The pH and pCO2 dependence of sulfate reduction in shallow-sea hydrothermal CO2 – venting sediments (Milos Island, Greece)

Elisa Bayraktarov1*,†, Roy E. Price2†, Timothy G. Ferdelman1,2 and Kai Finster3,4

1 Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, Bremen, Germany
2 Center for Marine Environmental Sciences, Bremen, Germany
3 Department of Biosciences, Section for Microbiology, University of Aarhus, Aarhus C, Denmark
4 Department of Physics and Astronomy, Studie Astrophysics Centre, University of Aarhus, Aarhus C, Denmark

INTRODUCTION

Microbial sulfate reduction (SR) is a dominant process for organic matter mineralization in sulfate-rich anoxic environments at neutral pH. Recent studies have demonstrated SR in low pH environments, but investigations on the microbial activity at variable pH and CO2 partial pressure are still lacking. In this study, the effect of pH and pCO2 on microbial activity was investigated by incubation experiments with radioactive 35S targeting SR in sediments from the shallow-sea hydrothermal vent system of Milos, Greece, where pH is naturally decreased by CO2 release. Sediments differed in their physicochemical characteristics with distance from the main site of fluid discharge. Adjacent to the vent site (T ∼40–75°C, pH ∼5), maximal sulfate reduction rates (SRR) were observed between pH 5 and 6. SR in hydrothermally influenced sediments decreased at neutral pH. Sediments unaffected by hydrothermal venting (T ∼26°C, pH ∼8) expressed the highest SRR between pH 6 and 7. Further experiments investigating the effect of pCO2 on SR revealed a steep decrease in activity when the partial pressure increased from 2 to 3 bar. Findings suggest that sulfate reducing microbial communities associated with hydrothermal vent system are adapted to low pH and high CO2, while communities at control sites required a higher pH for optimal activity.

Keywords: sulfate reduction, sulfate reduction rate, shallow-sea-hydrothermal vents, pH effect, pCO2 effect, microbial activity, extreme environment

Most studies of sulfate reducing bacteria (SRB) have focused on environments at circumneutral pH including marine and freshwater sediments (Widdel, 1988; Hao et al., 1996). However, there is now increasing evidence for SR to occur also in low pH habitats, such as acidic lakes and rivers, acidic soils, peat lands, acid rock, or mine tailings (Koschorreck, 2008 and references therein). SR was also observed in hydrothermal systems where the pH is lowered due to CO2 venting (Amend et al., 2004) but further knowledge on the activity of these microbial communities is limited. SR has a low yield of metabolic energy (Widdel, 1988). However, surprisingly SRB are active under acidic conditions where additional energy requirements to keep an elevated intracellular pH (Martin, 1990; Lowe et al., 1993), the toxicity of metabolic products (e.g., H2S or protonated fatty acids, Hao et al., 1996) and competition with other more resistant microbes (e.g., iron reducing bacteria or methanogens) may inhibit SR to occur (Koschorreck, 2008). Below a pH of 5, the metabolic product of SR is exquisitively present in its undissociated form H2S which is considered as the most toxic form of sulfide (Moosa and Harrison, 2006). As uncharged gas, H2S permeates the cell membrane where it

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*Correspondence: Elisa Bayraktarov, Department of Ecology, Labek Center for Tropical Marine Ecology, Fahrenheitstraße 6, 28359 Bremen, Germany; e-mail: elisa.bayraktarov@emi.bremen.de
†Present address: Elisa Bayraktarov, Department of Ecology, Labek Center for Tropical Marine Ecology, Fahrenheitstraße 6, 28359 Bremen, Germany; Roy E. Price, Department of Earth Sciences, University of Southern California, Los Angeles, CA, USA.
can react with free metal ions and metal containing functional groups of the electron carrier system of the cell (Hao et al., 1996). Amino acids and metabolic coenzymes inhibiting the functionality of the microbial cell (Koschorreck, 2008). Volatile fatty acids (VFA) are fermentation products of organic carbon and represent typical substrates of sulfate reducers. VFA exist as protonated organic acids under low pH. In their protonated form they can diffuse through the cytoplasmic membrane and act as "uncouplers" (Braun et al., 2010) leading to a collapse of the membrane potential (Baronofsky et al., 1984). In addition, protonated organic acids decrease the pH of the cytoplasm upon entering by diffusion. Free intracellular protons can impair processes such as DNA transcription, protein synthesis, and enzyme activities (Baker-Austin and Dopson, 2007). In nature, intermediates of fermentation, e.g., VFA and H₂, are generally maintained at low concentrations, showing a close coupling of terminal oxidation to fermentation (Finke and Jørgensen, 2008) countereacting an accumulation of acid and a subsequent decrease in pH.

In addition, SR at low pH is of special interest because this process can be applied for biogenic neutralization of acid rock drainage environments and for bioremediation (Kaksonen and Puhakka, 2007). The investigation of CO₂ venting hydrothermal sediments is of particular interest, as they represent a system in which the degassing effects of previously sequestered CO₂ can be investigated (Shitashima et al., 2008). Based on all these implications further knowledge is necessary to understand the biological and chemical dynamics in acidified sediments.

This study was conducted on sediments collected from a shallow submarine hydrothermal vent system in the Aegean Sea near the island of Milos (Greece), and focuses on the effect of pH and low submarine hydrothermal vent system in the Aegean Sea near and chemical dynamics in acidified sediments.

**MATERIALS AND METHODS**

**STUDY SITE**

The study site (36° 40′ 25N, 24° 30′ 58E; Figure 1A) was located ∼1 m from an active gaseous hydrothermal vent system at 4.5 m water depth in Paleochori Bay, Milos Island, Greece. The bay is situated at the southeastern coast of Milos in the Aegean Sea (Figure 1B). Free gases sampled from the site (collected in 2011) had a mean gas composition of 92.5% CO₂, 0.13% O₂, 0.67% N₂, 7 ppm He, 11450 ppm H₂, 0.7 ppm CO, and 916 ppm CH₄ (Price et al., 2013, this issue). These free gas values can be used as an approximation for the discharged gases of the seep. The vent sites displayed a characteristic zonation of colored surface deposits surrounding the gas outlets (Figure 2). The sediment next to the vent was covered by a layer of bright yellow-orange deposits containing the arsenic sulfide minerals similar to orpiment (Price et al., 2012). A zone covered by white flocculent material on top of black sediment termed "white zone" surrounded this area. The white precipitate consists of a mixture of amorphous silica and native S (Fitzsimons et al., 1997). It is assumed that this precipitate is associated with microbial mat communities (Dando et al., 1995; Fitzsimons et al., 1997). The white zone was surrounded by an area of gray sediment referred to as the "transition zone" and a brown background zone called the "brown zone" with hydrothermally unaffected sediment characteristics such as ambient temperature and pH. Sediments from the brown zone were used as a control.

**SAMPLING**

Sampling was carried out along a transect through different sediment zones by SCUBA diving in August 2010. Sediments at increasing distance (ca. 1, 2, and 5 m) from the gas emission zone were sampled underwater with 1 L preserving jars (Figure 2; J. WECK GmbH & Co. KG, Wehr, Germany) containing a butyl rubber stopper which prevented the contamination of the sample with oxygen. Samples were maintained dark and cold during shipping. Temperature measurements were conducted in situ at a sediment depth of ∼10 cm with a digital thermometer in a custom underwater housing (Fisher Scientific, Germany). The pH measurements integrated over 60 mL of sediment pore water. Measurements were performed ∼1 h after sampling from a 10 cm sediment depth at the main sampling locations using 60 mL syringes elongated by tubing connected to long perforated pipette tips. Pore water from the white zone was sampled down
to 12 cm sediment depth at 2 cm resolution for analysis of sulfide, sulfate, alkalinity and pH using rhizon soil moisture samplers (5 and 10 cm rhizons, pore size: 0.1 m, Rhizon core solution sampler (CSS), Rhizosphere Research Products, The Netherlands). For sulfide analysis, pore water samples were fixed with an equal volume of 50 mmol L$^{-1}$ zinc acetate (ZnAc) solution.

PHYSICOCHEMICAL MEASUREMENTS
Pore water sulfide fixed with ZnAc was analyzed spectrophotometrically according to Cline (1969). Concentrations of SO$_4^{2-}$ were measured on a Metrohm Compact 761 ion chromatograph equipped with a Metrohm Metrosep A column. The eluent was a 3.2 mmol L$^{-1}$ Na$_2$CO$_3$/1 mmol L$^{-1}$ NaHCO$_3$ solution and the flow rate was 0.7 mL min$^{-1}$. The standard deviation of repeated measurements was always below 2% of the measured concentration. Prior to measurement, samples were diluted 100-fold with distilled water. Blanks were used for background corrections.

MEDIUM
Artificial seawater medium for SRB (modified from Widdel, 1988) contained (mmol per liter): KBr (0.756), KCl (8.05); CaCl$_2$$\cdot$2 H$_2$O (10); MgCl$_2$$\cdot$6 H$_2$O (27.89); MgSO$_4$$\cdot$7 H$_2$O (11); NaCl (451); NH$_4$Cl (4.67) and KH$_2$PO$_4$ (1.47) and was prepared without the addition of NaHCO$_3$ as buffering solution to allow for easier pH adjustment. For incubation experiments the pH was adjusted to values of 3, 4, 5, 6, and 7 with sterile 1 mmol L$^{-1}$ phosphoric acid (H$_3$PO$_4$) or NaHCO$_3$ (Widdel, 1988) before sterilization for 25 min at 121°C and re-adjusted if necessary. Prior to sterilization, needles were inserted into the butyl stoppers of each medium bottle to allow escape of oxygen from the liquid at high temperature. The 75°C hot medium was allowed to cool down under constant stirring and flushing the headspace of the bottle with a mixture of N$_2$/CO$_2$ at a 9/1 ratio(v/v). The salinity of the SRB-media was 33.

SULFATE REDUCTION RATE MEASUREMENTS
To determine sulfate reduction rates (SRR) for the different incubation experiments, 20 μL of $^{35}$S-sulfate tracer containing 100 kBq were injected into 15 mL Hungate tubes containing 2 mL homogenized sediment and 3 mL of SRB medium in 1:1.5 (v/v). Samples were incubated at 40 or 75°C. Per experiment, three killed controls were prepared by transferring the sediment and the medium directly to Hungate tubes containing 5 mL of 20% (w/v) ZnAc solution in centrifuge tubes. The tubes were shaken and tracer was added to the killed slurries. Every sample was prepared in triplicate. Incubations were stopped by transferring the sediment slurries into centrifuge tubes containing 5 mL of a 20% (w/v) ZnAc solution.

Reduced inorganic sulfur compounds were removed from the fixed samples by single step cold acidic chromium distillation method as described by Fossing and Jørgensen (1989) and further modified by Kallmeyer et al. (2004). The $^{35}$S incorporated into the pool of total reduced inorganic sulfur was recovered as zinc sulfide in traps containing 7 mL of a 5% (w/v) ZnAc solution and finally counted in a scintillation counter (Packard Tri-Carb Liquid Scintillation Counter, MA, USA). SRR were determined as described in Kallmeyer et al. (2004). The porosity of each sediment type was determined by differential weighting of wet and dry sediment after drying for 2 days at 80°C to constant weight.

INCUBATIONS
All collected sediment samples were used for experiments on residual sulfate reducing activity 52 days after sampling as this was the time required for shipping of sampled material and installation of experimental setup. A pre-incubation for 18 h without tracer was followed by tracer incubation for 35 h. Incubations were performed either at 40 or 75°C. A SRB medium with 11 mmol L$^{-1}$ of sulfate and pH 5.3 was used to mimic the in situ characteristics of the white zone sediment.

A device for anaerobic slurry preparation and simultaneous pH measurement was constructed of sterilized material (15 min at 121°C). Slurry of 1:4 (v/v) ratio of sediment to medium was prepared and the pH was adjusted with phosphoric acid or NaHCO$_3$. After pH adjustment, 5 mL of slurry were transferred into pre-flushed oxygen-free Hungate tubes. The headspace of each tube was flushed with a mixture of N$_2$ and CO$_2$ at a 9/1 ratio (v/v) and the tube was sealed with a butyl rubber stopper and kept in place by a screw cap.

For experiments on stimulated SR, a mix of VFA (Widdel, 1988) was supplied to the sediment samples as additional electron donors.
donors. It contained formate, acetate, propionate, butyrate and succinate in equal-molar amounts providing a total fatty acid concentration of 1 mmol L\(^{-1}\). The mixture of VFA was added with a \(\text{Na}_2\text{CO}_3\)-flushed 1 mL syringe through the butyl stopper of the slurry bottles to produce a final slurry concentration of 1 mmol L\(^{-1}\) VFA. The pH was re-adjusted for each slurry bottle after fatty acid addition. For each experiment with VFA-supplemented slurries, three incubations were prepared as killed controls. All experiments were carried out in triplicates. For pH experiments, samples were incubated for 16–18 h at 40\(^\circ\)C after a short pre-incubation. Pre-incubation time was calculated as the time required for pH adjustment and the distribution of slurry into Hungate tubes prior to incubation with radioactive tracer. Sample preparation was conducted at room temperature and was completed in less than 4 h. For all incubation experiments, the surface sediment fractions (0–5 cm) of white and transition zone sediments and a deeper fraction (5–10 cm) of the brown zone sediment were used. For incubation experiments at pH of 3, 4, 5, 6, and 7, the phosphate buffering system, with \(pK_a\) values 2.35, 6.87, and 12.33 (Perrin, 1972) was appropriate. Before and after each incubation experiment, the pH of each tube was measured after gentle mixing. For pH measurement prior to incubation, 200 \(\mu\)L of the liquid were removed with a needle and a \(\text{Na}_2\text{CO}_3\)-flushed 1 mL syringe through the butyl stopper of the tube. The pH was measured after the incubation by inserting the pH electrode into each tube after gentle shaking. All pH values were determined in triplicates. The pH changed during the incubation between \(\pm 0.1\) and \(\pm 0.5\) pH units (horizontal error bars in Figure 4).

Additionally, incubation experiments were conducted to investigate the combined effects of pH and pCO\(_2\) on SR. Therefore, different CO\(_2\) partial pressures in the range of 0, 1, 2, and 3 bar were applied after a pre-incubation for 12 h at 40\(^\circ\)C. Prior to incubation experiments, CO\(_2\) partial pressures were adjusted to the gas phase of tubes (10 mL headspace in a total volume of 15 mL) using a pressure reducer. 

**Statistical Analyses**

Arithmetic means and standard errors were calculated for each triplicate set of experiments and are represented by vertical error bars in the activity diagrams (Figures 4 and 5). Statistical analysis was performed with SigmaPlot, 12.0, Copyright © 2011 Systat Software, Inc. A One-Way-ANOVA was used to test for statistically significant differences between the groups considering a triplicate at a certain pH value (3, 4, 5, 6, and 7) or CO\(_2\) partial pressure (0, 1, 2, and 3 bar) as a group. The dependent variable was SRR. Only values above the reported detection limit of 0.04 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\) were taken into account and values below this limit were set to 0. Degree of freedom df, F factor, and \(P\)-value (df, F, and \(P\)) are given. A Two-Way ANOVA was applied to test for differences between groups and treatment (untreated or supplemented sediment).

**Results**

**Geochemical Characterization**

In situ measurements of temperature and ex situ determination of pH demonstrated that the shallow-sea hydrothermal vent site constituted an extreme environment. The pH decreased with increasing proximity to the gas vent reaching values of 5.3 in the white and 7.6 in the pore water of brown zone sediment (dissolution displayed in Figure 2). Temperatures measured at 10 cm sediment depth were 75\(^\circ\)C in the white and 26\(^\circ\)C in the brown zone sediment. Temperature and pH measurements were not conducted in the transition zone sediment. The pore water concentration of hydrogen sulfide increased from 553 \(\mu\)M at a sediment depth of 2 cm to 608 \(\mu\)M at 4 cm; concomitantly, the sulfide concentration decreased from 11.3 to 10.5 mmol L\(^{-1}\) at the same depth.

The bulk seawater pH was 8.0 and the alkalinity 3.6 mmol kg\(^{-1}\). The pH in the white zone depth profile was between 5.2 and 5.3. Alkalinity decreased with depth from 11.3 to 10.5 mmol kg\(^{-1}\) in white sediment pore water (Price et al., 2012).

**Sulfate Reduction Rate Measurements**

Sulfate reduction rates determined at 40\(^\circ\)C in sediments that were collected close to the main venting site (white zone) were below 1 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\). At this temperature, SRR increased with distance from the gas discharge zone, reaching 24 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\) in surface sediment of the transition zone (Figure 3A). The highest activity at 40\(^\circ\)C was found within the brown zone sediment (control) with 35 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\). SR was mostly inhibited at 75\(^\circ\)C (Figure 3B). However, SR was measurable in brown zone sediment at 75\(^\circ\)C, with a SRR of 2.4 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\), which is approximately 15 times less than the rate measured at 40\(^\circ\)C. As SRR were below the detection limit of our method at 75\(^\circ\)C, all further incubation experiments were conducted at an intermediate temperature of 40\(^\circ\)C, which represents approximately the average found in hydrothermally influenced sediments.

The pH effect on Sulfate Reduction

The SRR increased with pH until they reached a pH optimum (\(pH_{\text{opt}}\)) defined as the pH at which the highest SRR was measured (Figures 4A–C). The different sediment types of Paleochori Bay varied in the level of SR activity, its pH dependence and \(pH_{\text{opt}}\). Among all tested sediments, the white zone sediment showed the lowest SR activity. The rates were statistically different for pH values between 3 and 7 (df = 4, \(F = 17.47, P < 0.001\)). Highest rates were measured at pH 6.3 with an arithmetic average of 0.2 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\) (Figure 4A). At pH 7 SRR were below the detection limit. This was also the case at pH < 4. At pH of 4.7 the rates were close to detection limit. The addition of 1 mmol L\(^{-1}\) of VFA to white zone sediment samples decreased SRR to below the detection limit at pH values < 4.5. The \(pH_{\text{opt}}\) remained at pH 6.4 but the corresponding SRR showed an increasing trend from 0.2 to 0.3 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\) after VFA addition. SR activity could also be detected at pH 7.3 with a rate of 0.1 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\) after VFA addition. In summary, the white zone sediment showed the highest SRR at pH values between 6.3 and 6.4 in treatments, both with and without VFA-amendment.

The transition zone sediments showed significantly different SRR for all pH values (df = 4, \(F = 19.89, P < 0.001\), Figure 4B). The highest rate was measured between pH 5.5 and 6.3 with rates of 6.5 nmol SO\(_2^-\) cm\(^{-1}\) d\(^{-1}\). The SRR measured at \(pH_{\text{opt}}\) were

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At pH < 5, SRR were below the detection limit. VFA amendment resulted in a significant increase (df = 1, F = 26.06, P < 0.001) and a different response as compared to the other sediments. The pHopt was 6.1 as was the case in the non-amended samples and the SRR increased from 2.3 nmol SO\textsubscript{4}\textsuperscript{2-} cm\textsuperscript{-3} d\textsuperscript{-1} with no amendment to 9.0 nmol SO\textsubscript{4}\textsuperscript{2-} cm\textsuperscript{-3} d\textsuperscript{-1}.
to 3.9 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ after VFA-addition. At pH 7 only a slight increase in SRR from 1.6 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ without amendment to 2.3 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ at after amendment was observed.

THE pCO$_2$ EFFECT ON SULFATE REDUCTION

The different pCO$_2$ partial pressures resulted in different pore water pH values in sediment samples obtained from the Paleochori Bay sites. In all sediment types a decreasing trend in SRR was observed with increasing pCO$_2$ (Figures 5A–C). A stimulation of SRR upon VFA amendment was observed for all sediment types at a pCO$_2$ of 1 bar. However, the rates decreased with increasing pCO$_2$ after VFA addition and were significantly different for the transition ($df = 2$, $F = 19.68$, $P < 0.001$) and the brown zone sediment ($df = 2$, $F = 31.51$, $P < 0.001$) and the brown zone sediment. We observed a steep decrease of SRR in all samples after the CO$_2$ gas pressure was increased from 2 to 3 bar even though the pH only decreased slightly, from 4.8 to 4.7 for the white zone sediment, from 4.7 to 4.6 for transition zone sediment and from 5.5 to 5.4 for the brown zone sediment (Figures 5A–C).

The sediment samples showed different buffering capacities, which were expressed as the relation between pCO$_2$ and subsequent pH decrease (Figure 6). The pore water of white zone sediment had an intermediate buffering capacity expressed by a pH decrease from 5.6 to 4.7 after addition of 3 bar of pCO$_2$. The pore water of transition zone sediment had the lowest buffering capacity since the pH decreased from 5.5 to 4.4 after addition of 3 bar of pCO$_2$. The pH value and buffering capacity was found in samples from the brown zone sediment which decreased from 6.1 to 5.2 after pCO$_2$ adjustment (Figure 6).

DISCUSSION

In this study, the highest SRR without VFA amendment were measured in the brown zone sediment with 35 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$. SRR determined in these control sediment samples were close to rates reported from temperate regions. For example, Jørgensen and Bak (1991) reported SRR between 5 and 20 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ from marine sediments of Kattegat, Denmark. Dando et al. (1995) studied a neighboring hydrothermal vent site in Paleochori Bay at a water depth of 10 m and determined SRR of between 5 and 80 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ applying a radio-tracer method (Dando et al., 1991) immediately after sampling. SRR peaked at 2 cm sediment depth with maximal rates of up to 80 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$, which were probably supported by residual organic carbon (Dando et al., 1995) and/or hydrogen gas from the vent fluids. The overall lowered rates in our sediment samples were most likely a consequence of electron donor limitation as the experiments could not be performed immediately after sampling. Intermediate activity was found in surface samples of transition zone sediment with a SRR of 24 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$. SRR in brown and transition zone sediment were in the range of SR determined in deep-sea hydrothermal vent sediments (19–61 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$) with in situ temperatures > 100°C (Jørgensen et al., 1992) but below the rates measured for the Logatchev hydrothermal field at the Mid Atlantic Ridge with 122–136 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ at temperatures between 65 and 100°C (Schauer et al., 2011). The lowest SRR was measured in the white zone sediment. There, SRR were below 1 nmol SO$_4^{2-}$ cm$^{-3}$ d$^{-1}$ after incubation at 40°C and below detection limit when the samples were incubated at 75°C, which is the in situ temperature at 10 cm sediment depth. The low SRR in the white zone may be due...
to substrate limitation, in particular lack of hydrogen gas from venting fluids.

The SRB are found in several phylogenetic groups, but mostly belong to the Deltaproteobacteria (e.g., 23 out of 68 genera), followed by the Firmicutes (Muyzer and Stams, 2008; Barton and Fauque, 2009). It is possible that the low SRB, especially in the white zone sediments, may be due to a smaller population size of SRB, at least relative to our other sediment types. Sievert et al. (1999) found mainly Cyanobacteria and sulfur oxidizing Thiomicrospira spp in a hydrothermal vent system that is located close to our study site. Our sediments were collected from a slightly higher temperature, 75 °C at 10 cm depth, whereas the white zone temperatures for Sievert et al. (1999) study reached only 50 °C at 3 cm sediment depth. Even if polymerase chain reaction (PCR) biases cannot be excluded, the elevated numbers of 16S rRNA sequences affiliated with the Deltaproteobacteria, to which most SRB belong, found in the transition and brown zone sediments (Sievert et al., 1999) are in agreement with our results as we found the highest SRR in these sediments.

In a more quantitative study, 16S rDNA clone libraries and phylogenetic data were obtained for samples collected in the same area and temperature as this study (Price et al., 2013, this issue). In the white zone with equivalent temperatures to our study, 7 out of 51 clones were affiliated with the order Desulfobacterales, one of the major groups known for SR (Muyzer and Stams, 2008). Price et al. (2013, this issue) also reported that members of the Firmicutes phylum became dominant in the deeper sediments, but were exclusively associated with Bacillus sp (at ~100% ID). In a pooled 0–6 cm sample from a lower temperature (~45 °C) white zone at the same site as this study, 24 out of 119 clones were affiliated with Desulfotomaculata, 12 of which were associated with the Desulfotherales order. In a pooled 0–6 cm sample from a nearby background brown area, 10 out of 97 clones were affiliated with Desulfotherales. Thus, in pooled samples of brown zone sediment, about 10% of the clones in the clone libraries affiliate with groups with the most potential for SO4 reduction, while 10% affiliated with SRBs in white zone sediments. In the 0–1.5 cm sediment sample, the contribution of 16S rDNA affiliated with SRB was 8%. Due to the small size of the libraries we do not consider the ratios to significantly different among sites. In future studies the microbial diversity in general and that of sulfate reducers should be addressed with next generation sequencing methods (Frank et al., 2013).

A possible explanation for the low SRR in white zone sediment could also be its high spatial instability due to transport of gas, venting and mixing in combination with oxygenation of the sediment layers which could lead to a removal of microorganisms. Mixing might also disturb the coupling between fermenters and sulfate reducers, which thrive on oxidizing fermentation products such as VFA and H2. Finke and Jørgensen (2008) observed that temperature had an effect on the coupling between fermentation and SR in sediment samples from Sturlaard and Wadden Sea as fermentation products accumulated in incubations above a critical temperature of 40 °C. The authors concluded that temperature is a factor that might disturb the metabolic link between both process types because sulfate reducers were inhibited at a lower critical temperature than fermenters (Finke and Jørgensen, 2008). In our study, a higher threshold for negative temperature effects is expected as the natural temperatures of the hydrothermal vent system ranged from 26 to 75 °C. Effects that reduce SRR can most likely be attributed to low pH leading to a protonation of acid anions, which short-cuts proton gradients in the cells and disrupts adenosine triphosphate (ATP) synthesis (Baronofský et al., 1984).

**THE pH EFFECT ON SULFATE REDUCTION**

Based on cell culture studies, it was suggested that SR may preferentially occur at pH between 6 and 8 (Widdel, 1988; Hooi et al., 1996). However, Dando et al. (1995) have documented that SR can take place at high rates in shallow submarine hydrothermal vent systems with low pH. Our results are in line with those reported by Dando et al. (1995), and demonstrate that hydrothermal sediments with lower pH and increased temperature harbor populations of SRB that respond distinctly to different pH conditions. In addition, our data indicate that hydrothermal sediments had different pH optima than SR populations of sediments with low temperature and neutral to slightly alkaline pH characteristics. We observed SR at pH < 5 in hydrothermally influenced, CO2-vented sediments. However, optimal pH of SR was found between pH 5 and 6 for the hydrothermally influenced white and transition zone sediments but between pH 6–7 for brown zone sediment that served as control.

Sulfate reducing activity under low pH conditions is often explained by the existence of microenvironments with more reduced and alkaline conditions than the acidic surroundings (Fortin, 1996). Knochelrock (2008) argued against the existence of neutral microniches formed by the alkalinity produced during SR. He calculated that a SRR of 1.8 × 10^4 nmol SO4 cm^-3 d^-1 is required to maintain circumneutral pH in a sphere with 100 μm diameter while the pH in the surrounding is 3. Applying this calculation to the hydrothermal vent sediments of Paleochori Bay,

![Buffering capacity of white, transition, and brown sediment depicted by the pH of each sediment type to the corresponding pCO2 values](image-url)
a SRR of 1.6 × 10^6 nmol SO_4^{2-} cm^{-3} d^{-1} would be needed to maintain the center of the sphere at pH 6 when the pH of the surrounding pore water was 5. This rate exceeds the SRR in the hydrothermal vent system of Paleochori Bay by five to six orders of magnitude and supports our argument that SRB populations are adapted to low pH rather than the presence of neutral microsites.

In previous studies, it was suggested that sulfate reducers are more susceptible to high VFA concentrations than methanogens (James et al., 1998), which might result in a shift from SR to methanogenesis at low pH (Koschorreck, 2008). A study on methanogenesis in a bioreactor at pH of 4.5 showed a 30% increase of methane yield as compared to neutral conditions, after a slow acclimation of the methanogens to lowered pH (Taconi et al., 2008). A competition between sulfate reducers and methanogens due to diluted sulfate concentrations by the vent outflow and higher availability of sulfate reducing community to low pH can be excluded as the abundance of Archaea as well as their diversity was low in white and brown zone sediments (Nitzsche, 2010; Price et al., 2013, this issue).

**THE EFFECT OF VOLATILE FATTY ACIDS ON SULFATE REDUCTION AT LOW pH**

Protonated short-chained fatty acids diffuse through the cell membrane and consequently act as protonophores and as uncoupling agents (Kell et al., 1981). This is not the case for their conjugate bases, which are excluded by their physical properties and charged head groups of lipids from the biological bilayer. Small fatty acids turn lipophilic under acidic conditions depending on their dissociation constant pK_a (Bruun et al., 2010). In this study, only the protonated fatty acid concentration at pH values of 3 to 7. The total concentration of protonated fatty acids of the VFA in the slurry is 4.3 mmol L^{-1} at pH of 3; 4.3 mmol L^{-1} at pH 4; 0.4 mmol L^{-1} at pH 5; 0.001 mmol L^{-1} at pH 6 and negligible for a pH 7 (0.004 mmol L^{-1}). In this study, only the protonated fatty acid concentration at pH values of 3 and 4 might become harmful to SRB (Baronofsky et al., 1984). This is consistent with our pH-experiment data in which the SRR of the VFA-amended slurry is lower than the rate measured in unamended sediment of white and transition zone sediment at pH < 5. At pH 6.4, the SRR measured after VFA addition to white zone sediment (0.3 nmol SO_4^{2-} cm^{-3} d^{-1}) exceeded the SRR (0.2 nmol SO_4^{2-} cm^{-3} d^{-1}) at a similar pH (pH 6.3) without addition. In brown zone sediment slurries, the limit for VFA stimulation was equal to unamended sediment at pH 5.8. The SRR at pH 6.2 of 2.3 nmol SO_4^{2-} cm^{-3} d^{-1} was stimulated by VFA amendment to a rate of 3.9 nmol SO_4^{2-} cm^{-3} d^{-1}. A SRR increase was also observed at neutral pH for brown zone slurries. SRR increased from 1.6 nmol SO_4^{2-} cm^{-3} d^{-1} without amendment to 2.3 nmol SO_4^{2-} cm^{-3} d^{-1} in amended slurries. Since VFA were added as a mixture to the slurries, no further differentiation between the individual fatty acids on SRR was possible. The presented results are consistent with studies on activity inhibition caused by organic acids at artificially lowered pH (Olesen et al., 2009) and fresh water seep systems (Bruun et al., 2010).

**THE EFFECT OF pCO2 ON SULFATE REDUCTION**

The content of CO_2 correlates with pH. Dissolved CO_2 reacts with water to form carbonic acid (H_2CO_3), which immediately dissociates to bicarbonate (HCO_3^-) and protons (H^+). HCO_3^- and H^+ can further dissociate to CO_3^{2-} and H^2O decreasing the pH in the solution. In this context, “carbonic acid” encompasses the species CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-} (Zeebe and Wolf-Gladrow, 2001). The existence of the different carbonic acid species depends on their concentrations, dissociation constants and pH as described by the Bjerrum plot (Zeebe and Wolf-Gladrow, 2001). In this study, the relation between pH and SRR relative to different pCO2 was examined. This is of particular interest in order to understand microbial functioning in CO_2-venturing sediments which are perfect natural laboratories for studies focused on CO_2 sequestration in which carbon dioxide is removed from the atmosphere and stored in sediments that should serve as long-term reservoirs (Shibata et al., 2008). After adjustment of CO_2 partial pressure to 0, 1, 2, and 3 bar, we found that a pCO2 increase resulted in different pH values among the sediment types. This may be a consequence of different chemical and biological buffering capacities of the sediment (Hejn et al., 1999). The mineral characteristics of the sediment (e.g., silicate, carbonate) and the bicarbonate content of the seawater dictate chemical buffering capacity of the sediment. The addition of CO_2 results in a decrease in pH (Zeebe and Wolf-Gladrow, 2001). SR produces bicarbonate alkalinity and consequently counteracts a decrease in pH. The data obtained from pCO2 incubation experiments provide a rough indication of the buffering capacity of the Paleochori Bay sediments: the brown zone sediment has the highest buffering capacity followed by white and finally transition zone sediments. This was also obvious from the amounts of phosphoric acid needed to decrease the pH in the pH experiments (data not shown).

Sulfate reduction rates responded to increase in pCO2 with a decrease in activity in nearly all experiments although effect of an increase in VFA from 2 to 3 bar on pH was negligible. A pCO2 increase from 1 to 2 bar reduced SRR from 0.2 to 0.1 nmol SO_4^{2-} cm^{-3} d^{-1} in the white zone sediment slurries, from 4.3 to 3.2 nmol SO_4^{2-} cm^{-3} d^{-1} in transition zone slurries and from 1.5 to 0.6 nmol SO_4^{2-} cm^{-3} d^{-1} for the brown zone sediment.
slurries. The increase in pCO₂ was accompanied by pH changes from 4.8 to 4.7, 4.8 to 4.6 and 5.5 to 5.4 in the three sediments, respectively. The effect of SR reduction on pCO₂ increase was even stronger when VFA were added to the slurries (Figure 5).

In our experiments we observed similar effects as in Debs-Louka et al. (1999) concluding that the reduction in pH due to increasing pCO₂ was not sufficient to account for antimicrobial activity. In this study microbial inactivation depended strongly on CO₂ partial pressure (tested for 15–55 bar), exposure time, the decomposition time and water content of the sample. A sudden increase in pCO₂ was observed to provoke cell ruptures and lead to reduced bacterial numbers (Debs-Louka et al., 1999). Additionally, it can be suggested that the content of CO₂ in the form of carbonic acid has a negative effect on the cell as it can permeate the membrane and dissociate in CO₃⁻ and H⁺ in the circumneutral cytoplasm, decreasing the intrinsic pH of the cell.

CONCLUSION

We observed a significant difference between SR in hydrothermally influenced and background sediments suggesting that microbial communities are adapted to low pH in the hydrothermal sediments of Milos. Sulfate reducing communities in hydrothermal sediments showed pH activity optima between 5 and 6. In contrast, SR in sediments with no hydrothermal influence exhibited pH optima between 6 and 7. The shallow-sea hydrothermal vent of Pal cosho Bay, Milos (Greece) with pH between 5 and 7, temperature range of 26–90°C and a high content of dissolved H₂S in the sediment pore water, represents an extreme environment in which SR may play an important role in the degradation of organic material. This study suggests that marine microbial SR communities that are specifically adapted to a life at high pCO₂ and low pH do exist.

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sulphate-reducing bacteria from Arctic sediments. Environ. Microbiol. 1, 497–507.

Koschorreck, M. (2008). Microbial sulphate reduction at a low pH. FEMS Microbiol. Ecol. 64, 328–342.

Lowe, S. E., Jain, M. K., and Zeikus, J. G. (1993). Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates. Microbiol. Rev. 57, 451–509.

Martin, A. (1990). Bioenergetic parameters and transport in obligate acidophiles. Biochim. Biophys. Acta 1018, 267–270.

Moss, S., and Harrison, T. L. (2006). Product inhibition by sulphide species on biological sulphate reduction for the treatment of acid mine drainage. Hydrochemistry 83, 214–222.

Møller, G., and Stams, A. J. (2008). The ecology and biotechnology of sulphate-reducing bacteria. Nat. Rev. Microbiol. 6, 441–454.

Nutikawa, K. (2010). Microbial Diversity of Ultrabasically Influenced Arsenic-Rich Sediments off the Coast of Miyake Island, Greece. thesis, TU Bergakademie, Freiberg, Germany.

Ottosen, L. D. M., Poulsen, H. V., Nielsen, D. A., Finster, K., Nielsen, L. P., and Revsbech, N. P. (2009). Observations on microbial activity in acidified pig slurry. Bioresour. Technol. 102, 291–297.

Perrin, D. D. (1972). Dissociation Constants of Organic Bases in Aqueous Solution London: Franklin Book Co.

Price, R. E., Savov, I., Planer-Friedrich, B., Bühring, S., Amend, J., and Pichler, T. (2012). Processes influencing extreme carbon enrichment in a shallow-sea hydrothermal fluid of Goupea, Greece. Geochem. Geophy. Geosys. doi: 10.1029/2012GC004881.

Price, R. P., Nitzsche, K., Lesniewski, R., Meyerdierks, A., Saltikov, C., Edwards, K., et al. (2013, this issue). Microbial diversity and arsenic metabolism in depth profiles from a shallow-sea hydrothermal vent near Milos Island (Greece). Environ. Microbiol. 13, 2633–2648.

Price, R. E., Savov, I., Planer-Friedrich, B., Bühring, S., Amend, J., and Pichler, T. (2012). Processes influencing extreme carbon enrichment in a shallow-sea hydrothermal fluid of Goupea, Greece. Geochem. Geophy. Geosys. doi: 10.1029/2012GC004881.

Schauer, R., Røy, H., Augustin, N., Gennerich, H. H., Peters, M., Wenzhoefer, F., et al. (2011). Bacterial sulfur cycling shapes microbial communities in surface sediments of an ultrasmall hydrothermal vent field. Environ. Microbiol. 13, 2633–2648.

Shitashima, K., Maeda, Y., Koike, Y., and Ohsumi, T. (2008). Natural analogue of the rise and dissolution of liquid CO2 in the ocean. Int. J. Gash. Geochem. Environ. 2, 95–114.

Sievert, S. M., Brinkhoff, T., Møller, G., Ziebis, W., and Finster, K. (1999). Spatial heterogeneity of bacterial populations along an environmental gradient in a shallow submarine hydrothermal vent near Milos Island (Greece). Appl. Environ. Microbiol. 65, 3836–3842.

Taconni, K. A., Zappi, M. E., French, W. T., and Brown, L. R. (2008). Methanogenesis under acidic pH conditions in a semi-continuous reactor system. Bioresour. Technol. 99, 8075–8081.

Widdel, F. (1988). “Microbiology and ecology of sulfatereducing bacteria,” in Biology of Anaerobic Microorganisms ed. A. J. B. Zeikus (New York: John Wiley), 469–586.

Zeebe, R. E., and Wolf-Gladrow, D. (2001). CO2 in Seawater: Equilibrium, Kinetics, Isotopes. Amsterdam: Elsevier Oceanography Book Series.