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ORIGINAL PAPER

Continuous enrichment culturing of thermophiles under sulfate and nitrate-reducing conditions and at deep-sea hydrostatic pressures

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Abstract A continuous culture bioreactor was developed to enrich for nitrate and sulfate reducing thermophiles under in situ deep-sea pressures. The ultimate objective of this experimental design was to be able to study microbial activities at chemical and physical conditions relevant to seafloor hydrothermal vents. Sulfide, sulfate and oxide minerals from sampled seafloor vent-chimney structures [East Pacific Rise (9°46′N)] served as source mineral and microbial inoculum for enrichment culturing using nitrate and sulfate-enriched media at 70 and 90°C and 250 bars. Changes in microbial diversity during the continuous reaction flow were monitored using denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rRNA gene fragments. Time series changes in fluid chemistry were also monitored throughout the experiment to assess the feedback between mineral–fluid reaction and metabolic processes. Data indicate a shift from the dominance of *epsilon Proteobacteria* in the initial inoculum to the several *Aquificales*-like phylotypes in nitrate-reducing enrichment media and *Thermodesulfobacteriales* in the sulfate-reducing enrichment media. Methanogens were detected in the original sulfide sample and grew in selected sulfate-enriched experiments. Microbial interactions with anhydrite and pyrrhotite in the chimney material resulted in measurable changes in fluid chemistry despite a fluid residence time only 75 min in the reactor. Changes in temperature rather than source material resulted in greater differences in microbial enrichments and mediated geochemical reactions.

Keywords Thermophiles · *Aquificales* · In situ pressure · Continuous culture

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Introduction

Deep-sea hydrothermal vents represent one of the most physically and chemically diverse habitats for microorganisms on Earth. Much of the initial studies at deep-sea vent ecosystems were focused on the endosymbionts associated with the invertebrates that colonize these deep-sea oases (Van Dover 2000, for review). However, the high temperature porous sulfide structures ('chimneys') that precipitate as a result of high temperature vent fluids mixing with cold seawater are ideal habitats for a wide range of physiological and phylogenetic diversity of thermophiles (Reysenbach et al. 2001; Takai and Fujiwara 2002). Both culture-dependent and culture-independent molecular phylogenetic approaches for diversity assessments are providing an inventory of the microbial diversity at deep-sea vents (e.g. Takai and Horikoshi 1999; Reysenbach et al. 2000, 2002; Takai et al. 2001; Huber et al. 2002b; Nercessian et al. 2003; Schrenk et al. 2003; Nakagawa et al. 2005a, b). However, these studies can only infer the geochemical impact that these microbes

have on the geology, geochemistry and ecology of hydrothermal vent ecosystems at the seafloor and in the subseafloor. Very few studies have attempted to explore the effects of microbial growth and metabolism of deep-sea vent microbes on geochemical processes at hydrothermal conditions relevant to seafloor vent systems (Marteinsson et al. 1999; Edgcomb et al. 2004; Lloyd et al. 2005). One approach to accomplish this is by deploying in situ devices at vents and within chimney structures on the seafloor (Alain et al. 2004; Reysenbach et al. 2000; McCliment et al. 2006; Nakagawa et al. 2005b; Takai et al. 2004a, b). These novel approaches are providing significant insights into the diversity and rates of succession and colonization, but do not effectively address how the microorganisms interact with and potentially change the geochemical and mineralogical substrate.

Simulating the actual physical and chemical conditions of seafloor hydrothermal systems in the laboratory is clearly challenging (Seewald et al. 1994; Seyfried et al. 1999). In particular, hydrothermal experiments must be designed to withstand high fluid pressures (up to 500 bars) and permit time series monitoring of changes in the composition of fluids coexisting with complex mineral assemblages at a wide range of reaction temperatures. Microbial growth experiments conducted at these simulated hydrothermal conditions, however, may increase our understanding of microbial diversity and biogeochemical cycles at mid-ocean ridges.

Closed system experiments have been conducted at hydrothermal conditions for several decades (Thornton and Seyfried 1987; Seyfried and Ding 1993) and flow-through devices have also been designed to study this environment (Jannasch et al. 1996). The experimental apparatus used in this study was designed to promote the growth of microbial populations at temperatures below 100°C and pressures of approximately 250 bars, while permitting time series monitoring of changes in fluid chemistry and microbial diversity. Although these initial experiments were designed to enrich for nitrate-reducing and/or sulfate-reducing thermophiles, this application is an effective tool to determine if differences in microbial communities caused by factors such as temperature, fluid chemistry, and chimney mineralogy result in corresponding changes in fluid–mineral reactions. Moreover, this type of apparatus may be used in future efforts to isolate novel organisms at high-pressure, variable conditions of fluid flow, and for time scales necessary to better study the formation of mineralogical biosignatures that could be preserved in the rock record.

Materials and methods

Experimental apparatus

An HPLC pump and linked ‘‘separator’’ continuously provided fluid reactants and dissolved gases at high pressure to columns made of PEEK® or ‘‘bioreactors’’ (0.75 cm id × 10 cm length) (4.41 cm^3 volume), which were maintained at a constant temperature by a series of band heaters external to the reactor and enclosed in an insulted tube furnace (Fig. 1). Four Watlow time-proportioning controllers provided temperature control in response to input from a series of thermocouples (type K) directly attached to the reactors in the furnace. The bioreactors were composed of PEEK® for chemical and physical stability. With the exception of flow control valves (Fig. 1), which were made from 316 stainless steel, all wetted parts of the fluid delivery and control system were composed of PEEK®, although buna o-rings were used as pressure seals throughout. Moreover, all tubing, PEEK® reactors and stainless valves were replaced between experiments to minimize contamination effects.

Fluid samples could be taken throughout experiments by activating manual and computer-controlled valves, which not only diverted sample fluid into the requisite gas-tight containers for processing, but also maintained the system at the desired pressure.

Reactants

DSRV *Alvin* collected samples of actively venting chimney structures at ‘L vent’ (sample 3753-1; maximum temperature at orifice 348°C, outer surface temperature 15°C) and at marker 19 (sample 3760-4; maximum temperature at orifice 351°C, outer surface temperature 114°C) on the East Pacific Rise (9°46′N). The first four digits of the sample number refer to the *Alvin* dive number during which the sample was obtained. In order to minimize contamination from ambient water, chimney samples were placed in the ‘biobox’ (insulated container in the submersible’s basket) during ascent to the sea surface.

Immediately after the chimney samples were recovered, a sub-sample was disaggregated and packed into a PEEK® bioreactor to be used for subsequent experiments. The remaining chimney material was stored in N_2-flushed gas-tight glass bottles sealed with butyl-rubber stoppers. Samples for mineralogical and geochemical analyses were stored at –20°C to inhibit oxidation of sulfide minerals.

The chimney material-filled PEEK® bioreactors were placed in line with the separator (Fig. 1), which

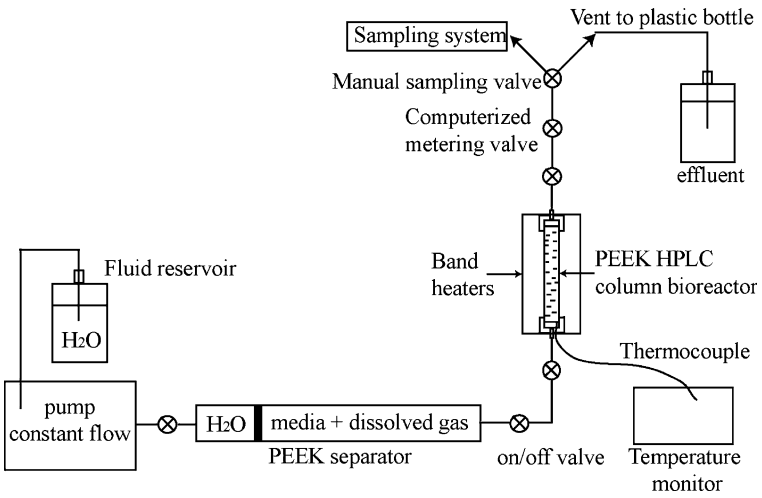

Fig. 1 Schematic drawing of the flow-through apparatus designed for this study. All parts were constructed of PEEK plastic, with the exception of the control-valves that were composed of stainless steel. The bioreactor in line with the fluid-delivery system contains the sulfide-chimney material used for the experiments. Two thermocouples were attached to the reactor, which in conjunction with a fully automated control system, maintained temperature and pressure within very narrow limits during the 100–180 h experiments

contained sterile media and dissolved gas. The media was composed largely of an artificial seawater solution (Table 1). The media, however, was further modified to facilitate two different microbial metabolic pathways: (1) nitrate reduction; and (2) sulfate reduction. Thus, in the first case (nitrate reduction) 20 mM $NaNO_3$ was added to the stock media, while excess dissolved sulfate and thiosulfate (Table 1) were added to media for experiments designed to better investigate metabolic processes involving sulfate reduction pathways.

Table 1 Major dissolved component concentrations of artificial seawater-based media used as the source fluid for experiments

Component	Component concentration (mM)
NaCl	499
KCl	6.7
$MgCl_2$	12.4
$CaCl_2$	2.63
NH_4Cl	3.7
$MgSO_4$	58
K_2HPO_4	0.99
Nitrate-enriched media+ $NaNO_3$	20
Sulfate-enriched media+ $MgSO_4$	58
$Na_2S_2O_3$	12.7

The pH of the synthetic seawater was adjusted to a value slightly above neutrality (pH~8) and purged with CO_2 (g) prior to experiments to achieve desired alkalinity. 10 ml/kg trace element solution (Boone et al. 1989) was added to assist microbial growth

The media was prepared under anaerobic conditions and similar conditions were maintained by bubbling CO_2 and H_2 through the media while filling the separator, which, by piston action, was used to achieve the high-pressure conditions needed for the experiments (Fig. 1). More importantly, the separator allows appropriate levels of gas to be dissolved in the media by filling a headspace with gas prior to fully pressurizing the system. Accordingly, the media used for all experiments was saturated with dissolved CO_2 and contained approximately 2.2 mM H_2. Water delivered at high pressure to the input side of the piston in the separator assured complete dissolution of the gas in the media, while pressurizing the media to the operational condition of 250 bar.

Experimental conditions and procedures

Circulation of fluid (gas-charged media) through the microbe-bearing chimney material at a constant pressure, temperature and fluid flow rate characterize the most basic elements of the operational experimental procedure. All experiments were performed at 250 bar, with temperatures of 70 or 90°C. Most experiments were performed at 70°C, however, with abundant chimney material recovered during Alvin dive 3573 (see above and Table 3). All experiments were conducted at a fluid flow rate of 0.03 ml/min. Thus, considering the volume of the reactor (4.41 cm^3), together with an assumed porosity of the contained chimney material of 50%, the residence time of fluid in the

reactor during the experiments was approximately 75 min.

The overall length of experimental run times ranged from approximately 100–180 h, and fluid samples were intermittently taken throughout. At sampling intervals, fluid from the bioreactor was allowed to enter previously sterilized, sealed and evacuated, and then slightly over pressured (N_2) Hungate tubes. These tubes were attached to the sampling valve by means of a syringe needle and removed immediately after the requisite volume of sample fluid had been obtained.

Reacted chimney minerals (sulfides) from the bioreactor following an experiment were extruded under anaerobic conditions by piston action into a sterile plastic tube, which allowed the original orientation of material during the experiment to remain intact. A representative sub-sample of material was taken from portions of the 'core' for molecular analysis and stored at –80°C. The remaining material was transferred to a gas-tight N_2-flushed Hungate tube and stored at –20°C for subsequent chemical analysis.

Geochemical analysis

Dissolved gas and coexisting aqueous species in fluid samples from experiments were analyzed as soon as possible. Gas from the headspace of the Hungate tubes was extracted and measured for CO, CO_2, CH_4, H_2, O_2, and N_2 by gas chromatography (Perkin Elmer 7600), using flame ionization and thermal conductivity detectors. Anions (Cl^-, $SO_4^=$, $S_2O_3^=$, NO_2^-, NO_3^-) and NH_4^+ were measured by ion chromatography (Dionex), cations (Fe, Cu, Zn) were analyzed by a ICP-MS (Thermo-elemental PQ Exell). Total dissolved sulfur was measured by ion chromatography following conversion to $SO_4^=$ by heating sample aliquots during reaction with excess hydrogen peroxide. Total dissolved sulfide was determined by summing sulfur oxyanions ($SO_4^=$, SO_3^-, and $S_2O_3^=$), and then subtracting from the concentration of total dissolved sulfur.

The stored solids from each experiment were analyzed by X-ray diffraction to determine mineralogical changes relative to previously examined starting chimney samples. Chemical analysis of the starting and reacted chimney material was conducted by ICP-MS following digestion in concentrated aqua regia.

Analytical uncertainties in the reported concentrations of aqueous species and composition of chimney components are estimated from duplicate measurements of samples and standards to be approximately ±2%, although uncertainties increase with decreasing concentration of analytes.

DNA extraction and PCR amplification of the 16S rRNA gene for denaturing gradient gel electrophoresis (DGGE)

Genomic DNA was extracted using the Ultraclean Soil DNA extraction kit according to the manufacturer's protocol (Mo Bio Laboratories, Solana Beach, CA, USA). DNA was stored at –20°C in 50 µl of 10 mM Tris–HCl, pH 8.0.

Fragments of the 16S rRNA gene were amplified using the 338F-GC clamp forward primer (5′-CGCC CGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCG CCCTCCTACGGGAGGCAGCAG-3′) and the reverse primer 519R (5′-ATTACCGCGGCTGCTGG-3′) for Bacteria, and the 344F-GC clamp forward primer (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGC CCCCGCCACGGGGCGCAGCAGGCGCGA-3′) and 744RA (5′-CCSGGGTATCTAATCC-3′) for Archaea. Community 16S rDNA was amplified from genomic DNA samples in a PCR mixture (50 µl) containing 5 µl of 10× Promega Mg-free PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.02 nM of each primer, and 1 U of Taq DNA Polymerase (Promega). Thermal cycling conditions were: 2 min at 94°C, followed by 35–40 cycles of 20 s at 94°C, 20 s at 50°C, and 45 s at 72°C and a final 10 min at 72°C.

PCR products were resolved on a denaturing gradient (30–70% urea/formamide gradient; 100% denaturant = 40% (vol/vol) formamide and 7 M urea) 6% acrylamide gel for 3.5 h (for 519R) or 4.5 h (744RA primers) at 200 V and 60°C in 1× TAE buffer (0.04 M Tris base, 0.02 M acetate, and 1.0 mM EDTA). Gels were stained for 30 min in SYBRGreen/1× TAE (1:20 000) and de-stained in 1× TAE for 10 min.

Sequencing of DGGE bands

Individual bands of DNA were touched with a sterile pipette tip and placed in 30-µl filter sterilized 10 mM Tris (pH 8) for 5 min. The Tris–DNA mixture (6 µl) was used as template for PCR using the PCR conditions described above. PCR products were purified using the Ultraclean PCR spin purification kit (MoBio) following the manufacturer's instructions. Purified re-amplified bands were sequenced using the Big Dye Terminator Cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol. Sequences obtained from bacterial and archaeal bands were compared to the GenBank sequence database using BLAST to identify the closest relative. The sequences were submitted to GenBank and are numbers DQ409062–DQ409076.

Results and discussion

Enrichment and growth of thermophiles was accomplished under elevated conditions of temperature and pressure in the flow through bioreactor at four different conditions using two different samples as initial source material. Chimney sample from dive 3753 was composed of 10.4% Ca and 26.5% Fe, together with minor amounts of Cu and Zn (1.8 and 5.4%, respectively). X-ray diffraction revealed gypsum, anhydrite, pyrite and pyrrhotite, with minor sphalerite and wurzite (ZnS). Sample 3760 was from a ''beehive-like'' structure (a spongy sulfide rock, resulting in diffuse flow with no major conduits for fluid flow) and contained 36.5% Fe, 21.8 wt% Zn, and only 1.1 wt% Cu. X-ray diffraction analysis confirmed the coexistence of pyrite, pyrrhotite and sphalerite, but no anhydrite, in keeping with the chemical data.

The mineralogical differences between the chimney samples were not manifested by differences in microbial diversity (Table 3). Although DGGE may not reveal all the complexity of the diversity (Muyzer and Smalla 1998; Moeseneder et al. 1999), similar observations were made in a detailed study of the phylogenetic diversity of chimneys along the East Pacific Rise (Reysenbach and Von Damm, unpublished data). Furthermore, we were not able to obtain sequence information for minor bands. However, as a goal of this study was to monitor the major shifts in the community structure under the growth conditions in the reactor, DGGE analysis provided a rapid means to follow these shifts. The initial microbial communities in both samples delineated by DGGE patterns were dominated by epsilon-proteobacterial components, one of the most predominant free-living microbial groups at deep-sea hydrothermal vents (Longnecker and Reysenbach 2001; Campbell et al. 2006). The archaeal diversity was surprisingly low, although typical inhabitants of sulfides, specifically thermophilic methanogens, heterotrophic sulfur reducers and uncultured deep-sea hydrothermal vent Archaea closely related to DHVE groups (Takai and Horikoshi 1999; Reysenbach and Shock 2002) were detected.

Unfortunately, it was not possible to run a negative control. Autoclaving the substrates changed the composition of the starting material so it was no longer comparable to the biological experiments. However, it was clear in biotic experiments that none of the chemical changes that were observed could occur by abiotic reactions alone.

Initial assemblage as a variable

Two experiments conducted at 70°C, in which dissolved sulfate and thiosulfate were enriched in the source fluid (medium), resulted in essentially the same cultures, even though the initial chimney material was different (3753III and 3760IV). Both cultures of microbial communities significantly altered the resulting fluid chemistry over time, creating very similar fluid compositions despite the difference in mineralogy and initial biodiversity.

Table 2 Experimental conditions and overview of molecular results

	Sample/experiment	# Bands Archaea	Sequences of major bands (Archaea)	# Bands bacteria	Sequences of major bands (bacteria)
3753	Original	3	Clone PS-A4 (94%, Page et al. 2004), clone FZ2bA42 (99%, Schrenk et al. 2003)	10	All epsilon proteobacteria most closely related to *Hydrogenimonas thermophila* and *Sulfurovum lithotrophicum* (98–100%)
3760	Original	2	*Thermococcus atlantis* (96%), *Methanocaldococcus* sp. Mc-S-85 (96%)	7	All epsilon proteobacteria most closely related to *Hydrogenimonas thermophila*, *Sulfurovum lithotrophicum* (98–100%) or the clone FT17B33 (Hoek et al. 2003)
3753-I	70°C, nitrate reducing	ND	ND	4	Aquificales: *Persephonella marina*, 100%; *Desulfurobacterium*, 86%; Aquificales Ob6, a relative of *Hydrogenivirga* (96%, Eder and Huber 2002)
3753-II	90°C, nitrate reducing	2	Clone PS-A4 (DHVE3, 94%, Page et al. 2004), pEPR119 (DHVE5, 98%, Nercessian et al. 2003)	3	Aquificales Ob6, a relative of *Hydrogenivirga* (97.6%, Eder and Huber 2002), *Aquifex* sp. NS85-1 (94%, Nakagawa et al. 2005b)
3753-III	70°C, sulfate reducing	ND	ND	5	*Thermodesulfatator indiensis* (90%)
3760-IV	70°C, sulfate reducing	1	*Methanocaldococcus vulcanius* (99%)	4	*Thermodesulfatator indiensis* (90%)

Percentage sequence similarity is provided in parentheses. Citations are provided for sequences without a described close relative in culture. Only good quality sequences (100–130 nucleotides) were used in the BLAST analysis

ND not detected

Table 3 Temperature, media enrichment, and initial primary mineralogy and composition of chimney samples used for microbial growth experiments

	3753-I	3753-II	3753-III	3760-IV
Temperature (°C)	70°	90°	70°	70°
Media-enrichment	Nitrate	Nitrate	Sulfate	Sulfate
Chimney mineralogy	An, Py, Po, Sph	An, Py, Po, Sph	An, Py, Po, Sph	Py, Po, Sph
Chimney composition	10.4% Ca and 26.5% Fe	10.4% Ca and 26.5% Fe	10.4% Ca and 26.5% Fe	36.5% Fe, and 21.8% Zn
Duration (h)	180	180	110	95

All experiments were conducted at 250 bar

An anhydrite, *Py* pyrite, *Po* pyrrhotite, *Sph* sphalerite

Microbial diversity

In both cases, the sequence of the dominant band in the DGGE gel was distantly related (89–90% similarity) to the deeply branching deep-sea vent SO_4-reducer *Thermodesulfatator* (Moussard et al. 2004), suggestive of a new genus in this sulfate-reducing lineage (Table 2). In this same experiment, the methanogen *Methanocaldococcus vulcanius* was also detected.

Microbially mediated fluid–mineral reactions

The similarity in geochemical changes during these two incubation studies may be caused by the common dominance of the *Thermodesulfatator* relative in both experiments. For example, both experiments showed clear evidence of $SO_4^{=}$ and thiosulfate reduction, and H_2S(aq) gain (Fig. 2). The increase of approximately 30 mM Ca during fluid reaction with chimney sample 3753 implicates anhydrite dissolution as an added source for sulfate reduction, while the sulfate-bearing media serves this purpose during fluid reaction with the anhydrite-free sample 3760 (Table 3, Fig. 2). The magnitude of sulfate reduction during both experiments (~15 mM $SO_4^{=}$), however, was about the same (Fig. 2). Thus, in spite of the abundant presence or complete lack of anhydrite in the chimney material, microbial respiration facilitated sulfate/thiosulfate reduction processes. In general, sulfate and thiosulfate reductions can be depicted by the following reactions:

$$\text{Pyrrhotite} + 0.375\,\text{H} + +0.1875\,\text{SO}_4^{--} = 0.25\,\text{Hematite} + 0.5\,\text{Pyrite} + 0.1875\,\text{H}_2\text{S}_{(\text{aq})} \quad (1)$$

and,

$$\text{Pyrrhotite} + 0.4\,\text{H} + +0.2\,\text{S}_2\text{O}_3^{--} = 0.2\,\text{Hematite} + 0.6\,\text{Pyrite} + 0.2\,\text{H}_2\text{S}_{(\text{aq})} \quad (2)$$

Pyrrhotite is used here as the primary reducing agent owing to the relative abundance of this mineral in the chimney material. Although dissolved Fe^{++} can also serve this purpose, the limited solubility of aqueous Fe-bearing species during the experiment implicates an Fe-bearing mineral. Furthermore, it has been recognized from in situ incubation studies (Edwards et al. 2003) that pyrrhotite and other Fe-monosulfide minerals provide an unusually effective substrate for microbial growth and colonization, which in turn enhances mineral dissolution along cryptographically favorable regions on the mineral surface. Although these studies largely involved chemical and physical conditions favorable for Fe-oxidizing bacteria at the chimney-seawater interface, the effectiveness of mineral surfaces in facilitating microbial metabolism and growth is almost certainly relevant to a wide range of chemical and biological systems.

It is clear from reactions 1 and 2, however, that the combined effects of sulfate and thiosulfate reduction would require the generation of approximately an equivalent amount of H_2S, something that was not observed in the fluid chemistry (Fig. 2), although non-stoichiometric effects involving mineral reactants and products could account for the relatively small discrepancy. Reactions such as these may also play a role in limiting the solubility of dissolved Fe, as observed (Fig. 2). Unfortunately, owing to the abundant presence of pyrite and pyrrhotite in the chimney material, it is difficult to confirm or rule out these reactions using routine analytical approaches that were available to us (X-ray diffraction). In any event, the overall changes in fluid chemistry in the sulfate-enriched media at 70°C suggest that a S-utilizing metabolism involving $SO_4^{=}$ or $S_2O_3^{=}$ is possible. Both experiments showed clear evidence of $SO_4^{=}$ and thiosulfate reduction, and H_2S(aq) gain (Fig. 2), which is largely (if not completely) lacking when nitrate is the primary electron acceptor (see below). Thus, regardless of the presence or lack of anhydrite in the chimney material, microbial respiration facilitated the sulfate/thiosulfate reduction processes. It is perhaps necessary to emphasize that in the absence of microbially induced processes, sulfate reduction at temperatures less than 100°C requires

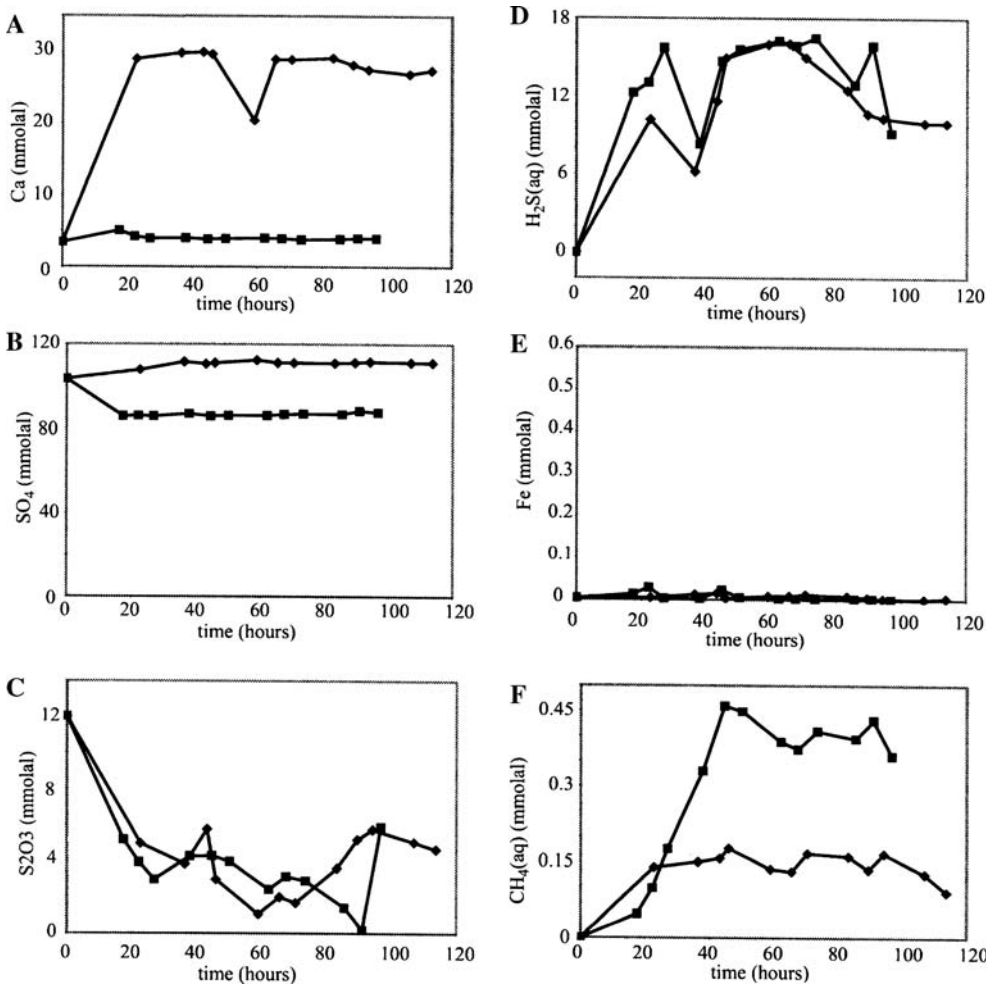

Fig. 2 Comparison of the change in fluid chemistry with time between experiments at 70°C involving media enriched in sulfate coexisting with different chimney-bearing mineral assemblages (3753-I, *diamonds*) and 3760-IV (*squares*). In particular, sample 3760 lacked anhydrite (see text)

time scales on the order of 10^4 years (Ohmoto and Lasaga 1983; Machel 2001).

The relatively high dissolved methane concentrations in both of these experiments (Fig. 2), is consistent with the observed presence of the methanogen, *Methanocaldococcus*. Once again, however, it is important to recognize that the growth of methanogens is likely constrained by the limited availability of dissolved H_2 (~2 mM). Thus, that the observed methane concentration did not exceed 0.5 mM (Fig. 2) is consistent with the predicted stoichiometry of methanogenesis fueled by dissolved H_2 and CO_2.

Temperature as a variable

Two experiments using the nitrate-enriched medium were conducted to investigate how a thermophilic community (enriched at 70°C) differs from a hyperthermophilic community (enriched at 90°C) in its resulting biogeochemical signature, given that all other variables are constant. The flow through conditions of these experiments enriched for different microbial communities that resulted in different geochemical reactions. Again, any abiotic geochemical reactions were masked by the microbially mediated fluid–mineral reactions, which in some cases provide evidence of microbial utilization of mineral substrates as electron donors.

Microbial diversity

After approximately 180 h of reaction at 70°C in the nitrate-enriched media (3753-I) (Table 3), several Aquificales related to the genera *Persephonella, Hydrogenivirga* and *Desulfurobacterium* were detected (Table 2). Both *Persephonella* and *Desulfurobacterium* are widespread at deep-sea vents, whereas *Hydrogenivirga* spp. were first isolated in shallow marine vents in Dijbouti (Eder and Huber 2002) and subsequently on the coast of Japan (Nakagawa et al. 2004). Thus, our report expands the distribution of this genus to deep-sea vents. *Persephonella* spp. and *Hydrogenivirga* spp.

can grow by hydrogen oxidation, sulfur oxidation or nitrate reduction (Gotz et al. 2002; Nakagawa et al. 2004). The Desulfurobacteriaceae generally reduce nitrate or sulfur (L'Haridon et al. 1998; Huber et al. 2002a).

At 90°C (3753-II), two different Aquificales were detected, one related to *Hydrogenivirga* and the other related to *Aquifex* sp. NS85-1. The hyperthermophilic Aquificales strain is most closely related to *A. aeolicus* that is unable to reduce nitrate (Deckert et al. 1998) (Table 2). The archaeal sequences that were detected do not have any close representatives in culture, although phylotype pEPR119 is most closely related to the methanogen *Methanotorris* (Burggraf et al. 1990; Takai et al. 2004a, b; Table 2).

Bacterial NO_3 reduction

At 70°C in nitrate enriched media, members of the Aquificales are capable of reducing nitrate in the absence of O_2(aq) (Huber et al. 1992; Reysenbach et al. 2001), which is consistent with the observed changes in fluid chemistry during the experiment (3753-I). After 70 h of incubation, for example, dissolved nitrate was quantitatively removed from solution and remained below detection for the remainder of the experiment, despite its continuous renewal by fluid recharge (Table 1, Fig. 3). Although changes in fluid chemistry at 90°C were similar, the extent of nitrate reduction was much less than at 70°C (Fig. 3), with nitrate reduction at 90°C (3753II) limited to approximately 2 mM. Since only one organism (*Hydrogenivirga*) capable of nitrate reduction was enriched at the higher temperature, it is not surprising that the rates of nitrate reduction were so low. The cultured isolates of *Hydrogenivirga* have an optimum temperature for growth at approximately 75°C, pH 7 (Eder and Huber 2002; Nakagawa et al. 2004), accounting perhaps for the temperature dependent variability in its growth observed during the present experiments.

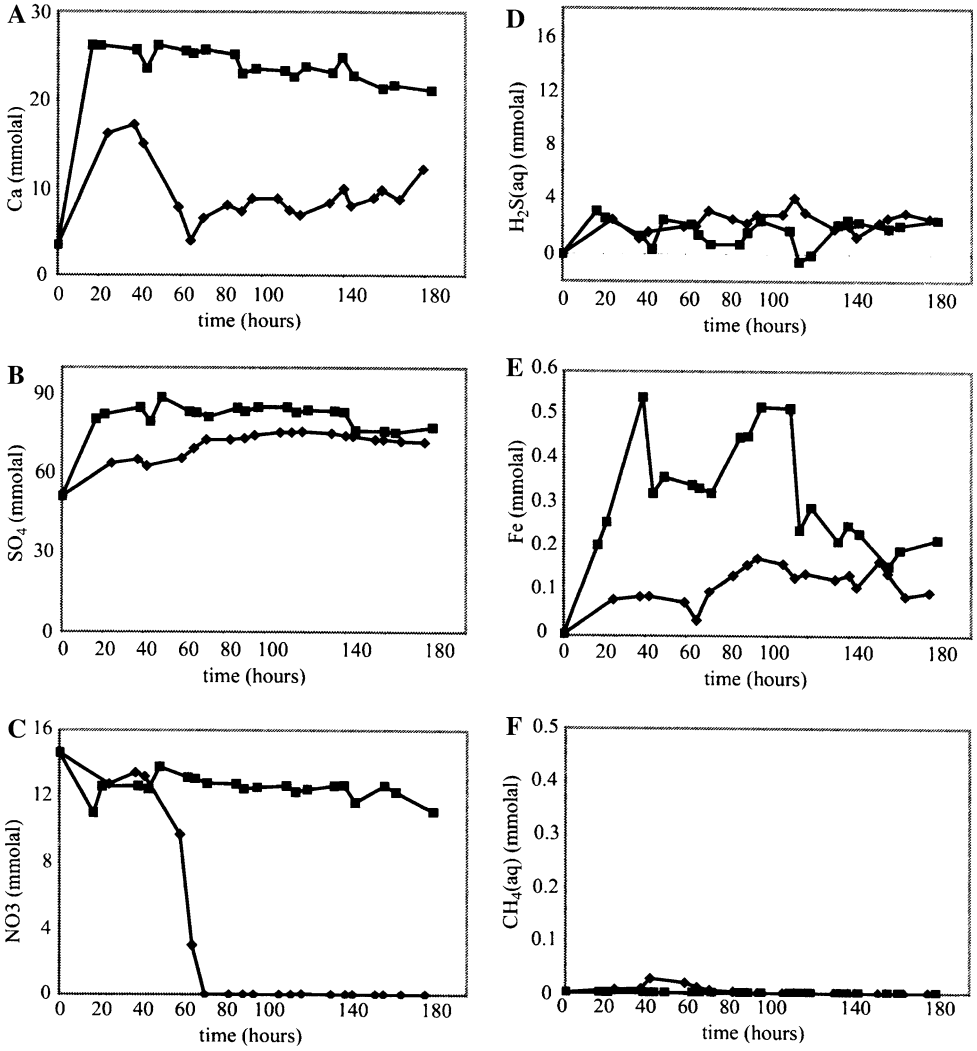

Fig. 3 Comparison of the change in fluid chemistry with time between experiment 3753-I (*diamonds*) (70°C) and 3753-II (*squares*) (90°C). Both experiments contained excess nitrate in the starting fluid

These results are consistent with the fact that although sulfate was present in the system, under the excess nitrate the microbial communities were dominated by H_2-oxidizing, nitrate-reducers, who would outcompete other H_2-utilizing metabolisms such as sulfate-reducers and methanogens due to their higher affinity for and catalytic velocities of H_2.

Archaeal methanogenesis

A small amount of methane (<0.025 mM) was produced between 40 and 70 h in the 70°C experiment (3753-I) (Fig. 3). Even though at 70°C we did not detect any archaeal 16S rRNA genes, it is possible that a methanogen was present but below our detection limits of DGGE and out competed by the Aquificales in this experiment. Likewise, since a potential methanogen, *Methanotorris*-like pEPR119, (Burggraf et al. 1990; Takai et al. 2004a, b) was detected in the 90°C experiment (3753II), this organism had to compete with the Aquificales for the limited availability of dissolved H_2. Furthermore, dissolved H_2 was reduced to a level below detection in both experiments in spite of the continuous addition to the reactor from the source fluid, suggestive that it was rapidly utilized by the microbes in the reactor.

Microbially mediated pyrrhotite oxidation at 70°C

The amount of sulfate produced at both 70 and 90°C exceeded the amount that could be contributed by anhydrite dissolution, as suggested by corresponding changes in dissolved Ca concentrations (Fig. 3). The decrease in dissolved Ca at 70°C after approximately 35 h of incubation, however, may indicate formation of calcite or aragonite, the only minerals that could account for Ca removal in the CO_2-saturated fluids. Accordingly, to better gauge the net increase in sulfate during the 70°C experiment we used the compositional trends subsequent to approximately 55–60 h of incubation. These data indicate a release of sulfate in excess of Ca of approximately 8 mM (Fig. 3), which implicates microbial metabolism through sulfide mineral oxidation (Fig. 3). Thus, the overall microbially mediated nitrate reduction process that resulted in sulfide oxidation can be illustrated, as follows:

$$NO_3^- + 1.1\,\text{Pyrrhotite} + 0.5\,N_{2(aq)} + 0.33\,\text{Hematite} + 0.30\,\text{Pyrite} + 0.5\,SO_4^{--} \quad (3)$$

This reaction illustrates a 2:1 nitrate/sulfate ratio, which is close to that observed (Fig. 3). Moreover, considering the abundant presence of pyrrhotite in the chimney material used for the experiment, this phase may serve as the reducing agent for nitrate reduction once the limited availability of dissolved H_2 is exhausted, thus leading to the prediction of hematite and pyrite formation as replacement products. Fluid chemical data indicate the absence of nitrite, while NH_4^+ was unchanged from initial media values. As the fluid samples were stored under a N_2 atmosphere, and air contamination during sample processing could not be entirely avoided, the measured N_2 could not be used to test unambiguously the stoichiometry predicted by reaction (3).

A reaction somewhat analogous to (3) has been documented in cultures of sulfide-oxidizing tube worm symbionts (Hentschel and Felbeck 1993). In this case, however, dissolved sulfide (H_2S) and H_2 clearly play a role in the nitrate reduction process. Here an additional electron donor is necessary owing to constraints imposed by the magnitude of change in dissolved nitrate (~15 mM) compared to the limited availability of dissolved H_2 and H_2S (see above and Fig. 3). Interestingly, there have been reports of some sulfate reducers such as *Desulfovibrio propionicus*, functioning in an unusual way by growing chemoautotrophically, while fueled by sulfide oxidation and nitrate reduction (Dannenberg et al. 1992; Cypionka 2000). Furthermore, in the warm fluids within the Ocean Drilling Programs deep-sea borehole ODP 1026B, the dominant clones recovered were nitrate reducers and sulfate reducers (Cowen et al. 2003). Here too, the levels of sulfide were lower than expected, and the authors predict that perhaps microbial sulfide oxidation was coupled to nitrate reduction, resulting in very low or undetected sulfide levels. Likewise, it is very possible that our enriched strains of *Persephonella, Hydrogenivirga* and *Desulfurobacterium*, while reported to reduce nitrate in batch laboratory cultures, can also couple this to sulfide oxidation. At this point one can only speculate on the extent to which these Aquificales contributed to the observed changes in fluid chemistry here or in related natural systems, but its existence is still compelling.

Microbially mediated iron reduction at 90°C

The most noticeable difference between the 70 and 90°C experiments, however, was the relatively high dissolved (Fe^{++}) concentration at 90°C, which is difficult to attribute entirely to inorganic processes at the conditions of the experiment; that is, a difference in reaction temperature of 20°C is unlikely to influence phase (mineral–fluid) relations sufficiently to account for the relatively large differences in dissolved Fe

between the two experiments. Iron reducing hyperthermophiles have been isolated from deep-sea vents (Kashefi et al. 2002; Kashefi and Lovley 2003), yet these are not related to the organisms we detected in our experiment. Although physiological and metabolic inferences need to be made with caution from phylogenetic data, several of the terrestrial members of the Aquificales are capable of iron reduction (Takai et al. 2003; Aguiar et al. 2004). It is therefore, possible that the enriched *Aquifex* or *Hydrogenivirga* may be able to reduce iron. Additionally, two archaeal phylotypes (bands) were detected whose sequence fell within clades without cultured relatives, namely DHVE3 and DHVE5 (Takai and Horikoshi 1999; Nercessian et al. 2003). The DHVE5 may be related to methanogens, but the DHVE3 have no known close relatives in culture. Hence it is tempting to speculate that these Archaea may be hyperthermophilic iron reducers that influenced the iron chemistry in this experiment. Many Archaea have been shown to reduce iron (Vargas et al. 1998) and it would indeed be interesting to enrich for iron reducers from this sample.

Conclusions

A first order observation from the present series of experiments is the over-riding importance of the nature of the electron acceptor (dissolved or mineral-associated) in the direction and magnitude of microbially induced geochemical processes. For example, methanogenesis appears to be limited in the presence of dissolved nitrate, being out-competed by sulfide oxidation pathways, but is not under sulfate-rich conditions. Microbial metabolism has also been shown to significantly alter fluid chemistry even in a flow through environment such as may be present within the walls of hydrothermal chimney structures or the surrounding subsurface. Regardless of the microbial mediated process that occurred during the present experiments, however, the geochemical response was not only immediate, but also exceeded by orders of magnitude changes predicted from constraints imposed by theoretical phase relations.

When the geochemical data are combined with simultaneous observations of microbial activity, the bioreactor flow system described here provides a very effective tool to investigate hydrothermal systems at temperatures, pressures and chemical conditions highly relevant to seafloor chimney deposits and their associated microbial communities. Although these initial experiments were designed to enrich for thermophilic organisms having specific microbial functions, which could be constrained by time series changes in solution chemistry, future efforts using the flow-through reactor described here may help to isolate novel organisms at elevated temperatures and pressures, while taking explicit account of constraints imposed by the chemistry of diffuse-flow vent fluids. Experiments performed under these conditions may simulate more precisely the role of fluid chemistry on the feedback between microbes and coexisting minerals, with clear implications for the determination of quantitative geochemical and microbiological processes difficult to obtain by other means.

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