## Chapter 2

## MINERAL-ASSOCIATED MICROBIAL ECOLOGY OF METHANE SEEP SEDIMENTS INVESTIGATED BY DENSITY SEPARATION

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### ABSTRACT

Minerals host subseafloor microbiomes, but it remains unknown if certain minerals provide niches for certain microbial taxa. Recent work has demonstrated that conductive minerals in particular may provide important conduits for extracellular electron transfer, a process central to the physiology of many syntrophic microorganisms that inhabit seafloor sediments. Multicellular consortia of anaerobic methanotrophic archaea (ANME) and partner sulfate-reducing bacteria (SRB) are a prominent example of syntrophic EET in marine sediments, but little is known about the extent to which these consortia associate with conductive minerals *in situ*. Here, we report the optimization of a heavy-liquids separation protocol and results from its application to methane seep sediment samples, which revealed preferential association between members of the ANME-3 and magnetite and/or pyrite mineral surfaces. We further demonstrate the capacity for this new protocol to improve detection of ecological interactions between microbial taxa.

Marine sediments comprise one of Earth's most energy-poor biomes. High-energy electron acceptors such as oxygen or nitrate diffusing downward into seafloor sediments from an aerobic water column are rapidly consumed by respiring microorganisms that inhabit sediments near the sediment-water interface (Froelich et al., 1979; Van Cappellen and Wang, 1996). In the subsurface biosphere, the presence of a mineral matrix reduces the rate at which electron acceptors can be replenished by diffusion or advection as occurs in the water column, leaving many subsurface microbial communities reliant upon lowerenergy electron acceptors to survive (Kallmeyer et al., 2012; Bowles et al., 2014). Organic carbon deposited on the seafloor provdes an abundant source of electrons for microbial communities buried in seafloor sediment, which in most coastal environments exceeds the supply of high-potential electron donors such as oxygen and nitrate (Jørgensen, 2000). The energetic challenge posed by this excess of electron donor is particularily acute in sediments proximal to seafloor methane seeps, where highly reducing, methane-charged fluid advects upwards from depth, inducing anoxia in sediment pore water within as little as a few millimeters beneath the sediment-water interface (Grünke et al., 2012).

The selective pressure imposed on microorganisms inhabiting highly reducing subseafloor environments has driven the evolution of adaptations that exploit diverse and unique electron acceptors or employ biochemical mechanisms of extracellular electron transfer (EET). Among these are sulfate ions as well as insoluble iron and manganese oxyhydroxides/oxides (Froelich et al., 1979; Van Cappellen and Wang, 1996) and humic substances (Lovley et al., 1996). Fe- or Mn-reducing microorganisms must pass electrons to an insoluble acceptor, presenting a physiological challenge in which the acceptor cannot be transported into the cell (Lovley, 1991), requiring these microorganisms to perform EET. EET is also thought to facilitate energy-poor syntrophic metabolisms important in the marine subsurface, such as the anaerobic oxidation of methane (AOM, (McGlynn et al., 2015; Wegener et al., 2015)), in which anaerobic methanotrophic archaea (ANME) couple methane oxidation to sulfate reduction in a syntrophic partnership with sulfate-reducing bacteria (SRB, (Boetius et al., 2000; Orphan et al., 2001b)). EET can facilitate both syntrophic partnerships and reduction of insoluble electron acceptors, in some instances in the same metabolism, such as the reduction of iron and manganese oxides during AOM syntrophy (Beal et al., 2009). Several biochemical mechanisms for EET to insoluble electron acceptors and/or syntrophic partners have been proposed, including conductive pili (El-Naggar et al., 2010; Summers et al., 2010; Pfeffer et al., 2012), cytochromes (McGlynn et al., 2015; Skennerton et al., 2017), and/or electron shuttles (Marsili et al., 2008; Scheller et al., 2016). EET through conductive pili and/or cytochromes requires direct physical contact with insoluble electron acceptors or syntrophic partners, and thus can be significantly influenced by the substrates to which cells employing EET are attached (Lovley, 2017).

Defining the inorganic matrix within which the marine subsurface microbiome is embedded, minerals hinder the diffusion of high-energy electron acceptors into the subsurface microbiome but also my provide conduits for EET to some sediment-dwelling microorganisms. Semiconductive minerals such as hematite ( $Fe_2O_3$ ), magnetite ( $Fe_3O_4$ ), greigite ( $Fe_3S_4$ ), and pyrite ( $FeS_2$ ) are mineral components of marine sediments and are

increasingly considered ecologically important for sediment-dwelling microorganisms (Rowe et al., 2015; Shi et al., 2016) by facilitating EET to mineral electron acceptors (Liu et al., 2015) and/or syntrophic partners (Kato et al., 2012b, 2012a; Li et al., 2015; Liu et al., 2015; Tang et al., 2016; Rotaru et al., 2018). The stimulation of syntrophic EET by magnetite specifically has been repeatedly demonstrated in cultured and uncultured microorganisms. Syntrophic acetoclastic methanogenesis, an important but underappreciated methanogenic process in seafloor sediments (Beulig et al., 2018), has been shown to be stimulated by magnetite amendments in incubations of rice paddy soil (Kato et al., 2012a) or marine sediments (Rotaru et al., 2018), by increasing the rate of EET between Geobacter sp. and Methanosarcina sp. partners. Defined Geobacter metallireducens/Methanosarcina barkeri co-cultures have corroborated these observations (Tang et al., 2016), and other studies have shown that magnetite stimulates syntrophic oxidation of acetate by nitrate in Geobacter sulfurreducens/Thiobacillus denitrificans cocultures (Kato et al., 2012b) and syntrophic oxidation of ethanol by fumarate in G. metallireducens/G. sulfurreducens co-cultures (Liu et al., 2015). It remains unknown, however, if related microorganisms or other syntrophs such as ANME-SRB consortia associate physically with conductive minerals *in situ* in methane seep sediments, where syntrophic EET modulates the flux of methane to the sediment-water interface (Reeburgh, 2007; Knittel and Boetius, 2009).

We sought to uncover *in situ* associations between microbial taxa and conductive minerals in marine sediments, targeting methane seep sediments—habitats for syntrophic microorganisms that are also rich in conductive minerals— as source material. In these

sediments, syntrophic ANME-SRB consortia perform AOM, and in so doing mediate the precipitation of authigenic carbonate (Aloisi et al., 2000; Luff and Wallmann, 2003; Naehr et al., 2007) and iron sulfide minerals. Of these, iron sulfide minerals such as pyrite (FeS<sub>2</sub>) and pyrrhotite (Fe<sub>(1-x)</sub>S, x = 0 - 0.2) are of particular interest, given their conductive nature (Sato and Mooney, 1960) and thus their potential involvement in microbial EET. Pyrite in particular is found in abundance at the SMTZ, the interface between sulfate-rich and methane-rich porewaters (Garming et al., 2005; Riedinger et al., 2005; März et al., 2008; Shi et al., 2017) where AOM rates are highest, precipitating from reaction of sulfide produced by AOM with dissolved Fe<sup>2+</sup> or with detrital iron oxides (e.g. hematite, magnetite) present in seep sediments (Roberts, 2015). We focused on methane seep sediments, given their abundance of conductive minerals and syntrophic ANME-SRB consortia, to explore potential interactions between syntrophic EET and conductive minerals.

Here, we present methods we optimized to separate minerals in marine sediments by density and the insights into mineral-associated microbial ecology obtained from our methods building on previous work by Harrison and Orphan (Harrison and Orphan, 2012). The dense nature of conductive minerals such as pyrite and magnetite ( $\rho >> 2.9$  g/cc) relative to the silicate clays ( $\rho \approx 2.0 - 2.5$  g/cc) that comprise most of marine sediments by mass enables separation based on mass. Carbonate minerals are also somewhat more dense than silicate clays ( $2.4 < \rho < 2.9$  g/cc), allowing for their separation from bulk sediments. Although not the focus of the current study, microbially-mediated carbonate precipitation is an important process in methane seep sediments, where it is responsible for the emplacement of large authigenic carbonate edifices and extensive pavements (Treude et al., 2007; Marlow et al., 2014; Case et al., 2015), and thus we designed our protocol to also separate carbonate minerals out of sediments. We obtained several density-separated fractions of methane seep sediment samples, and applied next-generation 16S rRNA amplicon sequencing (Degnan and Ochman, 2012) to these fractions to profile the microbial communities present in each minerallogically-distinct fraction (Fig. 1). This work revealed a novel association between members of a clade of anaerobic methanotrophic archaea termed ANME-3 and a dense sediment fraction enriched in pyrite and magnetite as well as trace clay minerals. We further hypothesize from correlation analysis that ANME-3 form a novel partership with SEEP-SRB1c, potentially coassociating on the surfaces of the conductive minerals magnetite and pyrite.

#### MATERIALS AND METHODS

#### Density Separation Using Sodium Metatungstate

To separate minerals in methane seep sediments, we used heavy-liquids initially based upon previously- published methods (Harrison and Orphan, 2012). We used sodium metatungstate hydrate (Na<sub>6</sub>W<sub>12</sub>O<sub>39</sub> · xH<sub>2</sub>O) given its non-toxic nature and the high densities that solutions of sodium metatungstate (SMT) can achieve without reaching saturation (up to ~3.1 g/mL). SMT and the related compound sodium polytungstate have previously been used to partition marine sediments by density to extract cells from sediment (Morono et al., 2013) and to isolate mineralogically-distinct density fractions (Harrison and Orphan, 2012). Here we present further development of these techniques to



**Figure 1.** Schematic depiction of heavy-liquids mineral separation for mineral-associated microbial ecology. Density separation using sodium metatungstate recovered mineralogically-distinct density fractions (demonstrated using XRD and FTIR) from which DNA was extracted and sequenced using high-throughput platforms. Downstream community analysis revealed taxa associated with particular minerals and amplifies correlations between taxa co-associating on mineral surfaces.

increase the mineralogical purity and reproducability of density fractions, as assayed by xray diffractometry (XRD) and fourier transform infrared spectroscopy (FTIR), and to prepare density fractions for downstream microbial community profiling by 16S rRNA amplicon sequencing using an Illumina platform. The density separation technique is presented in detail for ease of future application of this technique:

- 1. 250 g SMT powder (Sigma-Aldrich #377341) was mixed with deionized water at a ratio of 250 g/55.0 mL in a 100 mL beaker with a stir bar. To increase the rate of SMT dissolution, this beaker was placed on a hot plate at 50° C for 1 hr. Higher temperatures decompose SMT.
- 2. When all SMT was dissolved, SMT was sterilized using a 0.2 μm filter flask. The filtrate SMT was decanted into sterile 60 mL serum vials sealed with butyl stoppers.
- **3.** SMT in serum vials was placed under UV-C light (254 nm wavelength) for 2 hr to remove DNA contamination. 125 mL filter-sterile (0.2 μm) DI water was simultaneously UV sterilized.
- 4. After UV irradiation, butyl stoppers were removed and serum vials placed on scales to measure SMT density, target density  $\rho = 2.9$  g/mL. 1 mL SMT was removed with a sterile 2 mL pipette and the mass deficit measured for the serum vial used to estimate SMT density. After density measurement, SMT was pipetted back into the serum vial and pipette was discarded. If density was too high, appropriate volume of DI was added, using this formula:

$$V_w = V_0 \frac{\rho_f - \rho_0}{\rho_w - \rho_f}$$

where  $V_w$  is the volume of added to SMT,  $V_0$  is the current volume of SMT,  $\rho_f = 2.9$  g/mL,  $\rho_i$  is the measured current density of SMT, and  $\rho_w \approx 1$  g/mL. If density was too low, SMT decanted into clean nonsterile beaker for evaporative densification (see step 23 below), followed by resterilization and density measurement (repeat steps 2-4).

- 5. With  $\rho = 2.9$  g/mL achieved, SMT was decanted into 6 sterile 5 mL Eppendorf Tubes® (Cat. No. 0030119401), with 4 mL SMT into each tube.
- 6. Remaining SMT was diluted with filter- and UV-sterile DI from step 3 to achieve  $\rho = 1.5$  g/mL, volume determined by use of formula above.
- 7.  $A \sim 10$  mL sample of methane seep sediment slurry were extracted from incubations while maintaining anaerobic conditions in incubations, and placed in two 50 mL Falcon centrifuge tubes.
- 8. Samples were centrifuged for 10 min at 2000× g in a Beckman-Coulter Allegra X-14 set to room temperature. After centrifugation, supernatant was either frozen immediately at -20 °C for downstream DNA extraction or discarded. Dissolved calcium readily reacts with SMT to form insoluble precipitates that cannot be recovered (Krukowski, 1988), and therefore caution should be taken to avoid any contact between

seawater and SMT solution. Dissolved sulfides can also reduce tungstate, producing a blue solution, which can be remedied by re-oxidation of the solution using  $H_2O_2$  (Krukowski, 1988).

- 9. Pellets were mixed with SMT w/ $\rho = 1.5$  g/mL in a slurry at a ratio of 1 mL sediment per 2 mL  $\rho = 1.5$  g/mL SMT. Samples were vortexed and shaken to produce a slurry.
- 10. 10 x 1 mL of SMT-sediment slurry was sampled into 2 mL tubes for downstream DNA extraction, constituting samples of the 'bulk' sediment, effectively control samples for use in downstream community analysis. Exposure to 1.5 g/mL SMT was important for these control samples, as SMT can interact with downstream PCR (Harrison and Orphan, 2012).
- 11. Samples of SMT-sediment slurry were diluted with nonsterile DI and pelleted by centrifugation for 30 s at 10000× g. Supernatant was decanted into a 500 mL Pyrex bottle used later to recycle SMT (steps 22-23).
- 12. The following wash steps were performed simultaneously with step 13 below. Three washes of the pellets were then performed by addition of 1.5 mL 1X PBS to tubes and repeated centrifugation at 10000× g for 30 s. After washing, samples were frozen at -20°C for DNA extraction, fixed for 1 hr in 2% paraformaldahyde (PFA) at RT for FISH, or washed in DI and air dried for mineralogical analysis. PFA-fixed samples were washed 3 times with 1X PBS after fixing and resuspended in 1:1 1X PBS:EtOH and stored at -20°C.
- 13. 1 mL of SMT-sediment slurry was overlain on the 4 mL  $\rho$  = 2.9 g/mL SMT added to each 5 mL tube.
- 14. Density separation was then performed by centrifugation using a Beckman-Coulter Allegra X-14 at 2000× g for 2 min at RT. 2 min was determined as a sufficient centrifugation time that maximizes the proportion of pelleted material while minimizing time of exposure of microorganisms to SMT solution (Supp. Fig. 1).
- 15. Supernatant sediment, forming a cohesive layer, was pipetted out of 5 mL tubes and into new sterile 5 mL tubes using a 5000  $\mu$ L pipette, with the first 5 mm of the pipette tip cut off using a razor blade. This modification enables removal of the cohesive layer without perturbing the density gradient, improving the reproducability and mineralogical purity of the separation.
- **16.** After the cohesive layer is pipetted into new 5 mL tubes, the remaining SMT solution was pipetted out of the original tubes and into the new tubes along with the cohesive layer, taking care to not disturbe the pellet in the original tubes.
- 17. 1 mL filter- and UV-sterilized DI was then added to each of the new tubes, which diluted the SMT to  $\rho = 2.4$  g/mL.

- 18. Simultaneously, the new tubes with SMT  $\rho = 2.4$  g/mL were centrifuged as in step 14 and the original tubes (containing pellets w/ $\rho > 2.9$  g/cc) were washed and frozen following step 12, with the modification that samples were pooled in one 2 mL tube.
- 19. After centrifugation, supernatant sediment in new tubes with SMT  $\rho = 2.4$  g/mL (sediment fraction with 2.4 g/cc <  $\rho$  < 2.9 g/cc) were removed by 5000 µL pipette as in step 15 and pipetted into 2 mL tubes for subsequent washing as in step 12.
- 20. At the same time, pelleted material ( $\rho < 2.4$  g/cc) was carefully pipetted out into 2 mL tubes for washing following step 12.
- **21.** Remaining SMT and contaminating sediment was decanted into the 500 mL Pyrex bottle to be used for SMT recycling.
- 22. SMT recycling was performed by decanting sediment-contaminated SMT along with the remaining 1.5 g/mL SMT from step 6 into the 0.2  $\mu$ m filter flask used in step 2.
- 23. After vacuum filtration, SMT filtrate  $\rho \approx 2.4$  g/mL was densified by evaporation, by decanting filtrate into a 500 mL Pyrex bottle with stir bar placed on a hot plate set to 50° C. Densification then proceeded for the next ~4 hr by evaporation, assisted by flow of compressed air passed through a 0.2 µm filter at the end of a hose directed into the Pyrex bottle. Subsamples were taken every ~1 hr to track SMT density by pipetting out 1 mL SMT into 1 mL nonsterile DI in a weighed 2 mL tube, taking care to pipette up and down several times into DI to ensure all SMT was successfully pipetted out. The 2 mL tube was then weighed to assay SMT density.
- 24. After SMT was successfully redensified to  $\rho = 2.9$  g/mL, filter and UV sterilization was performed as in steps 2-3. SMT can be recycled many times but the formation of insoluble calcium tungstate will eventually remove enough tungstate to require purchase of new SMT powder.

Application of this protocol to incubations of methane seep sediment maintained under AOM conditions in the laboratory (see below) yielded three density fractions, the bulk sediment control, and the porewater fraction from initial centrifugation of sediment samples. For each sediment sample, seven replicate separations were performed for statistical robustness in downstream community analysis. Fractions from five of the separations were frozen at -20°C for DNA extraction, fractions from the fourth separation were not frozen but washed in DI and dried for mineralogical analysis, and fractions from the fifth separation was fixed for 1 hr with 2% PFA. Every separation yielded 2 replicate samples in each fraction. Thus, the five replicate separations for downstream DNA extraction yielded dense ( $\rho > 2.9$  g/cc, n = 5), intermediate ( $2.4 < \rho < 2.9$  g/cc, n =10), and light ( $\rho < 2.4$  g/cc, n = 10) fractions as well as samples of the bulk control (n =10). Only 5 samples of the dense fraction were acquired as the small mass of dense minerals in these samples required pooling for successful PCR amplification.

## Sample Collection, Processing, and Incubation

Sediment samples analyzed in this study were collected by push core using the ROV *Doc Ricketts* of the R/V *Western Flyer* during a cruise in October 2013 and the ROV *JASON* II of the R/V *Atlantis* in September 2011 (AT18-10) to seafloor methane seep sites in Santa Monica Basin, California, and Hydrate Ridge North, Oregon, respectively. Push core (PC) samples were recovered from seafloor methane seep sites in Santa Monica Basin (PC-KD: 33.640056, -118.800278, 898 mbsl) and Hydrate Ridge North (sample 5133: 44° 40.03N, 125° 6.00W, 600 mbsl). PC-KD, a long (24 cm) push core sample, was homogenized shipboard by preparation of a sediment slurry, and was sealed an Ar-sparged mylar bag and stored at 4° C for laboratory-based incubation experiments. Sample 5133 was derived from a PC sectioned by sediment depth horizons that were subsequently transferred to Pyrex bottles, slurried with filtered seawater, sparged with CH<sub>4</sub>, and sealed with butyl stoppers (Skennerton et al., 2016). Seawater samples were collected proximal to

methane seep sites with Niskin bottles, and collected seawater was filter-sterilized on board and placed in sterile 2 L Pyrex bottles. Laboratory incubation experiments were performed by mixing N<sub>2</sub>-sparged filter-sterile seawater with sediment samples at a ~2:1 sediment to seawater ratio in sterile 500 mL Pyrex bottles continuously sparged with N<sub>2</sub> to maintain anoxia. Incubations were sealed with butyl stoppers and pressurized with CH<sub>4</sub> to maintain AOM conditions. Every ~6 months, water from the bottle incubations was removed and were refreshed with filter-sterilized N<sub>2</sub>-sparged seawater to maintain a steady supply of sulfate and prevent buildup of sulfide.

## *X-ray Diffractometry*

The mineralogy of density-separated methane seep sediments was characterized in part by use of X-ray diffractometry (XRD). Air-dried samples of mineral fractions and the bulk control were resuspended in 1000  $\mu$ L DI and 200  $\mu$ L each were spotted onto round glass slides (diameter 1.5 cm) for fitting into the XRD holder. After air drying, samples were analyzed by a PANalytical X-Pert Pro (Malvern Panalytical, Malvern, UK) housed in the Department of Applied Physics and Materials Science at Caltech. Scans were performed between  $3.5 - 65^{\circ}$  2 $\theta$  with a step size of 0.008° 2 $\theta$  and step time of 1 s. The a Cu anode source was set to 45 kV and 40 mA. Peak-matching to JCPDS mineral standards was performed on XRD spectra using X'Pert Highscore software (Malvern Panalytical). Peakmatching was performed by first using the "Search Peaks" function with parameters: minimum significance = 25, minimum tip width = 0.5, peak base width = 1.5, method = minimum  $2^{nd}$  derivative. The "Search & Match" function was then used, with the following settings: minimum score = 20, search sensitivity = 1.8, smoothing range = 1.5, threshold factor = 3.

### Fourier Transform Infrared Spectroscopy

Samples analyzed by XRD were then measured by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) for further mineralogical characterization. Samples adhered by air drying to glass slides were inverted and placed onto an ATR diamond plate in a SensIR Durascope (SensIR Technologies, Danbury, CT) accessory mounted inside a Thermo Nicolet Magna 860 FTIR (Thermo Fischer Scientific, Waltham, MA, USA) housed in the Rossman Laboratory in the Division of Geological and Planetary Sciences at Caltech. Spectra were acquired at a resolution of 4 cm<sup>-1</sup> between 4000 to 400 cm<sup>-1</sup>. Comparison to mineral standards was then performed using the RRUFF database (rruff.info, (Lafuente et al., 2016)) and FTIR spectra aqcuired from API clay mineral standards by Prof. Rossman

(http://minerals.gps.caltech.edu/FILES/ATR/myweb5/clay.htm).

## Scanning Electron Microscopy

Sediment fractions were imaged by scanning electron microscopy (SEM) and analyzed for elemental composition by energy-dispersive X-ray spectroscopy (EDS). The remaining 800 µL of each mineral fraction suspended in DI prior to XRD and FTIR analysis (see 'Sample Collection, Processing, and Incubation' above) was concentrated into a ~1 mm diameter area on a 0.2 µm EMD Millipore white polycarbonate filter (code GTTP) using a pipette. The concentrated mineral sample was cut out of the filter using a razor blade and adhered to carbon tape attached to an SEM stand. Samples were then Pdcoated (10 nm thickness) using a Cressington Sputter Coater 208HR. Samples were imaged by a Zeiss 1550VP Field Emission SEM equipped with an Oxford INCA Energy 300 X-ray EDS system housed in the Division of Geological and Planetary Sciences at Caltech. SEM images were acquired using an electron beam energy of 10 eV and EDS point spectra were acquired with a 15 eV electron beam.

### DNA Extraction and Illumina MiSeq sequencing of 16S rRNA gene

DNA was extracted from samples of methane seep sediments using the Power Soil DNA Isolation Kit 12888 following manufacturer (Qiagen, Germantown, MD, USA) directions modified for sediment samples (Orphan et al., 2001a; Case et al., 2015). The V4-V5 region of the 16S rRNA gene was amplified using archaeal/bacterial primers (Parada et al., 2016) with Illumina (San Diego, CA, USA) adapters on 5' end (515F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GTGYCAGCMGCCGCGGTAA-3', 926R: 5' GTCTCGTGGGGCTCGGAGATGTGTATAAGAGACAG-

CCGYCAATTYMTTTRAGTTT-3'). PCR reaction mix was set up in duplicate for each

sample with Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs,

Ipswich, MA, USA) in a 15  $\mu$ L reaction volume according to manufacturer's directions with annealing conditions of 54°C for 30 cycles. Duplicate PCR samples were then pooled and 2.5  $\mu$ L of each product was barcoded with Illumina NexteraXT index 2 Primers that include unique 8-bp barcodes (P5 5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXX-TCGTCGGCAGCGTC-3' and P7 5'-

CAAGCAGAAGACGGCATACGAGAT-XXXXXXX-GTCTCGTGGGCTCGG-3'). Amplification with barcoded primers used the same conditions as above, except for a volume of 25 μL, annealing at 66°C and 10 cycles. Products were purified using Millipore-Sigma (St. Louis, MO, USA) MultiScreen Plate MSNU03010 with vacuum manifold and quantified using ThermoFisherScientific (Waltham, MA, USA) QuantIT PicoGreen dsDNA Assay Kit P11496 on the BioRad CFX96 Touch Real-Time PCR Detection System. Barcoded samples were combined in equimolar amounts into single tube and purified with Qiagen PCR Purification Kit 28104 before submission to Laragen (Culver City, CA, USA) for 2 x 250 bp paired end analysis on Illumina's MiSeq platform with PhiX addition of 15-20%.

## Processing of 16S rRNA gene MiSeq sequences

Sequence data was processed in QIIME version 1.8.0 (Caporaso et al., 2010) following published methods (Mason et al., 2015). Raw sequence pairs were joined and quality-trimmed using the default parameters in QIIME. Sequences were clustered into *de*  novo operational taxonomic units (OTUs) with 99% similarity using UCLUST open reference clustering protocol and the most abundant sequence was chosen as representative for each de novo OTU (Edgar, 2010). Taxonomic identification for each representative sequence was assigned using the Silva-119 database (Quast et al., 2013) clustered at 99% similarity. This SILVA database had been appended with 1,197 in-house high-quality, methane seep-derived bacterial and archaeal full-length 16S rRNA sequences. Any sequences with pintail values > 75 were removed. The modified SILVA database is available upon request from the corresponding authors. Further taxonomic assignment of OTUs assigned to the SEEP-SRB1 clade was performed by aligning these 411 bp amplicon sequences to the Silva 119 database in ARB (Ludwig et al., 2004) and construction of a phylogenetic tree from full-length and amplicon 16S rRNA sequences of SEEP-SRB1 and sister clades to delineate SEEP-SRB1 subgroups (Schreiber et al., 2010). Known contaminants in PCR reagents as determined by analysis of negative controls run with each MiSeq set were also removed (Salter et al., 2014) along with rare OTUs not present in any given library at a level of at least 10 reads.

## 16S rRNA Amplicon Community Analysis

The QIIME-produced table of OTUs detected in the bulk control and densityseparated fractions of methane seep sediment was then analyzed using community ecology packages in R versions 3.3.3 and 3.5.0 (R Core Team, 2017). Dissimilarity matrices for samples of bulk control and density-separated fractions were calculated using the BrayCurtis dissimilarity metric (Legendre and Legendre, 1998) by the R package *vegan* (Oksanen et al., 2009). Non-metric multidimensional scaling (NMDS) was applied to ordinate samples based on Bray-Curtis pairwise sample dissimilarities. OTUs differentially abundant in microbial communities associated with dense ( $\rho > 2.9$  g/cc) sediment fractions were detected using the R package *ANCOM* (Mandal et al., 2015), selected here for its low false discovery rate (Weiss et al., 2017). OTUs identified by ANCOM were used to explore the differential abundance of other OTUs of the same taxonomy assignment by regression of OTU relative abundances onto NMDS space using the *envfit* function in *vegan*, with statistical significance (p < 0.05) calculated by 999 permutations. Many additional analyses were performed to provide robustness to these results (see Supplemental File 1 for code)

### Correlation analysis of 16S rRNA amplicon libraries

A QIIME-produced table of OTUs detected in the density-separated fractions of methane seep sediment (excluding bulk controls) was further analyzed using the correlation algorithm SparCC (Friedman and Alm, 2012). A bash shell script (sparccWrapper.sh, written by Karoline Faust) was used to call SparCC Python scripts SparCC.py, MakeBootstraps.py, and PseudoPvals.py. First, SparCC.py calculated correlations between OTUs. MakeBootstraps.py then produced 100 shuffled OTU tables by random sampling from the real data with replacement and SparCC.py was used to calculate correlations in each of these 100 shuffled OTU tables. Finally, PseudoPvals.py calculated pseudo-*p* values for OTU correlations in the real dataset by comparison to

correlations calculated in the shuffled OTU tables. As described by Friedman and Alm, 2012, pseudo-*p*-values represent the fraction of correlation coefficients for a given pair of OTUs calculated from the 100 shuffled datasets that are greater than that calculated from the real datasets. Thus, a *pseudo*-p-values < 0.01 for a given pair of OTUs indicates that no correlation coefficient from any given shuffled dataset was greater than that calculated from our real data. Subsequent analysis of the produced tables describing magnitude and significance for OTU correlations was performed in R versions 3.3.3 and 3.5.0, using visualization packages *igraph* (Csardi and Nepusz, 2006), *circlize* (Gu et al., 2014), *ggplot2* (Wickham, 2009), and *RColorBrewer* (Neuwirth, 2014). In this study, only positive correlations (correlation coefficient > 0) between OTUs were used.

#### Fluorescence in situ Hybridization

To confirm the presence of microbial taxa in density-separated sediment fractions, as inferred by analysis of 16S rRNA amplicon datasets, we performed fluorescence *in situ* hybridization (FISH) using the following modification of previously published protocols (Orphan et al., 2002). Density-separated sediment fractions fixed by PFA were concentrated onto ~1 mm diameter areas on 0.2  $\mu$ m white polycarbonate filters. This area of the filter was then cut out with a razor blade and placed in a 200  $\mu$ L PCR tube for FISH labeling. FISH was performed overnight (18 hr) using the following modifications (G. Chadwick, pers. comm.) to previously-published protocols. A hybridization buffer at appropriate stringency was prepared along with accompanying wash buffer [33, 34] and

pre-warmed to 46°C and 48°C in a hybridization oven and a water bath, respectively. 5 µL of FISH probe stock (50 ng/µL) ANME-3-125 with cy3 dye (K. Dawson, pers. comm. 5'- TCCTAAGGGCAGGTTATCCA-3') and either Eub338 mix (Daims et al., 1999) or probe Seep1c-1309 (Schreiber et al., 2010) in cy5 was added to 40 µL hybridization buffer along with filter sections. After 18 hr hybridization, filters were removed and incubated for 20 min at 48°C in 200 µL wash buffer. Filter sections were then removed and briefly dipped in deionized water and placed on Superfrost Plus slides (Thermo Fischer Scientific) to dry at room temperature in the dark. 10 µL 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) dissolved in Citifluor (Electron Microscopy Sciences) was applied to filter sections and left to incubate for 15 min in the dark. A cover slip (No. 1.5, VWR, Radnor, PA, USA) was then placed on each filter. Hybridized samples were illuminated using an XCite Series 120Q fluorescence source and imaged with a Qimage QIClick camera attached to an Olympus BX51 epifluorescence microscope with 60x (Olympus PlanApo N Oil, N.A. 1.42) and 100x (Olympus UPlan FL N Oil, N.A. 1.30) objectives. Imaging software cellSens Dimension was used to acquire images. Composites of epifluorescence images were produced using the image processing software O-Capture Pro 7.

#### RESULTS

#### Density Separation by SMT Yields Three Mineralogically-Distinct Fractions

The efficacy of the density-separation protocol presented here was assayed by characterizing the mineralogy of density fractions. Methane seep sediment samples from both Hydrate Ridge North (sample 5133) and Santa Monica Basin (sample PC-KD) were

both separated by density and analyzed in parallel by XRD, FTIR, and SEM to identify mineral constituents of each fraction and by 16S rRNA amplicon sequencing to characterize the microbial communities present in each fraction. Here, we present mineralogical data from density separation of sample 5133 and microbial community data from sample PC-KD. Mineralogical data from only sample 5133 are presented here, as samples of fractions from PC-KD originally intened for mineralogical analysis were instead used for 16S rRNA amplicon sequencing to increase the size of the sequencing dataset. Sequencing data from sample 5133 as well as samples of seep sediment from Costa Rica (samples 10073, 10003-10007) are not shown as our separation protocol did not yield insight into microbial communities found in fractions rich in conductive minerals.

XRD analysis of dense ( $\rho > 2.9$  g/cc), intermediate (2.4 <  $\rho < 2.9$  g/cc), and light ( $\rho < 2.4$  g/cc) density fractions of methane seep sediment sample 5133 documented distinct mineralogical compositions in each fraction. Automated analysis of XRD spectra identified magnetite and pyrite in the dense fraction (Fig. 2, Table 1). Several significant peaks were not identified by X'Pert Highscore, most notably (counts > 30) those at  $2\theta = 8.4^{\circ}$ ,  $10.2^{\circ}$ ,  $12.2^{\circ}$ ,  $17.4^{\circ}$  (Table 1). XRD analysis of the intermediate fraction catalogued peaks assigned to quartz and calcite (Fig. 2, Table 2), and several without a peak assignment, including those at  $2\theta = 8.8^{\circ}$ ,  $12.4^{\circ}$ ,  $21.9^{\circ}$ ,  $24.1^{\circ}$ ,  $27.8^{\circ}$  (Table 2). XRD analysis of the light fraction identified only quartz and halite, with peaks not identified also observed with peak maxima at  $2\theta = 8.6^{\circ}$ ,  $12.3^{\circ}$ ,  $18.6^{\circ}$ ,  $19.5^{\circ}$ ,  $21.8^{\circ}$ ,  $23.5^{\circ}$ ,  $27.6^{\circ}$ , and  $29.6^{\circ}$  (Table 3). A large, broad peak not detected by the automated peak identification algorithm was also observed in the light fraction at  $2\theta \approx 6^{\circ}$  (Fig. 2).

Spectra collected by FTIR performed on of density-separated methane seep sediment samples complemented XRD analysis, revealing three mineralogically-distinct fractions. FTIR spectra collected from the dense fraction displayed absorbance peaks at 454, 509, 1012, 1451, 1636, 3424, and 3601 cm<sup>-1</sup>. Comparison to FTIR spectra from the RRUFF database (Lafuente et al., 2016) corroborated XRD characterization of this fraction as FTIR of pyrite (RRUFF ID R050070) and magnetite (R080025) closely matching a patten of increasing absorbance at wavenumbers >1200 cm<sup>-1</sup> (Fig. 3). FTIR detected clays in this fraction not observed in XRD spectra, with peaks at 454, 509, and 1012 cm<sup>-1</sup> matching a FTIR spectrum of illite (H-36, Morris, Illinois) closely (Fig. 3). The XRD spectrum for this fraction did not detect clay minerals, but this may have been a result of the absence of extensive clay mineral standards in the X'Pert Highscore database or the inherent challenges of characterizing clay minerals, the identification by XRD of which can require specialized techniques (Moore and Reynolds, 1997).

FTIR spectroscopy performed on the intermediate density fraction further corroborated XRD analysis of that fraction, with promenent absorbance peaks at 880 cm<sup>-1</sup> and 1422 cm<sup>-1</sup> matching absorbance peaks from a FTIR spectrum collected from a calcite sample (Fig. 3). FTIR spectra of this fraction also displayed spectroscopic features possibly indicating the presence of other Ca carbonates dolomite or aragonite, such as a shoulder to the 1418 cm<sup>-1</sup> peak in the sample spectrum at 1460 cm<sup>-1</sup> (Supp. Fig. 2); dolomite or aragonite was not detected by automated peak matching for XRD scans. There also appeared to be significant clay in this fraction, demonstrated by a close match between the sample spectrum and that of an illite standard (Fig. 3).



**Figure 2.** Representative X-ray diffraction (XRD) spectrum of the dense (*top*,  $\rho > 2.9$  g/cc), intermediate (*middle*, 2.4 <  $\rho$  < 2.9 g/cc), and light (*bottom*,  $\rho$  < 2.4 g/cc) mineral fraction sfrom density-separated methane seep sediment, with peaks identified and annotated by best-fit mineral from powder-diffraction standards.

FTIR spectroscopy corroborated XRD observations for the light density fraction, with prominent absorbances at 419, 524, 1000, 1461, and 1644 cm<sup>-1</sup>, and a broad peak at ~3430 cm<sup>-1</sup>, all matching equivalent absorbances from a montmorillonite standard (H-24, Otay, California; Fig. 3). While automated analysis XRD spectrum of this sample did not detect clay minerals, possibly for the same reasons discussed above for the intermediate density fraction, we inferred the presence of clays from the XRD spectrum in this fraction by the observation of a broad peak at  $2\theta \approx 6^{\circ}$  (Fig. 2), approximately equivalent to the basal 001 reflection for montmorillonite between  $2\theta = 6.3^{\circ} - 6.6^{\circ}$  (rruff.info, R110053).

Taken together, the XRD and FTIR data supported the inference that our densityseparation protocol yielded three fractions of sediment distinguished by mineralogy. The dense fraction was composed of pyrite and magnetite with minor (only observed by FTIR) clays, the intermediate fraction consisted of calcite and minor clays, and the light fraction contained only clays. We thus concluded that we could apply our protocol to acquire three mineralogically-distinct fractions: a silicate-rich fraction ( $\rho < 2.4$  g/cc), a carbonate-rich fraction ( $2.4 < \rho < 2.9$  g/cc), and a fraction rich in iron sulfides and oxides ( $\rho > 2.9$  g/cc). We used this nomenclature to describe the fractions for the remainder of the study.



**Figure 3.** Representative FTIR spectra of the dense (*top*, blue), intermediate (*middle*, green), and light (*bottom*, red) fractions of density-separated methane seep sediment. Spectra were normalized to maximum absorbance equal to 1. Spectra from minerals in the RRUFF database (Lafuente et al., 2016) and from API clay mineral standards are underlain for comparison; minerals were selected on the basis of their identification in XRD spectra collected from thesame density fractions (Fig. 2).

Given our successful enrichment of conductive minerals magnetite and pyrite in the iron sulfide/oxide-rich fraction, we wanted to characterize the microscale nature of this fraction by SEM. Optical microscopy performed on this fraction documented abundant 10-25  $\mu$ m diameter clusters of ~2-5  $\mu$ m diameter framboidal solid phases that appeared highly reflective under reflected light (Fig. 4). Epifluorescent imaging using a DAPI stain documented ~1  $\mu$ m diameter signal colocalized with outer surfaces of reflective framboids (Fig. 4). Subsequent correlated SEM imaging revealed DAPI signal to be correlated with ~1  $\mu$ m rods adhered to framboid surfaces. EDS point spectra characterized the framboidal phases as Fe and S rich (data not shown). Together with the FTIR and XRD data, we inferred from our correlated fluorescence and electron microscopy observations that the iron sulfide/oxide-rich fraction contained abundant pyrite framboids with adhered microbial cells.

## 16S rRNA Amplicon Sequencing of Density Fractions Reveals Mineral-Associated Microbial Communities

Application of the density separation protocol to methane seep sediments from Santa Monica Basin (sample PC-KD) yielded 5 iron sulfide/oxide-rich fractions, 10



**Figure 4.** Correlated epifluorescence (left) and scanning elecctron microscopy (to scale, right) imaging documenting an aggregate of Fe sulfides (composition confirmed by EDS, data not shown) in a sample of the dense ( $\rho > 2.9$  g/cc) sediment fraction, featuring attached cells visible by DAPI stain and under SEM (arrows).

carbonate-rich fractions, 10 silicate-rich fractions, and 10 bulk control samples. Of these, PCR amplification all using 30 PCR cycles was successful in all 5 iron sulfide/oxiderich fractions, 9 carbonate-rich fractions, 9 silicate-rich fractions, and 7 bulk sediment controls. QIIME processing of amplicon sequences prepared from these 30 samples yielded 875 OTUs after filtering in R.

16S rRNA amplicon sequencing of the bulk sediment controls revealed a microbial community in the bulk sediment controls dominated by ANME subtypes ANME-2a/b, ANME-2c and ANME-3 (Fig. 5). OTUs assigned to the common ANME partner SEEP-SRB1 (Schreiber et al., 2010) were found to be the second-most-abundant group of OTUs. OTUs assigned to archaeal Marine Benthic Groups B and D, the bacterial SEEP SRB2 clade, and to the deeply-branching Atribacteria were observed to be minor members of the bulk community.

Comparison of 16S rRNA community profiles between the bulk sediment controls and the iron sulfide/oxide-rich fraction revealed ANME-3 OTUs to be differentially abundant in this fraction. ANME-3 OTUs constitute a larger portion of the total number of reads in the iron sulfide/oxide-rich fraction, comprising  $15 \pm 2.6\%$  of all reads in this fraction compared to only ~2.5% of the reads in the bulk sediment controls (Fig. 5). Comparison of the relative abundance of the 78 ANME-3 OTUs across all density fractions documented higher ANME-3 OTU abundance correlated with fraction density (Fig. 6), with the ANME-3 OTU population dominated (~80% of ANME-3 reads) by 4 OTUs.



**Figure 5.** Stacked bar plot comparing relative abundance of OTUs, clustered by taxonomy assignment at a family level according to NCBI taxonomy assignments in Silva-119 (Quast et al., 2013), between bulk sediment controls and density-separated fractions rich in iron sulfides and iron oxides. Bars are replicate samples, and are ordered on increasing abundance of ANME-3 OTUs.

Silicate-rich density fractions, with average density lower than that of the bulk sediment, were shown to have fewer ANME-3 reads than bulk sediment controls (Fig. 6). Ordination of sample pairwise dissimilarity by NMDS identified the 16S rRNA community profiles for the iron sulfide/oxide-rich fraction to be more dissimilar to the bulk controls than other density fractions (Fig. 7). Regression of OTU relative abundance onto the NMDS ordination revealed all significant (p < 0.05) ANME-3 OTU vectors were correlated with iron sulfide/oxide-rich fractions (Fig. 7). Consistent with the observation that ANME-3



**Figure 6.** *Top*, stacked bar plots comparing relative abundance of ANME-3 OTUs across all density-separated sediment fractions, with each bar representing a replicate sample. Samples are ordered by increasing total abundance of ANME-3 OTUs and are annotated by density fraction. *Bottom,* mean relative abundance of all ANME-3 OTUs in density-separated fractions ordered by ANME-3 OTU abundance.



**Figure 7.** *Left*, NMDS plot displaying differences in 16S rRNA community composition of density-separated methane seep samples from Santa Monica basin, as measured by Bray-Curtis dissimilarity. Replicates are colored by the XRD- and FTIR-identified mineralogy of the density fraction from which DNA was extracted and amplified. Arrows represent regressions (using *envfit*) of ANME-3 OTU relative abundance onto Bray-Curtis NMDS space; light arrows are *envfit* regressions (p < 0.05) and dark arrows are *envfit* regressions for OTUs detected to be differentially abundant by ANCOM (p < 0.05, Mann-Whitney *U* test, B-H adjusted). *Right*, subset of OTUs detected to be differentially abundant in (p < 0.05, Mann-Whitney *U* test, B-H adjusted). *Right*, subset of OTUs detected to be differentially abundant in (p < 0.05, Mann-Whitney *U* test, B-H adjusted). *Right*, subset of OTUs detected to be differentially abundant in (p < 0.05, Mann-Whitney *U* test, B-H adjusted). *Right*, subset of OTUs detected to be differentially abundant in (p < 0.05, Mann-Whitney *U* test, B-H adjusted). *Right*, subset of OTUs detected to be differentially abundant in (p < 0.05, Mann-Whitney *U* test, B-H adjusted).



**Figure 8.** Stacked histograms illustrating the distribution of SparCC-calculated correlations between 16S rRNA amplicon sequences from all densityseparated fractions, excluding bulk sediment controls. *Left*, comparison of correlations deemed significant on the basis of bootstrap-calculated pseudop-values < 0.01 (green) with those not calculated to be significant ('*N.S.*', in gray). *Right*, significant correlations broken down by the taxonomy assignment of each OTU in the pair. 'SRB' are considered those taxa with assignments of known ANME-partner SRB (e.g. SEEP-SRB1) or any Deltaproteobacteria with genus-level taxonomy assignment containing the string 'Desulf' (e.g. *Desulfococcus* sp.).

OTU relative abundance is correlated with fraction density (Fig. 6), carbonate-rich fractions lie in NMDS space between iron sulfide/oxide-rich fractions and those of the bulk sediment controls (Fig. 7). The differential abundance R package ANCOM was then applied to detect differentially-abundant OTUs between the bulk sediment control and the iron sulfide/oxide-rich fraction. ANCOM detected 3 OTUs, all ANME-3, that were differentially-abundant in the iron sulfide/oxide-rich fraction (p < 0.05, Mann-Whitney U test, B-H adjusted; Fig. 7), supporting the inference from *envfit* regression that ANME-3 OTUs are preferentially found associated with magnetite and pyrite in our samples of Santa Monica Basin methane seep sediment.

# Correlation Analysis of Density-Separated Methane Seep Sediment Reveals Associations Between Microbial Taxa

With the association between ANME-3 OTUs and magnetite/pyrite established by community analysis, we turned to investigate the potential partnerships between ANME-3 and different bacterial SRB clades that could possibly be detected with higher fidelity due to their co-association on pyrite and/or magnetite surfaces. We applied the correlation tool SparCC (Friedman and Alm, 2012) to the 30 16S rRNA amplicon datasets prepared from density-separated methane seep sediments, calculating 383,250 pairwise correlations between the 875 OTUs detected in our 30 samples. Of these pairs, 4.4% (17,196) had pseudo-*p*-values < 0.01, indicating the coefficients for each of these correlations exceeded that calculated for that same OTU pair in any of the 100 bootstrapped datasets (Fig. 8). The taxonomic assignment of the constituent OTUs of correlations with pseudo-p < 0.01were then inspected: 33% (5,740) of correlations with pseudo-p < 0.01 describe those involving ANME.

From this set of significant correlations, a network diagram was constructed to identify the OTUs correlated with ANME-3 OTUs, representing the microbial community adhered to conductive mineral surfaces that may be interacting with ANME-3 via coattachement to these minerals. Examination of clusters of OTUs within this network revealed two primary communities of OTUs (Fig. 9). Of these communities, one consisted of ANME-2c, ANME-2a/b, and SEEP-SRB1 OTUs and the other centered on ANME-3 and SEEP-SRB1 OTUs (Fig. 9). SEEP-SRB1 OTU representative sequences were extracted and compared with SEEP-SRB1 full-length 16S rRNA sequences used by Schreiber and coworkers to assign subtype taxonomy (e.g. SEEP-SRB1a, SEEP-SRB1c) to these sequences (data not shown). This analysis revealed SEEP-SRB1 OTUs associated with ANME-3 OTUs in network analysis are predominantly SEEP-SRB1c, while those associated with ANME-2a/b and -2c in the network are a mix of SEEP-SRB1a and SEEP-SRB1c. Additionally, ANME-3 associate with other non-SRB taxa in the network, including several OTUs assigned to the Atribacteria and Marine Benthic Group B (now referred to as Lokiarchaeota).

To validate the presence of ANME-3 consortia in the iron sulfide/oxide-rich fraction, we performed fluorescence in situ hybridization (FISH). Epifluorescence microscopy of an aliquot of the iron sulfide/oxide-rich fraction documented several ANME-3 consortia of ~10 µm diameter (Fig. 10). Other microbial aggregates stained only by DAPI counterstain (deemed to be putative ANME-SRB consortia) were also observed, but quantification of the relative abundance of ANME-3 consortia as a proportion of total consortia was precluded given the small sample size, with total consortia observed  $\leq 25$ . The propensity for the SMT density matrix to form precipitates via reaction with  $Ca^{2+}_{(ac)}$ presented significant challenges for our FISH experiments, as these precipitates, often forming  $\sim 100 \,\mu\text{m}$  long crystals (data not shown), interfered significantly with FISH reactions. Reflected light microscopy revealed several ANME-3 consortia with reflective domains inferred to be reflective pyrite or magnetite on the basis that iron sulfides with composition confirmed by EDS (Fig. 4) appeared reflective. These consortia were typically  $\sim$ 10-15 µm in diameter, and featured ANME-3 cocci forming aggregates with a shell-type morphology similar to that previously observed for ANME-3 consortia (Lösekann et al., 2007). Application of FISH probes targeting bacterial 16S rRNA did not successfully hybridize to cells in consortia, but DAPI staining of non-ANME-3 cells in consortia suggested that bacterial cells were likely present but not labeled.



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- SparCC correlations, pseudo-*p* < 0.05 (100 bootstraps)
- SparCC correlations w/ coefficients > any bootstrap, pseudo-p < 0.05 (100 bootstraps)



**Figure 9.** Network diagrams constructed from SparCC-calculated (pseudo-p < 0.01) pairwise correlations between OTUs in density-separated methane seep sediment. *Top*, network diagram with vertices (OTUs) colored by taxonomy assignment. OTUs found to be particularily highly-correlated include ANME-3 OTUs that were found to be differentially-abundant in the iron sulfide/oxide-rich fraction (*bottom inset*); these OTUs are correlated with specific SEEP-SRB1 OTUs that fall within the SEEP-SRB1c lineage as previously defined (Schreiber et al., 2010).

## DISCUSSION

Associations between sediment-dwelling microorganisms and minerals have proven challenging to parse, but are likely are important for the physiology of these microorganisms given the observations from cultures (Kato et al., 2012b; Liu et al., 2015; Tang et al., 2016) and environmental incubations (Kato et al., 2012a; Rotaru et al., 2018) that conductive minerals stimulate syntrophic EET. Previous methods and results to examine mineral-associated microbial ecology in marine sediments reported by Harrison and Orphan described density-separation protocols using SMT to yield a dense ( $\rho > 2.8$ g/cc) and a light ( $\rho < 2.8$  g/cc) fraction from methane seeps, but the mineralogical purity of these fractions was only examined by the elemental composition of these fractions using EDS, and microbial communities in each fraction were identified by terminal restriction fragment length polymorphism (Harrison and Orphan, 2012). Other work has used SMT to separate marine sediments by density and characterized the mineralogical purity of density fractions by XRD (Poppe et al., 1991; Poppe and Commeau, 1992; Totten et al., 2002), but no work to date has used SMT separation in tandem with next-generation Illumina sequencing of 16S rRNA amplicon libraries to reveal in situ microbe-mineral associations in marine sediments.







**Figure 10.** Reflected light and epifluorescent microscopy imaging of ANME-3—SRB consortia extracted from the iron sulfide/oxide-rich fraction. ANME-3 were labeled using FISH probe ANME-3-125 (K. Dawson, pers. comm.). Reflective domains embedded in the extracellular matrix appear as white from reflected light.

Here, we present the successful enrichment of iron sulfide/oxide-rich and carbonate-rich mineral fractions from methane seep sediments by application of SMTbased density separation to methane seep sediments. XRD (Fig. 2) and FTIR (Fig. 3) spectra acquired from the dense ( $\rho > 2.9$  g/cc) fraction were fit by pyrite and magnetite standards. FTIR spectroscopy of the intermediate-density fraction, in contrast, documented spectra from this fraction well-matched by calcite standards (Fig. 3), corroborating XRD measurements (Fig. 2). FTIR spectra of these fraction also contained features characteristic of clay minerals, indicating either the presence of particularily dense illite (Totten et al., 2002) and/or lower-density clays adhered to magnetite or pyrite surfaces. However, we inferred that clays were significantly less abundant in these fractions in comparison with the light fraction, as XRD spectra of the dense and intermediate fractions did not detect the peak at  $2\theta \approx 6^{\circ}$  observed in the light fraction (Fig. 2), interpreted as reflective of clays at significant abundance to produce prominent signal at low  $2\theta$ . Successful enrichment of these mineral fractions by density separation not only made possible downstream ecological study of mineral-associated microbial communities, but may also prove useful for future geochemical study of carbonate and sulfide mineral authigenesis in methane seep sediments.

16S rRNA amplicon sequencing of the density fractions revealed a particularily unique community associated with the iron sulfide/oxide-rich fraction. Differential abundance analysis by ANCOM detected 3 OTUs, all assigned to the ANME-3, that were significantly abundant relative to the bulk sediment (Fig. 7); no other OTUs were detected as differentially abundant in any other fraction relative to the bulk sediment control. As an enrichment rather than purification technique, density separation using SMT results in some cross-contamination across density fractions; the elevated abundance of ANME-3 in the carbonate-rich fraction (Figs. 9, 10) is likely a consequence of this. An interpretation of the differential abundance of ANME-3 in the dense fraction to be a result of intracellular minerals such as magnetosomes that have been observed in some SRB partners to ANME-2 (McGlynn et al., 2018) seems less likely, as the volume of magnetite or pyrite adhered to or inside of a given cell must be >86-90% of the volume of the cell to achieve mean particle density > 2.9 g/cc. However, given the exceedingly high salinity of SMT solutions, dehydration could significantly reduce cell volumes during density separation. Further EM examination of ANME-3 associated with the dense fraction will be required to characterize the nature of the association.

ANME-3, first discovered in the Haakon Mosby mud volcano in the Barents Sea (Niemann et al., 2006), have also been observed at methane seep sites in the Congo Basin (Pop Ristova et al., 2012) and the Eel River Basin (Orphan et al., 2001a), but relatively less is known about their physiology compared to more common ANME subtypes such as ANME-2a. Given their preferential association with the iron sulfide/oxide-rich fraction, which by mass constituted only a small percentage (~1%) of the total mass of the bulk sediment, we inferred that ANME-3 may appear rare when characterizing the microbial community in bulk samples due to their preference for an ecological niche provided by relatively rare magnetite or pyrite grains. We speculated that this niche may provide a condiut for EET and facilitate survival in electron-donor poor AOM sediments, aiding electron transport from ANME-3 to syntrophic partners or to other electron acceptors. In a

similar manner as that observed for in syntrophic co-cultures of *Geobacter* sp. and *Methanosarcina* sp. (Rotaru et al., 2018), conductive minerals may aid in the transfer of electrons in AOM. Future research should leverage insights from recent work indicating that magnetite in particular stimulates syntrophic EET and focus on extracting magnetic minerals from marine sediments, as has been attempted previously (Harrison and Orphan, 2012. While magnetic mineral concentrations are depleted at the SMTZ relative to their concentration in sediments above and below due to diagenetic reduction of magnetite by sulfide (Roberts, 2015), these trace minerals may still host local populations of microorganisms employing mineral-facilitated EET. Anaerobic extraction methods for magnetite from marine sediments developed in this thesis (see Appendix A) were moderately successful, but future work could build on these methods to extract live microbial communities attached to magnetic minerals for downstream culturing and molecular work.

Density separation of methane seep sediments also provided new hypotheses about microbe-microbe interactions. By extracting fractions of sediment enriched in a particular mineral, density separation amplified ecological signals resulting from the co-association of certain microbial taxa on mineral surfaces. Here, we observed improved signal from correlation analysis performed on density fractions from Santa Monica Basin seep sediments (Fig. 8) relative to that observed from application of the same analysis to a ecological survey of Costa Rica seep sediments (Ch. 1, Fig. 1A, 1B). In the density-separated samples, correlation analysis detects fewer total significant correlations but those correlations have higher coefficients, indicating improved ecological signal. Microbial

communities are readily detected in density-separated samples, requiring no further thresholding of the correlation analysis to obtain insights; in contrast, performing the same analysis on ecological survey data requires a much larger dataset (~300 vs 30 samples) and more post-analysis processing to obtain ecological insights. Furthermore, ecological insights from density separation techniques describe microscale niche differentiation within a single sample, critical information for the investigation of microbial ecology in situ. Here, we infer a preferential association between ANME-3 and SEEP-SRB1c in the iron sulfide/oxide fraction, notable as previous microscopy study had not observed SEEP-SRB1c in association with ANME (Schreiber et al., 2010). Attempted FISH labeling of SEEP-SRB1c using published probe Seep1c-1309 (Schreiber et al., 2010) was unsuccessful, but we inferred this failure did not reflect the absence of association between ANME-3 and SEEP-SRB1c but rather a failure of the probe, designed on the basis of 8 full-length 16S rRNA sequences, to hybridize SEEP-SRB1c 16S rRNA in our samples resulting either from sequence divergence or interference from SMT. Future work should identify the SRB partner for ANME-3, which has been identified in different seep environments as SEEP-SRB1a (Schreiber et al., 2010), SEEP-SRB1c (hypothesized in this study), *Desulfobulbus* sp. (Lösekann et al., 2007), or SEEP-SRB4 (Ruff et al., 2015).

#### CONCLUSIONS

Here, we demonstrate statistically-significant associations between certain 16S rRNA amplicon sequences and specific minerals by application of a heavy-liquids protocol to separate minerals in marine sediments by density compatible with downstream molecular analyses. Application of this protocol to samples of methane seep sediment yielded three mineralogically-distinct fractions of methane seep sediment, with mineralogy of each fraction validated by XRD and FTIR. Community analysis of each density fraction documented a statistically-significant association between ANME-3 OTUs and a density fraction rich in magnetite and pyrite. The presence of ANME-3 in this fraction was then confirmed by FISH microscopy, and the microbial community correlated with ANME-3 in this fraction was detected by analysis of a network constructed from the using the correlation analysis tool SparCC (Friedman and Alm, 2012). Comparison of the correlation analysis results from density-separated seep sediments to those from an ecological survey of seep sediments demonstrated the capacity for this method to amplify ecological signal from microorganisms co-associating on mineral surfaces. The future application of this technique to other sediments and soils holds promise to reveal the microscale interactions between microorganisms and minerals as well as those between microorganisms.

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- Aloisi, G., Pierre, C., Rouchy, J.-M., Foucher, J.-P., and Woodside, J. (2000). Methane-related authigenic carbonates of eastern Mediterranean Sea mud volcanoes and their possible relation to gas hydrate destabilisation. *Earth Planet. Sci. Lett.* 184, 321–338. doi:10.1016/S0012-821X(00)00322-8.
- Beal, E. J., House, C. H., and Orphan, V. J. (2009). Manganese- and Iron-Dependent Marine Methane Oxidation. Science 325, 184–187. doi:10.1126/science.1169984.
- Beulig, F., Røy, H., Glombitza, C., and Jørgensen, B. B. (2018). Control on rate and pathway of anaerobic organic carbon degradation in the seabed. *Proc. Natl. Acad. Sci.* 115, 367– 372. doi:10.1073/pnas.1715789115.
- Boetius, A., Ravenschlag, K., Schubert, C. J., Rickert, D., Widdel, F., Gieseke, A., et al. (2000). A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 407, 623. doi:10.1038/35036572.
- Bowles, M. W., Mogollón, J. M., Kasten, S., Zabel, M., and Hinrichs, K.-U. (2014). Global rates of marine sulfate reduction and implications for sub-sea-floor metabolic activities. *Science* 344, 889. doi:10.1126/science.1249213.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi:10.1038/nmeth.f.303.
- Case, D. H., Pasulka, A. L., Marlow, J. J., Grupe, B. M., Levin, L. A., and Orphan, V. J. (2015). Methane Seep Carbonates Host Distinct, Diverse, and Dynamic Microbial Assemblages. *mBio* 6. doi:10.1128/mBio.01348-15.
- Csardi, G., and Nepusz, T. (2006). The igraph software package for complex network research. *InterJournal* Complex Systems, 1695.
- Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. (1999). The Domainspecific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. Syst. Appl. Microbiol. 22, 434–444. doi:10.1016/S0723-2020(99)80053-8.
- Degnan, P. H., and Ochman, H. (2012). Illumina-based analysis of microbial community diversity. *ISME J.* 6, 183–194. doi:10.1038/ismej.2011.74.
- Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi:10.1093/bioinformatics/btq461.
- El-Naggar, M. Y., Wanger, G., Leung, K. M., Yuzvinsky, T. D., Southam, G., Yang, J., et al. (2010). Electrical transport along bacterial nanowires from Shewanella oneidensis MR-1. Proc. Natl. Acad. Sci. 107, 18127. doi:10.1073/pnas.1004880107.

- Friedman, J., and Alm, E. J. (2012). Inferring Correlation Networks from Genomic Survey Data. *PLOS Comput. Biol.* 8, e1002687. doi:10.1371/journal.pcbi.1002687.
- Froelich, P. N., Klinkhammer, G. P., Bender, M. L., Luedtke, N. A., Heath, G. R., Cullen, D., et al. (1979). Early oxidation of organic matter in pelagic sediments of the eastern equatorial Atlantic: suboxic diagenesis. *Geochim. Cosmochim. Acta* 43, 1075–1090. doi:10.1016/0016-7037(79)90095-4.
- Garming, J. F. L., Bleil, U., and Riedinger, N. (2005). Alteration of magnetic mineralogy at the sulfate-methane transition: Analysis of sediments from the Argentine continental slope. *Phys. Earth Planet. Inter.* 151, 290–308. doi:10.1016/j.pepi.2005.04.001.
- Grünke, S., Lichtschlag, A., Beer, D. de, Felden, J., Salman, V., Ramette, A., et al. (2012). Mats of psychrophilic thiotrophic bacteria associated with cold seeps of the Barents Sea. *Biogeosciences* 9, 2947–2960. doi:https://doi.org/10.5194/bg-9-2947-2012.
- Gu, Z., Gu, L., Eils, R., Schlesner, M., and Brors, B. (2014). Circlize implements and enhances circular visualization in R. *Bioinformatics* 30, 2811–2812.
- Harrison, B. K., and Orphan, V. J. (2012). Method for Assessing Mineral Composition-Dependent Patterns in Microbial Diversity Using Magnetic and Density Separation. *Geomicrobiol. J.* 29, 435–449. doi:10.1080/01490451.2011.581327.
- Jørgensen, B. B. (2000). "Bacteria and Marine Biogeochemistry," in *Marine Geochemistry*, eds. H. D. Schulz and M. Zabel (Berlin, Heidelberg: Springer), 173–207. doi:10.1007/978-3-662-04242-7\_5.
- Kallmeyer, J., Pockalny, R., Adhikari, R. R., Smith, D. C., and D'Hondt, S. (2012). Global distribution of microbial abundance and biomass in subseafloor sediment. *Proc. Natl. Acad. Sci.* 109, 16213. doi:10.1073/pnas.1203849109.
- Kato, S., Hashimoto, K., and Watanabe, K. (2012a). Methanogenesis facilitated by electric syntrophy via (semi)conductive iron-oxide minerals. *Env. Microbiol* 14, 1646–54. doi:10.1111/j.1462-2920.2011.02611.x.
- Kato, S., Hashimoto, K., and Watanabe, K. (2012b). Microbial interspecies electron transfer via electric currents through conductive minerals. *Proc. Natl. Acad. Sci.* 109, 10042. doi:10.1073/pnas.1117592109.
- Knittel, K., and Boetius, A. (2009). Anaerobic Oxidation of Methane: Progress with an Unknown Process. Annu. Rev. Microbiol. 63, 311–334. doi:10.1146/annurev.micro.61.080706.093130.
- Krukowski, S. T. (1988). Sodium Metatungstate: A New Heavy-Mineral Separation Medium for the Extraction of Conodonts from Insoluble Residues. J. Paleontol. 62, 314–316.

Lafuente, B., Downs, R. T., Yang, H., and Stone, N. (2016). The power of databases: The RRUFF project. *Highlights Mineral. Crystallogr.*, 1–29. doi:10.1515/9783110417104-003.

Legendre, P., and Legendre, L. (1998). Numerical Ecology. 2nd ed. Amsterdam: Elsevier.

- Li, H., Chang, J., Liu, P., Fu, L., Ding, D., and Lu, Y. (2015). Direct interspecies electron transfer accelerates syntrophic oxidation of butyrate in paddy soil enrichments. *Environ. Microbiol.* 17, 1533–47. doi:10.1111/1462-2920.12576.
- Liu, F., Rotaru, A. E., Shrestha, P. M., Malvankar, N. S., Nevin, K. P., and Lovley, D. R. (2015). Magnetite compensates for the lack of a pilin-associated c-type cytochrome in extracellular electron exchange. *Env. Microbiol* 17, 648–55. doi:10.1111/1462-2920.12485.
- Lösekann, T., Knittel, K., Nadalig, T., Fuchs, B., Niemann, H., Boetius, A., et al. (2007). Diversity and Abundance of Aerobic and Anaerobic Methane Oxidizers at the Haakon Mosby Mud Volcano, Barents Sea. *Appl. Environ. Microbiol.* 73, 3348–3362. doi:10.1128/AEM.00016-07.
- Lovley, D. R. (1991). Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev. 55, 259-287.
- Lovley, D. R. (2017). Syntrophy Goes Electric: Direct Interspecies Electron Transfer. Annu. Rev. Microbiol. 71, 643–664. doi:10.1146/annurev-micro-030117-020420.
- Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P., and Woodward, J. C. (1996). Humic substances as electron acceptors for microbial respiration. *Nature* 382, 445–448. doi:10.1038/382445a0.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, et al. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res.* 32, 1363–1371. doi:10.1093/nar/gkh293.
- Luff, R., and Wallmann, K. (2003). Fluid flow, methane fluxes, carbonate precipitation and biogeochemical turnover in gas hydrate-bearing sediments at Hydrate Ridge, Cascadia Margin: numerical modeling and mass balances. *Geochim. Cosmochim. Acta* 67, 3403– 3421. doi:10.1016/S0016-7037(03)00127-3.
- Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., and Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26, 27663. doi:10.3402/mehd.v26.27663.
- Marlow, J. J., Steele, J. A., Ziebis, W., Thurber, A. R., Levin, L. A., and Orphan, V. J. (2014). Carbonate-hosted methanotrophy represents an unrecognized methane sink in the deep sea. *Nat. Commun.* 5, 5094. doi:10.1038/ncomms6094.

- Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., and Bond, D. R. (2008). Shewanella secretes flavins that mediate extracellular electron transfer. *Proc. Natl. Acad. Sci.* 105, 3968–3973. doi:10.1073/pnas.0710525105.
- März, C., Hoffmann, J., Bleil, U., de Lange, G. J., and Kasten, S. (2008). Diagenetic changes of magnetic and geochemical signals by anaerobic methane oxidation in sediments of the Zambezi deep-sea fan (SW Indian Ocean). Mar. Geol. 255, 118–130. doi:10.1016/j.margeo.2008.05.013.
- Mason, O. U., Case, D. H., Naehr, T. H., Lee, R. W., Thomas, R. B., Bailey, J. V., et al. (2015). Comparison of Archaeal and Bacterial Diversity in Methane Seep Carbonate Nodules and Host Sediments, Eel River Basin and Hydrate Ridge, USA. *Microb. Ecol.* 70, 766– 784. doi:10.1007/s00248-015-0615-6.
- McGlynn, S. E., Chadwick, G. L., Kempes, C. P., and Orphan, V. J. (2015). Single cell activity reveals direct electron transfer in methanotrophic consortia. *Nature* 526, 531–535. doi:10.1038/nature15512.
- McGlynn, S. E., Chadwick, G. L., O'Neill, A., Mackey, M., Thor, A., Deerinck, T. J., et al. (2018). Subgroup Characteristics of Marine Methane-Oxidizing ANME-2 Archaea and Their Syntrophic Partners as Revealed by Integrated Multimodal Analytical Microscopy. *Appl. Environ. Microbiol.* 84, e00399-18. doi:10.1128/AEM.00399-18.
- Moore, D. M., and Reynolds, R. C. (1997). X-Ray Diffraction and the Identification and Analysis of *Clay Minerals*. New York: Oxford University Press Available at: https://www.cambridge.org/core/article/div-class-title-m-span-class-sc-oore-span-dm-andamp-r-span-class-sc-eynolds-span-r-c-jr-1997-span-class-italic-x-ray-diffractionand-the-identification-and-analysis-of-clay-minerals-span-2nd-ed-xviii-378-pp-oxfordnew-york-oxford-university-press-price-27-95-spiral-bound-paperback-isbn-0-19-508713-5-div/36DBF262516EC65CD0665FE59E361B57.
- Morono, Y., Terada, T., Kallmeyer, J., and Inagaki, F. (2013). An improved cell separation technique for marine subsurface sediments: applications for high-throughput analysis using flow cytometry and cell sorting. *Environ. Microbiol.* 15, 2841–2849. doi:10.1111/1462-2920.12153.
- Naehr, T. H., Eichhubl, P., Orphan, V. J., Hovland, M., Paull, C. K., Ussler, W., et al. (2007). Authigenic carbonate formation at hydrocarbon seeps in continental margin sediments: A comparative study. *Deep Sea Res. Part II Top. Stud. Oceanogr.* 54, 1268– 1291. doi:10.1016/j.dsr2.2007.04.010.
- Neuwirth, E. (2014). RColorBrewer: ColorBrewer Palettes. Available at: https://CRAN.R-project.org/package=RColorBrewer.
- Niemann, H., Lösekann, T., Beer, D. de, Elvert, M., Nadalig, T., Knittel, K., et al. (2006). Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink. *Nature* 443, 854. doi:10.1038/nature05227.

- Oksanen, J., Kindt, R., Legendre, P., Hara, B., Simpson, G., Solymos, P., et al. (2009). The vegan Package.
- Orphan, V. J., Hinrichs, K.-U., Ussler, W., Paull, C. K., Taylor, L. T., Sylva, S. P., et al. (2001a). Comparative Analysis of Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in Anoxic Marine Sediments. *Appl. Environ. Microbiol.* 67, 1922–1934. doi:10.1128/AEM.67.4.1922-1934.2001.
- Orphan, V. J., House, C. H., Hinrichs, K.-U., McKeegan, K. D., and DeLong, E. F. (2001b). Methane-Consuming Archaea Revealed by Directly Coupled Isotopic and Phylogenetic Analysis. *Science* 293, 484–487. doi:10.1126/science.1061338.
- Orphan, V. J., House, C. H., Hinrichs, K.-U., McKeegan, K. D., and DeLong, E. F. (2002). Multiple archaeal groups mediate methane oxidation in anoxic cold seep sediments. *Proc. Natl. Acad. Sci.* 99, 7663–7668. doi:10.1073/pnas.072210299.
- Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* 18, 1403–1414. doi:10.1111/1462-2920.13023.
- Pfeffer, C., Larsen, S., Song, J., Dong, M., Besenbacher, F., Meyer, R. L., et al. (2012). Filamentous bacteria transport electrons over centimetre distances. *Nature* 491, 218. doi:10.1038/nature11586.
- Pop Ristova, P., Wenzhöfer, F., Ramette, A., Zabel, M., Fischer, D., Kasten, S., et al. (2012). Bacterial diversity and biogeochemistry of different chemosynthetic habitats of the REGAB cold seep (West African margin, 3160 m water depth). *Biogeosciences* 9, 5031– 5048. doi:https://doi.org/10.5194/bg-9-5031-2012.
- Poppe, L. J., and Commeau, J. A. (1992). Mineralogy of the silt fraction in surficial sediments from the United States mid-Atlantic shelf, slope and rise. *Mar. Geol.* 103, 185–197. doi:10.1016/0025-3227(92)90015-A.
- Poppe, L. J., Commeau, J. A., and Valentine, P. C. (1991). Mineralogy of the silt fraction in surficial sediments from the outer continental shelf off southeastern New England. J. Sediment. Res. 61, 54–64. doi:10.1306/D4267686-2B26-11D7-8648000102C1865D.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi:10.1093/nar/gks1219.
- R Core Team (2017). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing Available at: https://www.R-project.org/.
- Reeburgh, W. S. (2007). Oceanic Methane Biogeochemistry. *Chem. Rev.* 107, 486–513. doi:10.1021/cr050362v.

- Riedinger, N., Pfeifer, K., Kasten, S., Garming, J. F. L., Vogt, C., and Hensen, C. (2005). Diagenetic Alteration of Magnetic Signals by Anaerobic Oxidation of Methane Related to a Change in Sedimentation Rate. *Geochim. Cosmochim. Acta* 69, 4117–4126. doi:10.1016/j.gca.2005.02.004.
- Roberts, A. P. (2015). Magnetic mineral diagenesis. *Earth-Sci. Rev.* 151, 1–47. doi:10.1016/j.earscirev.2015.09.010.
- Rotaru, A.-E., Calabrese, F., Stryhanyuk, H., Musat, F., Shrestha, P. M., Weber, H. S., et al. (2018). Conductive Particles Enable Syntrophic Acetate Oxidation between Geobacter and Methanosarcina from Coastal Sediments. *mBio* 9. doi:10.1128/mBio.00226-18.
- Rowe, A. R., Chellamuthu, P., Lam, B., Okamoto, A., and Nealson, K. H. (2015). Marine sediments microbes capable of electrode oxidation as a surrogate for lithotrophic insoluble substrate metabolism. *Front. Microbiol.* 5. doi:10.3389/fmicb.2014.00784.
- Ruff, S. E., Biddle, J. F., Teske, A. P., Knittel, K., Boetius, A., and Ramette, A. (2015). Global dispersion and local diversification of the methane seep microbiome. *Proc. Natl. Acad. Sci.* 112, 4015–4020. doi:10.1073/pnas.1421865112.
- Sahin, M., Ayranci, K., Kosun, E., and Ayranci, E. (2009). Density, sound velocity and viscosity properties of aqueous sodium metatungstate solutions and an application of these solutions in heavy mineral separations. *Chem. Geol.* 264, 96–100. doi:10.1016/j.chemgeo.2009.02.016.
- Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., et al. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 12, 87. doi:10.1186/s12915-014-0087-z.
- Sato, M., and Mooney, H. M. (1960). The electrochemical mechanism of sulfide self-potentials. *Geophysics* 25, 226–249. doi:10.1190/1.1438689.
- Scheller, S., Yu, H., Chadwick, G. L., McGlynn, S. E., and Orphan, V. J. (2016). Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. *Science* 351, 703–707. doi:10.1126/science.aad7154.
- Schreiber, L., Holler, T., Knittel, K., Meyerdierks, A., and Amann, R. (2010). Identification of the dominant sulfate-reducing bacterial partner of anaerobic methanotrophs of the ANME-2 clade. *Environ. Microbiol.* 12, 2327–2340. doi:10.1111/j.1462-2920.2010.02275.x.
- Shi, L., Dong, H., Reguera, G., Beyenal, H., Lu, A., Liu, J., et al. (2016). Extracellular electron transfer mechanisms between microorganisms and minerals. *Nat. Rev. Microbiol.* 14, 651–662. doi:10.1038/nrmicro.2016.93.
- Shi, M., Wu, H., Roberts, A. P., Zhang, S., Zhao, X., Li, H., et al. (2017). Tectonic, climatic, and diagenetic control of magnetic properties of sediments from Kumano Basin,

Nankai margin, southwestern Japan. Mar. Geol. 391, 1–12. doi:10.1016/j.margeo.2017.07.006.

- Skennerton, C. T., Chourey, K., Iyer, R., Hettich, R. L., Tyson, G. W., and Orphan, V. J. (2017). Methane-Fueled Syntrophy through Extracellular Electron Transfer: Uncovering the Genomic Traits Conserved within Diverse Bacterial Partners of Anaerobic Methanotrophic Archaea. *mBio* 8, e00530-17. doi:10.1128/mBio.00530-17.
- Skennerton, C. T., Haroon, M. F., Briegel, A., Shi, J., Jensen, G. J., Tyson, G. W., et al. (2016). Phylogenomic analysis of Candidatus 'Izimaplasma' species: free-living representatives from a Tenericutes clade found in methane seeps. *ISME J.* 10, 2679–2692. doi:10.1038/ismej.2016.55.
- Summers, Z. M., Fogarty, H. E., Leang, C., Franks, A. E., Malvankar, N. S., and Lovley, D. R. (2010). Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. *Science* 330, 1413–5. doi:10.1126/science.1196526.
- Tang, J., Zhuang, L., Ma, J., Tang, Z., Yu, Z., and Zhou, S. (2016). Secondary Mineralization of Ferrihydrite Affects Microbial Methanogenesis in Geobacter-Methanosarcina Cocultures. *Appl. Environ. Microbiol.* 82, 5869–5877. doi:10.1128/AEM.01517-16.
- Totten, M. W., Hanan, M. A., Knight, D., and Borges, J. (2002). Characteristics of mixed-layer smectite/illite density separates during burial diagenesis. *Am. Mineral.* 87, 1571–1579. doi:10.2138/am-2002-11-1207.
- Treude, T., Orphan, V., Knittel, K., Gieseke, A., House, C. H., and Boetius, A. (2007). Consumption of Methane and CO2 by Methanotrophic Microbial Mats from Gas Seeps of the Anoxic Black Sea. *Appl. Environ. Microbiol.* 73, 2271–2283. doi:10.1128/AEM.02685-06.
- Van Cappellen, P., and Wang, Y. (1996). Cycling of iron and manganese in surface sediments; a general theory for the coupled transport and reaction of carbon, oxygen, nitrogen, sulfur, iron, and manganese. Am. J. Sci. 296, 197–243. doi:10.2475/ajs.296.3.197.
- Wegener, G., Krukenberg, V., Riedel, D., Tegetmeyer, H. E., and Boetius, A. (2015). Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. *Nature* 526, 587–590. doi:10.1038/nature15733.
- Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., et al. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5, 27. doi:10.1186/s40168-017-0237-y.
- Wickham, H. (2009). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York Available at: http://ggplot2.org.

Pos. [°2θ]	Height [cts]	FWHM Left [°2θ]	d-spacing [Å]	Rel. Int. [%]	Tip Width	Matched by
8.6211	71.39	0.3149	10.25700	11.86	0.3779	
12.2542	194.17	0.3149	7.22293	32.25	0.3779	
18.5588	52.08	0.2519	4.78103	8.65	0.3023	
19.5450	120.24	0.3779	4.54197	19.97	0.4534	
20.7623	90.62	0.4093	4.27834	15.05	0.4912	01-089-1961
21.7652	117.54	0.2519	4.08342	19.52	0.3023	
23.4771	38.52	0.3149	3.78939	6.40	0.3779	
26.3782	547.67	0.2676	3.37885	90.97	0.3212	01-089-1961
27.6269	184.78	0.3779	3.22890	30.69	0.4534	
29.6342	87.23	0.3779	3.01460	14.49	0.4534	
31.4440	602.03	0.2676	2.84510	100.00	0.3212	00-005-0628
36.3486	44.62	0.3149	2.47167	7.41	0.3779	01-089-1961
45.2137	353.49	0.2834	2.00553	58.72	0.3401	01-089-1961
						00-005-0628
49.9841	49.85	0.4408	1.82473	8.28	0.5290	01-089-1961
59.6788	42.63	0.3779	1.54940	7.08	0.4534	01-089-1961

Ref. Code	Score	Compound Name	Displacement [°2Th.]	Scale Factor	Chemical Formula
01-089-1961	70	Silicon Oxide	-0.199	0.850	SiO <sub>2</sub>
00-005-0628	55	Sodium Chloride	-0.219	0.949	NaCl

**Table 3.** Peaks detected (*top*) and corresponding mineralogical identification (*bottom*) by X'Pert Highscore from a representative XRD spectra collected from a sample of the light ( $\rho < 2.4$  g/cc) fraction of density-separated methane seep sediment (Fig. 2).

Pos. [°2θ]	Height [cts]	FWHM Left	d-spacing	Rel. Int. [%]	Tip Width	Matched by
		[°20]	[Å]			
8.7742	57.33	0.3149	10.07834	3.47	0.3779	
12.3601	174.51	0.2519	7.16130	10.55	0.3023	
20.7157	277.06	0.2519	4.28786	16.75	0.3023	01-089-1961
21.8928	159.28	0.3149	4.05990	9.63	0.3779	
23.5424	152.92	0.4408	3.77903	9.24	0.5290	01-086-2341
24.1270	127.08	0.3779	3.68878	7.68	0.4534	
25.0271	143.98	0.3779	3.55811	8.70	0.4534	
26.5142	1654.45	0.3149	3.36183	100.00	0.3779	01-089-1961
27.8307	461.98	0.3779	3.20572	27.92	0.4534	
29.8100	1396.58	0.3464	2.99722	84.41	0.4156	01-086-2341
36.4232	349.49	0.2519	2.46678	21.12	0.3023	01-089-1961
						01-086-2341
37.6643	54.39	0.3779	2.38830	3.29	0.4534	
39.9014	318.50	0.4408	2.25941	19.25	0.5290	01-089-1961
						01-086-2341
42.3318	164.52	0.2834	2.13515	9.94	0.3401	01-089-1961
43.7411	244.94	0.3779	2.06957	14.80	0.4534	01-086-2341
48.2544	198.28	0.4408	1.88602	11.98	0.5290	
49.2895	228.52	0.4408	1.84881	13.81	0.5290	
50.0550	280.77	0.2519	1.82232	16.97	0.3023	01-089-1961
54.7251	69.45	0.2519	1.67734	4.20	0.3023	01-089-1961
59.8555	222.53	0.2834	1.54525	13.45	0.3401	01-089-1961

Ref. Code	Score	Compound Name	Displacement [°2Th.]	Scale Factor	Chemical Formula
01-089-1961	76	Silicon Oxide	-0.094	0.990	SiO <sub>2</sub>
01-086-2341	52	Calcium	0.501	0.731	$Ca(CO_3)$
		Carbonate			

**Table 2.** Peaks detected (*top*) and corresponding mineralogical identification (*bottom*) by X'Pert Highscore from a representative XRD spectra collected from a sample of the intermediate ( $2.4 < \rho < 2.9$  g/cc) fraction of density-separated methane seep sediment (Fig. 2).

Pos. [°2θ]	Height [cts]	FWHM Left [°20]	d-spacing	Rel. Int. [%]	Tip Width	Matched by
8.4469	38.59	0.2204	10.46805	7.75	0.2645	
10.1959	497.73	0.2362	8.67596	100.00	0.2834	
12.2320	97.94	0.1889	7.23598	19.68	0.2267	
17.3895	57.20	0.3149	5.09980	11.49	0.3779	
19.4890	15.92	0.2519	4.55488	3.20	0.3023	
26.3485	91.17	0.3149	3.38259	18.32	0.3779	
28.2200	307.19	0.2519	3.16237	61.72	0.3023	00-024-0076
29.5466	145.63	0.1889	3.02334	29.26	0.2267	01-089-0950
32.7266	133.66	0.1889	2.73647	26.85	0.2267	00-024-0076
36.6646	58.47	0.3779	2.45110	11.75	0.4534	00-024-0076
						01-089-0950
56.0246	58.28	0.3779	1.64148	11.71	0.4534	00-024-0076
						01-089-0950

Ref. Code	Score	Compound Name	Displacement [°2Th.]	Scale Factor	Chemical Formula
00-024-0076	56	Iron Sulfide	-0.322	0.353	FeS <sub>2</sub>
01-089-0950	34	Iron Oxide	-0.559	0.280	Fe <sub>3</sub> O <sub>4</sub>

**Table 1.** Peaks detected (*top*) and corresponding mineralogical identification (*bottom*) by X'Pert Highscore from a representative XRD spectra collected from a sample of the dense ( $\rho > 2.9$  g/cc) fraction of density-separated methane seep sediment (Fig. 2).



**Supplemental Figure 1.** Spherical particle size pelleted from a 2 min spin at  $2000 \times$  g as a function of the density of that particle and the density and viscosity of different SMT solutions, showing the time in minutes (contours) required for a particle of given diameter to pellet at the bottom of a 5 mL tube, assuming Stokes' settling. Viscosities for different SMT densities were adapted from the literature (Sahin et al., 2009).



**Supplemental Figure 2**. FTIR spectrum collected from the intermediate density fraction comparing this sample spectrum to those of several carbonate standards (cf. Fig. 3).