Seafloor incubation experiment with deep-sea hydrothermal vent fluid reveals effect of pressure and lag time on autotrophic microbial communities

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Abstract:
Depressurization and sample processing delays may impact the outcome of shipboard microbial incubations of samples collected from the deep sea. To address this knowledge gap, we developed an ROV-powered incubator instrument to carry out and compare results from in situ and shipboard RNA Stable Isotope Probing (RNA-SIP) experiments to identify the key chemolithoautotrophic microbes and metabolisms in diffuse, low-temperature venting fluids from Axial Seamount. All the incubations showed microbial uptake of labeled bicarbonate primarily by thermophilic autotrophic Epsilonbacteraeota that oxidized hydrogen coupled with nitrate reduction. However, the in situ seafloor incubations showed higher abundances of transcripts annotated for aerobic processes suggesting that oxygen was lost from the hydrothermal fluid samples prior to shipboard analysis. Furthermore, transcripts for thermal stress proteins such as heat shock chaperones and proteases were significantly more abundant in the shipboard incubations suggesting that depressurization induced thermal stress in the metabolically active microbes in these incubations. Together, results indicate that while the autotrophic microbial communities in the shipboard and seafloor experiments behaved similarly, there were distinct differences that provide new insight into the activities of natural microbial assemblages under near-native conditions in the ocean.

Importance:
Diverse microbial communities drive biogeochemical cycles in Earth’s ocean, yet studying these organisms and processes is often limited by technological capabilities, especially in the deep ocean. In this study, we used a novel marine microbial incubator instrument capable of in situ experimentation to investigate microbial primary producers at deep-sea hydrothermal
vents. We carried out identical stable isotope probing experiments coupled to RNA sequencing both on the seafloor and on the ship to examine thermophilic, microbial autotrophs in venting fluids from an active submarine volcano. Our results indicate that microbial communities were significantly impacted by the effects of depressurization and sample processing delay, with shipboard microbial communities more stressed compared to seafloor incubations. Differences in metabolism were also apparent and are likely linked to the chemistry of the fluid at the beginning of the experiment. Microbial experimentation in the natural habitat provides new insights into understanding microbial activities in the ocean.

Introduction:

At deep-sea hydrothermal vents, low temperature (i.e., diffuse) hydrothermal fluids emanating directly from igneous rock are hot spots of microbial primary production and provide access points to subseafloor habitats. Diffuse vents are formed when cold, oxidized seawater mixes with hot chemically reduced hydrothermal fluids at and below the seafloor, creating steep geochemical gradients that support increased microbial biomass, activity, and diversity relative to the surrounding deep ocean (1-5). These fluids are dominated by chemolithoautotrophic bacteria and archaea that carry out a variety of metabolisms utilizing hydrogen, sulfur compounds, nitrate, and methane (6-12). However, our understanding of the impact of different microbial metabolisms on ocean biogeochemistry and the extent of carbon production from these reactions are nascent. This is partially due to the challenges associated with the collection and transfer of samples from the deep ocean to the surface for experimentation. Samples transferred from the deep ocean to the sea surface are subject to changes in temperature and pressure and usually involve a long lag time between collection, sample
recovery, and shipboard processing. Deep-sea devices designed for filtering seawater and other fluids at depth have been used to minimize these issues through *in situ* filtration, cell concentration, preservation, and analysis (reviewed in 13, 14). The outgassing of compounds such as hydrogen and carbon dioxide impacts microbial measurements from deep-sea hydrothermal vents; therefore, samples often need to be maintained at *in situ* pressures or temperatures when possible (reviewed in 15). For example, McNichol et al. (4, 16) used an isobaric gas tight fluid sampler to conduct shipboard carbon fixation experiments with diffuse vent fluids maintained at *in situ* pressures and temperatures. However, these and other such experiments have sample processing delays and lack *in situ* preservation. This could be critical when sampling microbial communities in diffuse fluids that are in an extreme state of chemical disequilibrium and will likely undergo redox reactions between sampling and arrival in shipboard labs, regardless of the temperature and pressure conditions maintained in the sampling device.

A limited suite of samplers have been developed to carry out experiments while deployed in the ocean, keeping the instrument submerged for the duration of the experiment and fixing the samples post-experiment, before instrument recovery. This avoids biases related to sample collection lag and depressurization, although other experimental artifacts, such as bottle effects still remain (reviewed in 14, 17). This includes the automated micro-laboratory designed to allow one to conduct multiple (in-series) tracer incubation studies during cabled or free-drifting deployments (18-21), as well as a modification of the instrument termed the Microbial Sampler-Submersible Incubation Device (MS-SID), allowing for *in situ* grazing incubation experiments together with *in situ* microbial sampling and preservation (13, 22, 23). Another instrument is the Environmental Sample Processor (ESP) unit which includes a molecular component that carries
out sample homogenization and subsequent detection of particular microbial groups using quantitative PCR, sandwich hybridization, or competitive ELISA (24). A version of the ESP has successfully been deployed in the deep ocean, including in venting hydrothermal fluids (10) and methane seeps (25).

We recently developed a shipboard RNA Stable Isotope Probing (RNA-SIP) procedure combined with metatranscriptomics to identify the active chemolithoautotrophs and metabolic processes being performed during uptake of DIC at deep-sea hydrothermal vent ecosystems (6, 12). In the present study, the method was extended to the seafloor by running RNA-SIP experiments in a newly developed incubator that collects, heats, incubates, manipulates, and preserves seawater and vent fluids to allow for in situ experimentation while powered by a remotely operated vehicle. The major advantage of using the seafloor incubator is the ability to complete an entire experiment at specific temperatures immediately after sample collection, while also controlling for both pressure changes and sample processing delays. As such, the results of the in situ incubation experiment were compared with parallel shipboard experiments in order to determine the effect of pressure changes and lag time on microbial metabolism.

Herein, we describe the new in situ incubator and the results of metatranscriptomic sequencing of the shipboard and seafloor RNA-SIP experiments to provide new insights into the activities of natural microbial assemblages under near-native conditions in the deep ocean.

Methods

Fluid collection for ‘Omics

Low temperature (41°C) hydrothermal vent fluid was collected from Marker 33 vent at Axial Seamount, a submarine volcano located off the coast of Oregon, USA (45.93346, -
129.98225, 1516 m depth) on 26 August 2015 on board the R/V Thomas G. Thompson using ROV Jason II. Fluids were collected using the Hydrothermal Fluid and Particle Sampler (HFPS, 1), which has an integrated temperature sensor to continuously monitor fluid temperature during intake. Collection and processing of diffuse vent fluid samples for RNA-SIP are described below. For collection of filtered vent fluid for microbial community DNA and RNA analyses, 3 L of diffuse fluid was pumped through a 0.22 µm pore size, 47 mm diameter GWSP filter and preserved immediately in situ with RNALater to be used in metagenomic and metatranscriptomic library preparation as described previously (7). Separate fluid samples were collected and analyzed for alkalinity and hydrogen sulfide, ammonia, methane, and hydrogen concentrations following methods described previously (1). The oxygen concentration and pH of the fluid were measured during intake using a Seabird 63 Optical oxygen sensor and an AMT deep-sea glass pH electrode that were integrated into the HFPS.

Shipboard RNA stable isotope probing experiments

Shipboard RNA-SIP experiments were performed as previously described (6). The HFPS was used to collect 4 L of diffuse vent fluid into an acid washed Tedlar bag. Approximately 17 hours later, the vehicle was recovered, and ~30 minutes later, diffuse fluid was pumped from the Tedlar bag into four evacuated 500 mL Pyrex bottles and filled to capacity (530 mL). Prior to filling, 12C-labeled sodium bicarbonate or 13C sodium bicarbonate was added separately to a pair of bottles to reach a final added concentration of 10 mM bicarbonate. After adding the fluid sample to each bottle, 1 mL of 1.2 M HCl was added to counteract the added bicarbonate and ensure a pH similar to unamended vent fluid. H2 (900 µmol) was then added to each bottle. A pair of 13C- and 12C-labelled bottles was then incubated at 55°C for 12 h while another pair was
incubated for 16 h. After incubation, the fluid from each bottle was filtered separately through 0.22 µm pore size Sterivex filters, preserved in RNALater, and frozen at -80°C.

Seafloor incubator RNA stable isotope probing experiments

The seafloor incubator units were incorporated as a module on the HFPS (Figure 1) and designed to pull in vent fluid using the existing HFPS framework A/C powered by the submersible. The incubations occurred concurrently with other HFPS fluid collection and dive operations. The main components of a single incubator unit consisted of an insulated incubator bottle containing the primary sample bag (4 mil thick Tedlar bag), an RTD (Resistance Temperature Detector) probe, and a 250 W heating rod and a final bottle containing a secondary sample bag (2 mil thick Tedlar bag) and a titanium shutoff valve situated between the fluid intake lines and incubator bottle. Four insulated incubation units were loaded onto one rack of the HFPS (Figure 1).

Prior to deployment, $^{12}$C-labeled sodium bicarbonate or $^{13}$C sodium bicarbonate was added separately to a pair each of primary incubation bags to reach a final concentration of 10 mM added bicarbonate upon filling with 800 mL of vent fluid. The lines running to each bag were primed with 1.5 mL of 1.2 M HCl to ensure a pH similar to unamended vent fluid as well as 900 µmol of pure H$_2$ to match the shipboard incubations. Approximately one hour prior to fluid sampling on the seafloor, the insulated incubator chambers were heated to 55°C. This incubation temperature was selected based on the high abundances of thermophiles at Marker 33 in previous studies (7, 26) and matched the incubation temperature of the shipboard RNA-SIP experiments described above. Once at temperature, the primary sample bags were filled with 800 mL of diffuse vent fluid using the HFPS as described above and a shut off valve was
hydraulically closed to prevent further intake from the sample manifold. An RKC MA901
Proportional-Integral-Derivative (PID) temperature controller housed in a separate titanium case
recorded and controlled incubator temperature from an RTD thermometer situated next to the
bag and maintained a constant temperature at a set point (± 2°C) by supplying variable power to
the heating rod located beneath the Tedlar incubation bag inside the incubator (Figure S1). The
PID control algorithm was tuned to the incubator bottle prior to deployment using the MA901
autotune feature. The heating rod induced convection in the incubation chamber resulting in an
even temperature distribution. The temperature distribution within the incubator sample bag was
monitored during pre-deployment laboratory experiments and was found to vary less than 2°C
(Table S4).

A pair of insulated incubator chambers containing $^{12}$C and $^{13}$C bicarbonate were
incubated for 12 h while an identical pair of chambers were incubated for 16 h. At the end of
each incubation, fluid was pumped from the primary incubator bag, through a 0.22 μm pore size
PES filter (Millipore) into a secondary bag that was surrounded by ambient seawater (~ 2°C).
Filters were preserved immediately *in situ* with RNALater. Once shipboard, the fluids in the
secondary sample bags were analyzed for pH and the filters were frozen at -80°C.

 Fractionation of RNA-SIP experiments, RT-qPCR, library preparation, and analysis

RNA from the incubator and shipboard SIP experiments was extracted, quantified, and
fractionated after isopycnic centrifugation as described in (6) and Supplemental Material. 16S
rRNA copy number was determined for each fraction via RT-qPCR with universal primers
Pro341F and Pro805R (27) as described in the Supplemental Material. This measurement was
used for comparison between the $^{12}$C and $^{13}$C samples and for determination of $^{13}$C enrichment.
Four fractions from each of the $^{12}$C and $^{13}$C samples from the shipboard and incubator samples were sequenced, including fractions with the maximum amount of 16S rRNA and a few fractions on either side of the peak, for a total of 16 metatranscriptomic libraries. RNA-SIP metatranscriptomic library preparation and downstream analyses were completed as described in detail in the Supplemental Material. Briefly, double stranded cDNA was constructed from each RNA-SIP metatranscriptome and used for library preparation via the NuGen Ovation Ultraslow Library DR multiplex system, following manufacturer’s instructions. Ribosomal RNA was not removed prior to library construction. For the RNA-SIP metatranscriptomes, taxonomy, overall transcript abundance, and hierarchical clustering is displayed for all 16 libraries. For visualization of key metabolic processes, the 16 libraries were collapsed into their corresponding experiments: 12C Shipboard, 13C Shipboard, 12C Incubator, and 13C Incubator. Transcript abundance across fractions was summed for each experiment.

**Marker 33 Metagenomic and Metatranscriptomic library preparation and analysis**

The in-situ preserved 47 mm diameter flat filters were cut in half with a sterile razor with each half used for DNA and RNA extractions, respectively, and corresponding libraries prepared for sequencing as described in the Supplemental Material and (7). Briefly, extracted RNA was treated using a Turbo-DNase kit (Ambion), purified, and concentrated using the RNAeasy MinElute kit (Qiagen). Ribosomal RNA removal, cDNA synthesis, and metatranscriptomic library preparation was carried out using the NuGen Ovation Complete Prokaryotic RNA-Seq DR multiplex system following manufacturer instructions. Metagenomic library construction was completed using the NuGen Ovation Ultralow Library DR multiplex system following manufacturer instructions.
Differential Expression Analysis

Differential Expression (DE) analysis was run to identify specific transcripts whose abundance was significantly higher or lower (adjusted p-value < 0.01) between the shipboard and seafloor RNA-SIP experiments. DE analysis was run using the interactive tool DEBrowser in R (28). Within DEBrowser, differential expression analysis was run using normalized transcript abundances for KO annotated genes for all 16 RNA-SIP libraries with Limma, an R package used for the analysis of gene expression data (29). Low count transcripts, defined as the maximum normalized abundance for each transcript across all samples being less than 10, were removed from the analysis. Resulting tables, heatmaps, and plots showing significance were generated within DEBrowser.

Mapping to thermophilic Epsilonbacteraeota MAGs

Metagenome Assembled Genomes (MAGs) were assembled and taxonomically identified from Axial Seamount metagenomic data as described in (7). In this study we determined the mean coverage of the 10 previously identified MAGs classified as thermophilic Epsilonbacteraeota via Phylosift (30) and CheckM (31) within the dataset (Table S3). All reads from the Marker 33 metagenome, Marker 33 metatranscriptome, and all RNA-SIP metatranscriptomes were mapped to each of the ten MAGs using Bowtie2 with an end-to-end alignment and default parameters (v2.0.0-beta5, 32). Mean coverage for each MAG within the Marker 33 metagenome was calculated via Anvi’o (33). Mean coverage for each MAG within the Marker 33 metatranscriptome and RNA-SIP metatranscriptomes was calculated via samtools (34). For ease of visualization, the 16 RNA-SIP metatranscriptomes were collapsed and mean coverage for each MAG within fractions was averaged for each of the four experiments: $^{12}$C.
Shipboard, $^{13}$C Shipboard, $^{12}$C Incubator, and $^{13}$C Incubator. Heatmaps of mean coverage were constructed in R using the package heatmap3 (v3.3.2, 35).

Data deposition

Raw sequence data are publicly available through the European Nucleotide Archive (ENA), with project number PRJEB38697 for RNA-SIP metatranscriptomes and PRJEB19456 for the Marker 33 metagenome and metatranscriptome. Assembled contigs for the Marker 33 metagenome and RNA-SIP metatranscriptomes are publicly available via IMG/MER under submission numbers 78401, 97537-97540, and 97583-97594. Contigs for the 10 Epsilonbacteraeota MAGs are available through FigShare at DOI: 10.6084/m9.figshare.12445976

Results

$^{13}$C Enrichment observed in RNA-SIP experiments

Diffuse hydrothermal fluid at Marker 33 vents directly from cracks in basalt along the eruption zone on the southeast side of the Axial caldera. Chemical analysis of this fluid is shown in Table S1. The fluid was 85% seawater and 15% hydrothermal end-member fluid based on magnesium concentration (7). The temperature was monitored throughout the experiment, and temperature records showed that the incubator rapidly heated the chambers to 55°C and maintained temperature within 2°C for the length of the incubations (Figure S1). Upon recovery, the mass of each secondary bag was determined to indicate how much incubated sample was pulled through the RNA preservative filter at the end of the incubation. In general, the secondary bags were full or nearly full and the primary incubator bags were empty or nearly empty. The pH
of the filtered fluids in the secondary bags and from the shipboard incubation bottles was near 6
462 (Table S2).

Both the 12 h and 16 h shipboard and incubator experiments showed $^{13}$C enrichment
463 (Figure 2, Figure S2). Only the 12 h samples were sequenced to avoid heterotrophic cross-
464 feeding from prolonged incubations. The maximum amount of 16S rRNA occurred at higher
465 RNA densities in the $^{13}$C experiments versus the $^{12}$C controls (Figure 2) indicating that dissolved
466 inorganic carbon (bicarbonate) was incorporated into RNA during the incubations. For the
467 shipboard experiment, the maximum amount of 16S rRNA occurred at densities of 1.788 and
468 1.804 for the $^{12}$C control and $^{13}$C experiment, respectively. For the incubator experiment,
469 maximum 16S rRNA occurred at lower RNA densities overall, with peak amounts occurring at
470 densities of 1.778 and 1.785 for the $^{12}$C-control and $^{13}$C-experiment, respectively (Figure 2).

**Taxonomic composition of RNA-SIP experiments**

The taxonomic composition of the RNA-SIP experiments was determined based on the
473 relative abundance of 16S rRNA sequences and nearly all were primarily composed (96.7% to
474 98.2%) of thermophilic bacteria belonging to the Epsilonbacteraeota (Figure 3A). The
475 thermophilic genus *Caminibacter* was most abundant within all SIP experiments, with a relative
476 abundance of 80.1%, 95.7% and 83.7% for the $^{12}$C shipboard, $^{12}$C incubator, and $^{13}$C incubator
477 metatranscriptomes, respectively (Figure 3A). For the $^{13}$C shipboard experiment, *Caminibacter*
478 was also the most abundant group but to a lesser extent, comprising 59.8% of the community, as
479 this experiment also had a higher relative abundance of both *Nautilia* and *Hydrogenimonas* 16S
480 rRNA sequences (Figure 3A). In the $^{13}$C shipboard experiment, *Nautilia* comprised 21.4% and
481 *Hydrogenimonas* comprised 19.0% of the 16S rRNA sequences on average, indicating a different
community composition in the $^{13}$C shipboard compared to the other experiments.

$^{13}$C Hydrogenimonas was more abundant in the shipboard community compared to the incubator community, where it only comprised 0.4% of the $^{12}$C and 8.0% of the $^{13}$C incubator communities on average (Figure 3A). This pattern was also observed in the taxonomic composition of the annotated transcripts (Figure 3B). While Caminibacter comprised close to 50% of annotated transcripts in the $^{12}$C shipboard, $^{12}$C incubator, and $^{13}$C incubator metatranscriptomes, transcripts classified as Nautilia comprised a high percentage of total annotated non-rRNA transcripts in all experiments (Figure 2B).

Mean coverage of Metagenome Assembled Genomes (MAGs) was used to determine the extent to which the MAGs were represented and active within the RNA-SIP experiments. Previously, 10 MAGs classified as thermophilic Epsilonbacterota (either Nitratifactor sp. or more broadly to the family Nautiliaceae) were identified from Marker 33 vent metagenomic assemblies as described in (7). The 10 MAGs range from 13% to 94% completeness, as determined via Anvi’. A heatmap depicting mean coverage of each MAG showed that these genomes were represented to varying degrees in the 2015 Marker 33 metagenome and were active in the Marker 33 metatranscriptome, as well as in the RNA-SIP metatranscriptomes (Figure S3). Due to the higher coverage of the MAGs within the Marker 33 metagenome, patterns among the SIP experiments were masked, and therefore a second heatmap was constructed showing only mean coverage across the SIP experiments (Figure 4). Results showed that three MAGs (Axial Epsilon Bins 1, 8, and 9) had the highest coverage across all SIP experiments. These three MAGs were broadly classified as belonging to the family Nautiliaceae (Figure S7). The $^{13}$C shipboard experiment showed additional coverage of two other MAGs (Axial Epsilon Bin 2 and 7), both also classified to the family Nautiliaceae (Figure 4).
Hierarchical clustering of all 16 RNA-SIP metatranscriptomes based on normalized KEGG ontology (KO) abundance of annotated transcripts showed that the $^{12}$C controls for the incubator and shipboard experiment clustered together, indicating functional similarity between the shipboard and incubator SIP experiments (Figure S4). The $^{13}$C experiments for the incubator and shipboard clustered separately from the $^{12}$C controls and from each other, with the four $^{13}$C shipboard metatranscriptomes forming a separate cluster (Figure S4).

When examining only the most abundant annotated transcripts expressed across all metatranscriptomes, the same clustering pattern was observed (Figure S5). The most abundant transcripts were annotated to genes related to cell growth, translational processes, and energy metabolism. The gene to which the most annotated transcripts mapped was peroxiredoxin, a gene involved in reducing oxidative stress and thus cell damage. Other highly abundant transcripts were annotated to genes for elongation factors and molecular chaperones, indications that translational machinery was active across all SIP experiments. In addition, transcripts for a key gene in the reductive TCA (rTCA) cycle, 2-oxoglutarate ferredoxin oxidoreductase, were also abundant, indicating carbon fixation was occurring (Figure S5). Additional transcripts for carbon fixation within the SIP experiments were also observed (Figure S6). As observed in the taxonomic profiles, examination of the most abundant annotated transcripts shows that the $^{13}$C shipboard metatranscriptome was slightly different compared to the other three experiments and clustered separately from the other metatranscriptomes (Figure S5).

Differential expression (DE) analysis was run to determine significant differences in annotated transcript abundance across the 16 RNA-SIP metatranscriptomes (Figure 5). Results
showed 233 genes were significantly differentially expressed (adj. p-value < 0.01) in shipboard vs. incubator RNA-SIP libraries, with all but one being more highly expressed in shipboard experiments compared to incubator experiments (Figure 5B). Annotated transcripts with the greatest difference in abundance (>10 log2 fold change) in shipboard vs. incubator experiments included transcripts of genes related to translation, DNA replication, purine synthesis, and motility (Table S5).

The abundance of annotated transcripts involved in important metabolic processes differed between the shipboard and incubator SIP experiments (Figure 6), although DE analysis revealed that many of these differences were not significant. The metatranscriptome of diffuse fluids reflects the diversity of metabolic processes that occur at a single vent, with the presence of annotated transcripts for aerobic respiration, denitrification, aerobic methane oxidation, methanogenesis, hydrogen oxidation, sulfur reduction, and sulfur oxidation. The reduced metabolic complexity, observed within the RNA-SIP metatranscriptomes highlights the specific organisms and their metabolisms active under experimental conditions. Transcripts for the mainly anaerobic process of denitrification, specifically nirS, norBC, and nosZ, were only observed in the shipboard experiments. Conversely, transcripts for cytochrome c oxidases, important for aerobic respiration, were only observed in one of the incubator experiments (Figure 6). Transcripts for methane metabolism differed, albeit not significantly, across experiments. Transcripts for methyl-coenzyme M reductase (mcrA gene), important for anaerobic methanogenesis, were only observed in the shipboard SIP experiments. Aerobic methane oxidation transcripts, however, showed an average of 1.35 log2 fold increase in incubator experiments when compared to the shipboard experiments (Figure 6). For hydrogen oxidation, transcripts annotated to genes for Group 1 Ni-Fe hydrogenases (hydA3 and hyaC) were more...
abundant in the shipboard experiments compared to the incubator experiments (adj. p-value < 0.01). Sulfur metabolism transcripts for polysulfide and thiosulfate reduction showed a significantly higher abundance (adjusted p-value < 0.01) in the shipboard compared to the incubator experiments (Figure 6).

Because DE analysis indicated that abundance of transcripts related to stress was significantly higher in the shipboard experiment compared to the incubator (Table S5), we further examined the abundance of transcripts related to stress (chaperones, proteases, and other heat shock proteins) within the samples (Figure 7). An increase in abundance of transcripts annotated to heat shock chaperone genes *dnaK/dnaJ* and *GroES/GroEL* was observed in both the shipboard and incubator experiments when compared to the metatranscriptome of diffuse fluids (Figure 7). However, *dnaJ* had a significantly higher abundance within the shipboard experiment (adjusted p-value < 0.01). Additionally, transcripts for the heat shock protein gene *htpX* were only expressed in the shipboard SIP metatranscriptomes. Proteases, which play an important role in protein degradation during times of stress, were in general more highly expressed in the shipboard SIP experiments when compared to the incubator. Specifically, transcripts for protease genes *clpX, clpP, ftsH, and hslU* all showed significantly higher abundance in the shipboard experiments (adj. p-value < 0.01, Figure 7, Table S5).

**Discussion:**

There are extreme technical challenges to understanding microbial life in the deep sea, and results and interpretations depend heavily on the experimental approach taken. Motivated by the hypothesis that chemical reactions and microbial activity that occur in sample containers between the time of sampling and the start of an experiment will affect experimental results...
significantly, we designed and built an in situ incubator to eliminate depressurization and lag time between sampling and experiment in order to better capture the in situ microbial activity at deep-sea hydrothermal vents where diffusely venting fluids exit the seafloor. We successfully demonstrated the ability to study thermophilic microbes close to their seafloor and subseafloor habitats and highlighted differences between shipboard and seafloor incubations. To probe these communities, an RNA-SIP methodology coupled to mRNA sequencing was applied to examine which organisms and metabolism are responsible for autotrophy under experimental conditions that reflect those in the subseafloor (6). However, many different types of incubations at temperatures from ambient to at least 80°C are possible with this instrument, making it a valuable new tool for marine microbial ecology.

Marker 33 vent was chosen as the site for the seafloor incubator testing due to the consistent presence of thermophilic bacteria and archaea detected in previous studies (7, 26, 36-38). We chose RNA-SIP for the demonstration of in situ autotrophic activity of subseafloor microbial communities, and these experiments must mimic the physical and chemical conditions of the environment as closely as possible, which is inherently challenging in any aquatic sample. For example, it may be hours before fluid collected on the seafloor can be dispensed into shipboard bottles and incubated, thus increasing the likelihood of changes to the microbial community. This time lag combined with pressure and temperature changes during transport of diffuse fluids to the surface in unpressurized vessels may also result in outgassing of key redox species such as methane, hydrogen sulfide, hydrogen, and carbon dioxide (16, 39), death of pressure- and temperature-sensitive organisms (40), or loss of oxygen to microbial consumption or chemical reactions in the sample container (41). Performing experiments in situ on the...
seafloor may help ameliorate many of the biases introduced with shipboard experiments, but few
direct comparisons between in situ versus shipboard experiments exist.
Except for location (seafloor or shipboard) and timing after fluid sampling, all other
conditions (temperature, pH, concentration of hydrogen and DIC label added, length of
incubation) were identical between experiments. The pH of the incubations in both sets of
experiments was similar to the vent fluid from Marker 33. Both the shipboard and seafloor
incubator experiments showed $^{13}$C enrichment relative to their $^{12}$C control with maximum 16S
rRNA occurring at higher RNA densities. However, the RNA densities of the two experiments
were slightly different with peak 16S rRNA occurring at lower RNA densities overall in the
incubator experiment. The reason for the lower level of enrichment in the seafloor incubator is
unclear but may be due to differences in the dominant microbial genera present in each
experiment or stochastic effects. The majority of the rRNA from all SIP experiments, both
shipboard and incubator, was comprised of thermophilic Epsilonbacteraeota oxidizing hydrogen
and reducing nitrate while fixing carbon, consistent with the native community present at the
Marker 33 site in 2015, as well as numerous -omic surveys at diffuse vents, indicating these
organisms and metabolism often dominate in the reducing, warm subseafloor habitat (4, 6, 7, 9,
10, 12, 42). There was a higher percentage of rRNA classified to the genera Hydrogenimonas
and Nautilia in the shipboard experiments relative to the incubator (Figure 3A) including two
Nautilia populations only observed in the $^{13}$C shipboard experiment (Figure 4). Based on
publicly available genomes, Nautilia species have a higher GC-content in their genomic DNA
(average 34.8%) compared to Caminibacter (average 28.9%), which may account for the higher
peak RNA density in the $^{13}$C shipboard compared to the $^{13}$C incubator experiment.
Differences in metabolism were apparent between the shipboard and incubator experiments and may be linked to the chemistry of the fluid at the beginning of the experiment. For example, transcripts annotated for denitrification (*nirS, norB, nosZ*) and methanogenesis (*mcrA*) were only observed in the shipboard experiments. Additionally, significantly higher abundance of hydrogen oxidation transcripts (*hyaA3, hyaC, adj. p-value < 0.01*) was observed shipboard compared to the seafloor incubations. Although not significant, there was a higher abundance of transcripts annotated for aerobic methane oxidation (*pmoA*) and oxygen utilization (*cox* and *cco*) in the incubator experiments compared to shipboard (Figure 6). We hypothesize that during the lag time between sample collection and beginning the experiment shipboard (approximately 17.5 hours), oxygen was consumed in the vent fluids by aerobic microorganisms and abiotic reactions with the high concentration of dissolved sulfide and reduced metals in the samples. Therefore, by the time the fluid was used in the shipboard incubations, there was little to no oxygen left. For the seafloor experiment, incubations of samples that were approximately 85% deep seawater (Table S1) contained oxygen at the start of the experiment and oxygen-consuming microbes grew. Aerobic oxidation of methane and sulfur species are important microbial metabolisms in hydrothermal vent plumes, as well as in many venting fluids where deep, oxygen-rich seawater mixes with the reducing vent fluids (43-45). For example, our metatranscriptomic study from multiple vent sites at Axial Seamount, including Marker 33 in 2015, showed transcription of cytochrome c oxidases and methane monooxygenase at this site, indicating these processes were occurring *in situ* in the venting fluids (7). Additional *in situ* experiments focused on assessing the metatranscriptome of the incubated vent fluid over a shorter time scale might resolve an initial aerobic stage from a later anaerobic stage and capture some of the dynamic spatial variability in microbial activity around diffuse vent sites. Overall,
these results highlight the importance of performing incubations in situ and demonstrate that incubations performed shipboard may underestimate aerobic metabolisms due to the consumption of oxygen during sample recovery.

In addition to differences in microbial metabolism, we found significantly higher abundance of transcripts annotated to heat-shock proteins, proteases, and chaperones in the shipboard experiments compared to the incubator, which may indicate that the shipboard microbial community was under more thermal stress (46). Chaperones can aid in protein folding and prevent protein denaturation that occurs during environmental stress (46, 47). The abundance of transcripts for chaperone encoding genes in both shipboard and incubator experiments was higher compared to the Marker 33 metatranscriptome (Figure 7), an indication that experimental incubations, be it on the seafloor or shipboard, enact some stress on microbial communities. However, transcripts annotated as proteases and heat shock proteins were significantly more abundant in the shipboard experiments (adj. p-value < 0.01), particularly in the $^{13}$C experiment (Figure 7). The increased environmental stress could be due to transport to atmospheric pressure, manipulation of fluid into glass bottles, or any number of differences that occur when carrying out incubations shipboard as compared to incubating the fluid in situ on the seafloor. Another possibility is that the incubations were performed at temperatures near the optimal growth temperatures of \textit{Caminibacter} (55-60°C, 48, 49, 50), \textit{Nautilia} (53-60°C, 51, 52, 53), and \textit{Hydrogenimonas} (55°C, 54), which may induce transcription of thermal stress proteins in these organisms. Growth at pressures found at deep-sea vents increased the optimal growth temperature (55-57) and raised the thermal induction temperature (56) in hyperthermophilic archaea. Therefore, \textit{in situ} incubation of vent fluids in this study may similarly ameliorate thermal stress in Epsilonbacteraeota relative to shipboard incubations.
In conclusion, this study showed the effects of depressurization and sample processing delays using a new *in situ* incubator instrument to carry out RNA-SIP experiments in situ on the seafloor. The taxonomic and functional gene differences observed between shipboard and incubator experiments were likely due to slight differences in the chemistry of the fluid at the start of the experiment, and more specifically, the availability of oxygen in the incubator experiment. Microbial populations were also more stressed in shipboard experiments. Although the shipboard and incubator experiments were similar, the slight differences between the two suggest that use of a seafloor incubator may give a more accurate account of the microbial metabolic processes occurring within diffusely venting fluids due to reduced lag time, depressurization, and stress, as well as limiting both abiotic and biotic reactions that modify the chemistry of the fluids during transport to the ship.

Use of instrumentation like the seafloor incubator is an important step in understanding and constraining the roles microbial communities play in the deep ocean, with potential applications well beyond those described here. The incubator can collect seawater, cold seep fluids, or vent fluids and their associated microbial communities and immediately amend the fluids while keeping them *at in situ* pressure and a controlled temperature before filtering and preserving the microbial biomass. Future experiments with the incubator will focus on performing quantitative time series measurements of microbial, viral, and geochemical activity for various biogeochemical processes, as well as nutrient amendment experiments to measure the effect of substrate concentration on reaction rates, chemical signatures, and microbial and viral community composition and function. Thus, our study expands our understanding of the activities of natural microbial assemblages under near-native conditions at deep-sea.
hydrothermal vents and allows for future deployments to better constrain marine microbial biogeochemistry in the ocean.

Acknowledgements

This work was funded by the Gordon and Betty Moore Foundation Grant GBMF3297, the NSF Center for Dark Energy Biosphere Investigations (C-DEBI) (OCE-0939564), NOAA/PMEL, contribution number 5182, and Joint Institute for the Study of the Atmosphere and Ocean (JISAO) under NOAA Cooperative Agreement NA15OAR4320063, contribution number 2020-1113. We thank the captains and crews of the R/V Thomas G. Thompson and R/V Brown, and in particular the ROV Jason II group for assistance with incubator integration on the vehicle. The RNA SIP methodology used in this work was developed during cruise FK010-2013 aboard R/V Falkor supported by the Schmidt Ocean Institute. Andra Bobbit, Bill Chadwick, Kevin Roe, Susan Merle, and Ryan Wells provided critical support at sea. We also thank Paula Pelayo who helped with RT-qPCR assay development. NOAA/PMEL supported this work with ship time in 2014 and through funding to the Earth Ocean Interactions group. NSF provided shiptime for the 2015 expedition through OCE-1546695 to DAB and OCE-1547004 to JFH.
Figure 1. Incubator setup for the in situ RNA Stable Isotope Probing (RNA-SIP) experiments (A-D). Each of the four incubation chambers was heated to a chosen set point temperature. Fluid was pulled into the insulated incubation chamber from the manifold of the Hydrothermal Fluid and Particle Sampler (HFPS) through a custom titanium shutoff valve, pulling hydrogen gas and buffering acid into the chamber as it filled. After the incubation period, the fluid was pulled from the incubation chamber through a 0.22 µm filter (A) with passive addition of RNA preservative. A cutaway view of the incubation chamber (B) shows the incubation bag over the heating element, with the RTD used to monitor chamber temperature near the end of the bag. The fully assembled incubator module (C, as deployed in 2015) slides into the HFPS sample rack (D). Fluid transfer is accomplished with the HFPS sample pump and selection valve.
Figure 2: 16S rRNA abundance in density gradient fractions of (A) Shipboard and (B) Incubator RNA-SIP experiments at 12 hours. Buoyant density (g ml⁻¹) of each fraction is depicted on the x-axis and amount of 16S rRNA as determined by RT-qPCR is on the y-axis. Diamond and square symbols denote fractions sequenced for the 12C control and 13C experiment respectively. Amount of 16S rRNA is displayed as the ratio of maximum quantity in order to compare between RNA-SIP experiments.
Figure 3: Taxonomic classification of (A) 16S rRNA reads and (B) functionally (KO) annotated non-rRNA transcripts from RNA-SIP metatranscriptomes.
Figure 4: Heatmap of mean coverage across the RNA-SIP experiments of metagenome assembled genomes (MAGs) taxonomically identified as thermophilic Epsilonbacteriaota, specifically either the genus *Nitritifactor* (orange) or the family Nautiliaceae (purple) as described in (7). Fractions from each of the four RNA-SIP experiments have been collapsed and mean coverage summed. Scale depicts range of mean coverage across MAGs. MAGs were clustered based on similarity of coverage within the RNA-SIP experiments.
Figure 5: Heatmap (A) showing the 233 KO annotated genes that were differentially expressed across fractions (adj. p-value < 0.01). Volcano plot (B) of fold change in abundance vs. adjusted p-value. Genes that were significantly up regulated (adj. p-value < 0.01) in the Incubator vs. Shipboard fractions are colored in red, down regulated genes are colored in blue.
### Figure 6: Normalized abundance of key genes and transcripts for oxygen, nitrogen, methane, hydrogen, and sulfur metabolisms within the 2015 Marker 33 metagenome, metatranscriptome, and shipboard and incubator RNA-SIP experiments. Fractions from each of the four RNA-SIP experiments have been collapsed to reflect the normalized abundance of each gene in the entire experiment. Normalized abundances of metatranscriptomes were transformed to the same scale as the Marker 33 metagenome. Black stars indicate a significant difference in transcript abundance (adjusted p-value < 0.01) between shipboard and incubator RNA-SIP experiments. Refer to Table S5 for the specific subunits identified as significant.
Figure 7: Normalized abundance genes and transcripts annotated to cell stress, including genes for protein chaperones, heat-shock proteins, and proteases within the 2015 Marker 33 metagenome, metatranscriptome, and shipboard and incubator RNA-SIP experiments. Fractions from each of the four RNA-SIP experiments have been collapsed to reflect the normalized abundance of each gene in the entire experiment. Normalized abundances of metatranscriptomes were transformed to the same scale as the Marker 33 metagenome. Black stars indicate a significant difference in transcript abundance (adjusted p-value < 0.01) between shipboard and incubator RNA-SIP experiments. Refer to Table S5 for the specific subunits identified as significant.
References:


