

## ORIGINAL ARTICLE

# Characterizing the distribution and rates of microbial sulfate reduction at Middle Valley hydrothermal vents

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Few studies have directly measured sulfate reduction at hydrothermal vents, and relatively little is known about how environmental or ecological factors influence rates of sulfate reduction in vent environments. A better understanding of microbially mediated sulfate reduction in hydrothermal vent ecosystems may be achieved by integrating ecological and geochemical data with metabolic rate measurements. Here we present rates of microbially mediated sulfate reduction from three distinct hydrothermal vents in the Middle Valley vent field along the Juan de Fuca Ridge, as well as assessments of bacterial and archaeal diversity, estimates of total biomass and the abundance of functional genes related to sulfate reduction, and *in situ* geochemistry. Maximum rates of sulfate reduction occurred at 90 °C in all three deposits. Pyrosequencing and functional gene abundance data revealed differences in both biomass and community composition among sites, including differences in the abundance of known sulfate-reducing bacteria. The abundance of sequences for *Thermodesulfovibro*-like organisms and higher sulfate reduction rates at elevated temperatures suggests that *Thermodesulfovibro*-like organisms may have a role in sulfate reduction in warmer environments. The rates of sulfate reduction presented here suggest that—within anaerobic niches of hydrothermal deposits—heterotrophic sulfate reduction may be quite common and might contribute substantially to secondary productivity, underscoring the potential role of this process in both sulfur and carbon cycling at vents.

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## Introduction

Deep-sea hydrothermal vent ecosystems are complex dynamic habitats characterized by steep gradients in temperature and geochemistry (Jannasch and Mottl, 1985). In these habitats, as hot hydrothermal fluid mixes with cold seawater, the precipitation of minerals creates large and complex hydrothermal chimney deposits. Within these permeable mineral structures, the continued mixing of chemically reduced, vent-derived fluids and oxidized seawater provides favorable conditions that support the growth of endolithic microbial communities (Schrenk *et al.*, 2003).

Sulfide oxidation is considered to be one of the most important microbial chemosynthetic pathways at ridge ecosystems, as evidenced by the ubiquity of sulfide-oxidizing *Epsilon*- and *Gammaproteobacteria* at ridge environments (Nakagawa *et al.*,

2004, 2005; Campbell *et al.*, 2006; Huber *et al.*, 2007; Nakagawa and Takai, 2008). To date, significantly less attention has been paid to the distribution and magnitude of sulfate reduction at vents, though sulfate-reducing bacteria and archaea have frequently been isolated from deep-sea hydrothermal environments (Jannasch *et al.*, 1988; Blöchl *et al.*, 1997; Alazard *et al.*, 2003; Audiffren *et al.*, 2003; Houghton *et al.*, 2007). Moreover, analyses of functional genes that express key proteins required for sulfate reduction suggest there is a high diversity of sulfate-reducing organisms at vents, higher than predicted via 16S ribosomal RNA (rRNA) gene analyses alone (Nakagawa *et al.*, 2004; Nercessian *et al.*, 2005).

From a biogeochemical and bioenergetic perspective, both sulfide oxidation and sulfate reduction would be favored at hydrothermal vents, although to varying degrees as a function of environmental chemistry. Sulfide oxidation is most favorable when coupled to oxygen or nitrate as an electron acceptor (Amend and Shock, 2001). Around vents, sulfide is typically in micromolar to millimolar concentrations (Butterfield *et al.*, 1994a, b), while oxygen and nitrate are around 110 and 40  $\mu\text{M}$ , respectively (Johnson *et al.*, 1986). In contrast, sulfate reduction

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is highly favored in anoxic niches at vents, as it is in other marine anaerobic environments (Muyzer and Stams, 2008). Here, as in most marine systems, sulfate is abundant at 28 mM (Dittmar, 1884), two to three orders of magnitude higher than oxygen. At vents, sulfate reduction would occur in regimes where seawater-derived sulfate is still present but oxygen is absent as, for example, within hydrothermal vent deposits. Sulfate-reducing microorganisms commonly use hydrogen and/or dissolved organic matter as electron donors, both of which are found within hydrothermal fluids (Cruse and Seewald, 2006; Lang *et al.*, 2006). Sulfate reduction—as a function of its extent and magnitude—could readily influence the cycling of sulfur and sulfur isotopes, as well as carbon, within hydrothermal environments.

To date, studies have quantified rates of sulfate reduction in hydrothermal-influenced sediments (Jorgensen *et al.*, 1992; Elsgaard *et al.*, 1994a,b, 1995; Weber and Jorgensen, 2002; Kallmeyer and Boetius, 2004) and isolated vent microorganisms (Hoek *et al.*, 2003). In contrast to the numerous studies of sulfate reduction in marine sediments (Canfield, 1989), studies of sulfate reduction in hydrothermal deposits are few (Bonch-Osmolovskaya *et al.*, 2011), due in part to the challenges associated with sampling and studying the heterogeneous and consolidated sulfide deposits typical of hydrothermal vent chimneys.

Here we present rates of microbially-mediated sulfate reduction from three distinct hydrothermal mineral deposits from active hydrothermal ‘chimneys’ found in the Middle Valley field along the Juan de Fuca Ridge, as well as assessments of bacterial and archaeal diversity, estimates of total biomass and the abundance of functional genes related to sulfate reduction, and *in situ* geochemistry. These analyses further our understanding of sulfate reduction (including rates, diversity, and distribution of known sulfate-reducing microbes) in vent ecosystems. Moreover, they underscore the potential role of heterotrophic sulfate reduction in hydrothermal systems and constrain its potential influence on both sulfur and carbon cycling.

## Materials and methods

### *Geologic setting and sampling of hydrothermal deposits*

Middle Valley (48°27'N, 128°59' W) is an intermediate spreading, axial rift valley located along the Endeavour Segment of the Juan de Fuca Ridge in the Northwest Pacific ocean. Layers of continentally-derived sediments cover Middle Valley, although active hydrothermal vents remain prominent above the sediments. Hydrothermal deposits were collected from three active hydrothermal spires during dive 4625 with the *HOV Alvin (R/V Atlantis expedition AT15-67, July 2010)* and brought to the

surface in a sealed, temperature-insulated polyethylene box. Samples were recovered from actively venting sulfide deposits at Needles (48.45778, -128.709, 2412.212 m,  $T_{\max}=123^{\circ}\text{C}$ ), Dead Dog (48.45603, -128.71, 2405.268 m,  $T_{\max}=261^{\circ}\text{C}$ ), and Chowder Hill (48.455543, -128.709, 2398.257 m,  $T_{\max}=261^{\circ}\text{C}$ ) vents. Once on board ship, samples were directly transferred to sterile anaerobic seawater and handled/processed using appropriate sterile microbiological techniques. Subsamples were immediately transferred to gastight jars (Freund Container Inc., Lisle, IL, USA), filled with sterile anaerobic seawater containing 2 mM sodium sulfide at pH 6, and stored at 4 °C. On return to the laboratory, all samples were maintained in anaerobic seawater (0.2 μm filter-sterilized before use) supplemented with 2 mM  $\Sigma\text{H}_2\text{S}$  (defined as the sum of  $\text{H}_2\text{S}$ ,  $\text{HS}^-$  and  $\text{S}^{2-}$ ) and adjusted to pH 6. The vent-like media was replenished every 8 to 12 weeks, and all samples were kept in the dark at 4 °C before incubation.

### *Vent fluid volatile geochemistry via in situ mass spectrometry*

*In situ* concentrations of dissolved volatiles (e.g.,  $\text{H}_2\text{S}$ ,  $\text{H}_2$ ,  $\text{CO}_2$ ,  $\text{O}_2$  and others) were measured at each site with an *in situ* mass spectrometer as previously described (Wankel *et al.*, 2011). Briefly, dissolved volatiles were quantified *in situ* by sampling vent effluent for up to 10 min, until partial pressures reached steady state (data were monitored in real-time within the submersible). Concentrations were determined from empirically derived calibrations and validated by comparison with discrete samples collected using titanium gastight samplers.

### *Measuring sulfate reduction rates (SRRs)*

Hydrothermal deposits were homogenized in a commercial blender (Xtreme blender, Waring Inc., Torrington, CT, USA) under a nitrogen atmosphere. Anaerobic homogenization was designed to minimize fine-scale geochemical and microbial heterogeneity and facilitate more accurate experimental replication. Hydrothermal homogenate (made up of both mineral deposit and interstitial fluid) was aliquoted volumetrically (7.5 mL, *ca* 29 g wet weight and *ca* 20 g dry weight) into Balch tubes in an anaerobic chamber. The tubes were supplemented with 15 mL of sterile artificial vent fluid media designed to mimic the geochemical composition of fluids within the pores of a sulfide deposit (pH 6, 14 mM  $\text{SO}_4^{2-}$ , 2.3 mM  $\text{NaHCO}_3$ , 1 mM  $\text{H}_2\text{S}$ , and 10 μM each of pyruvate, citrate, formate, acetate, and lactate). Organic acid concentrations are comparable to those measured *in situ* along the Juan de Fuca Ridge (Lang *et al.*, 2006). Sufficient  $^{35}\text{SO}_4^{2-}$  was added to achieve 555 kBq (15 μCi) of activity. Owing to technical difficulties with post processing methodology, shipboard incubations using fresh material were not successful. The data

presented here were generated using samples that had been maintained in sulfidic vent-like effluent (as described above) for 1 year. Samples were incubated anaerobically for 7 days at 4 °C, 30 °C, 40 °C, 50 °C, 60 °C, 80 °C, and 90 °C. Controls for sulfate reduction consisted of samples amended with 28 mM molybdate, a competitive inhibitor of sulfate reduction (Saleh *et al.*, 1964; Newport and Nedwell, 1988). Six biological replicates were run for each treatment, along with three biological replicates for each control. Reactions were quenched with the injection of 5 mL 25% zinc acetate (which is ~20-fold more zinc than the maximum sulfide concentration), and all samples were frozen at –20 °C for further analysis.

To determine SRRs, samples were thawed and the supernatant was removed and filtered through a 0.2 µm syringe filter. The crushed deposits that remained in the tube were washed three times with deionized water to remove any remaining sulfate. One gram (wet weight) of crushed deposit was analyzed via chromium distillation (see Supplementary Methods), and SRRs were calculated as in Fossing and Jorgensen (1989) using the following calculation:

$$SRR = \frac{nSO_4^{2-} \cdot a \cdot 1.06}{(a + 1 + A) \cdot t} \quad (\text{Eq. 1})$$

Where  $nSO_4^{2-}$  is the quantity (in moles) of sulfate added to each incubation (14 mM × 15 mL = 210 µmol),  $a$  is the activity (d.p.m.) of the trapped sulfide, 1.06 is the fractionation factor between the sulfide and sulfate pools,  $A$  is the activity of the sulfate pool at the completion of the incubation, and  $t$  is the incubation time (days). The rates are presented in units of nmol S g<sup>-1</sup> day<sup>-1</sup>.

#### DNA extraction

Immediately before conducting the rate experiments, a subsample of the homogenized hydrothermal deposit was removed and frozen at –80 °C for molecular analysis. This approach ensures that the resulting sequences best represent those communities responsible for the observed activity. DNA was extracted from this crushed deposit sample with a protocol modified from (Santelli *et al.*, 2008). Subsamples were washed with 0.1 N HCl, followed by two rinses with a sterile solution containing 10 mM Tris (pH 8.0) and 50 mM EDTA. A known mass of material was added to PowerSoil beadbeating tubes (MoBio Laboratories, Carlsbad, CA, USA), incubated at 70 °C for 10 min, and then amended with 200 ng of poly-A. Subsamples were subjected to beadbeating, followed by three freeze-thaw cycles to further lyse cells. Nucleic acids were extracted using hot phenol (60 °C for 3 min), followed by two chloroform:isoamyl alcohol phase extractions and a final precipitation in ethanol. DNA was resuspended in TE (pH 8.0) and quantified using the Qubit fluorometer (Life Technologies, Grand Island, NY, USA).

*Enumeration of gene abundance via quantitative PCR*  
Quantitative PCR (qPCR) was used to determine the abundance of bacterial and archaeal 16S rRNA genes. To provide additional constraints on the abundance of sulfate-reducing microbes, qPCR was also used to enumerate (A) the adenosine 5'-phosphosulfate reductase (*aprA*) gene using primers that target both sulfate-reducing bacteria and archaea (Christophersen *et al.*, 2011); (B) bacterial dissimilatory sulfite reductase (*dsrA*) using primers that target bacteria (Kondo *et al.*, 2004); and (C) *Deltaproteobacteria* via primers that target their 16S rRNA genes (Stults *et al.*, 2001). Quantification was performed in triplicate with the Stratagene MX3005p qPCR System (Agilent Technologies, Santa Clara, CA, USA) using the Perfecta SYBR FastMix with low ROX (20 µl reactions, Quanta Biosciences, Gaithersburg, MD, USA), specific primers and annealing temperatures (Table 1), and 10 ng of template genomic DNA. The thermal cycling conditions for all assays was 94 °C for 10 min, 35 cycles of 94 °C for 1 min, the annealing temperature for 1 min (Table 1), extension at 72 °C for 30 s, and fluorescence read after 10 s at 80 °C. Following amplification, dissociation curves were determined across a temperature range of 55–95 °C. Ct values for each well were calculated using the manufacturer's software. Plasmids containing bacterial and archaeal 16S rRNA and functional gene inserts (amplified from *Arcobacter nitrofigulis* (ATCC 33309), *Methanosarcina acidovorans*, and *Desulfovibrio vulgaris* Hildenborough (ATCC 29579/NCIMB 8303/AE017285, respectively) were used as standards for calibration (see Supplementary Methods for more detail).

#### Sequencing and phylogenetic analysis via 454 pyrosequencing

DNA samples were sequenced using 454 pyrotag methods similar to those described previously (Dowd *et al.*, 2008). All samples were sequenced at the Research and Testing Laboratory (Lubbock, TX, USA) using a 454FLX instrument (Roche Inc., Indianapolis, IN, USA) with Titanium reagents. The resulting bacterial and archaeal 16S rRNA (bacterial V1–V3 and archaeal V3–V4 of the 16S rRNA genes; primers are shown in Table 1), as well as *dsrB* sequences, were analyzed via Mothur (Schloss *et al.*, 2009). Sequences were trimmed, quality checked, aligned to the SILVA-compatible alignment database reference alignment (*dsrB* gene data sets were aligned to a *dsrB* gene database generated from the Ribosomal Database Project (RDP)), analyzed for chimeras, classified against the Greengenes99 database, and clustered into operational taxonomic units (OTUs; see Supplementary Methods for more detail). Rarefaction curves were used to examine the number of OTUs as a function of sampling depth. Alpha diversity was assessed by generating values from the Chao1 richness estimator and the inverse Simpson diversity index.

**Table 1** Primers used for the enumeration of 16S rRNA and sulfate reduction functional genes

Target gene	Forward primer	Conc (nM)	T (°C) <sup>a</sup>	Reverse primer	Conc (nM)	T (°C) <sup>a</sup>	Reference
Bacterial 16S	Bact1369F- 5'-CGGTGA ATACGTTCYCGG-3'	1000	59	Prok1541R- 5'-AAGGAGGT GATCC RGCCGCA-3'	1000	59	Suzuki <i>et al.</i> (2001)
Bacterial 16S <sup>b</sup>	Gray28F -5'-GAGTTT GATCNTGGCTCAG-3'	500	54	Gray519R- 5'- GTNTTACNCGCGCKGCTG-3'	500	54	Modified from <i>Frias-lopez et al.</i> (2002); modified from Manefield <i>et al.</i> (2002)
Archaeal 16S	Arch1-1369F- 5'-CGGTGA ATACGTCCCTGC-3' Arch2- 1369F-5'-CGGTGAA TATGCCCCCTGC-3'	500 (1:1 Mix)	59	Prok1541R- 5'-AAGGAGGT GATCC RGCCGCA-3'	1000	59	Suzuki <i>et al.</i> (2001)
Archaeal 16S <sup>b</sup>	Arch349F- 5'-GYGCAS CAGKCGMGAAW-3'	500	54	Arch806R- 5'- GGACTACVSGGGTATCTAAT	500	54	Takai and Horikoshi (2000)
$\delta$ -Proteobacteria <sup>c</sup>	Delta361GF- 5'-AAGCCT- GACG CASCAA-3'	600	55	Delta685R- ATCTACGGATTT CACTCCTACA-3'	600	55	Stults <i>et al.</i> (2001)
Disimilatory <sup>d</sup> sulfite reductase	DSR1-F + -5'-ATCGGN CARGCNTTYCCNTT-3'	400	58	DSR-R- 5'- GTGGMRCCGTGCAKRTTGG- 3'	600	58	Kondo <i>et al.</i> (2004)
Disimilatory sulfite reductase <sup>e</sup>	DSR2060F 5'-CAACATCG- TYC ATACMCAGGG-3'	500	50	DSR4R- 5'-GTGTAGCAGT- TACC GCA-3'	500	50	Wagner <i>et al.</i> (1998); Oakley <i>et al.</i> (2011)
Adenosine 5'-phosphosulfate reductase	aps3F 5'-TGGCAGATCATG WTYAAAYGG-3'	400	55	aps2R- 5'-GCGCCGTAACCRT CYTTRAA-3'	400	55	Christophersen <i>et al.</i> (2011)

<sup>a</sup>Annealing temperature.

<sup>b</sup>Primers used for 454-pyrotag sequencing, all other primers used for qPCR.

<sup>c</sup>Primers Delta361F and Delta685R allowed for quantification of iron- and sulfate-reducing genera within the  $\delta$ -Proteobacteria including *Geobacter*, *Pelobacter* (including fermentative species), *Desulfovibrio*, *Desulfomicrobium*, *Desulfuromusa*, and *Desulfuromonas* (including dissimilatory S reducers) (Stults *et al.*, 2001).

<sup>d</sup>Although these primers amplify bacterial *dsrA* gene sequences, they may not detect some gram-positive species and cannot detect select thermophilic bacterial and archaeal sulfate-reducing lineages (Kondo *et al.*, 2004).

<sup>e</sup>Targets both bacterial and archaeal *aprA* gene (Christophersen *et al.*, 2011).

### Sequence accession numbers

The 16S rRNA and *dsrB* gene sequences reported in this study have been submitted to the Sequence Read Archive under the accession numbers SRX154520 through SRX154528.

## Results

### Physical and geochemical characteristics of the study sites

The hydrothermal deposits sampled from Middle Valley were all relatively friable and were composed predominantly of anhydrite (CaSO<sub>4</sub>, M Tivey, personal communication). Chowder Hill and Dead Dog had the highest observed venting fluid temperatures (measured *in situ* at 261 °C), followed by Needles (123 °C). *In situ* measurements of dissolved hydrogen sulfide (H<sub>2</sub>S) revealed significant differences in hydrothermal fluid composition among hydrothermal deposits. Unfortunately the inline pH probe with the *in situ* mass spectrometer malfunctioned during the dive. Using previously reported pH values (Butterfield *et al.*, 1994a,b), Chowder Hill would have the highest *in situ* measurement of total sulfide (3.9 mM), followed by Dead Dog (2.2 mM) and Needles (0.59 mM) (Table 2). These concentrations are within the same magnitude of previously

reported H<sub>2</sub>S in focused vent fluids at Middle Valley (Butterfield *et al.*, 1994a). Chowder Hill did exhibit the highest *in situ* concentration of hydrogen (1.86 mM), followed by Dead Dog (1.66 mM) and Needles (~1.42 mM). These values are also consistent with previous studies (Cruse and Seewald, 2006), as well as gastight samples collected and analyzed shipboard (M Lilley, personal communication).

### Sulfate reduction rates

Sulfate reduction was observed in all samples at temperatures ranging from 4 °C to 90 °C (Figure 1). Maximal rates of sulfate reduction were observed between 88 °C and 90 °C (2670 nmol g<sup>-1</sup> day<sup>-1</sup> at Needles, 1090 nmol g<sup>-1</sup> day<sup>-1</sup> at Chowder Hill and 142 nmol g<sup>-1</sup> day<sup>-1</sup> at Dead Dog; Figure 1). Notably, the highest SRRs were observed in Needles samples, which were ~20-fold higher than those observed at Dead Dog, and ~2-fold greater than at Chowder Hill. Many of the rates exhibit large deviations because of the high variability among the biological replicates, most likely due to persistent mineralogical and microbiological heterogeneity across incubations, even after homogenization. Sulfate reduction was also observed in molybdate amended experiments, although we suspect that molybdate was scavenged

by minerals that attenuated the effect of the inhibitor as has been previously observed in metal-rich environments (Bostick *et al.*, 2003; Xu *et al.*, 2006).

### Quantification of taxonomic and functional genes

Microbial density (as estimated by 16S rRNA gene copies  $\text{g}^{-1}$  mineral) was greatest at Needles and lowest at Dead Dog. Microbial communities at each site were dominated by archaea (Figure 2a), with Needles showing the highest ratio of archaea to bacteria (227:1 as compared with 14:1 at Dead Dog or 17.5:1 at Chowder Hill). Assuming an average of 4.19 copies of 16S rRNA gene per bacterium and 1.71 copies of 16S rRNA gene per archaeon genome (Klappenbach *et al.*, 2001; Lee *et al.*, 2009), Needles hosts a microbial community of  $4.12 \times 10^8$  cells  $\text{g}^{-1}$  sample, three orders of magnitude higher than

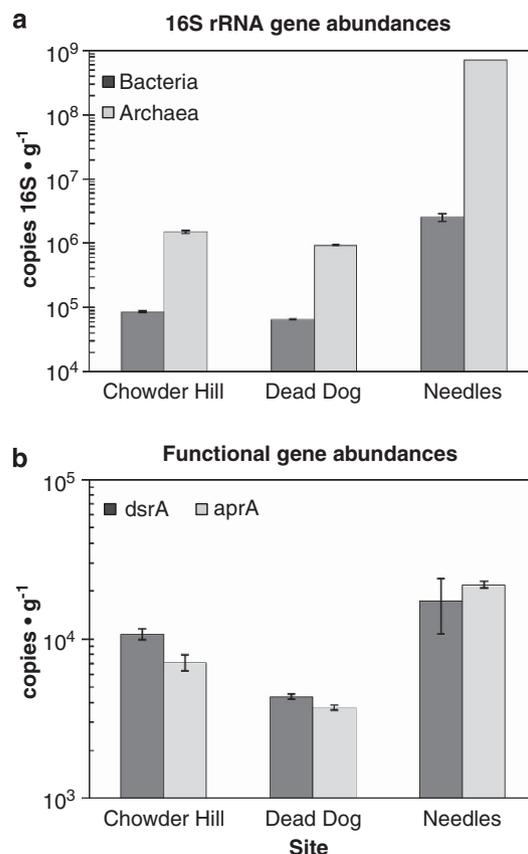
**Table 2** In situ  $\sum\text{H}_2\text{S}$  measurements compensated for a range of hydrothermally relevant pH

pH	Chowder Hill $\text{H}_2\text{S}$ (mM)	Dead Dog $\text{H}_2\text{S}$ (mM)	Needles $\text{H}_2\text{S}$ (mM)
Not pH compensated <sup>a</sup>	3.91	2.10	0.660
3.5	3.9	2.1	0.55
4.0	3.9	2.1	0.55
4.5	3.9	2.1	0.55
5.0	3.9 <sup>b</sup>	2.1	0.56
5.5	4.0	2.2 <sup>b</sup>	0.56 <sup>b</sup>
6.0	4.2	2.3	0.59
6.5	4.8	2.6	0.68
7.0	6.8	3.7	0.96
7.5	13	7.1	1.9
8.0	33	18	4.7
8.2	50	27	7.1

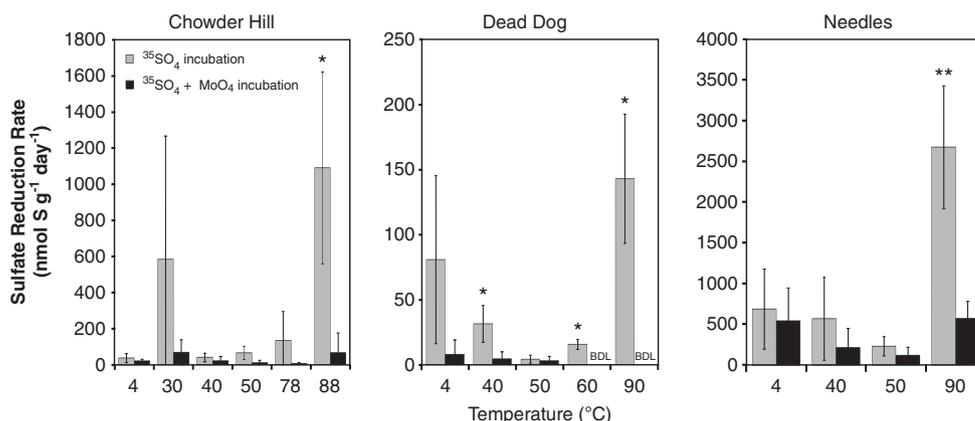
Values represent the median values of the 10 highest sampling points.  
<sup>a</sup>The first row of values refers only to  $(\text{H}_2\text{S})$  and not  $\sum\text{H}_2\text{S}$ .

<sup>b</sup>Total sulfide calculation at the most environmentally relevant pH (Butterfield *et al.*, 1994).

Chowder Hill ( $8.96 \times 10^5$  cells  $\text{g}^{-1}$  sample) and Dead Dog ( $5.65 \times 10^5$  cells  $\text{g}^{-1}$  sample).



**Figure 2** Abundance of (a) 16S rRNA genes and (b) functional gene markers for sulfate reduction across three massive sulfide deposits. Samples of hydrothermal sulfide material were collected from each of the three vent sites, frozen on return to the surface, and DNA was extracted using described protocols. Bacterial and archaeal 16S rRNA, *dsrA*, and *aprA* genes were enumerated by qPCR using published primer sets and normalized to grams of extracted sulfide. Error bars represent 1 s.d.



**Figure 1** Temperature-dependent sulfate reduction rates in three hydrothermal deposits (Chowder Hill, Dead Dog, and Needles) recovered from Middle Valley. Slurried samples were incubated for 7 days with (■) or without (□) the addition of 28 mM molybdate (a competitive inhibitor for sulfate reduction). Error bars represent 1 s.d. from the average. Statistical significance from the controls (Wilcoxon-Mann-Whitney) are shown as \* $P < 0.1$ , \*\* $P < 0.05$ . Scintillation measurements of 0 c.p.m. for radioactive sulfide ( $\text{Zn}^{35}\text{S}$ ) were considered below the detection limit (BDL).

16S rRNA gene primers specifically targeting ribotypes allied to *Desulfovibrio*, *Desulfomicrobium*, *Desulfuromusa*, and *Desulfuromonas* were used to enumerate *Deltaproteobacteria* known to mediate sulfate reduction in many marine systems (Stults *et al.*, 2001). However, given the difficulty in amplifying 16S rRNA genes from deep-sea thermophiles with typical primer sets—because of mismatches with limited sequence representation in GenBank—it is probable that these assays also underestimate abundances in these environments (Teske and Sorensen, 2008). *Deltaproteobacterial* abundance at Needles was approximately  $4.48 \times 10^5$  copies  $g^{-1}$  sample (approximately 26% of the entire bacterial population), although none were detected at Chowder Hill or Dead Dog (data not shown). The abundance of both functional genes for sulfate reduction, *dsrA* and *aprA*, was greatest at Needles and lowest at Dead Dog (a pattern similar to that seen in the 16S rRNA gene abundance estimates; Figure 2b). If we assume an average of one *dsrA* gene copy per genome (Klein *et al.*, 2001; Kondo *et al.*, 2004), the proportion of sulfate-reducing bacteria in the bacterial population is only 2.7% in Needles as compared with 28% in Dead Dog and 53% at Chowder Hill.

#### Microbial diversity

454 pyrotag sequencing, rarefaction analyses, and diversity metrics all revealed measureable differences in microbial community composition among the three hydrothermal deposits (Table 3). Via these assessments, Needles hosts the least diverse assemblage of bacteria and archaea, while Chowder Hill and Dead Dog host communities of comparable diversity. Examination of OTUs at 97%, 95%, and 92% sequence similarity further reveal differences in microbial community composition among the three sites. Among archaea at the 97% level, only two archaeal OTUs (1% of all archaeal OTUs classified) are shared among the hydrothermal deposits. The sequences classified to these OTUs represent 69%, 48%, and 18% of all library sequences from Needles, Dead Dog, and Chowder Hill, respectively. One of these OTUs is allied to the ammonium-oxidizing archaeal Candidatus *Cenarchaeum* in the phylum *Thaumarchaeota*, and accounts for 35% of Needles and <5.0% of Dead Dog and

Chowder Hill library sequences. The other OTU is allied to a thermophilic sulfur-respiring archaeon within the class *Thermoplasmata*. Nearly 40% of the archaeal sequences from Dead Dog were allied to this archaeon. Methanogens allied to *Methanocaldococcus* comprised about 1.0% of the total archaeal sequences from Dead Dog and were not represented in the libraries from Chowder Hill or Needles. Most of the archaeal diversity at Chowder Hill (80% of sequences) and Dead Dog (50% of sequences) was unclassified. No sequences allied to true sulfate-reducing archaeal lineages such as *Archaeoglobus fugilis* or *Aciduliprofundus boonei* were recovered. However, the potential diversity of thermophilic sulfate-reducing archaea in these samples is likely much greater than suggested here. This may be explained in part by biases in primer binding, sequencing, or even DNA extractions. For example, the archaeal sequencing primers used in this study only target about 34% of the *Archaeoglobus*-like sequences contained in the RDP database (as assayed by Probe Match; Cole *et al.*, 2005). Furthermore, the primers may miss members of the dominant *Thermoplasmatales*, as *in silico* analysis only returns 48% (1715/3558 sequences) of the RDP reported sequences. Together, these archaeal sequencing primers (349F–806R) miss 42% of the total archaeal sequences (67 713/117 373 sequences) in the RDP database. Similar bias has been reported in other studies in the deep-sea and deep-subsurface biotopes (for example, Dhillon *et al.*, 2003, 2005, Teske *et al.*, 2008).

Among bacteria at the 97% similarity level, 54 of the classified bacterial OTUs (7.0%) were shared among all hydrothermal deposits and account for 84%, 80%, 71% of the sequences from Chowder Hill, Needles, and Dead Dog, respectively (Figure 4). One of these OTUs accounted for 44%, 36%, and 25% of the sequences from Chowder Hill, Dead Dog, and Needles, respectively. Aligning representative sequences from this OTU via Blastn (Altschul *et al.*, 1997) revealed a best match to *Thermodesulfovibrio*, an anaerobic, thermophilic, sulfate-reducing bacterium from the phylum *Nitrospira* (81% identity). Given its abundance, we postulate that it likely contributes substantially to the high thermophilic SRRs. Furthermore, the *dsrB* gene library was dominated by sequences phylogenetically allied to *Thermodesulfovibrio* (Supplementary Table S1).

**Table 3** 16S rRNA sequence tag and alpha diversity characteristics among sites

Sample	Trimmed reads	Sampled reads	OTU (97%)	Coverage	Npshannon	Simpson	Chao
Chowder Hill-bacteria	3997	3544	340	0.948	3.30	0.168	647.23
Dead Dog-bacteria	3544	3544	341	0.957	3.69	0.141	572.12
Needles-bacteria	3983	3544	278	0.966	3.19	0.203	458.02
Chowder Hill-archaea	783	308	76	0.877	3.59	0.095	134.58
Dead Dog-archaea	308	308	58	0.903	3.34	0.071	97.55
Needles-archaea	2587	308	35	0.938	2.24	0.233	63.5

Abbreviation: OTU, operational taxonomic unit.

Other dominant groups of bacteria include members of the *Gammaproteobacteria*, *Bacteroidetes*, *Deltaproteobacteria*, and *Alpha proteobacteria*. Via Blastn, most of the unclassified sequences matched to partial 16S rRNA gene sequences from hydrothermal vent fluid communities (Nunoura *et al.*, 2010; Sylvan *et al.*, 2012). Sequences classified as *Deltaproteobacteria* comprised 5.5%, 8.2%, and 14% of the total population of Chowder Hill, Needles, and Dead Dog, respectively. Although the Dead Dog library had the highest proportion of *Deltaproteobacteria*, sequences related to known sulfate reducers within the *Deltaproteobacteria* (*Desulfobacteraceae*, *Desulfobulbus rhabdoformis*, and *Desulfovibrio*) were only found at Needles and comprised 1.1% of the 16S rRNA gene library. The majority of the sequences classified as *Deltaproteobacteria* in each of the three sites were from one unclassified *Deltaproteobacterial* OTU and comprised of 4.4%, 5.3%, and 13% of the sequences from Chowder Hill, Needles, and Dead Dog, respectively.

## Discussion

SRRs measured in deposits recovered from the Middle Valley vent field reveal the potential for active sulfate reduction within hydrothermal deposits. The magnitude of all measured rates (from 15.7 nmol g<sup>-1</sup> day<sup>-1</sup> at Dead Dog at 60 °C to 2670 nmol g<sup>-1</sup> day<sup>-1</sup> at Needles at 90 °C) is comparable in magnitude to those previously observed in hydrothermally influenced sediments (for example, Guaymas basin or Lake Tanganyika; Elsgaard, *et al.*, 1994a, b; Weber and Jorgensen, 2002; Kallmeyer and Boetius, 2004), although the availability of organic carbon is markedly higher in those hydrothermal vent sediments, with Guaymas having up to 200-times greater concentrations of organic carbon (Chen *et al.*, 1993; Cruse and Seewald, 2006; Lang *et al.*, 2006). However, these rates are measurably higher than those typically observed in non hydrothermal deep-sea sediments (0.1–10 nmol g<sup>-1</sup> day<sup>-1</sup>, converted here for comparison by assuming an average sediment density of 2 g cm<sup>-3</sup>; Elsgaard, *et al.*, 1994a; Weber and Jorgensen, 2002; Joye *et al.*, 2004). To date, the only other measurement of sulfate reduction from sulfide deposits along the East Pacific Rise exhibited rates comparable to those reported here, (Bonch-Osmolovskaya *et al.*, 2011), but it should be noted that their samples were incubated under a pure H<sub>2</sub> atmosphere and likely represented autotrophic sulfate reduction.

Notably, the maximum rates of sulfate reduction in Middle Valley sulfides occurred at 90 °C in all three deposits. This is in contrast to measurements of sulfate reduction in hydrothermal sediments, where the greatest rates are often observed between 40 °C and 70 °C, and more modest rates of sulfate reduction have been reported between 80 °C and 91 °C (Elsgaard, *et al.*, 1994a, b; Weber and

Jorgensen, 2002). The relatively low or insignificant SRRs between 4 °C and 80 °C suggest that Middle Valley deposits harbor a high proportion of hyperthermophilic sulfate-reducing microbes.

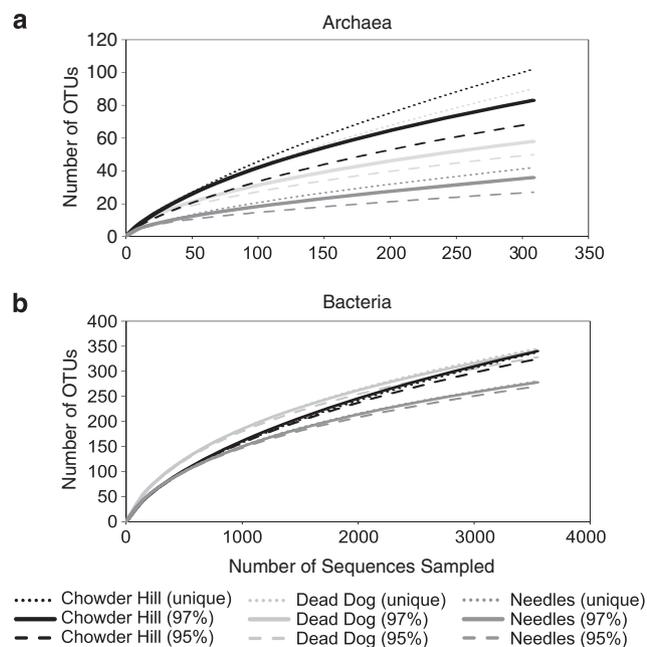
The significant differences in the rates we observed among deposits (Kruskal–Wallis,  $P < 0.0001$ ) are likely due to differences in biomass and the composition of microbial communities. These differences in density and composition are, in turn, a reflection of the availability of relevant substrates and the physico-chemical conditions at each site. Indeed, microbial biomass (as estimated by 16S rRNA genes) directly correlates to rates of activity and is likely one of the strongest factors affecting the observed rates of sulfate reduction (Pearson correlation coefficient  $r = 0.879$ ,  $P < 0.0005$ ). Needles had both the highest observed rates as well as the highest cell density (Figures 2 and 3). Of all the deposits sampled, Needles had the lowest venting fluid temperature (123 °C) resulting in the largest zone of microbial habitability. Consistent with this observation, Needles also had the greatest abundance of *dsrA* and *aprA* genes per gram, suggesting a larger potential sulfate-reducing community. Here, *Deltaproteobacteria* allied to *Desulfovibrio*, *Desulfobulbus*, *Desulfobacteria*, and *Desulfuromonas* accounted for 25.7% of the bacterial community. These clades of *Deltaproteobacteria* were not observed at Chowder Hill or Dead Dog by either qPCR or pyrosequencing. Cultured representatives from some of these *Deltaproteobacterial* clades (*Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*) have been shown to reduce sulfate at high rates (ranging from 10 to 1340 nmol min<sup>-1</sup> mg<sup>-1</sup> protein) with varying electron donors (Cypionka and Konstan, 1989; Fitz and Cypionka, 1991).

*Thermodesulfovibrio*-like organisms dominated the bacterial communities within each hydrothermal deposit (35–44%; Figure 4). *Thermodesulfovibrio* sp. are considered obligately anaerobic, thermophilic bacteria that can reduce sulfate and other sulfur compounds (Garrity and Holt, 2001). In pure cultures, members of this genus are able to link growth with hydrogen and a limited range of organic carbon molecules (formate, pyruvate, and lactate), maintaining optimal growth between 55 °C and 70 °C (Sekiguchi *et al.*, 2008). Needles had a greater proportion of sequences (from pyrosequencing) related to *Thermodesulfovibrio*-like species than the other two deposits. The combination of sequences related to a thermophilic sulfate-reducing bacteria and higher rates of sulfate reduction at elevated temperatures (90 °C) suggests that *Thermodesulfovibrio*-like organisms may have a substantial role in sulfate reduction in warmer environments. However, constraining the relative proportion of sulfate reduction by *Thermodesulfovibrio*-like organisms in these mixed communities was beyond the scope of this study.

It is unclear why Chowder Hill and Dead Dog exhibited large differences in rates of sulfate reduction despite other similarities in geochemistry and

biomass. One plausible explanation might be that different types of biological interactions (e.g., syntrophy or competition) occur because of differences in the composition and distribution of microbial communities within the mineral matrix of each deposit. Slight differences in community composition, such as Dead Dog having a higher representation of sequences related to sulfur-respiring (*Thermoplasmata*) and methanogenic (*Methanocaldococcus*-like) archaea than Chowder

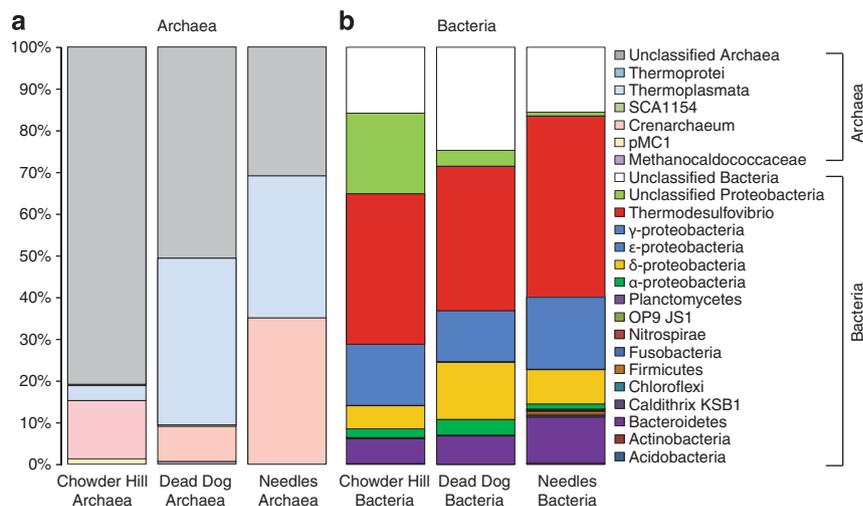
Hill, may reflect differences in biological interactions, which have implications for rates of sulfate reduction in each deposit. Also, substrate competition for H<sub>2</sub> or consumption of locally produced DOC (Oremland and Polcin, 1982; Lovley and Phillips, 1987) may be more prevalent in one deposit over another. Future experiments should aim to better resolve how specific interactions between populations, for example, syntrophy or competition for a common substrate, may influence sulfate reduction.



**Figure 3** Rarefaction analysis of archaeal (a) and bacterial (b) sequences from each site at OTU clustering at the 95%, 97% and unique level. All 16S rRNA libraries were randomly sampled down to the smallest sample size, (a)  $n=308$  (Dead Dog), (b)  $n=3544$  (Dead Dog). For both bacteria and archaea, Needles had the least diverse populations.

#### *The potential role of heterotrophic sulfate reduction in productivity and biogeochemistry*

Heterotrophic sulfate reduction is likely a prominent metabolic mode within Middle Valley sulfides and sediments, and the SRR data herein (which solely measure heterotrophic sulfate reduction) support that supposition. Sedimented vent fields typically contain allochthonous organic carbon that could readily support heterotrophy. Indeed, at Middle Valley, bottom waters contain 3.5 mg DOC·L<sup>-1</sup> (about seven-fold higher than the overlying surface seawater), whereas porewater concentrations range from 0.1 to 84.0 mg DOC·L<sup>-1</sup> at sediment depths to 200 mbsf (Ran and Simoneit, 1994). Based on data from culture studies of *Desulfovibrio* strains, including the H<sup>+</sup>/H<sub>2</sub> ratio of 1.0 for *Desulfovibrio vulgaris* Marburg (Fitz and Cypionka, 1991), a P/2e<sup>-</sup> ratio of 1/3 for *Desulfovibrio gigas* (this is the number of ATPs produced for every two electrons transferred to an electron acceptor (Barton *et al.*, 2003), and the assumption that 10% of ATP production supports growth (20 mmol ATP per gram biomass), our estimates suggest that—at our maximum empirically measured rates—heterotrophic sulfate reduction could support 140 g biomass yr<sup>-1</sup> ( $\sim 1.5 \times 10^{14}$  cells) at Chowder Hill (volume = 109 900 cm<sup>3</sup>), 16 g biomass yr<sup>-1</sup> ( $\sim 1.7 \times 10^{13}$  cells) at



**Figure 4** Archaeal (a) and Bacterial (b) taxonomic distribution among the different hydrothermal deposits revealed differences in microbial community composition. Bacterial V1–V3 region and archaeal V3–V4 regions of the 16S were sequenced by 454, analyzed with Mothur, and all libraries were randomly sampled down to the smallest sample size, (a)  $n=308$  (Dead Dog), (b)  $n=3544$  (Dead Dog).

Needles (volume = 5495 cm<sup>3</sup>), and 2.1 g biomass yr<sup>-1</sup> (~2.2 × 10<sup>12</sup> cells) at Dead Dog (volume = 12 560 cm<sup>3</sup>). These values are modest in comparison to global estimates of chemoautotrophic biomass production on the global ridge system (10<sup>10</sup>–10<sup>13</sup> g of biomass yr<sup>-1</sup>; McCollom and Shock, 1997; Bach and Edwards, 2003), yet may be significant in the context of local secondary productivity. Moreover, with respect to the sulfur cycle, the sulfide produced by these heterotrophic sulfate reducers could represent up to 3% of the H<sub>2</sub>S flux from Middle Valley deposits (given previously published vent fluid flow rates from the Main Endeavor field; Wankel *et al.*, 2011), which can influence, for example, sulfur isotope biogeochemistry. Additional rate measurements that represent the diversity of physicochemical conditions found within deposits or ridge systems are necessary to better constrain the contribution of heterotrophic sulfate reducers to global vent biomass and geochemistry.

Hydrothermal vents are dynamic environments where carbon and sulfur cycling are intimately linked. Both autotrophic and heterotrophic sulfate-reducing microbes have been isolated from vents, and the data shown here are among the first to constrain the potential for heterotrophic sulfate reduction at vents (in particular those with higher organic carbon loads), as well as the relationship between SRRs, temperature, microbial biomass, and community density and composition. These data, as well as the vent field estimates of sulfate reduction, underscore the relevance of sulfate reduction in hydrothermal ecosystems and further indicate the need for continued studies of sulfur cycling along ridge systems.

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