Primary productivity below the seafloor at deep-sea hot springs

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Below the seafloor at deep-sea hot springs, mixing of geothermal fluids with seawater supports a potentially vast microbial ecosystem. Although the identity of subseafloor microorganisms is largely known, their effect on deep-ocean biogeochemical cycles cannot be predicted without quantitative measurements of their metabolic rates and growth efficiency. Here, we report on incubations of subseafloor fluids under in situ conditions that quantitatively constrain subseafloor primary productivity, biomass standing stock, and turnover time. Single-cell-based activity measurements and 16S rRNA-gene analysis showed that Campylobacteria dominated carbon fixation and that oxygen concentration and temperature drove niche partitioning of closely related phylotypes. Our data reveal a very active subseafloor biosphere that fixes carbon at a rate of up to 321 μg C L−1 d−1, turns over rapidly within tens of hours, rivals the productivity of chemosynthetic symbioses above the seafloor, and significantly influences deep-ocean biogeochemical cycling.

I n 1977, the discovery of deep-sea hot springs revealed unusual ecosystems vastly more productive than other regions in the energy-limited deep sea (1). This productivity is sustained by chemosynthetic microorganisms that harness chemical energy made available when oxidizing seawater and reducing hydrothermal fluid mix. It has long been recognized that the habitat for such organisms may extend far below the seafloor to vast regions of the ocean crust where fluid mixing takes place (1). Fluids exiting this subseafloor biosphere are enriched in microbial biomass relative to surrounding seawater (2) and contain active microorganisms (2–4) that are physiologically and metabolically diverse (5–7). Despite the early realization that subseafloor ecosystems likely contribute significantly to overall chemosynthetic primary productivity (1) and provide nutrition to the surrounding food-limited deep sea, their extent, productivity, and biological dynamics remain poorly constrained (1, 2, 8). It has been generally assumed that above-seafloor production (i.e., microbe-animal symbiotic associations) exceeds production below the seafloor (9). However, this assumption has not been rigorously tested by empirically quantifying subseafloor productivity.

Quantifying subseafloor productivity at submarine hot springs requires knowledge of both the amount of chemical energy that can be supplied by hydrothermal fluids and the efficiency by which microbial communities convert this chemical energy into biomass. Although the chemical composition of hydrothermal fluids is well-described and provides strong indirect evidence for high biological activity in the subseafloor (10, 11), the actual amount of carbon fixed in situ is highly uncertain. Although in situ growth yields can be estimated from pure cultures (12), results from laboratory cultures may not be relevant to complex and largely uncultivated natural communities growing under different physical and chemical conditions. Bulk carbon fixation rates reported for mixed subseafloor microbial communities could likewise be used to constrain subseafloor primary productivity, but only one such measurement has been previously obtained under realistic temperature and pressure conditions (4). Another important consideration is that electron acceptors such as oxygen and nitrate rapidly become limiting during incubation experiments with vent fluids (13, 14), which may lead to carbon fixation rates being greatly underestimated (13). However, it is difficult to ascertain the extent of this bias for existing studies because electron acceptor consumption has not typically been measured alongside carbon fixation. Theoretical estimates of primary productivity have also been derived by combining geochemical measurements with thermodynamic models (15, 16). However, these studies rely on a number of untested assumptions necessary to convert the available energy into biomass.

To overcome these limitations, we used a well-studied low-temperature hot spring known as Crab Spa at the 9°N hydrothermal vent field on the East Pacific Rise (EPR) as a model...
system to constrain subseaﬂoor chemosynthetic production. Data from incubations conducted under simulated in situ conditions were combined with in situ chemistry and ﬂuid ﬂow rate (14, 17) to arrive at empirical estimates of subseaﬂoor primary productivity in a deep-sea hydrothermal system.

Results and Discussion

Fluids emanating from the subseaﬂoor at Crab Spa are characterized by a temperature of ∼24 °C, microbial abundances substantially elevated relative to bottom seawater, and a chemical composition for most aqueous species that reﬂects formation by conservative subseaﬂoor mixing of the high-temperature end-member ﬂuid with seawater (14). Dissolved concentrations of redox reactive sulﬁde, hydrogen, nitrate, and oxygen, however, are substantially depleted relative to values expected for conservative mixing (Table 1), indicating microbial consumption below the seafloor (10, 11, 14). Sixteen independent incubation experiments with Crab Spa ﬂuids were conducted at in situ pressure and temperature (24 °C, ∼246 bar) for ∼24 h, using isoar bic gas-tight (IGT) ﬂuid samplers (18); an additional two samples were incubated at in situ pressure and an elevated temperature of 50 °C. For all experiments, natural ﬂuids were amended with NaH13CO3 as a tracer to measure/assess autotrophic carbon ﬁxation. Three experiments were left unmodiﬁed as controls, whereas the remaining 15 received additional additions of nitrate, oxygen, hydrogen, or combinations of these substrates (14). This approach allowed us to quantify the rates and the stoichiometry of redox reactions supporting microbial metabolism and the resulting inorganic carbon ﬁxed into biomass (14). From these measured parameters, we directly calculated the efﬁciency of energy conversion into biomass in situ. We also explored how variations in environmental parameters inﬂuenced primary productivity and microbial community composition.

Microbial Community Composition and Rate Measurements. In all incubations, Campylobacteria (7) were the dominant microorganisms, as shown by catalyzed-reporter deposition ﬂuorescence in situ hybridization (CARD-FISH) cell counts [Fig. 1A and SI Appendix, Table S1; 94 ± 11% of total cells (14)] and the proportion of 16S rRNA gene sequences (97 ± 3.7%; SI Appendix, Fig. S1). Identiﬁed sequences were related to known chemolithoautotrophs, and a pronounced switch from known mesophilic to thermophilic Campylobacteria occurred in the incubation at 50 °C (Fig. 1A and SI Appendix, Fig. S1 and Table S1). Active carbon ﬁxation was conﬁrmed by speciﬁcally measuring H13CO3− tracer incorporation in campylobacterial cells with halogen in situ hybridization-nanoscale secondary ion mass spectrometry [HISH-SIMS (19); Fig. 2]. HISH-SIMS also demonstrated that amendments increased relative CO2 ﬁxation rates, especially for oxygen or a combination of nitrate and hydrogen (Fig. 3). Given that Campylobacteria dominate the natural community in sampled ﬂuids (∼80% of total cells; Fig. 1A and ref. 14) and make up the vast majority of microbes found in the incubations (Fig. L4 and SI Appendix, Fig. S1 and ref. 14), we conclude this group dominates primary productivity in the chronically electron acceptor-limited subseaﬂoor environment at Crab Spa (14), and likely also at other deep-sea vent sites similarly dominated by Campylobacteria (5, 6).

Because CARD-FISH underestimates carbon ﬁxation as a result of tracer loss during sample preparation (20), we also quantiﬁed 13CO2 incorporation into bulk microbial biomass by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) (19). Bulk carbon incorporation was, on average, 45% higher than HISH-SIMS, consistent with previous estimates of tracer loss (20). Carbon ﬁxation rates were consistently high (∼17–320 μg C-L−1-d−1; Table 2 and SI Appendix, Fig. S2), far exceeding values reported from previous microbial incubations of hydrothermal ﬂuids by factors of ∼2–650 (3, 4). This likely reﬂects the fact that our sampling and incubation approach minimized electron acceptor limitation (13) and changes to the physicochemical environment experienced by microbes (14). The carbon ﬁxation rates

Table 1. Predicted versus observed concentrations of potential electron donors and acceptors at Crab Spa

|                  | Predicted* | Observed |
|------------------|------------|----------|
| H2S, μmol/L      | 552        | 184      |
| H2, μmol/L       | 29         | <2       |
| CH4, μmol/L      | 8.1        | 6.3      |
| NH4+, μmol/kg    | 0.2        | 11.9     |
| O2, μmol/L       | 107        | 3.6      |
| NO3−, μmol/L     | 32         | 6.3      |
| SO42−, mmol/kg   | 25.8       | 26.5     |

*Values are based on fluid mixing calculations previously described in ref. 14.

Fig. 1. Bacterial community composition at the end of incubations. (A) Taxonomic composition inferred from CARD-FISH counts, and (B) Nonmetric multidimensional scaling (NMDS) plot showing the similarity of Sulﬁruminas 97% OTU composition between experimental treatments. Each dot represents a diﬀerent biological replicate for incubations carried out at 24 °C and is colored according to the initial P O2. All CARD-FISH data are averaged by treatment, and errors are presented as SDs (n = 3) or ranges (n = 2) except for the Nautilales probe in the 110 μM O2 treatment (n = 1). Validation of newly designed probes (Nautilales = NAUT921 and Sulﬁruminas = SFMN287; SI Appendix, Table S2) are described in the Materials and Methods, and speciﬁcity tests are shown in SI Appendix, Figs. S5 and S6. Campylobacteria in A corresponds to the combined probes EPS549 and EPS914.
Sulfurimonas area of Crab Spa colonized by giant tubeworms (Riftia pachyptila), which are the dominant megafaunal species on the East Pacific Rise and the most productive symbiosis described to date (ref. 25 and Table 2 and SI Appendix, SI Text).

Our results can also be used to provide an independent minimum bound on seafloor biomass standing stock by assuming that cell-specific rates of nitrate and oxygen respiration we reported previously are maximum values for in situ microbial communities (on average, 463 and 70 fmol/cell/d for O$_2$ and NO$_3^-$, respectively (14)). Combining chemical depletions of oxygen/nitrate at Crab Spa (14) with fluid flux (17), we determined total chemical consumption resulting from seafloor microbial metabolism and the minimum number of cells ($1 \times 10^{14}$ cells or $\sim 29$ g C) necessary to account for these depletions (Table 2 and SI Appendix, SI Text). These values provide empirical constraints on biomass standing stock of the deep-sea vent subseafloor biosphere.

**Chemosynthetic Growth Efficiency.** Because substrate amendments influenced chemosynthetic activity during incubations (Figs. 2 and 3 and SI Appendix, Fig. S2), we normalized activity to changes in fluid composition (Dataset S1) to determine the amount of carbon fixed per electron acceptor reduced. This parameter, which we term community chemosynthetic growth efficiency (CGE), is the fraction of electron equivalents derived from electron donors used to reduce CO$_2$ into biomass:

$$CGE = \frac{E_{Q_{\text{CFIX}}}}{E_{Q_{\text{CFIX}}} + E_{Q_{\text{DISS}}}} [1]$$

where $E_{Q_{\text{CFIX}}}$ = electron equivalents to carbon fixation and $E_{Q_{\text{DISS}}}$ = electron equivalents to dissimilatory electron acceptors.

CGE values are a direct empirical constraint on how efficiently hydrothermal vent microbes convert available chemical potential energy into biomass, and also demonstrate which conditions have the largest effect on their ecophysiology. As shown by 16S rRNA gene amplicon data (SI Appendix, Fig. S1) and CARD-FISH counts reported here are between two and five orders of magnitude higher than carbon production by aerobic ammonia and nitrite oxidation, the two main chemosynthetic processes in the dark pelagic ocean (21, 22), and are in the range of rates measured for carbon production in the photic zone of the coastal and open ocean (23).

**Constraints on the Productivity, Turnover, and Standing Stock of the Deep-Sea Vent Subseafloor Biosphere.** With knowledge of chemical depletions and fluid flux, CGE provides a foundation to quantitatively constrain the extent, productivity, and biological dynamics of deep-sea vent subseafloor ecosystems. Using treatment-averaged CGE as upper and lower bounds (0.06–0.13), we estimate that 104–253 $\mu$g C is fixed per liter of mixed fluid at our study site. This is equivalent to 1.4–3.5 mg C fixed per liter of end-member hydrothermal fluid (Table 2), which comprises $\sim$7% of Crab Spa mixed fluids (14). Considering fluid flux (17), annual production at Crab Spa amounts to 6.1–15 kg C (Table 2). This is comparable to the amount of carbon likely fixed in the $\sim$1 m$^2$ area of Crab Spa colonized by giant tubeworms (Riftia pachyptila), which are the dominant megafaunal species on the East Pacific Rise and the most productive symbiosis described to date (ref. 25 and Table 2 and SI Appendix, SI Text). These values provide empirical constraints on biomass standing stock of the deep-sea vent subseafloor biosphere.
stock of the subseafloor biosphere at deep-sea vents and provide insight into its biological dynamics. For example, if we assume that the amount of subseafloor biomass is at steady state, biomass residence time will be short (17–41 h), within the range of doubling times for cultured chemosynthetic Campylobacteria (refs. 8 and 16 and SI Appendix, SI Text). As both the ambient deep-sea water entering the ocean crust and the endmember hydrothermal vent fluids do not contain any significant numbers of Campylobacteria, yet they constitute the dominant biomass in the fluids exiting the seafloor at Crab Spa (14), their growth must have occurred below the seafloor.

**Vent Field and Global Estimates of Productivity and Standing Stock.**

On a larger scale, export of biomass into the food-limited deep sea can be assessed by multiplying low-temperature fluid flux of the 9°N EPR vent field (26) with our volumetric primary productivity values (Table 2). Estimated subseafloor chemosynthetic productivity in the vent field ranges from ~380 to 9,300 g C m⁻² y⁻¹, values that are at least two to four orders of magnitude greater than the amount of photosynthetic biomass reaching this depth [0.4–4 g C m⁻² y⁻¹ (27)]. For the entire 9°N EPR vent field area of 10⁻³–10⁺⁴ m² (26), this corresponds to 3.8–9.3 Mg C y⁻¹. Although we do not know what proportion of newly produced subseafloor biomass reaches the surrounding deep-sea water column, even a small amount would vastly increase the availability of labile carbon for heterotrophic consumers locally, making the deep ocean in the vicinity of vent fields hot spots of microbial activity (28).

Although this study was confined to one hydrothermal vent site, we believe our results are applicable to other subseafloor hydrothermal systems. Although some aspects of fluid chemistry differ between vent fields, there are also some striking similarities. Similar to Crab Spas, most other subseafloor vent fluids are typically enriched in dissolved inorganic carbon and electron donors and contain limited abundances of electron acceptors (9, 11, 15). Under such conditions, it is known that Campylobacteria dominate the in situ microbial community (6, 8, 24, 29). In contrast, the other main group of sulfur-oxidizing chemosynotrophs found at vents, the Gammaproteobacteria, are typically found at interfaces where warm vent fluids and ambient seawater mix turbulently and oxygen concentrations are higher (6, 8, 24, 29). Other potential autotrophic metabolisms could occur in the subseafloor at higher temperatures (such as hydrogenotrophic methanogenesis), but they are likely to be of minor importance quantitatively in basalt-hosted systems compared with aerobic/denitrifying oxidation of sulfide/hydrogen (15, 16). For the example of methanogenesis, fluid composition data for Crab Spas suggest that methane is being consumed in the subseafloor, rather than being produced (Table 1). Collectively, these observations suggest that most moderate-temperature (~15–60 °C), sulfidic, and oxygen/nitrate-limited subseafloor ecosystems will be dominated by Campylobacteria. Because autotrophic Campylobacteria share fundamental physiological attributes [and therefore core mechanisms of energy conservation (6, 7)], we believe the CGE values derived here for mesophilic and thermophilic communities can be reasonably extrapolated to other systems.

To extend our quantitative estimates of productivity and standing stock to a global perspective, we used low-temperature (5 °C) and high-temperature (350 °C) fluid flux values to calculate the lower and upper bound on global subseafloor productivity, respectively (Table 2) (30). These estimates suggest that subseafloor chemosynthetic productivity at deep-sea hot springs amounts to at most 1.4 Tg C y⁻¹ (Table 2), which is somewhat lower than previous theoretical estimates (refs. 15 and 16 and SI Appendix, SI Text) and representing at most 0.43% of photosynthetic primary productivity reaching depths >2,000 m (27). We also calculate a value for global subseafloor standing stock of 1.4–2.7 Gg C, more than three orders of magnitude lower than previous theoretical estimates (Table 2), which assumed that microbes in the subseafloor are in maintenance mode (16) rather than actively growing as shown here (SI Appendix, SI Text).

**Conclusions**

Although the paradigm of chemosynthetic microbes transferring geothermal energy to higher trophic levels at deep-sea hot springs has become well-established, the significance of the subseafloor
ecosystem to global ocean biogeochemistry is difficult to estimate without reliable quantitative data. Using direct measurements of chemosynthetic growth efficiency and metabolic rates under in situ conditions, our data show that the standing stock of the chemosynthetic subseafloor biosphere is relatively small and turns over rapidly. On the basis of our estimates, subseafloor carbon fixation rivals highly productive animal–microbe symbioses above the seafloor and could therefore constitute a significant source of labile carbon to the otherwise food-limited deep sea. We also identified temperature and oxygen as critical factors driving the niche partitioning of natural communities composed of closely related and physiologically similar taxa, showing how deep-sea hot spring microbes interact with and are shaped by their unique environment.

Moving forward, similar measurements at other vent sites, including additional chemosynthetic processes, and better constraints on the overall fluid flux will be needed to refine our estimates. Although we can now better constrain subseafloor chemosynthetic productivity, how this newly produced organic carbon affects the deep ocean food web and biogeochemistry remains to be determined. Broad application of high-pressure incubations such as those reported here represents a powerful approach to gain quantitative insight into microbiologically mediated processes in the underexplored deep ocean.

**Materials and Methods**

**Experimental Design.** Fluid samples for all analyses were collected from the Crab Spas vent with the ROV Jason II deployed from the R/V Atlantis during research cruise AT26-10 in January 2014. Crab Spa is located at a depth of 2,506 m at 9°50.3981N, 104°17.4942W. Shipboard incubations of fluids were carried out at in situ pressure in IGT samplers for ~18–24 h with amendments of H2, NO3, O2, and H2N-O2 in addition to NaH13CO3 as an isotope tracer for carbon fixation. With the exception of 2 NO3-H2 incubations carried out at 50 °C, all incubations were conducted at 24 °C, which is the in situ fluid temperature at Crab Spas. During incubations, cell abundance and concentrations of selected chemical species (H2S, H2O, NO3, NH4, and O2) were measured every ~6 h. Full details of sampling, incubation procedures, chemical measurements, cell counts, and rate measurements are described in ref. 14.

**DNA Analyses.** At the end point of each experiment (~24 h), the remaining volume of fluid in the IGT (~40 mL) was drawn into a clean, sterile, and DNA-free syringe (Norm-Ject), filtered through a 0.2 μM Sterivex filter cartridge, dried under filtered nitrogen gas, and frozen immediately at ~80 °C. DNA was extracted as previously described (31). Amplicons were subsequently sequenced using bacterial primers 27Fmod and 519Rmodbio and 454-pyrosequencing technology (Molecular Research LP).

Pyrotag sequences were analyzed using the QIME pipeline (32). Sequences were subjected to QC filters (libraries.pyw 50–0.300-L 1,000–a 0-H 6–B 8–z truncate, only), then denoised from 454 workflows (denoise_wrapper.py). After denoising, chimeras were removed using the script “identify_chimeric.seqs.py,” with USEARCH as the method. This yielded 3,597 ± 1,371 (SD) sequences per sample. Next, 97% OTUs were picked de novo, using the script “pick_otus.py,” with USEARCH as the method and classified with the script “assign_taxonomy.py,” using the SILVA v119 database as a reference. Raw sequences in .stf format are deposited at NCBI under accession number SRP079342.

**CARD-FISH and HISH-SIMS 13C Incorporation.** Aliquots of ~10 mL of fluid were taken from the IGTs at 16 or 24 h after the addition of labels/amendments and preserved with paraformaldehyde (1%, 1 h at room temperature). Fluids were then filtered under moderate vacuum on Au/Pd-sputtered 0.2 μM polycarbonate filters, washed 2× with 10 mL 1x PBS, air-dried, and stored at ~20 °C before further analysis.

Filters were embedded in low-melting-point agarose, endogenous peroxidases were inactivated by immersion in 3% H2O2 for 10 min, and cells were permeabilized for 30 min at 37 °C in a 10 mg mL−1 solution of lysozyme in TE buffer. Hybridization and tyramide amplification were conducted at 46 °C for 3 h and 20 min, respectively. Oregon Green 488-X was used for tyramide amplification, which contains two atoms of fluorine per molecule. All newly designed probes and their formamide concentrations are shown in SI Appendix, Table S1. Newly designed probes were tested with positive and negative control cultures across a melting curve to determine both the potential for nonspecific hybridization and the optimum concentration of formamide (SI Appendix, Table S2). Probes were additionally tested to ensure specificity by doing a double hybridization with both the EPSIS49-914 combination and the newly designed probes on natural environmental samples from other oceanic locations (34).

Because cells hybridized with NAUT921 and SFMN287 were also hybridized with the EPSIS49-914 probe, this was additional confirmation that these probes are specific to Campylobacteria (SI Appendix, Figs. S5 and S6).

Once hybridized, 5-mm-diameter circular sections were cut out from each filter, and regions of interest were marked with a laser-dissecting fluorescence microscope (Zeiss) with a 63× (NA, 0.75) objective. The remaining portions of filters were used to count the percentage of DAPI-stained cells hybridized to each specific probe. Seven grids were analyzed per sample, amounting to 400–700 DAPI-stained cells.

Regions of interest or random grids hybridized with the EPSIS49-914 probes were analyzed on NanoSIMS 50L, ionprobe from CAMECA (AMETEK), detecting the following secondary ions: 13C, 12C, 13C3N, 12C13C, 13C2F, Au, and 13F. An average of 49.6 target cells were analyzed per IGT incubation for the EPSI probes, with a range of 22–96. A subset of three samples was also analyzed with the NAUT921 probe (between 14 and 21 cells per sample).

**13C Isotope Incorporation into Bulk Biomass.** At the last point during the incubation experiment, a known volume of fluid (~20 mL) was filtered onto a precombusted GF-75 glass fiber filter (0.3 μM pore size; Advantec), wrapped in combusted aluminum foil, and stored at ~80 °C before further analysis.

Filters were subsequently acidified to remove carbonates by exposure to HCl vapor for 3 d at 60–65 °C and then dried for 1 d at the same temperature. Immediately before combustion, dried filters were wrapped with tin foil (Costech part # 041073) and folded into pellets. Samples were combusted in a Carlo Erba/Fisons 1107 Elemental Analyzer “EA” (fitted with a Costech Zero-Blank” carousel). The EA is attached via Finnigan-MAT ConflO-II interface to a DeltaPlus stable isotope mass spectrometer. Data were acquired using the isocod (version 2.5) software.

**Carbon Fixation Rate Determinations.** For all incubations, 13C-labeled dissolved inorganic carbon (DIC) was supplied as a H13CO3− solution dissolved in filtered bottom seawater and added into low-temperature hot spring fluid (14). The fraction of total DIC as 13C label was determined using a HISTO-CARD-FISH for background and was used for normalization in all cases (14). A conversion factor derived from these label percentages was used in both rate determinations below to derive total CO2 fixation rates.

For rate determinations from bulk isotope incorporation measurements, background 13C from an average of background (uncultivated) samples was subtracted from detected 13C and normalized as described earlier to determine total CO2 fixed. Rates were determined by dividing total carbon fixed by the time from label addition to when samples were taken.

For HISH-SIMS-derived rates, data were processed with Look@NanoSIMS (33) to demarcate regions of interest for EPSIS49-914-hybridized cells based on the 19S signal. Cell biovolume was estimated using the area and length: width ratio parameters for each region of interest, which was then combined with cell carbon density previously reported (34) to estimate carbon content for each cell. The amount of CO2 fixed per cell was then determined corresponding 13C14N212N212O reverse electron transport (12) by multiplying CO2 concentrations in fluids. This value was then multiplied by EPSI-hybridized cells mL−1 and normalized by time to yield total CO2 fixed per volume per time.

**CGE Determinations.** CGE represents the proportion of electrons transferred from energy-yielding oxidation half-reactions (e.g., hydrogen or sulfur oxidation) that are used to reduce CO2 into cell carbon (assuming biomass oxidation state of 0). The inverse proportion, equivalent to the “y” parameter estimated by Klatt and Polerecky (12), is the fraction of electrons transferred to energy-yielding dissimilatory metabolism (e.g., oxygen and nitrogen reduction).

Total carbon fixed from bulk isotope measurements was determined as described earlier. The consumption of nitrogen and oxygen were also measured, likely the only electron acceptors of importance during incubations (14). The means by which electron equivalents used to reduce these substrates was calculated has been previously described (14). Total electrons oxidized from sulfide and hydrogen were not directly measurable as a result of incomplete oxidation of sulfide (14), so this value was inferred by taking the sum of electron equivalents to carbon fixation and electron equivalents to electron acceptors. CGE was then derived by dividing total carbon fixed by this sum (Dataset S1).

**Statistical Analysis.** Correlations of community composition with environmental parameters was carried out with a subset of total sequences and according to statistical analyses that are implemented in scripts of the QIME.
pipeline. *Sulfurimonas* 97% OTUs found in 24 °C incubations were first normalized within each sample as the percentages of total *Sulfurimonas* sequences. Next, beta diversity was calculated using beta_diversity.py with UniFrac as the distance metric. A tree of sequences necessary for the UniFrac metric was generated by aligning sequences using MUSCLE (align_seq.py) and building a tree using default parameters (make_phylogeny.py). The divergence between these different communities of *Sulfurimonas* was visualized by using the script nmds.py to generate values for a 2-D Nonmetric Multidimensional Scaling plot. Next, the script compare_categories.py was used with the adonis method to investigate the effect of the following variables (at the beginning of incubations) on final *Sulfurimonas* OTU composition: pH, [H$_2$], [H$_2$S], [NH$_3$], [NO$_3$]$_2$, PO$_4$ (PO4) cell density and time from seafloor until the beginning of incubations. Finally, the script observation_metadata.correlation.py with Pearson correlations was used to look for the effect of PO$_4$ on individual *Sulfurimonas* OTUs.

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