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Project: Event response to an eruption at Axial Seamount (NeMO2015)

Sampling and Analytical Methodology:

In August 2012, September and October 2013, August 2014, and August 2015, 7-40°C diffuse hydrothermal fluids were collected from 10 vent sites at 1515-1716 m depths from Axial Seamount on the Juan de Fuca Ridge. Descriptions of the fluid sample temperatures and the sample sites are provided in Table 1. The fluid samples were drawn into 650 ml Tedlar plastic bags with polyethylene valves within rigid housings using the NOAA Hydrothermal Fluid and Particle Sampler. The sampler pumped vent fluid through a titanium nozzle and recorded the temperature of the fluid within the intake nozzle once every second during pumping. Samples were collected using the research submarines *Jason* II and *ROPOS*. Background seawater was collected by shipboard hydrocasts at 1500 m depth directly over the caldera (25 m above the bottom) and 3 km west of the summit with 10 L Niskin bottles. The hydrothermal fluid and background seawater samples were divided for cultivation-dependent Most Probable Number (MPN) concentration estimates of thermophiles and hyperthermophiles (100 ml), microcosm incubations (400 ml), and total cell counts (40 ml). All operations at sea occurred on the research vessels *Marcus G. Langseth*, *Thomas G. Thompson*, *Falkor*, and *Ronald H. Brown*.

Microcosm Incubations:

Description of microcosms

Group	Years	Description
Set A (high H_2)	All	200 kPa H_2:CO_2 (80%:20%)
Set B (low H_2)	All	200 kPa N_2:CO_2 (80%:20%), 1 ml of headspace replaced with 1 ml of H_2:CO_2
Set C (no H_2)	All	200 kPa N_2:CO_2
Set D (high H_2 + NH_4^+)	2012-2013	200 kPa H_2:CO_2 plus 4.7 mM NH_4Cl (2012) or 47 µM NH_4Cl (2013)
Set E (no H_2, tryptone added)	2014-2015	200 kPa N_2:CO_2 plus 0.5% tryptone and 0.01% yeast extract

Each 60 ml serum bottle contained 25 ml of low-temperature diffuse hydrothermal fluid that was incubated in pairs at 55°C and 80°C.

For each sample site, 25 ml of hydrothermal fluid or background seawater was added without exposure to air to each of 16 sealed 60 ml serum bottles that had been pre-flushed with either H_2:CO_2 (80%:20%) or N_2:CO_2 (80%:20%), depending on the headspace composition used for incubation (**see table above**). The bottles were divided into four sets of four bottles with a pair of bottles from each set incubated at 55°C and 80°C for up to a week or until visibly turbid. Three of the four sets of microcosms (sets A-C) were incubated each of the four study years. Set A was flushed and filled with 200 kPa of H_2:CO_2 yielding an estimated aqueous H_2 concentration of 1.2 mM at their incubation temperatures based on calculations using the geochemical prediction software Geochemist's Workbench. Sets B and C were flushed and filled with 200 kPa of N_2:CO_2, and half of these bottles

(set B) were given 1 ml of H_2:CO_2 in exchange for 1 ml of N_2:CO_2 to produce an estimated aqueous H_2 concentration of 20 mM at their incubation temperatures. In 2012 and 2013, the remaining four serum bottles (set D) were amended with 4.7 mM NH_4Cl (2012 only) or 47 mM NH_4Cl (2013 only) and flushed and filled with 200 kPa of H_2:CO_2 to test for growth stimulation by ammonium. The NH_4Cl concentration was based on that added to our defined methanogen growth medium (see below). In 2014 and 2015, the remaining four serum bottles (set E) were amended with 0.5% (wt vol^{-1}) tryptone plus 0.01% (wt vol^{-1}) yeast extract and flushed and filled with 200 kPa of N_2:CO_2 to test for H_2 syntrophy. All samples were reduced with 0.025% (wt vol^{-1}) each of cysteine-HCl and Na_2S•$9H_2O$. Growth of methanogens was determined by analyzing for CH_4 in the headspace using gas chromatography once the cells in the bottle had reached stationary growth phase. In 2015, an aliquot of the 80°C and 55°C tryptone/no H_2 samples (set E) that showed CH_4 production were filtered onto 0.2-μm pore size nucleopore filters prestained with Irgalan black (Sterlitech, Kent, WA, USA), stained with acridine orange, and examined using epifluorescence microscopy. In 2015, the 80°C and 55°C tryptone/no H_2 samples from the Marker 113 vent site were also separately filtered through Sterivex GP 0.22 μm sterile filter units (Millipore, Billerica, MA, USA) and frozen at -80°C until analyzed. In2015, 10 ml of hydrothermal fluid was added to sealed Balch tubes without exposure to air, amended separately with 0.1% (wt vol^{-1}) sodium formate and 0.5% (wt vol^{-1}) sodium acetate, flushed and filled with 200 kPa N_2:CO_2, and incubated in duplicate at 80°C and55°C for up to seven days to determine if these substrates can support methanogenesis at high temperatures.

Total cell counts in the original hydrothermal fluids were done by preserving in duplicate 18 ml of hydrothermal fluid with 1.8 ml of 37% formaldehyde. Samples were stored at 4°C for less than a month prior to counting by epifluorescence microscopy as described above.

DNA Extractionand16SrRNAAmplicon Sequencing

In this study, Marker 113 vent showed the highest concentrations of methanogens and methanogenesis at Axial Seamount. Therefore, DNA from each 2015 Marker 113 microcosm that had been amended with tryptone (i.e., set E) and concentrated with a Sterivex filter was extracted and eluted using the MoBio PowerWater DNA extraction kit (MoBio, Carlsbad, CA, USA) as described by the manufacturer to determine which methanogens and other microorganisms were present following the microcosm incubations.The DNA was quantified using a Nanodrop 2000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) and stored at -20°C. The v4v5 regions of the 16S rRNA gene were amplified separately for bacteria and archaea and prepared for Illumina sequencing from the DNA extractions. Bacterial amplification was carried out as previously described (Huse et al., 2014). The archaeal v4v5 16S rRNA gene was targeted by a combination of five forward primer variants (517F; GCCTAA AGCATCCGTAGC, GCCTAAARCGTYCGTAGC, GTCTAAAGGGTCYGTAGC, GCTTAAAGNGTYCGTAGC, GTCTAAARCGYYCGTAGC) and a single reverse primer (958R; CCGGCGTTGANTCCAATT). Amplification primers were designed based on information from probeBase and the SILVA database. 16S rRNA amplicon sequencing was performed using an Illumina MiSeq Benchtop sequencer (Illumina, San Diego, CA, USA) at the Marine Biological Laboratory in Woods Hole, MA as described on the Visualization and Analysis of Microbial Population Structures(VAMPSs) website (https://vamps.mbl.edu/resources/primers.php). Paired- end sequences were assessed for quality and merged using the code base previously described (Eren et al., 2013). The sequences were binned into operational taxonomic units (OTUs) using subsampled open reference OUT picking method at 97% sequence identity based on the Greengenes database and taxonomies assigned using the RDP Classifier (Wang et al., 2007) with minimum

confidence score 0.8 in QIIME (Caporaso et al., 2010). Sequences are available at the NCBI Sequence Read Archive under accession number SRP071807.

Media Used:
The defined methanogen growth medium for laboratory experimentation and MPN analyses was a modification of DSM 282 medium, which contained (per liter in ddH_2O): 0.14 g of K_2HPO_4, 0.14 g of $CaCl_2$•7H_2O, 0.25 g of NH_4Cl, 3.4 g of $MgSO_4$•7H_2O, 5.1 g of $MgCl_2$•6H_2O, 0.34 g of KCl, 0.05 mg of $NiCl_2$•6H_2O, 0.05 mg of Na_2SeO_3•5H_2O, 30 g of NaCl, 1 g of $NaHCO_3$, 1 g of NaS_2O_3, 0.24 g of Na_2MoO_4•2H_2O, 10 ml of Wolfe's minerals, 10 ml of Wolfe's vitamins, and 0.25 mg of resazurin. For the 2012 MPNs, 0.24 g of Na_2MoO_4•2H_2O was also added to suppress sulfate reduction but was omitted in subsequent years. The medium was pH balanced to 6.0, reduced with 0.025% each of cysteine-HCl and Na_2S•9H_2O, and pressurized with 200 kPa of H_2:CO_2 headspace. The autotrophic sulfur-reducer medium was the same as the methanogen medium except that 10 g l^{-1} of elemental sulfur were added and the medium was reduced with 3.2 mM dithiothreitol (DTT). The heterotroph medium for MPN estimates was based on the Adams medium (Adams et al., 2001) and contained 0.5% tryptone plus 0.01% yeast extract. It was pH balanced at 6.8, reduced with 0.025% each of cysteine-HCl and Na_2S•9H_2O, and pressurized with 100 kPa of N_2:CO_2 headspace. The heterotroph-methanogen co-culture medium was the modified DSM282 medium with 0.1 ml of 10 mM Na_2WO_4 •H_2O, 1 ml of 0.2% $(NH_4)_2Fe(SO_4)_2$-$(NH_4)_2Ni(SO_4)_2$, and 0.5% tryptone plus 0.01% yeast extract added with 200 kPa of N_2:CO_2 headspace. The medium was pH balanced to 6.8.

Most Probable Number (MPN) Cell Estimates:
Three-tube MPN analyses were used by adding 3.3, 0.33, and 0.03 ml of the hydrothermal fluid samples in triplicate to the methanogen, autotrophic sulfur reducer, and heterotroph media. After inoculation, the tubes were incubated at 80°C and 55°C for up to 7 days. Growth in the tubes was confirmed using phase-contrast light microscopy. Growth of methanogens and H_2-producing heterotrophs was verified by analyzing all of the tubes for CH_4 and H_2, respectively, in the headspace using gas chromatography. Total and H_2-producing heterotroph cell concentration estimates were scored and reported separately based on tubes that had cells versus those with H_2. In order to estimate the concentration of non-methanogenic autotrophs in the autotrophic sulfur medium, the estimated number of methanogens in the autotrophic sulfur medium MPN tubes was subtracted from the estimated concentration of total cells.