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Characterizing Microbial Community and Geochemical Dynamics at Hydrothermal Vents Using Osmotically Driven Continuous Fluid Samplers

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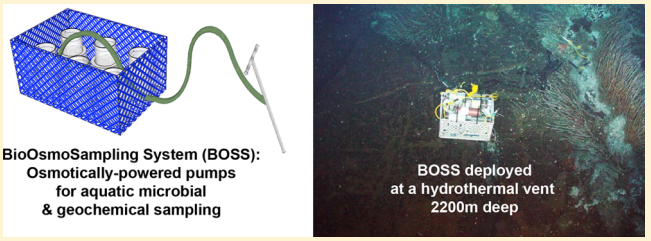
Supporting Information

ABSTRACT: Microbes play a key role in mediating aquatic biogeochemical cycles. However, our understanding of the relationships between microbial phylogenetic/physiological diversity and habitat physicochemical characteristics is restrained by our limited capacity to concurrently collect microbial and geochemical samples at appropriate spatial and temporal scales. Accordingly, we have developed a low-cost, continuous fluid sampling system (the Biological OsmoSampling System, or BOSS) to address this limitation. The BOSS does not use electricity, can be deployed in harsh/remote environments, and collects/preserves samples with daily resolution for >1 year. Here, we present data on the efficacy of DNA and protein preservation during a 1.5 year laboratory study as well as the results of two field deployments at deep-sea hydrothermal vents, wherein we examined changes in microbial diversity, protein expression, and geochemistry over time. Our data reveal marked changes in microbial composition co-occurring with changes in hydrothermal fluid composition as well as the temporal dynamics of an enigmatic sulfide-oxidizing symbiont in its free-living state. We also present the first data on in situ protein preservation and expression dynamics highlighting the BOSS's potential utility in meta-proteomic studies. These data illustrate the value of using BOSS to study relationships among microbial and geochemical phenomena and environmental conditions.



INTRODUCTION

Marine biogeochemical cycles are governed primarily by microbes, which influence the cycling of elements from carbon to metals.[1,2] Aquatic microbiologists and biogeochemists recognize the complexity of microbial interactions with their geochemical milieu even in putatively homogeneous, stable environments. Recent studies illustrate that − even in seemingly homogeneous deep-sea environments − there is tremendous fine-scale heterogeneity in microbial community composition.[3]

There is growing interest in better understanding this microbial heterogeneity and its significance to biogeochemical cycling. In light of the complex interplay between microbes and their habitat geochemistry, we posit that the relationship between biological and geochemical phenomena can be addressed by examining co-occurring spatial and temporal changes in both microbial diversity/physiology and geochemistry. To date, electromechanical fluid and particulate collection systems have been used to conduct studies in both shallow- and deep-water environments for analysis of microbial community composition and diversity in relation to changes in geochemistry and temperature (e.g., the McLane sampler, refs 4−6). Other more complex in situ autonomous systems have been used to successfully monitor events such as harmful algal blooms (reviewed in ref 7) and changes in marine picoplankton.[8−10] These samplers are highly effective but are large, costly, require substantial electrical power, and can be challenging to operate in harsh, remote environments.

To complement existing sampling technologies and further enable higher spatial and temporal resolution sampling of fluids for coregistered microbial and geochemical analyses, we developed the Biological OsmoSampling System (or BOSS). Based upon existing osmotic pumping systems[11,12] (Figure S1 of the Supporting Information), the BOSS continuously collects fluid samples in aquatic environments, and preserves those samples in situ for molecular microbiological analyses. Like other osmotic pumping systems, the BOSS can be deployed from weeks to years (depending on the quantity and quality of biomolecules required), requires no electrical power,

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and is small enough to be deployed in space-constrained environments such as boreholes and groundwater aquifers. Deploying the BOSS concurrently with an osmotic sampler configured for geochemical preservation enables coregistered microbial and geochemical analyses.

Here, we present the results of a series of laboratory assessments over 1.5 years, which establish the efficacy of commercially available reagents in preserving DNA and proteins for phylogenetic and proteomic analyses over time. We also present the results of two field studies, in which we deployed two BOSSes in low-temperature (<15 °C) marine hydrothermal vents to assess the performance of these preservatives − as well as the sampling system − in the field. These in situ data reveal marked changes in community composition, total bacterial and archaeal abundance, and protein expression over time. Collectively these data demonstrate the successful performance of the BOSS and illustrate the degree to which it enables concurrent microbial and geochemical sampling in aquatic ecosystems including remote environments.

METHODS

General Description of OsmoSamplers. OsmoSamplers are osmotic pump-based samplers that have been successfully used to study temporal changes (~1 day resolution) in fluid composition in a number of environments including seafloor boreholes, hydrocarbon seeps, and hydrothermal vents (ref 11 for a recent summary). Briefly, OsmoSamplers consist of two compartments separated by water-permeable membranes (as in ref 12). Prior to deployment, one compartment is filled with deionized water and the other with a saturated sodium chloride solution (part A of Figure S1 of the Supporting Information). Membranes allow water to flow along the osmotic gradient from the compartment with deionized water to the compartment with the brine. The flow rate is primarily governed by temperature, membrane type, and surface area. To enable continuous fluid sampling over time, a substantial length of sterile acid-washed small bore tubing − typically hundreds of meters of fluorinated ethylene propylene (FEP) tubing with a 0.8 to 1.10 mm ID − is filled with sterile deionized water and attached to the compartment containing the deionized water. The other end of the tubing is placed into the environment of interest, and the osmotic force within the pump draws sample fluid into the tubing. To enable preservation in situ, an additional tubing coil is precharged with a solution (e.g., biocide) and attached to the brine compartment of an osmotic pump with a slower flow rate. The other end is connected to a three-way PEEK tee connector coupled to the sample coil (part A of Figure S1 of the Supporting Information). Thus, when a deployment does not exceed the intended duration, samples are stored within the tubing and never enter the membrane chamber. This two-pump system continuously preserves and samples fluids in situ for months to years depending on operational constraints. Previous deployments of osmotic pumps for geochemical sampling[12] have used pumps and tubing that acquire approximately 1 mL of fluid per day in 1 m length of tubing, with 0.1 mL of preservative per 1 mL of sample.[13] These flow rates can be scaled down to 0.1 mL·day^{-1} or up to 5 mL·day^{-1} as needed. Given the aforementioned controls on pumping rate, deployments in environments where temperature is essentially constant (e.g., deep ocean below 100 m) results in a uniform rate of sampling for the duration of the deployment. If temperature varies, pumping rate can be readily calculated as long as temperature is logged for the duration of the deployment.[12,14] Upon recovery, the Teflon tubing is cut into lengths as determined by the temperature record taking care to cut the tubing using sterile techniques and decant the contents into sterile tubes for later analyses (note that capillary action helps retain the fluid in the tubing coil during sampling).

Assessing the Efficacy of Biomolecular Preservatives. We conducted a series of laboratory experiments that examined DNA and protein degradation in various preservatives over 1.5 years. We tested five different preservatives: (A) RNAlater (Ambion Inc., Austin, TX), (B) UmFix (Sakura FineTek USA, Inc., Torrance, CA), (C) a glycerol/phosphate buffered saline solution, as well as (D) phosphoric, and (E) hydrochloric acid. These acids have previously been used to keep metals in solution during deployments, and we were interested in ascertaining if they could be used in nucleic acid preservation. To test the efficacy of these preservatives, *Vibrio harveyi* (strain ATCC BAA-1116) was grown in 1 L of *Vibrio* minimal media (50.0 mM Tris, 50.0 mM $MgSO_4$, 10.0 mM $CaCl_2$, 300 mM $CaCl_2$, 300 mM NaCl, 10.0 mM KCl, 0.33 mM K_2PO_4, 18.5 mM NH_4Cl, 32.6 mM glycerol) to achieve densities of 10^6 cells·mL^{-1}. At the start of the experiment, twenty 2 mL samples were taken from the culture while stirring and stored at −80 °C. These represent our T_0 samples and were used as controls. The remaining culture was divided into eight subsamples and dispensed into sterile Erlenmeyer flasks equipped with filtered plugs for aeration. Each flask contained 50 μg·mL^{-1} kanamycin and 340 μg·mL^{-1} chloramphenicol. A single candidate preservative was added to each flask as follows: 1:1 volume RNALater, 1:1 volume UmFix, 1:10 volume UmFix, 15% glycerol, 30% glycerol, 10% H_3PO_4, or 0.1N HCl. The eighth flask did not include a preservative. These subsamples were incubated at 4 °C in the dark. After vigorous shaking to homogenize the cultures, 2 × 2.5 mL aliquots were taken for analyses at predetermined intervals for the first eight weeks, and less frequently thereafter for over a year. During sampling on days 15 and 45, 100 μL of each of the preserved subsamples was streaked on lysogeny broth (LB) agar and *Vibrio* minimal media and incubated at 4 °C, 24 °C, and 37 °C to screen for contamination.

Assessing DNA Preservation. DNA was extracted from a 500 μL subsample using the MoBio Tissue DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA). DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). The extent of degradation was evaluated by embedding preserved 2 mL subsamples and T_0 samples in agarose plugs.[15] Equal lengths of plugs were sliced and analyzed on a 0.3% agarose gel to determine the extent of DNA degradation by visualization of molecular weight using SYBRSafe DNA stain and low and high molecular weight ladders (Invitrogen Life Technologies, Carlsbad, CA).

Assessing Protein Preservation. Proteins were extracted from each subsample by detergent lysis [16] and quantified by the Bradford method, using bovine serum albumin dilutions as standards (0−10 000 μg·mL^{-1}). Percent recovery was evaluated based on concentrations relative to −80 °C preserved T_0 culture extractions. To examine the extent of degradation over time, extracted proteins were electrophoresed on a 10−15% gradient SDS-polyacrylimide gel (Biorad Inc., Hercules, CA). Protein quality was evaluated based on the size of proteins and visual assessment of degradation in each lane in comparison to T_0 samples.

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Proof-of-Concept Field Deployments of the BOSS. To test performance in situ, two complete systems were fabricated (part B of Figure S1 of the Supporting Information) and deployed on the Juan de Fuca Ridge during expeditions in 2007 and 2008. Each system contained two BOSSes (two osmotic samplers configured to sample and preserve microbes) as well as two osmotic samplers configured for geochemical analyses as previously described. One BOSS used RNAlater as a preservative at a concentration to achieve a 1:1 dilution ratio. The other BOSS used 80% glycerol as a preservative at a concentration to achieve a 1:5 dilution ratio. The two geochemical OsmoSamplers included a Teflon sample coil for geochemistry and a copper sample coil for dissolved gas analyses.[11] The geochemistry OsmoSamplers were configured to deliver 6N trace-metal free HCl and Terbium (an element used as a tracer to calculate dilution) into the seawater sampling stream at 1:10 dilution. Antibiotic solutions were delivered to the two BOSSes and one dissolved gas OsmoSampler at a final concentration of 250 $\mu g \cdot mL^{-1}$ kanamycin and 340 $\mu g \cdot mL^{-1}$ chloramphenicol. All four samplers were configured with 500 m of sampling coil and mounted in a single plastic milk crate (75 × 30 × 30 cm). A 2 m length of tubing from each sampling system was bundled inside a Tygon tubing sheath and connected to a titanium tee handle to enable accurate placement on the seafloor by the submersible. An in situ temperature recorder (Vemco, Inc., Billings, MT) was attached to this tee handle and was programmed to sample every 5 min for the duration of the deployment.

One system was deployed using the ROV *ROPOS* on the flank of the Hulk sulfide mound (47.9501 latitude, −129.0970 longitude, 2200 m) at the Juan de Fuca Ridge on August 8, 2007. The inlet was placed among an aggregation of *Ridgeia piscesae* tubeworms from which diffuse hydrothermal fluids emanated. The package was recovered 22 days later by the DSV *Alvin*. To examine long-term protein preservation and expression, the second system was deployed using the DSV *Alvin* at the flank of the Grotto sulfide mound (47.9532 latitude, −129.0427 longitude, 2210 m) at the Juan de Fuca Ridge on August 30, 2008. For this deployment, the inlet was placed in a crevice among microbial mats and *Ridgeia piscesae* tubeworms, from which diffuse hydrothermal fluids also emanated. The system was recovered 375 days later by the DSV *Alvin*.

BOSS Field Deployments: Microbial Diversity, Quantification, and Geochemical Analyses. Onboard ship, sample tubing coils were immediately detached from pumps, sealed, and kept at 4 °C until further processing (within hours of recovery). The sample tubing from each system was cut into 1 m long segments (representing approximately 1 day resolution), and the resulting 1 mL aliquots were dispensed into sterile 1.5 mL epppendorf tubes and frozen at −80 °C. Aliquots for geochemical analyses were expelled into acid-washed tubes and maintained at 4 °C. To test the efficacy of DNA recovery from BOSS samples, DNA was extracted from 350 μL of glycerol-preserved biological sample recovered early during the deployment (a sample that was likely comprised of seawater sampled during transport to the seafloor) using a Geneclean DNA extraction kit (MP Biomedicals, Santa Ana, CA). Extracted DNA was diluted to 10 $ng \cdot \mu L^{-1}$ and PCR amplified using bacterial 16S primers 27F and 1492R,[17] and cloned and sequenced as described below.

In addition, given the small volumes recovered from the BOSS, we examined whether multiple displacement amplification (MDA) could be used to generate representative libraries while retaining sample for other analyses, for example quantitative PCR. Accordingly, we constructed sequence libraries using both unamplified and MDA-amplified genomic DNA (gDNA) as template for 16S rRNA PCR. For the traditional 16S clone libraries, unamplified gDNA was diluted to 10 $ng \cdot \mu L^{-1}$ and PCR amplified as described above. For MDA, three 16.5 μL aliquots from three adjacent samples recovered from 1 m lengths of tubing were pooled and spun at 17 000 g at 4 °C to pellet cells. Cell pellets were then resuspended, lysed via osmotic-thermal lysis in 10× Tris-EDTA buffer at 95 °C for five minutes and amplified by MDA using the Repli-G Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. To minimize amplification bias, MDA extension was limited to 3 h rather than the recommended 16 h. Negative controls were run concurrently with all MDA and 16S PCR amplification to rule out any contamination (none of these negative controls amplified). Cycling conditions were 95 °C for 10 min, followed by 30 cycles (9 cycles for MDA template) of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s. MDA product was then PCR amplified as described above. Amplicons from both MDA and non-MDA 16S PCR were cloned into One Shot TOP10 cells (Invitrogen Life Technologies, Carlsbad, CA). Between 96 and 192 clones from each library were plasmid prepped (Axygen, Inc., Union City, CA) and then bidirectionally sequenced by the Dana Farber/Harvard Cancer Center Resource Core. Resulting sequences were analyzed via the Ribosomal Database Project and BLAST.[18,19] For comparison, the libraries resulting from traditional as well as MDA-based amplification are depicted in Figure S3 of the Supporting Information.

Quantitative PCR (qPCR) assays were employed to enumerate *Gamma-* and *Epsilonproteobacterial* ribotypes and the *Gammaproteobacterial* symbiont of *Ridgeia piscesae*, a hydrothermal vent tubeworm symbiotic with sulphide-oxidizing bacteria (primers are presented in 20−22). Primers were screened, optimized for efficiency, checked for cross-reactivity as in ref 23, and validated by comparison to all available 16S rRNA gene sequences within ARB and BLAST (www.arbhome.de, http://www.ncbi.nlm.nih.gov). All qPCR assays were performed on a Stratagene MX3005p sequence detector (Agilent Technologies, Inc., Santa Clara, CA) in 96-well optical grade plates. Each 30 μL qPCR reaction contained 5 μL of template as well as the following reagents at final concentrations: 1× Taqman Universal PCR Master Mix (Applied Biosystems Life Technologies, Carlsbad, CA), appropriate concentrations of target-specific oligonucleotide primers (as in the previous references), and 100 nM of each TaqMan probe. ROX fluorescent dye was added to a final concentration of 30 nM to improve the signal-to-noise ratio. Amplification was carried out after 2 min at 50 °C and 10 min at 95 °C, with 45 cycles of 15 s at 95 °C and 1 min annealing at temperatures between 55 and 64 °C. Data analyses were carried out with the *MXPro* software (Agilent Technologies, Inc., Santa Clara, CA). All reactions (standards, samples, and blanks) were performed in triplicate and included a no-template control.

Fluid subsamples were analyzed for dissolved ions using an ICP mass spectrometer (as in ref 24). For analysis of dissolved volatiles, samples were recovered by crimping the metal tubing into 1 m lengths while unspooling and then piercing each end of the resulting section with a saddle valve. One valve was connected to an argon source, whereas the other end was connected to a pre-evacuated 10 mL gastight vial. Positive

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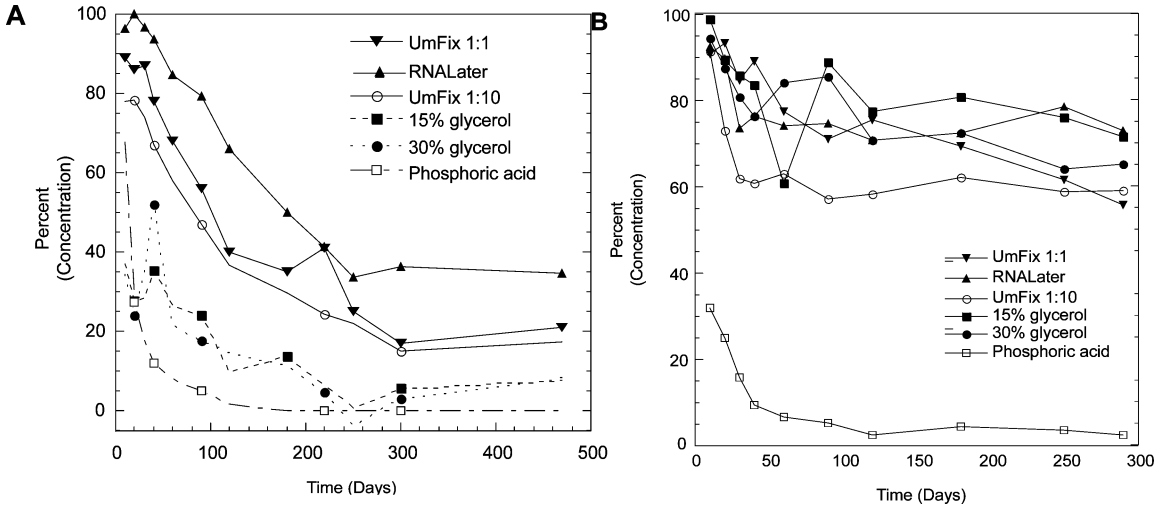

Figure 1. Preservation of DNA over time in six different preservatives. Plot of (A) % DNA concentration and (B) % protein concentration (relative to T_0) over time as recovered from 1 L *Vibrio harveyi* cultures grown under identical conditions, preserved with six different preservatives (legend), and subsampled over 500 and 300 days respectively.

pressure was used to dispense the fluid sample and dissolved gas from the tubing. Methane was measured via gas chromatography (as in ref 23).

Proteomics Analyses from in Situ BOSS Deployment. A subset of samples recovered from the second BOSS deployment (in RNALater) were used to determine the volume of sample required for proteomic analyses. These samples were pooled into 5, 10, 15, 20, 25, and 30 mL aliquots. Proteins were extracted (Supporting Information), and the concentration and quality of each pool was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Results indicated that between 15 and 25 mL of sample (depending on microbial biomass) provided sufficient protein (ca. 10 to 25 pM) for proteomic analyses. Subsequently, 25 mL of BOSS subsample (comprising 20 subsamples selected evenly from time-points 17 through 110) were pooled together for analyses. These are hereafter referred to as the Early series. A 25 mL subsample also was recovered from evenly dispersed aliquots of time-points 150 through 247 and is referred to as the Late series. For more details on sample processing and protein extraction, see the Supporting Information.

Analysis by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Digested proteins for Early and Late samples were analyzed in triplicate using a reverse phase HPLC separation coupled to a hybrid linear ion trap and an LTQ-Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA). Briefly, separated peptides were ionized (positive) using an electrospray ionization interface that consisted of chemically etched electrospray emitters (150 mm o.d. Twenty mm i.d). The LTQ-Orbitrap was operated using a heated capillary temperature and spray voltage of 200 °C and 2.2 kV, respectively. Measured MS/MS spectra were analyzed using both the PepNovo[25] sequencing algorithm and SEQUEST.[26] For more details, see the Supporting Information.

RESULTS

Efficacy of Biomolecular Preservatives. UMFix and RNALater yielded the highest quality DNA (high molecular weight DNA, or HMW-DNA) (part A of Figure 1, part A of Figure S2 of the Supporting Information). The modest increases in total DNA yields observed over the first few weeks from some preservatives were likely the result of continuing cell lysis and more efficient DNA extraction after two weeks of preservation. After 1.5 years in UMFix and RNALater, we recovered 35% and 25% of the total DNA measured at T_0, respectively (~38 and 27 $\mu g \cdot mL^{-1}$ via spectrophotometric analyses, part A of Figure 1). UMFix diluted 1:10 and 15% glycerol showed modest performance with less than 10% recovery of DNA after 1.5 years. This observation suggests that, if effective preservatives are used, sufficient DNA can be recovered from less than 1 mL of sample for conventional analyses (e.g., PCR and PCR-based sequencing, blots, and autoradiography) after one year (the poor efficacy of both the hydrochloric acid and the no preservative treatments made DNA quantification impractical, and as such they are not shown in Figure 1). Gel electrophoretic assessments demonstrate that HMW-DNA is well preserved for the first 60 days and still present to some extent up to 1 year as illustrated by the retention of DNA in the wells (part A of Figure S2 of the Supporting Information). This suggests the possibility of constructing large-insert libraries (e.g., cosmid or BAC libraries) by pooling several milliliters of sample.[15,27] It remains to be determined whether there is sufficient HMW-DNA present to construct large-insert libraries from >60 day old samples. Given the increasing and widespread use of RNALater as a preservative and its relatively low cost per unit volume, we suggest that RNALater be used for longer-term deployments in which DNA and protein recovery is desired. Further, in longer term deployments, we have used 0.2% mercuric chloride as an additional precaution to arrest metabolism, though to date we it remains to be determined how effective this was, and the degree to which it might influence DNA or protein recovery. For shorter-term deployments, ca. 10 to 15 days, glycerol (along with an appropriate antibiotic cocktail) appears suitable for both DNA and protein recovery. Acids were ineffective at preserving biological material, and we cannot recommend their use for this application. It should be noted that, in mixed cultures or natural microbial assemblages, there may be differences in the rate of degradation among microbial types, and this will likely vary within and among environments. Because microbial

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community composition varies widely in nature, we suggest that future deployments consider characterizing heterogeneity in cell lysis and nucleic acid degradation and perhaps identify better approaches to mitigating this effect.

Total protein preservation was exemplary (part B of Figure 1 and part B of Figure S2 of the Supporting Information). Visual observation of proteins on an SDS-PAGE gel reveals high protein integrity after one year of incubation in most of the preservatives, with the exception of UMFix, hydrochloric acid, and phosphoric acid (greater than 55% of the starting concentration, part B of Figure 1). In contrast to DNA, protein concentration in the samples appeared more variable, which is likely due to the limitations of protein quantification via absorption spectroscopy.

Co-Registered Phylogenetic and Geochemical Analyses from the BOSS Field Deployment. The first BOSS package was deployed at a diffuse flow hydrothermal site with the inlet sampling fluids from among *Ridgeia piscesae* tubeworms. To ensure consistent pumping, the pumps were deployed in ambient seawater, where the temperature was a nearly constant 2 °C. Next, to find a suitable location for the sample inlet, a thermocouple on the DSV *Alvin* was used to find a position with a temperature between 10 and 20 °C. The wand was deployed, and the onboard temperature logger revealed that temperatures at the BOSS inlet for 6 days after deployment were ∼15 °C. From days 7 to 11, there was a decrease in temperature from ∼15 to 12 °C, accompanied by larger oscillations in temperature (part A of Figure 2). This was followed by periods of nearly uniform temperatures around 15 °C with much less scatter, similar to the first 6 days of deployment. In contrast, during days 7−11, the decrease in seawater-derived magnesium and the concomitant increase in vent-derived methane are consistent with a greater proportion of hydrothermal fluids during this period (part B of Figure 2). Although the temperature and geochemistry initially appear contradictory, one must consider that temperature and dissolved ions are transported by conduction and convection as discussed in ref 14. The elevated temperatures, with minimal fluctuations, suggest that the temperature logger is in close proximity or in contact with the metal sulfide and basaltic substrate and is being influenced by conductive heat transfer during periods with little to no hydrothermal flow. As hydrothermal flow increases around the inlet, the influence of hydrothermally convected fluids dominates the temperature record and the temperature approaches that measured in the visible diffuse flows observed by the DSV *Alvin*. Thus, the data reveal that the BOSS was entraining a mixture of bottom, cold seawater and hydrothermal fluid, and the proportions of these two varied over time as described above. The observed oscillations during this time are also consistent with previous studies of diffuse flow (e.g., ref 28). On the basis of these data and the consistency of the geochemical and microbial data (discussed below), future deployments must consider the configuration of the intake and temperature logger with respect to the environment to provide a better coupling among thermal, chemical, and microbial data and changes.

The 16S rRNA clone libraries shown in Figure 2 reveal that, from days 6 through 21, all libraries were dominated by *Gammaproteobacteria*, particularly those allied to known seawater and sediment psychrophilic *Pseudoalteromonas* and *Pseudomonas* ribotypes (part C of Figure 2).[4,29] Fluctuations in representation among the libraries suggest temporal heterogeneity in the abundance of ribotypes including *Pseudoalteromonas* (though library representation is not quantitative). In addition, ribotypes allied to known hydrothermal and endolithic organisms were evident in the libraries consistent with the samples being a mix of seawater and hydrothermal fluids. During the 9−12 day interval, the abundance of vent endemic microbes increased. The bacterial symbiont of *Ridgeia piscesae*, vent tubeworms that produce large aggregations around diffuse flows, accounted for 21% of the sequences during this interval (part C of Figure 2).

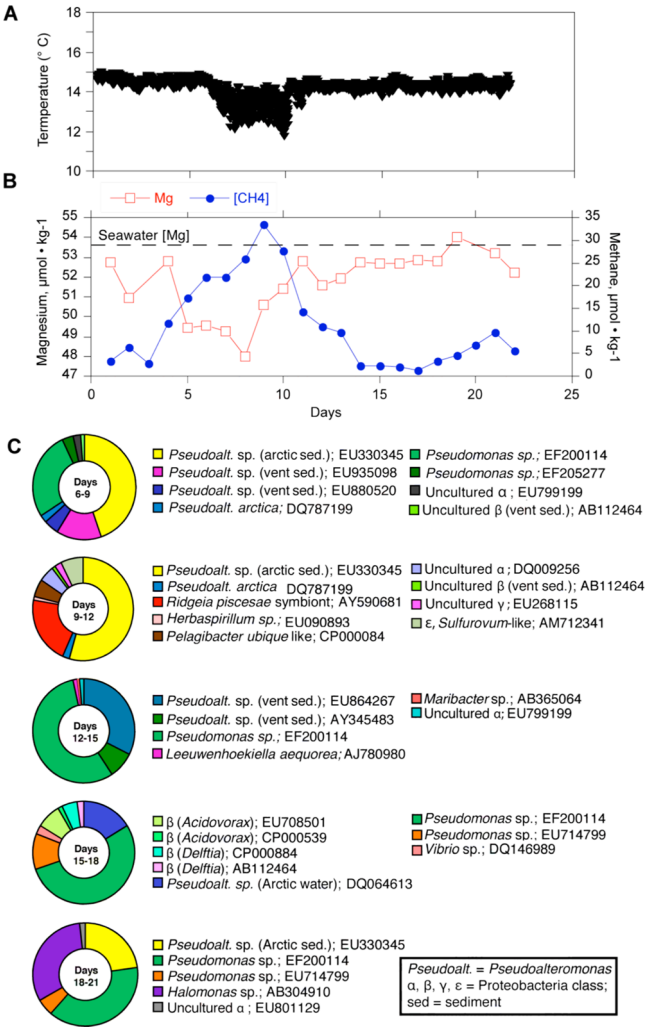

Figure 2. Physical, geochemical, and community composition data from BOSS deployment at the diffuse zone near the Hulk sulfide mound. (A) BOSS sample inlet temperature, sampled every five minutes over the course of the pilot deployment in a diffuse vent. (B) Changes in methane and magnesium concentrations in the recovered fluids over the same time period. (C) 16S rRNA clone libraries constructed from PCR amplicons from non-MDA amplified samples recovered over the duration of the deployment (each library represents a 3-day period starting from days 6−9).

To determine if clone libraries generated via MDA could be used to generate representative libraries while retaining sample for other analyses, we constructed both an MDA-based library as well as a traditional 16S rRNA PCR library (Figure S3 of the Supporting Information). Community composition in the MDA-amplified library was largely comparable to the non-MDA-amplified 16S rRNA clone library at the class level, though both *Epsilon-* and *Betaproteobacteria* were absent in the MDA-amplified clone library (they comprised of 21% and 5%

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respectively of the non-MDA amplified library; as seen in Figure S3 of the Supporting Information). It does appear that even this modified MDA protocol biases amplification, and in future studies where diversity assessments are a priority we recommend using nonamplified genomic DNA for clone library construction.

Using qPCR, we enumerated total bacteria, archaea, *Gammaproteobacteria*, *Epsilonproteobacteria*, and *Ridgeia piscesae* tubeworm symbionts in the glycerol-preserved samples. DNA yields from the samples used for these analyses ranged from 13.2 to 55.9 ng/μL. The efficiencies of all assays were between 93 and 103% (103% efficiency is due to the assay reporting a higher copy number from the standard curve than was added as template), and the limit of detection for all assays were ~200 copies·mL^{-1}. We also assume an average of 4.19 copies of 16S rRNA gene per bacterium and 1.71 copies of 16S rRNA gene per archaeon (Lee et al., 2009; Klappenbach et al., 2001). Because of minor inhibition observed in three samples (equivalent to a 10% reduction in efficiency), we chose not to include those samples in our analyses.

Total bacterial abundance (expressed as copies·mL fluid^{-1}) typically varied from 10^6 to 10^7 over time, with an excursion to ~1×10^5 from days 10−12 (part A of Figure 3). Total archaeal abundance exhibited an opposing pattern showing an increase in abundance around day 9 (part A of Figure 3). *Gammaproteobacteria* showed a modest increase in abundance from days 5−7, whereas *Epsilonproteobacteria* exhibited a substantial increase in abundance from days 7−12 (part B of Figure 3). *Ridgeia piscesae* symbionts typically varied beween 10^4 and 10^5 copies·mL fluid^{-1} but exhibited a striking increase during days 10−12 reaching a peak abundance of 4.1×10^7 copies·mL fluid^{-1} (part C of Figure 3).

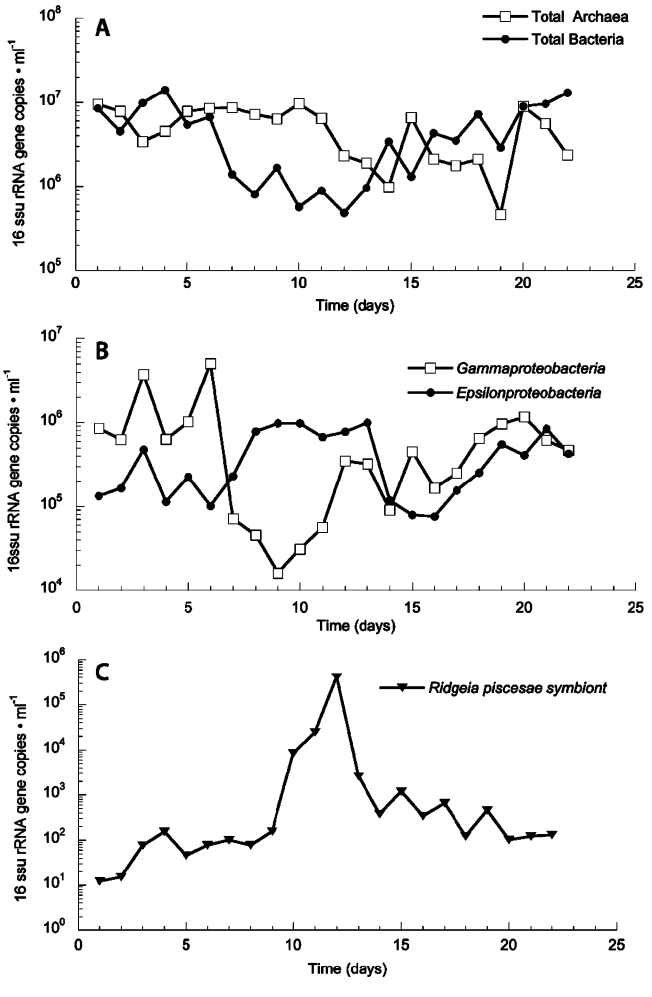

Figure 3. Changes in target microbial abundance during the BOSS deployment at Hulk hydrothermal vent site. Changes over time in (A) total bacterial and archaeal abundance, (B) *Epsilon-* and *Gammaproteobacterial* abundance, (C) *Ridgeia piscesae* (free-living) symbionts.

Proteomics Analyses from in Situ BOSS Deployment. Proteomics data were obtained from samples from the second BOSS deployment at Grotto mound. The proteomic analyses revealed unique features (peptides) from the Early and Late samples (Table S1 of the Supporting Information). From the genomic database of concatenated organism sequences (including common contaminants), a total of 1151 and 820 unique peptides, passing spectral probability filter criteria, were recovered from the Early and Late samples, respectively (Figure S3 of the Supporting Information). De novo sequencing using *PepNovo*[25] increased the number of peptide sequence assignments to 6113 for the Early sample and 4279 for the Late sample and preserved the 1.4 peptide ratio of Early to Late peptides identified using concatenated set of organism sequences (Table S2 of the Supporting Information). This de novo analysis revealed a large number of quality MS/MS spectra that could not be assigned a peptide sequence when just using the concatenated set of organisms.

Results from protein BLAST[19] against de novo derived peptide sequences identified a diverse set of bacterial ribotypes allied to the *Gammaproteobacteria*, Firmicutes, and high GC Gram-positive bacteria, all consistent with bacteria living in the deep sea and around hydrothermal vents (Figure 4). Protein BLAST assigned 10% to 13% of peptides to expected protein

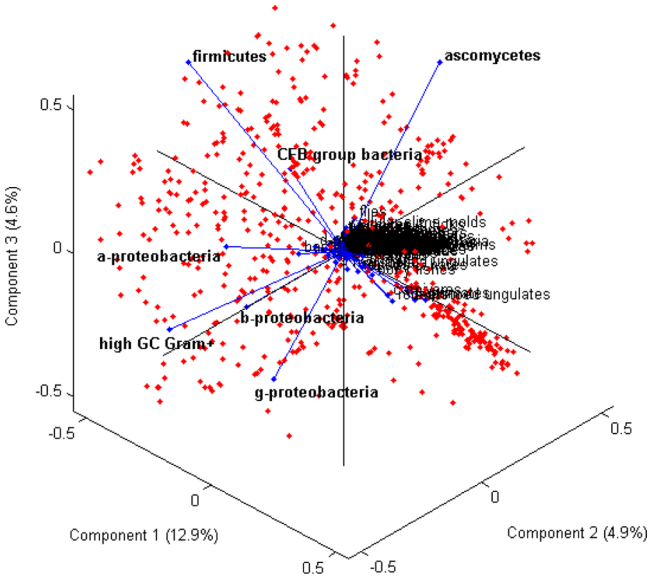

Figure 4. Proteomic analysis of microbial communities recovered during the second BOSS deployment at the Grotto hydrothermal vent site. Biplot of ribotypes identified via protein BLAST of de novo derived peptide sequences from the BOSS Late sample. Values in parentheses next to each component are percent of variance represented by that component. With the exception of the vector for ascomycetes (a known contaminant from the previous instrument run), vectors for bacterial ribotypes principally explain protein BLAST results.

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contaminants (keratin, trypsin, etc.) including proteins from ascomycetes (which unfortunately were carried over from a previous instrument run). Excluding identified contaminants, and requiring a minimum of two unique peptides per protein, 32 Early and 24 Late proteins were confidently identified (Table S1 of the Supporting Information). Some of these proteins are associated with energy metabolism (e.g., hydrogenases) though most are involved in biosynthesis and cellular maintenance. The samples from this long-term deployment have not yet undergone phylogenetic or geochemical analyses, so the precise significance of these patterns remains to be determined.

DISCUSSION

These data demonstrate the utility of osmotic fluid samplers in preserving microbial samples for year-long deployments as well as their capacity to collect microbial and geochemical samples to associate temporal changes in fluid composition with microbial community composition, density, and protein expression. These data further reveal that several fixatives are effective in preserving DNA and cellular structure, though we suggest that − in this context − RNALater may be the most appropriate preservative for DNA and protein preservation as it is both effective and readily available.

The results of the pilot study demonstrate that measurable (and likely ecologically relevant) shifts in microbial diversity are evident even with small changes in the physical and geochemical regime. As mentioned, the collected fluids represent a mixture of hydrothermal effluent and bottom water (as in refs 28,30). Despite the limitations of the temperature data, the geochemical and microbial community compositions are consistent with an increase in vent fluids around days 6−9 when methane concentrations increase and *Epsilonproteobacteria* appear soon after. These data are in agreement with qPCR temporal data, which show an increase in *Epsilonproteobacteria* near that same period. Specifically, the clone libraries were replete with both *Gamma-* and *Epsilonproteobacteria* allied to known psychrophiles. Santelli et al.[29] observed that basaltic and deepwater communities contained ∼90% *Gammaproteobacteria* including the *Pseudoalteromonas* recovered in our libraries. *Alpha-*, *Beta-*, and *Epsilonproteobacteria*, as well as *Flavobacteria*, which were recovered throughout our libraries, have been observed in previous studies of diffuse hydrothermal flows.[4,31−34] However, the clone library and qPCR analyses revealed that vent-endemic ribotypes, including *Epsilonproteobacteria* known to be sulfur-cycling bacteria, were most abundant on days 9−12 (part B of Figure 3), with some general agreement with methane concentrations. Most striking is the observation that *Ridgeia piscesae* symbionts exhibit a marked increase in abundance during this same period (part C of Figure 3). *Ridgeia piscesae* are the dominant megafauna found around the Juan de Fuca Ridge hydrothermal vents and are obligately symbiotic with chemoautotrophic bacteria. After the worm larvae settle, it is believed that free-living symbionts colonize the surface of the larvae, penetrate their skin, and establish the mutualistic endosymbiosis.[35] To date, little is known about the distribution of these symbionts ex hospite (in the water column)[36] though a previous study of a closely related worm (*Riftia pachyptila*) suggests that free-living symbionts can be found in the water surrounding vents.[35] The data shown here, however, are the first to reveal temporal variation in symbiont abundance in diffuse vent fluids, and the collection of this data was enabled by the capacity to examine changes in microbial composition, including *Ridgeia* symbiont abundance, over time. Future BOSS studies will be aimed at interrogating their metabolic activity ex hospite.

Our proteomics analyses are the first of their kind and, using de novo tools and concatenated organism sequences, we were able to assign amino acids to a number of high quality MS/MS spectra. However, the absence of metagenomes from this environment severely limited protein assignment restricting our ability to provide a more in-depth comparison of Early and Late proteomics results. Nevertheless, these results establish that protein samples recovered via the BOSS are sufficient both in quality and quantity to enable proteomic analyses and resolve differences in peptide identity among samples, though pooling of samples is required. Future efforts should employ metagenomics to build a reference database as well as use more sample to ensure greater recovery of proteins and higher resolution data.

The challenge of allying microbial phylogeny, activity, and biogeochemistry is daunting, and a major hindrance in marine microbiology continues to be our inability to sample both microbiology and geochemistry simultaneously at the appropriate spatial and temporal scales. Whereas these data have established that the BOSS is capable of collecting and preserving nucleic acids and proteins for up to one year and that longer durations may be possible (and are the subject of ongoing investigations), they also illustrate how little we know about the activity of these microorganisms and how changes in geochemistry might influence their distribution. As a low-cost, high-resolution fluid sampler, the BOSS enables higher-resolution studies, which can shed light on such questions. Whereas it is limited in the amount of material it can recover per day, its relatively low cost, reliability, and ease of use make the BOSS a promising tool for use in other remote aquatic ecosystems such as terrestrial and marine subsurface aquifers, high altitude lakes, high latitude oceans, or other less accessible areas. The BOSS may also be effective in long-term monitoring of anthropogenic influences on aquatic ecosystems. Also, in concert with larger systems, the BOSS could be used to extend the footprint of sampling natural microbial communities over a wide range of temporal (days to years) scales and spatial (micrometers to kilometers) scales. These attributes will help further our knowledge of how microbial diversity relates to physiological activity and biogeochemical processes in aquatic ecosystems.

ASSOCIATED CONTENT

Supporting Information

Mass spectrometric proteomics methodology, BOSS schematic, gel electrophoresis from lab-based assessments, and comparison of sequence library construction methods as well as proteomic analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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