxygen isotope where there is full oxygen isotope equilibrium between the ambient water and sulfate. Previous studies explain as the tangent of a concaved shape toward equilibrium (Brunner et al., 2013). According to the δ18O3H2O of the groundwater which ranged between -3.3‰ and 3.9‰, the oxygen isotopic equilibrium would be expected to be +25‰ - +35‰ (Fig. 9). Theoretically, the linear correlation between δ18O3SO4 vs. δ34S3SO4 and the fact that δ18O3SO4 is still not at equilibrium suggest that the kinetic isotope effects control both sulfur and oxygen isotope evolution during bacterial sulfate reduction. In traditional bacterial sulfate reduction, lower rates of sulfate reduction with more re-oxidation of sulfate intermediates lead to an equilibrium curve with δ18O3SO4 value of sulfate exchange with water and oxidation. However, the linear relationship can also be explained as the tangent of a concaved shape toward equilibrium (Brunner et al., 2005; Antler et al., 2013), and therefore, oxygen isotopic equilibration between sulfate and water cannot be entirely ruled out.

The slope of δ18O3SO4 vs. δ34S3SO4 has been related to the rate of bacterial sulfate reduction (Böttcher et al., 1998; Antler et al., 2013). The slope of δ18O3SO4 vs. δ34S3SO4 found in this study, 0.76 (Fig. 9), is close to the range reported by Aharon & Fu (2000) for sulfate-driven AOM in the high salinity cold seeps from the Gulf of Mexico (0.3–0.7). In their work, the rate of bacterial sulfate reduction varied over almost two orders of magnitude (~5 × 10^-4 to ~2 × 10^-6 mol cm^-3 year^-1). Although these rates were not directly measured but were modeled from the pore fluid sulfate concentration profile, they are much higher than those that have been observed in deep-sea sediments (up to 7 orders of magnitude higher—Turcyn et al., 2006; Wortmann et al., 2007; Antler et al., 2013). Therefore, even though we were not able to calculate the rate of bacterial sulfate reduction directly in this study due to the poorly constrained physical properties of our system, we suggest, based on the slope of δ18O3SO4 vs. δ34S3SO4, that the rate of bacterial sulfate reduction is comparable to that observed at gas and oil seeps (Aharon & Fu, 2000, 2003; Rubin-Blum et al., 2014) or estuaries (Antler et al., 2013), supporting our link between bacterial sulfate reduction and AOM. In addition, it has recently been shown that during the AOM in seeps or estuaries, a linear correlation between δ18O3SO4 vs. δ34S3SO4 is observed (Antler et al., 2014).

**Microbial taxon**

Overall, the microbial community supports the geochemical evidence, suggesting that the aquifer supports coupled AOM and sulfate reduction. The bacterial community in particular is indicative of bacterial sulfate reduction and association with ANME in general (Knittel & Boetius, 2009; Rabus et al., 2013). Desulfohalobiaceae sequences are commonly observed in libraries sequenced from hypersaline environments (La Cono et al., 2011), and while strains of this class have been observed to reduce sulfate in culture (Jakobsen et al., 2006), none have been observed to be a part of an ANME-SRB consortium. The abundant taxon Desulfovibacteraceae has been shown to be associated with ANME previously (Pernthaler et al., 2008).

Despite the high relative abundance of Euryarchaeota, Methanomicrobia was the only taxon containing marine ANME groups to be observed at either site (Fig. 5B, 0.67% EG19, 4.58% EG20). This result is less surprising in light of previous studies showing hypersaline sites to be sources of previously unidentified diversity among methan-oxidizing microbes (Scholten et al., 2005). The Euryarcheota Thermoplasma is a taxon that might also be involved with methane oxidation at this site. Included in this group is marine benthic group B or deep-sea archaeal group. This taxon has been implicated in AOM previously,
as it is frequently identified in methane-hydrate-bearing sediments (Inagaki et al., 2006b; Roalkvam et al., 2011).

SUMMARY

The anoxic part of the FSI zone in the DS aquifer was used to explore sulfur and oxygen isotope systematics of sulfate in hypersaline groundwater (up to 6300 mS) due to sulfate-driven AOM. Correlation between $\delta^{34}$S$_{SO_4}$ and sulfate depletion indicated bacterial sulfate reduction. The anaerobic conditions and the isotope mass balances suggest that the observed decrease in $\delta^{13}$C$_{DIC}$ is mainly a result of methanogenesis (AOM) through this BSR. The microbial taxon observed support the geochemical evidence. In particular, the bacterial community is indicative of bacterial sulfate reduction and association with ANME. The apparent net bacterial sulfate reduction rate has been calculated. The extent of sulfate removal by reduction is correlated with the salinity and methane concentrations, suggesting that the two major factors controlling the variable reduction in the DS area are the salinity and methane availability. According to the Rayleigh equation, the sulfur isotope fractionation during bacterial sulfate reduction in the FSI zone is $17 \pm 3.5_{\text{per}}$ considerably lower than that found in the DS in the past ($^{34}s = 30_{\text{per}}$) for bacterial sulfate reduction that is not associated with AOM. This low value ($17 \pm 3.5_{\text{per}}$) may indicate a higher oxidation rate of methane compared with that of organic matter as suggested in previous reports. In this study, the plot of $\delta^{18}$O$_{SO_4}$ vs. $\delta^{34}$S$_{SO_4}$ shows a linear trend without reaching the equilibrium value between the ambient water and sulfate. This could be a result of a kinetic isotope effect, although the equilibrium effect cannot be entirely ruled out. In addition, the slope value of 0.76 suggests a high rate of bacterial sulfate reduction. Improvement of the parameters controlling oxygen isotope ratios in the future by defining precisely the oxygen isotope fractionation during bacterial sulfate reduction likewise increasing identification of methane-oxidizing microbes diversity in hypersaline environments could improve our understanding of the AOM reaction in the DS area.

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