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ORIGINAL ARTICLE

Functional diversity of bacteria in a ferruginous hydrothermal sediment

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A microbial community showing diverse respiratory processes was identified within an arsenic-rich, ferruginous shallow marine hydrothermal sediment (20–40 °C, pH 6.0–6.3) in Santorini, Greece. Analyses showed that ferric iron reduction with depth was broadly accompanied by manganese and arsenic reduction and FeS accumulation. Clone library analyses indicated the suboxic–anoxic transition zone sediment contained abundant Fe(III)- and sulfate-reducing *Deltaproteobacteria*, whereas the overlying surface sediment was dominated by clones related to the Fe(II)-oxidizing zetaproteobacterium, *Mariprofundus ferroxydans*. Cultures obtained from the transition zone were enriched in bacteria that reduced Fe(III), nitrate, sulfate and As(V) using acetate or lactate as electron donors. In the absence of added organic carbon, bacteria were enriched that oxidized Fe(II) anaerobically or microaerobically, sulfide microaerobically and aerobically and As(III) aerobically. According to 16S rRNA gene analyses, enriched bacteria represented a phylogenetically wide distribution. Most probable number counts indicated an abundance of nitrate-, As(V)- and $Fe(III)_{(s,aq)}$-reducers, and dissolved sulfide-oxidizers over sulfate-reducers, and FeS-, As(III)- and nitrate-dependent Fe(II)-oxidisers in the transition zone. It is noteworthy that the combined community and geochemical data imply near-surface microbial iron and arsenic redox cycling were dominant biogeochemical processes.

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Introduction

Marine hydrothermal sediments are thought to host an abundance of bacteria and archaea that exploit the steep geochemical gradients formed at the confluence of reduced, metal-rich hydrothermal fluids and oxidized seawater (Jannasch and Mottl, 1985; Zierenberg *et al.*, 2000; Luther *et al.*, 2001). However, information on the metabolic diversity of prokaryotes and their effect on redox cycling remains limited, not the least in iron-rich deposits. Hydrothermal deposits dominated by iron-rich mineral phases are found in the vicinity of seafloor spreading centers, intra-plate seamounts and island arcs. A majority of these have been identified in deep-sea environments (reviewed in Rona, 1988), but shallow marine examples are also known (for example, Santorini, Aegean Volcanic Arc; Ambitle Island, Papua New Guinea; Holm, 1987; Alt, 1988; Pichler and Veizer, 1999). Formation proceeds either by the *in situ* precipitation of iron from ascending hydrothermal fluids or the fall out of iron oxides formed in hydrothermal plumes (Alt, 1988; Mills *et al.*, 1993), with temperatures typically ranging from that of ambient seawater to 100 °C (see references in Little *et al.*, 2004).

These ferruginous deposits are of particular interest owing to their ability to scavenge and retain trace elements from seawater (German *et al.*, 1991), as well as the analogies that may be drawn between their development and that of Banded Iron Formations, which formed under oxygen limitation during the Precambrian (Krapez *et al.*, 2003). The oxidation of Fe(II) within contemporary iron oxide deposits at circumneutral pH is increasingly attributed to bacteria that catalyze the transformation of Fe(II) to Fe(III) under anaerobic or microaerobic conditions. Currently, much of the evidence for marine microbial Fe(II) oxidation is based on the association of stalk- or sheath-like structures within hydrothermal

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iron deposits that are reminiscent of the Fe(II) oxidizers *Gallionella* and *Leptothrix*, respectively (for example, Alt, 1988; Juniper and Fouquet, 1988; Hanert, 2002; Kennedy *et al.*, 2003; Little *et al.*, 2004). Both of these taxa are better known from terrestrial environments. Only a very limited number of marine Fe(II) oxidizers have been isolated to date, namely the zetaproteobacterium *Mariprofundus ferrooxydans* and several alphaproteobacteria and gammaproteobacteria strains related to the genera *Hyphomonas* and *Marinobacter* (Emerson and Moyer, 2002; Edwards *et al.*, 2003a; Rogers *et al.*, 2003; Lysnes *et al.*, 2004; Emerson *et al.*, 2007).

The nature of other hypothetical biogeochemical processes in this type of deposit may include the microbial respiration of Fe(III), SO_4^{2-} and NO_3^-, and the biogeochemical cycling of trace elements (for example, Mn, Cu, U, Cd, As, Ag and Au) typically enriched in hydrothermal deposits (cf. Glynn *et al.*, 2006; Severmann *et al.*, 2006). Many of these trace elements may be reduced or oxidized by prokaryotes to conserve energy for cell growth or maintenance, or for toxicity resistance (reviewed in Lloyd, 2003). Of these, arsenic is highly enriched in numerous ferruginous deposits (Cronan, 1972; Rona, 1988), including those at Santorini and Tutum Bay, Papua New Guinea (Varnavas and Cronan, 1988; Price and Pichler, 2005). Although the microbial reduction of As(V) and the oxidation of As(III) have been studied extensively in terrestrial freshwater and geothermal brine environments (Lloyd and Oremland, 2006), little is currently known regarding these processes in marine settings.

In this study, we examine the phylogenetic diversity and geochemical effect of prokaryotic activity within a temperate shallow marine hydrothermal sediment at Santorini, Greece that is iron- and arsenic-rich (Varnavas and Cronan, 1988). The sediment was examined using microbiological, molecular phylogenetic and geochemical techniques. In particular, enrichment culturing was used to analyze the capacity of the indigenous prokaryotic community to transform a range of geochemically important inorganic species (that is, Fe, Mn, As, S and N).

Materials and methods

Sampling and analytical methods

Samples were collected from a shallow embayment ($\geqslant$0.3 m water depth, 20–40 °C; Bostrom and Widenfalk, 1984) on the western margin of Nea Kameni island, within the Santorini flooded caldera. Several cores were extracted in a spatially confined area using hand-push tubes (10 cm internal diameter $\times$ 50 cm long), and sealed with air-tight caps. One core was selected for geochemical analyses. Down-core Eh and pH measurements, initial enrichment inoculations and sediment sectioning for geochemical analyses (at 5 cm intervals from 0–35 cm depth) were conducted directly after collection under N_2 in a glove bag. Sediment was transported immediately to Manchester on ice, where pore-waters were separated by centrifugation (4100 rpm, 4 min), and filtered (0.45 μm) under N_2. Pore-water was acidified with HNO_3 for dissolved element analyses, or kept anaerobic and chilled for rapid analysis of anions and cations.

Total pore-water element concentrations were measured by ICP-AES (Perkin-Elmer Optima 5300 Dual View, Waltham, MA, USA). Anions were measured using a Dionex DX600 ion chromatograph, fitted with a high-capacity ion exchange column (AS9-HC) and an AG9-HC guard column (Sunnyvale, CA, USA), eluted at 1.4 ml min^{-1} with 12 mM isocratic Na_2CO_3 (2600–2800 psi). Dissolved organic carbon was measured using Shimadzu TOC 5050A total carbon analyzer (Milton Keynes, UK). Ammonium concentrations were measured after reaction with Nessler's reagent (at 420 nm). Iron species were quantified by the ferrozine method (Lovley and Phillips, 1986, 1987; Anderson and Lovley, 1999).

Solid-phase elements were quantified by an Axios Sequential Wavelength-dispersive X-ray fluorescence spectrometer (XRF; PANalytical, Almelo, The Netherlands). Total carbon was determined in duplicate by flash combustion of freeze-dried finely powdered samples using a Carlo Erba EA 1108 elemental analyzer (CE Elantech, Lakewood, NJ, USA). Total inorganic carbon was determined on a Coulomat 702 C/S analyzer (Ströhlein, Karst, Germany). The difference between total carbon and total inorganic carbon was taken to be total organic carbon. Sediment was imaged with a Philips FEG-XL30 environmental scanning electron microscope (Eindhoven, The Netherlands), fitted with a PRISM EDS detector and Spirit software (PGT, Princeton, NJ, USA). Samples were imaged unwashed or washed ($\times$ 3) with MilliQ H_2O, with or without fixation by 2.5% glutaraldehyde.

Enrichment cultivation

Anaerobic, microaerobic and aerobic enrichment media were inoculated in triplicate with 10% (v/v) wet sediment from the suboxic to anoxic transition zone (5–20 cm depth), incubated in the dark at 25 °C for $\geqslant$1 month, and subcultured repeatedly before community composition analysis. An anaerobic marine minimal medium (MMM) (described in Handley *et al.*, 2009a), supplemented with 10 mM acetate or lactate as the electron donor and carbon source, was used for the reduction of 10 mM amorphous Fe(III) oxyhydroxide (FeOOH) (Lovley and Phillips, 1986), soluble Fe(III)-nitrilotriacetic acid (NTA) or nitrate (KNO_3), or 5 mM As(V) ($Na_2HAsO_4.7H_2O$). Fe(III)-NTA stock (100 mM) was prepared as described by (Fredrickson *et al.*, 2000), sparged with N_2 and filter sterilized. $FeSO_4.7H_2O$ (20 mM) with KNO_3 (10 mM) was used to test

anaerobic Fe(II) oxidation. Arsenite oxidizers were enriched in aerobic MMM using 5 mM Na_3AsO_2, acidified with HCl to pH 6.8–7.0. Marine Postgate Medium B, with 20 mM acetate or 30 mM lactate was used for sulfate reducer growth (Postgate, 1984).

Oxygen gradient tubes using FeS plugs and an agarose slush overlayer of Modified Mineral Wolfe's Medium in artificial seawater were used to cultivate microaerophilic Fe(II) oxidizers following the methods given by Kucera and Wolfe (1957) and modifications by Hanert (1992) and Emerson and Moyer (1997, 2002), with vitamins and minerals as for MMM. Tubes targeting sulfide oxidizers received 1% agarose plugs containing 10 mM neutralized Na_2S and 25% anoxic artificial seawater (Jannasch *et al*., 1985). The overlayer consisted of artificial seawater, 1 ml l^{-1} vitamins and minerals (MMM), 0.001% phenol red (Jannasch *et al*., 1985) and 0.1% agarose. A 25 µl stab inoculum was added to overlayers 24 h after formation.

No organic carbon was added to media using inorganic electron donors.

Most probable number counts (MPN)
Enumeration of different microbial functional groups was achieved through the MPN method, using 10-fold serial dilutions from 10^{-1} to 10^{-8}. Media for cultures was as described for enrichment cultures, with no organic carbon added to media with inorganic electron donors, but acetate and lactate added in combination to media requiring organic electron donors. Cell numbers were estimated from the three-tube table published in de Man (1983).

Isolation of bacteria
Sulfate reducers were isolated from enrichment cultures anaerobically using Postgate Medium E agar plates with acetate or lactate (Postgate, 1984). Anaerobic Fe(III)-NTA MMM agar plates were used to isolate from a lactate-dependant FeOOH enrichment culture. Fe(III)-reducing bacteria from Fe(III)-NTA and FeOOH/acetate enrichment cultures were isolated on Luria–Bertani agar plates. As(V)- and As(III)-oxidizers were isolated on Luria–Bertani and *Marinobacter* medium (DSMZ 970) agar plates. Anaerobic nitrate MMM agar plates were used to isolate from the lactate-metabolizing nitrate enrichment. Dual-layer plates, based on the gradient tube method, but substituting 1.5% agarose in both layers (cf. Wirsen *et al*., 2002) were used to isolate aerobic sulfide-oxidizers. The metabolism of each isolate was subsequently tested in the appropriate liquid medium.

Analytical techniques for detection of growth on target substrates
The reduction or oxidation of iron in cultures were determined by the ferrozine method, and were also evident owing to: the visible formation of the white ferric iron-bearing mineral vivianite $[Fe_3(PO_4)_2.8H_2O]$ (confirmed by XRD) from Fe(III)-NTA; the transformation of FeOOH from orange to a dark-brown or green precipitate; or the change of Fe(II)/NO_3^- medium from green to orange. Ferrous iron oxidation in FeS gradient tubes was indicated by a visible horizon of cell growth stained with an orange ferric precipitate. Similarly, Na_2S oxidation was indicated by a horizon of cell growth, and color change in phenol red (to pale yellow). Sulfate reduction was evident from the development of FeS. Nitrate reduction was established by an increase in optical density, and NO_3^-, NO_2^- and NH_4^+ concentrations. Arsenate and arsenite levels were measured by the molybdenum blue colorimetric method (see Handley *et al*., 2009b).

DNA extraction and amplification
Genomic DNA from sediments, enrichment cultures ($\geqslant$3rd subculture of the initial 10% v/v dilutions), and sulfate-reducing colonies imbued with FeS was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). DNA from other isolates was amplified directly from colonies. Bacterial 16S rRNA genes were amplified using the primers 8F and 519R and PCR settings according to Holmes *et al*., (2002) with 10-min final extensions. Dissimilatory sulfite reductase (*dsr*) gene primers, DSR1F and DSR4R (Wagner *et al*., 1998) were also used for analysis of sulfate-reducing communities. Reaction conditions were: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 54 °C for 1 min, and 72 °C for 2 min; and 72 °C for 10 min. PCR products were purified using a QIAquick purification kit (Qiagen, Crawley, UK), and verified on an ethidium bromide stained 1% agarose tris-borate-EDTA gel.

Cloning and restriction fragment length polymorphism analysis (RFLP)
Purified PCR products were cloned using Invitrogen TA Cloning and Top10 kits (Paisley, UK). Colonies were screened using the vector primers 1F (5′-AGTG TGCTGGAATTCGGCTT-3′) and 1R (5′-ATATCTGCA GAATTCGGCTT-3′). Between 47 and 57 clones were sampled from enrichment libraries, 57 and 100 clones were sampled from surface and transition zone sediment libraries, respectively, and 6 and 25 from *dsr* gene libraries. Sequence diversity was determined by RFLP (Weidner *et al*., 1996) analysis using endonucleases EcoRI or Sau3A and MspI (Roche Diagnostics, Lewes, UK), and separation within 3% agarose tris-borate-EDTA gels.

DNA sequencing and phylogenetic analysis
DNA from pure cultures and multiple representatives of each RFLP pattern were sequenced using the reverse PCR primers and the ABI Prism BigDye

Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). Electrophoresis was performed using an ABI Prism 3100 Genetic Analyzer. Sequences were verified for chimeras using Chimera Check v. 2.7, RDP-II (Cole *et al.*, 2003). The phylogenetic affiliations of the nucleotide partial sequences were determined using BLAST analysis (Altschul *et al.*, 1990). Sequences were aligned with phylogenetically related sequences from GenBank, and a neighbor-joining phylogenetic tree was constructed with 1000 boot-strap replicates, based on evolutionary distances estimated using MEGA v4 (http://www.megasoftware.net/index.html) with the Maximum Composite Likelihood method (Tamura *et al.*, 2004, 2007).

Sequence accession numbers
Sequences are deposited in GenBank under the following accession numbers: EU983110–EU983129 (surface sediment), EU983130–EU983155 (transition zone sediment), EU983213–EU983262 (enrichment cultures) and EU983263–EU983274 (pure cultures).

Intergenic spacer profiling
DNA fingerprinting of down-core sediment bacterial communities was undertaken by amplifying the 16S (small subunit) and 23S (large subunit) rRNA intergenic spacer region, using the primers SD-Bact-1522-b-s-20 and LD-Bact-132-a-A-18 (Normand *et al.*, 1996; Ranjard *et al.*, 2000). These primers yield approximately 20 and 130 bp of 16S and 23S rRNA genes (Ranjard *et al.*, 2000), respectively, along with the intergenic spacer region, which ranges from approximately 150–1500 bp in prokaryotes (for example, Fisher and Triplett, 1999; Cardinale *et al.*, 2004). PCR settings were: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. Purified product was separated within a 3% agarose tris-borate-EDTA gel.

Results and discussion

Sediment geochemistry
The sediment was 25 °C during sampling (February 2006), owing to warm geothermal fluids, and comprised three distinct geochemical and mineralogical zones as it graded downwards from suboxic to anoxic. Suboxic surface sediment (zone one, 0–5cm depth) was largely characterized by Fe(II) oxidation and Eh values near zero (Figure 1). This surface sediment was unconsolidated and rust-colored with a filamentous microtexture (Figure 2) typical of iron oxide deposits (cf. Little *et al.*, 2004 and references therein). Deeper anoxic sediment was marked by increasingly negative Eh values, and the reduction of alternative electron acceptors, such as Fe(III) and Mn(IV). The suboxic to anoxic transition zone (zone two, approximately 5–20 cm depth, Eh −60 to −140 mV) graded from poorly to well-consolidated green–brown mud with localized (predominantly millimeter-scale) areas of black precipitate, most likely $FeS_{(am)}$, which continued through the lower anoxic zone (zone three, 20–35 cm depth). From $\geqslant$35 cm depth the sediment was gravel-rich.

Sediment contained 44–52% Fe, 349–424 ppm As and minor enrichments of Mo (48–56 ppm), P (0.2–1.5%) and S (0.3–0.5%) (Supplementary Tables 1 and 2). Silicon values were low, although not unusual for similar hydrothermal deposits, and elements typically enriched in hydrothermal sediments, such as Mn, Zn and Cu, were depleted (cf. El Wakeel and Riley, 1961; Bostrom and Widenfalk, 1984; Alt, 1988). Inorganic carbon comprised 1.0% of the sediment at 0–5 cm depth, 4.0% at 5–20 cm, and 3.4% at 20–30 cm. Organic carbon was 0.1%, 0.5% and 0.3% at the respective sediment depths.

Pore-water was significantly enriched in dissolved Fe (144.2–316.8 mg l^{-1}), and contained elevated levels of V (1.07–1.2 mg l^{-1}), Mn (0.01–0.27 mg l^{-1}), Si (6.34–17.21 mg l^{-1}) and P (0–0.95 mg l^{-1}) (Supplementary Table 3). Peaks in pore-water Mn and Fe (as ferrous iron) occurred at 5–10 cm and 10–15 cm depth, respectively, while seawater concentrations of sulfate (2680–3040 mg l^{-1}) remained constant over the 30 cm depth analyzed (Figure 1). High levels of ammonium (20–36 mg l^{-1}) were also present throughout the core (Figure 1, Supplementary Table 4). No nitrate or nitrite were detected. Total dissolved inorganic carbon concentrations were consistently low (0–1 mg l^{-1}), whereas dissolved organic carbon was substantially elevated in the surface sediment (495 mg l^{-1}) (Figure 1, Supplementary Table 4).

In situ *bacterial community and carbon sources*
Results from ribosomal intergenic spacer analysis (RISA) illustrate a gradational shift in the bacterial community structure with increasing sediment depth (Figure 3). An exploration of the surface sediment (0–5 cm depth), based on 16S rRNA gene clone sequencing after RFLP sorting, suggests the community was dominated by bacteria related to the marine Fe(II)-oxidizing zetaproteobacterium, *Mariprofundus ferrooxydans* (33% library abundance; Emerson and Moyer, 2002; Emerson *et al.*, 2007), the Fe(III)- and nitrate-reducing deltaproteobacterium, *Geothermobacter* sp. (23%; Kashefi *et al.*, 2003) and *Chloroflexi* (16%; Figures 3 and 4, Supplementary Table 5). In the transition zone sediment (5–20 cm depth) bacterial community, the greatest proportion of clone sequences (46%) were most similar to *Deltaproteobacteria*, with 23% of these sequences similar to bacterial species within the *Desulfuromonadales*, an order characterized by Fe(III)- and S^0-reducers (Lovley *et al.*, 2004), and 16% similar to the *Desulfobulbus* genus, known for sulfate and Fe(III) reduction (for example, Sass *et al.*, 2002; Holmes *et al.*, 2004a). These data suggest a broad shift with

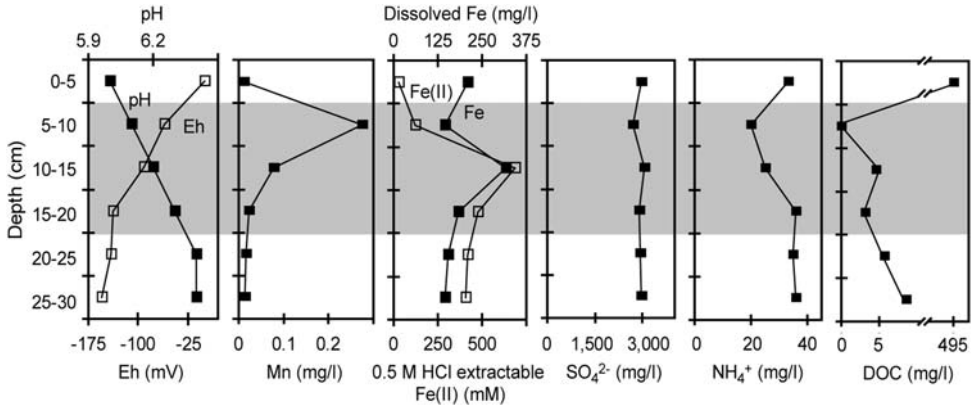

Figure 1 Pore-water profiles with sediment depth, depicting pH (solid symbols) and Eh values (open symbols), and dissolved concentrations of total iron (solid symbols), manganese, sulfate, ammonium and organic carbon (DOC). The 0.5 M HCl soluble (bioavailable) Fe(II) fraction extracted from the sediment is depicted by open symbols. The transition zone is shown in gray.

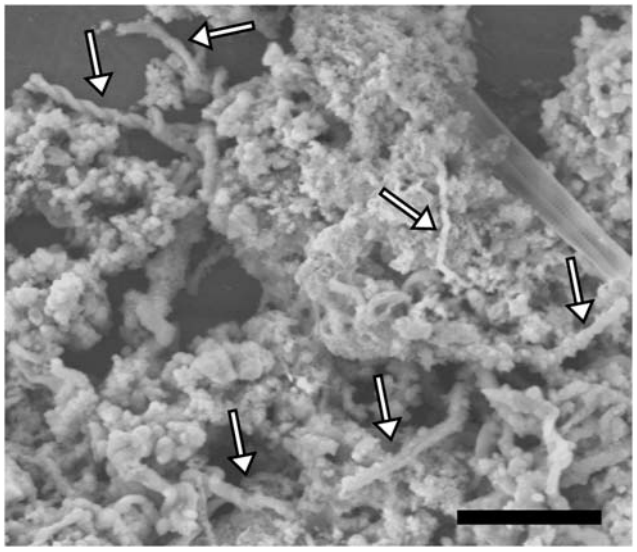

Figure 2 Environmental scanning electron microscope (ESEM) image of the oxidized surface sediment layer, depicting numerous filamentous (including helical) cells (arrows), associated with the Fe(III) oxyhydroxide. Scale bar, 10 μm.

depth from microbial Fe(II) oxidation (by the *M. ferrooxydans*-like bacterium) to Fe(III) reduction (for example, by *Desulfuromonodales*).

Reactions driven by heterotrophy may have in part derived organic carbon from photosynthetic bacteria, such as *Chloroflexi*, owing to the shallow water depth. Other pools of organic carbon originate from algae, indicated by algal biomarkers (sterols) from this sediment, and allochthonous terrestrial plant matter, indicated by the presence of higher plant biomarkers (for example, *n*-alkanols and *n*-alkanoic acids; Handley, unpublished data). Configurations of organic matter indicated a mixture of immature plant and microbial material that is associated with sedimentation, and mature material that has likely been altered by diagenetic and hydrothermal processes. Current sediment temperatures do not account for the maturation, especially given the presence of the well-preserved immature compound classes. Heterotrophic bacteria might not only use these organic inputs, but could also use metabolites arising from their decay. In fact, the very low dissolved organic matter concentrations in the transition zone (Figure 1), suggest that the heterotrophs in this layer are consuming dissolved substrates (that is, metabolites) rather than organic matter associated with the solid phase. Lithoautotrophy may be supported by inorganic carbon from hydrothermal fluids/gases and seawater (that is, CO_2/HCO_3^-).

Cultivated organisms

Although bacteria enriched in the first dilution (10% inocula) do not necessarily represent the most abundant members of their respective functional groups in the Nea Kameni sediment (Dunbar *et al.*, 1997; Fry, 2003), enrichment experiments clearly showed a range of microbial functional processes that potentially drive key inorganic reactions in the transition zone and beyond. Prokaryotes performing these reactions (Figures 4 and 5) were enumerated by MPN techniques (Table 1). No transformations were detected in uninoculated or heat-killed controls. Reactions involving the oxidation of the inorganic electron donors were without added organic carbon, and can be attributed to either autotrophy supported by bicarbonate buffer in the media, or heterotrophic consumption of metabolites produced by the enrichment community, while utilizing inorganic electron donors as auxiliary energy for cell maintenance.

Fe(III) reduction. In experiments with ferric-$NTA_{(aq)}$, iron was reduced to vivianite. Clone libraries indicated a dominance of bacteria with similar 16S rRNA gene sequences to soluble/insoluble Fe(III)-reducers, *Malonomonas rubra* (97%, 480/491 bp identity) and *Shewanella algae* (100%, 508/508 bp)

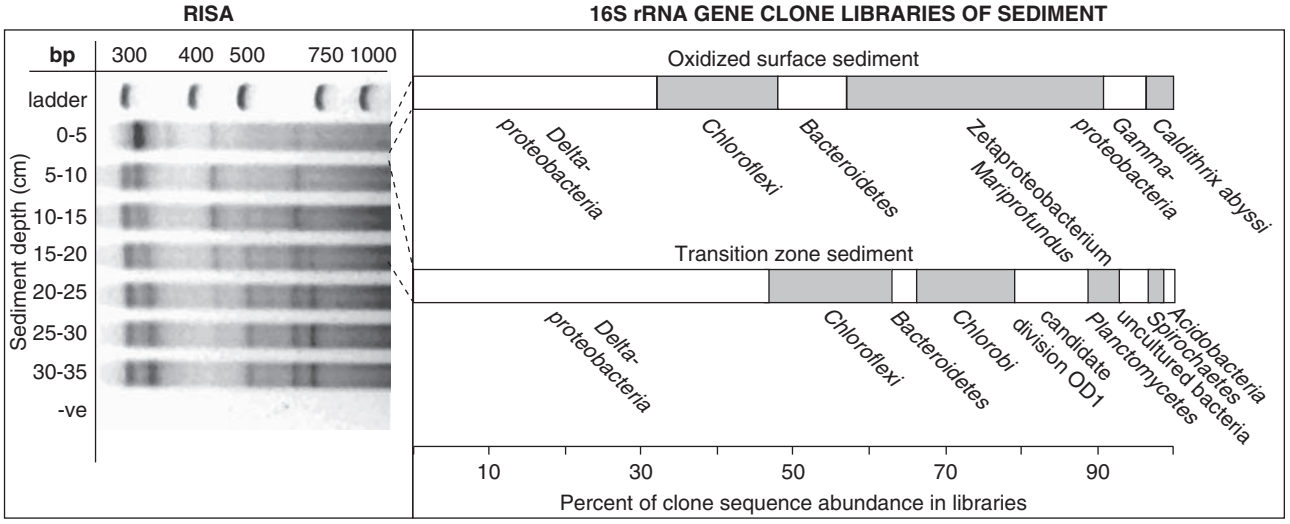

Figure 3 Electrophoresis gel showing the community diversity of bacteria at different sediment depths indicated by RISA (left). DNA ladder, top row; and negative control, –ve. Graphs depicting the phylogenetic diversity of bacteria in the oxidized surface and transition zone sediments, indicated by 16S rRNA gene clone library analyses (right).

grown with acetate and lactate, respectively (Caccavo *et al*., 1992; Holmes *et al*., 2004b). An isolate from the Fe(III)-NTA/lactate enrichment (NKiF3NL1), 99% (482/485 bp) similar to *S. algae*, readily reduced soluble and insoluble Fe(III).

The Fe(III) in cultures, as $FeOOH_{(s)}$, transformed into a dark Fe(II)-bearing, non-magnetic precipitate. FeOOH and lactate enriched for a bacterium related to a soluble Fe(III)-reducing genus, *Desulfuromusa* (95% similar to strain Fe30-7C-S, 469/490 bp; Liesack and Finster, 1994). An isolate (NKiF3GL1) from this culture, 95% (435/455 bp) similar to *Desulfuromusa* sp. Fe30-7C-S, also reduced FeOOH readily. Ferric iron reduction was comparatively slow with acetate (weeks vs days). A small proportion of clones shared 88% sequence identity (442/501 bp) with *M. rubra*. The majority of clones were similar to *Paracoccus homiensis* (97%, 439/451 bp) and *Marinobacter koreensis* (98%, 494/502 bp). *Paracoccus* species can enzymatically reduce Fe(III) (Mazoch *et al*., 2004), although no reduction of $Fe(III)_{(aq,s)}$ occurred in the presence of an isolate (NKiF3GA1) from the FeOOH/acetate enrichment related to *Paracoccus* sp. 88/2–4 (97%, 425/437 bp). *Marinobacter* species have not previously been shown to reduce Fe(III). However, an isolate (NKiA3O1) from an As(III)-oxidizing enrichment, which had 98% (449/456 bp) 16S rRNA gene similarity to *M. koreensis*, also reduced Fe(III)-NTA, in a medium supplemented with lactate and 0.004% yeast extract, to an Fe(II)-bearing green rust-like precipitate.

Arsenic reduction and oxidation. Arsenate cultures with acetate and lactate enriched for dissimilatory As(V)-reducing bacteria 98% (469/477 bp) and 90% (463/514 bp) related to 16S rRNA gene sequences of *M. koreensis*-like bacteria, respectively. As(III)-oxidizing cultures were dominated by a bacterium 98% (498/505) similar to *M. koreensis*. Growth was maintained in As(III) cultures through >9 subcultures without added organic carbon. An isolate from the As(V)/lactate enrichment (*Marinobacter santoriniensis* NKSG1^T; Handley *et al*., 2009a) was shown to derive energy for growth from both the anaerobic reduction of As(V) coupled with lactate or acetate oxidation, and to enzymatically oxidize As(III) in aerobic media supplemented with lactate as a carbon source (Handley *et al*., 2009b). The genus was not previously known to reduce or oxidize arsenic.

Nitrate reduction. In nitrate-reducing enrichments, visible cell growth corresponded with the complete disappearance of NO_3^- in media with either acetate or lactate as electron donors. No NO_2^- or NH_4^+ was detected, most likely owing to complete reduction to N_2. Relatives of the denitrifiers, *Sedimenticola selenatireducens* (97% similar, 503/515 bp), *Marinobacter* sp. CAB (95%, 487/509 bp) and *Thalassospira profundimaris* (98%, 443/452 bp), were enriched in acetate-supplemented cultures (Rontani *et al*., 1997; Narasingarao and Häggblom, 2006; Liu *et al*., 2007). In lactate-amended cultures bacteria were affiliated with *S. selenatireducens* (98%, 504/513 bp), the nitrate-reducer *M. koreensis* (98%, 496/502 bp), and the *Desulfuromusa* genus (strain Fe30-7C-S, 95%, 476/498 bp), also capable of nitrate reduction (Liesack and Finster, 1994; Kim *et al*., 2006). The enrichment isolates *M. santoriniensis* and *Desulfuromusa*-like bacterium NKiF3GL1 also reduced nitrate in culturing experiments.

Sulfate reduction. Sulfate reduction in Postgate B cultures resulted in FeS and $H_2S_{(g)}$ formation. Sulfate-reducing bacteria were identified based on

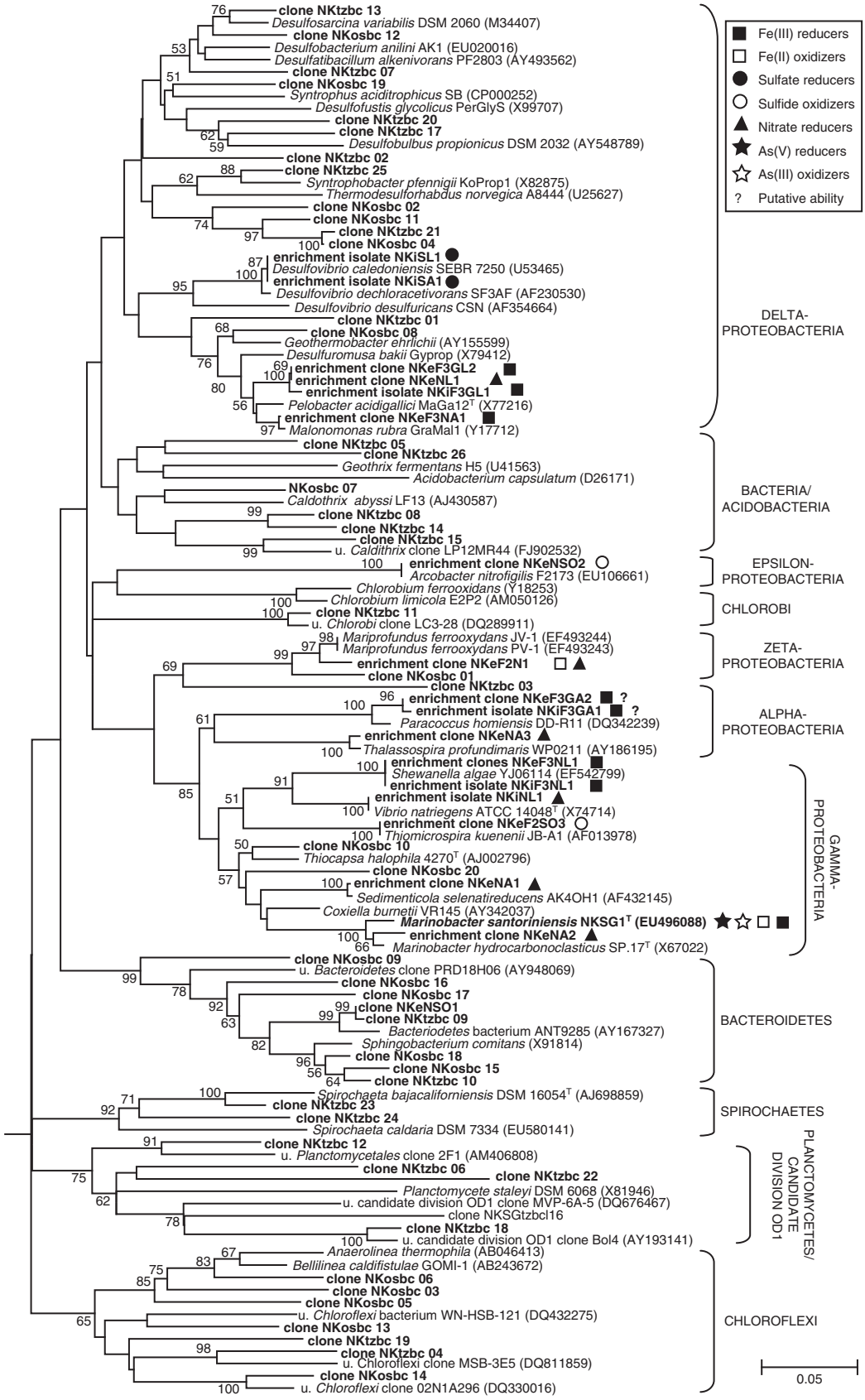

Figure 4 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of sediment and enrichment culture clones and isolates. Only bootstrap values >50% are shown. The scale bar represents changes per base position. The outgroup is *Thermus aquaticus* YT-1 (L09663).

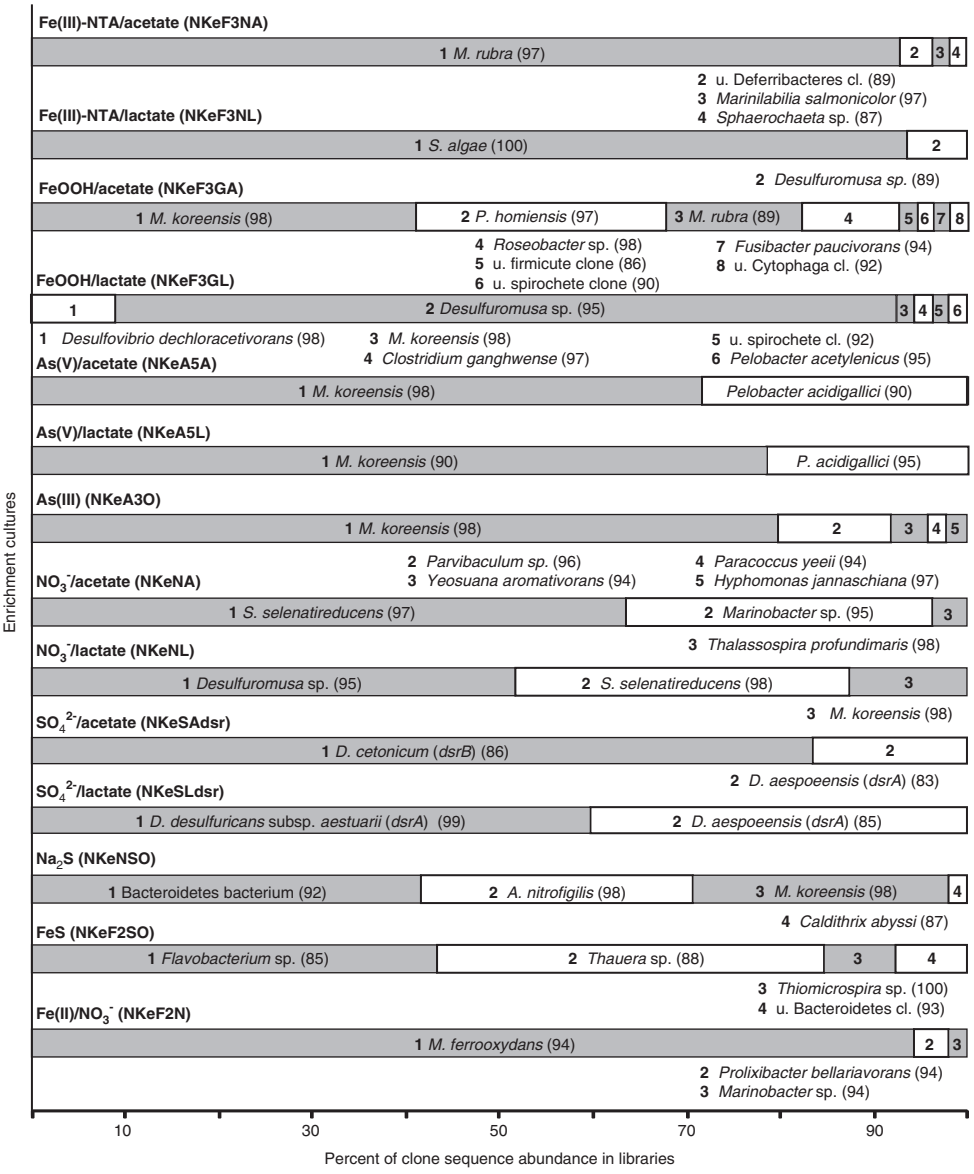

Figure 5 Graphs of 16S rRNA and *dsrAB* gene clone library analyses depicting bacterial enrichment communities. Closest BLAST matches and affinities (%) are shown.

Table 1 MPN estimates of cells per ml of transition zone sediment determined according to metabolic function (listed from highest to lowest abundance)

Respiratory process	*MPN (cells ml^{-1})*	*⩾95% CI*	*⩾99% CI*
NO_3^- red.	7.50×10^7	1.7–19.9	1.1–27.0
As(V) red.	2.40×10^6	0.4–9.9	0.3–15.2
Fe(III)-NTA red.	9.30×10^5	1.8–36.0	1.2–43.0
FeOOH red.	9.30×10^5	1.8–36.0	1.2–43.0
Na_2S ox.	3.60×10^5	0.4–9.9	0.3–15.2
FeS ox.	3.60×10^4	0.4–9.9	0.3–15.2
SO_4^{2-} red.	9.30×10^3	1.8–36.0	1.2–43.0
As(III) ox.	2.40×10^3	0.4–9.9	0.3–15.2
Anaer. Fe(II) ox.	2.30×10^2	0.5–9.4	0.3–14.2

Abbreviations: CI, confidence interval; MPN, most probable number.

the putative partial DsrA or DsrB amino acid sequences deduced from *dsrAB* gene clone libraries, which yielded matches with *Desulfobacterium cetonicum* (86%, 647/744 bp) and *Desulfovibrio aespoeensis* (83%, 711/847 bp) in SO_4^{2-} and acetate medium, and *Desulfovibrio desulfuricans* sub sp. *aestuarii* (99%, 929/934 bp) and *D. aespoeensis* (85%, 824/961 bp) in SO_4^{2-} and lactate medium. Sulfate-reducing strains (NKiSA1, NKiSA2 and NKiSL1), 97–99% (459/470–500/507 bp) related to *Desulfovibrio dechloracetivorans* based on 16S rRNA gene sequences, were isolated from acetate and lactate containing cultures. *Desulfovibrio* species are not known to couple the oxidation of acetate to sulfate (Kuever *et al.*, 2005a, b), and may instead have been supported by yeast extract (Steger *et al.*, 2002) and products generated by the community in the acetate-amended cultures. The 16S rRNA gene clone libraries of the sulfate-reducing enrichments were dominated by fermentative bacteria, although in the acetate-oxidizing culture, clones closely

related to *Desulfosarcina variabilis* (98%, 512/519 bp) and *Desulfobacterium niacin* (98%, 506/515 bp) were also present as minor components of the library (data not shown).

Sulfide and Fe(II) oxidation. The Na_2S gradient medium developed an aerobic horizon of cell growth that was accompanied by acidification of the medium, turning the pH indicator pale yellow. A significant proportion of library clones were related to *Arcobacter nitrofigilis* (98%, 482/487 bp), the genus of which includes autotrophic sulfide-oxidizers (for example, Wirsen *et al.*, 2002). An isolate of the *Arcobacter*-like bacterium (NKiNSO1) was also obtained on sulfide over-layer plates. In the FeS gradient medium, distinct rust-colored opaque cell horizons formed mid-way between the FeS plug and the air-agarose interface. No distinct horizons formed in uninoculated media. Abiotic oxidation resulted in broad, diffuse Fe(III) bands. The enrichment was equally dominated by clones yielding sequence matches to *Flavobacterium* sp. AKB-2008-JO5 (85%, 434/506 bp), and the autotrophic sulfur-oxidizing genus, *Thiomicrospira* (strain JB-A1, 100%, 506/506 bp; for example, Kuenen and Veldkamp, 1972; Brinkhoff and Muyzer, 1997). Analyses of cloned 16S rRNA genes from the anaerobic Fe(II)-oxidizing/nitrate-reducing enrichment culture revealed it was dominated by a bacterium most closely related to the autotrophic Fe(II)-oxidizer, *Mariprofundus ferrooxydans* JV-1 (94%, 476/505 bp; Emerson *et al.*, 2007). Growth of the enrichment through successive subcultures (>10 times) occurred without organic carbon supplementation. Nitrate-dependant Fe(II) oxidation was also attained by the FeS enrichment and *M. santoriniensis* (Handley *et al.*, 2009a) with 5 mM acetate. Autotrophy was not confirmed for any Fe(II)- or sulfide-oxidizing enrichment culture bacterium.

Implications for biogeochemical processes

Neutrophilic Fe(II) iron-oxidizing bacteria are though contribute to the formation of hydrothermal ferruginous deposits, and alteration of iron sulfides and exposed spreading ridge basalt (for example, Emerson and Moyer, 2002; Kennedy *et al.*, 2003; Edwards *et al.*, 2003a, b; Little *et al.*, 2004), and bacteria phylogenetically related to Fe(II)-oxidizing *Gallionella ferruginea* are known to be present in deep-sea vent sediments (Rogers *et al.*, 2003). Nevertheless, there is increasing evidence that other bacteria also have a major role in marine Fe(II) oxidation, in particular zetaproteobacteria and *Marinobacter* species (Rogers *et al.*, 2003; Edwards *et al.*, 2003a; Lysnes *et al.*, 2004; Emerson *et al.*, 2007; Kato *et al.*, 2009; M. Müller, personal communication; this study). Structures resembling the freshwater Fe(II) oxidizers *Gallionella* and *Leptothrix* have previously been recognized in iron-rich mud of Palaea Kameni, the island adjacent to Nea Kameni at Santorini (Hanert, 2002), but neither were identified using molecular techniques in this study. In contrast, bacteria related to the Fe(II)-oxidizing stalk-former, *M. ferrooxydans* (Emerson *et al.*, 2007), were abundant in the Nea Kameni surface-most sediment (Figure 5), and other bacteria potentially also contribute to Fe(II) oxidation within this sediment, such as rod-shaped *Marinobacter* species (Handley *et al.*, 2009a).

The geochemistry and microbiology of the Nea Kameni sediment are consistent with observations that Fe(II)-oxidizing bacteria are important in the development of ferruginous sediments. However, results also illustrate the importance of other biogeochemical processes in these sediments, notably, complete redox cycling of iron. Ferruginous deposits also contain enrichments of other elements of interest, with high levels of arsenic not being uncommon. In sediments at Tutum Bay, similar to those at Santorini, Price and Pichler (2005) found only a very small proportion of arsenic was associated with the easily extractable, 'bioavailable' iron oxide fraction. Ferric iron-reducing bacteria, however, are able to consume more recalcitrant forms of iron as well—although at a slower rate—potentially freeing up structurally bound arsenic. Arsenic in the Nea Kameni sediment was found to be over 60% As(V) in the near surface, but only 5% As(V) in the transition zone (Handley *et al.*, unpublished XAS study). This significant change in the proportion of solid-phase As(III) and As(V) between the surface and transition zones occurs in the presence of, and likely owing to bacteria capable of redox cycling arsenic. We know of only one other example of arsenic metabolism (dissimilatory arsenate reduction) recorded in a deep-sea hydrothermal environment (iron sulfide black smoker vent chimney, Takai *et al.*, 2003).

Biogeochemical zones

Geochemical cycling in marine sediments characteristically occurs across the oxic-anoxic gradient by the transformation of redox couples in order of decreasing energy yield, for example, O_2/H_2O, NO_3^-/NO_2^-, Mn(IV)/Mn(II), Fe(III)/Fe(II), SO_4^{2-}/H_2S and then CO_2/CH_4, producing biogeochemical stratification with depth (Froelich *et al.*, 1979; Jørgensen, 2006). Geochemical data indicated the successive reduction of Mn(IV), Fe(III) and some sulfate (to FeS) with depth in the transition zone sediment at Nea Kameni (cf. Burdige, 2006; Jørgensen, 2006). Sediment-bound arsenic was reduced in the transition zone as described above. It is also likely that seawater nitrate was reduced rapidly at the sediment-water interface, but was not detected at the 5 cm resolution of our analyses. The relative abundances of organisms enriched tend to correspond with sediment geochemistry. MPNs are subject to media-biases, and may underestimate abundances; however, results broadly suggest a higher likelihood

for microbial As(V), Fe(III) and nitrate reduction and sulfide oxidation over sulfate reduction and As(III) and Fe(II) oxidation within the transition zone (Table 1).

Iron and manganese transformations. Neutrophilic microbial oxidation of Fe(II) coupled with oxygen or nitrate reduction, while forming rust-colored iron-oxyhydroxides, out-competes abiotic oxidation at low oxygen fugacities and produces sufficient energy to support growth (for example, Straub *et al*., 1996; Supplementary Table 6). Sharp increases in first aqueous manganese and then iron in the deeper transition zone imply the onset of microbial Mn(IV) and then Fe(III) reduction. In support of this, a range of bacteria capable of both Fe(III) and Mn(IV) respiration (for example, *Shewanella* species, Lovley *et al*., 2004) were present in the transition zone. The depth-related order in which these species were reduced corresponds with the respective energetics of each transformation, whereby the Mn(IV)-Mn(II) transformation tends to be energetically more favorable (for example, Thauer *et al*., 1977; Supplementary Table 6), and Mn(IV) is consequently used in preference to Fe(III) where both substrates are present.

Arsenic oxidation and reduction. Both the enrichment and isolation of As(III)-oxidizing bacteria suggests As(V) in the surface sediment may be attributed to the enzymatic oxidation of As(III), arising from mixotrophy or chemolithotrophy coupled with O_2 or an alternative electron acceptor. In contrast, the abiotic oxidation of As(III) can be exceptionally slow (months–years) even under fully aerobic conditions (Smedley and Kinniburgh, 2002, and references therein). Bacteria also are able to access, and reduce, structurally bound and surficially complexed As(V) associated with sediments and minerals (for example, Newman *et al*., 1997; Zobrist *et al*., 2000), potentially accounting for As(III) in the transition zone. Arsenate respiration generates energy yields similar to those for Fe(III) reduction (Supplementary Table 6), although these values will be dependent on several environmental factors including concentrations of the electron acceptors and mineralogical constraints (Cutting *et al*., 2009). Arsenate reduction to As(III) has been shown to occur before, concomitant with, or subsequent to Fe(III) reduction, depending on the model system studied (for example, Ahmann *et al*., 1997; Zobrist *et al*., 2000; Islam *et al*., 2004; Campbell *et al*., 2006). Conversely, the release of iron-bound As(III), after As(V) reduction, may occur alongside or subsequent to Fe(III) reduction (Islam *et al*., 2004).

Sulfur cycling. It is unclear from available data whether sulfide oxidation occurs primarily in the surface or transition zone sediment, although the greater abundance of sulfide-oxidizers over sulfate-reducers in the transition zone (Table 1) may indicate the latter. Evidence for microbial sulfide oxidation in the transition zone sediment is also afforded by the cultivation of sulfide-oxidizers closely related to *Arcobacter* and *Thiomicrospira*. Concentrations of hydrothermally generated H_2S are either minor or non-existent in the Santorini hydrothermal sediments (Bostrom and Widenfalk, 1984; Minissale *et al*., 1997). However, sulfide may also be generated by microbial sulfate reduction.

Consistently high levels of pore-water sulfate throughout the sediment column suggests sulfate-reducing conditions are never fully attained over the sampled sediment depth, even where putative FeS visibly formed within the lower portion of the transition zone. This is corroborated by the low numbers estimated for transition zone sulfate-reducers (Table 1). Sulfur concentrations in the sediment did not increase with depth, suggesting FeS was relatively negligible. Low levels of sulfate reduction may be due to the relatively high Eh values (above −200 mV; Postgate, 1984), and the inexhaustion of other electron acceptors, notably Fe(III), which possess a higher energy yield (Thauer *et al*., 1977; Supplementary Table 6).

Nitrate reduction. Rapid reduction of seawater-derived nitrate by bacteria, immediately at or below sediment-water interfaces, forms part of the characteristic geochemical profile within marine sediments (Froelich *et al*., 1979). It is plausible that this process also occurs at the Nea Kameni sediment-water interface, owing to the absence of detectable seawater nitrate, and abundant nitrate-reducing bacteria in the sediment. The high reducing capacity of nitrate, mean it is the inorganic electron acceptor typically used after oxygen depletion (Supplementary Table 6), and may be coupled with the oxidation of organic carbon, or a range of inorganic electron donors, such as Fe(II), As(III) or sulfide (for example, Straub *et al*., 1996; Oremland *et al*., 2002; Cardoso *et al*., 2006).

Conclusions

Considering the extent to which microorganisms are capable of catalyzing geochemical reactions, it is palpable that while ferruginous deposits undoubtedly serve as an important substrate for microbial Fe(II) oxidation, results here illustrate they also are home to a range of other geochemically significant microbial processes. Bacteria cultivated from the Santorini sediment transformed a range of relevant marine and hydrothermal inorganic electron donors and acceptors, particularly Fe(II), Fe(III), As(V) and As(III), but also nitrate, sulfide and sulfate. Findings also contribute to the limited information currently available on bacteria performing Fe(II) and As(III) oxidation and As(V) reduction in the marine environment, owing especially to the cultivation of

novel bacteria able to transform both these chemical species.

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