Assessing Microbial Processes in Deep-Sea Hydrothermal Systems via Incubations at *In Situ*
Temperature and Pressure

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Abstract:

At deep-sea hydrothermal vents, a large source of potential chemical energy is created when
reducing vent fluid and oxidizing seawater mix. In this environment, chemolithoautotrophic microbes
catalyze exergonic redox reactions which in turn provide the energy needed to fuel their growth and the
fixation of CO₂ into biomass. In addition to producing new organic matter, this process also consumes
compounds contained both in vent fluid and entrained seawater (e.g. H₂, NO₃⁻). Despite their
biogeochemical importance, such reactions have remained difficult to quantify due to methodological
limitations. To address this knowledge gap, this study reports a novel application of isobaric gas-tight
fluid samplers for conducting incubations of hydrothermal vent fluids at *in situ* temperature and
pressure. Eighteen ~24 h incubations were carried out, representing seven distinct conditions that

24 examine amendments consisting of different electron donors and acceptors. Microbial activity was
25 observed in all treatments, and time series chemical measurements showed that activity was limited by
26 electron acceptor supply, confirming predictions based on geochemical data. Also consistent with these
27 predictions, the presence of nitrate increased rates of hydrogen consumption and yielded ammonium as
28 a product of nitrate respiration. The stoichiometry of predicted redox reactions was also determined,
29 revealing that the sulfur and nitrogen cycles are incompletely understood at deep-sea vents, and likely
30 involve unknown intermediate redox species and possibly novel biochemical pathways. Finally, the
31 measured rates of redox processes were either equal to or far greater than what has been reported in
32 previous studies where *in situ* conditions could not be maintained. In addition to providing insights into
33 deep-sea hydrothermal vent biogeochemistry, the methods described herein also offer a practical
34 approach for the incubation of any deep-sea pelagic sample under realistic *in situ* conditions.

35

36 **Keywords:** chemolithoautotrophy; *Epsilonproteobacteria*; hydrothermal vent; microbial ecology; rate
37 measurements; high-pressure incubations

38

39 **1. Introduction:**

40 At deep-sea hydrothermal vents, entire ecosystems are supported by primary production in the
41 absence of sunlight. This process, known as chemosynthesis or chemolithoautotrophy, can occur due to
42 chemical disequilibria between reducing hydrothermal vent fluids and oxidizing seawater.
43 Chemosynthetic microbes catalyze thermodynamically-favorable redox reactions and couple this
44 chemical energy to CO₂ fixation, thereby transforming an inorganic energy source into biomass
45 (Jannasch and Mottl, 1985). In addition to supporting productive ecosystems, this process has
46 significant biogeochemical implications. For example, chemosynthetic microbes not only consume

47 reduced inorganic compounds, but they also remove nitrate and oxygen from seawater that mixes with
48 vent fluid.

49 In the past two decades, the analysis of nucleic acids obtained directly from natural microbial
50 communities as well as the characterization of newly isolated strains of chemolithoautotrophic
51 microbes has revealed insights into the taxonomy, abundance and metabolic potentials of deep-sea vent
52 chemolithoautotrophs (e.g. Huber et al., 2007; Nakagawa and Takai, 2008; Sievert and Vetriani, 2012
53 and references therein). Studies conducted thus far have shown that fluid composition can exert
54 important controls on microbial community structure and function (Amend et al., 2011; Flores et al.,
55 2011; Flores et al., 2012; Dahle et al., 2015; Hentscher and Bach, 2012), but the reverse question, i.e.
56 how microbes themselves affect fluid composition has received less attention. Although microbial
57 metabolism can be inferred indirectly in low temperature hydrothermal fluids by measuring deviations
58 from the conservative mixing line between high temperature endmember fluids and seawater
59 (Butterfield et al., 2004; Von Damm and Lilley, 2004; Proskurowski et al., 2008; Wankel et al., 2011),
60 this approach cannot provide unequivocal proof that these signatures are microbial nor determine the
61 rates of processes. Therefore, the lack of direct measurements of microbial metabolism hinders our
62 understanding of the biogeochemical role of chemosynthetic processes within vent ecosystems,
63 including their primary productivity (Sievert and Vetriani, 2012).

64 In order to identify active metabolic pathways and their rates at deep-sea vents, experiments
65 that simulate the natural environment as closely as possible are needed (Sievert and Vetriani, 2012). A
66 conceptually straightforward approach to accomplish this goal is to directly incubate fluids collected
67 from the environment with their resident microbes. This approach is complicated, however, by both
68 inherent challenges of working with deep-sea vent fluids as well as difficulties designing experiments
69 to realistically infer *in situ* processes. Indeed, previous studies have shown that deep-sea

70 microorganisms are sensitive to a decrease in pressure (Bartlett, 2002), suggesting that decompression
71 can affect biological rate measurements. For example, Bianchi et al (1999) showed that bacterial
72 production rates are underestimated by approximately one half in decompressed pelagic water samples
73 from depths greater than 850 m.

74 In addition to pressure, the chemical environment is also fundamentally important since the
75 concentration of chemical species directly influences which microbial assimilatory/dissimilatory redox
76 reactions can occur and the rates at which these reactions proceed. The chemical environment in fluid
77 samples collected at deep sea vents may change dramatically if pressure is not maintained during
78 transport to the sea surface due to the loss of volatile species (i.e. H_2 , H_2S , CH_4 , CO_2). This results in
79 the removal of potential energy sources and alteration of key parameters such as pH, which in turn may
80 affect the composition and activity of the indigenous microbial communities. Artifacts may also be
81 introduced during fluid collection from the natural environment if ambient seawater is entrained. The
82 presence of exogenous seawater not only alters the chemical environment, but also introduces a
83 compositionally distinct microbial community not representative of the vent system.

84 In addition to technical challenges in obtaining representative samples of vent fluid, deriving
85 accurate information on *in situ* processes from incubations of this fluid is complicated by the fact that
86 chemolithoautotrophic microorganisms will have already affected the geochemistry of fluids prior to
87 sampling, depleting some chemicals while producing others (Butterfield et al., 2004; Von Damm and
88 Lilley, 2004; Proskurowski et al., 2008; Wankel et al., 2011). The extent of this effect is poorly known,
89 but must be a function of fluid flow rate, absolute concentrations of chemical species, and the
90 abundance of microorganisms and their metabolic rates. Because diffuse-flow hydrothermal
91 environments are characterized by a continuous replenishment of substrate-bearing fluids, microbial
92 communities can be sustained *in situ* even if relevant metabolic redox couples are present at very low

93 concentrations in sampled fluids. Therefore, in closed-system (batch) growth experiments, low
94 concentrations of reactants in energy-yielding redox reactions is problematic since it will be difficult (if
95 not impossible) to measure some processes, despite the fact that they likely occur in the natural
96 environment.

97 To circumvent the aforementioned issues, we used an existing hydrothermal fluid sampler to
98 conduct microbial incubations under simulated sea-floor conditions. Isobaric gas-tight (IGT) fluid
99 samplers (Seewald et al., 2002) were designed to maintain fluids at seafloor pressure following
100 collection. They are ideal for sampling low-flow diffuse vents because their slow fill rate (~ 75
101 $\text{mL} \cdot \text{min}^{-1}$) minimizes the entrainment of ambient seawater and a thermocouple co-located with the
102 sampler inlet snorkel provides real-time temperature information to precisely position the fluid intake
103 in the vent environment. As part of this new application, we developed methods for fluid withdrawals
104 from and additions to the IGT sampler, which allowed both initial chemical amendments and
105 monitoring of substrate concentrations and cell numbers over time, all the while maintaining seafloor
106 pressure. Below, we report the results of shipboard experiments designed to improve the understanding
107 of microbial metabolism in deep-sea hydrothermal vent fluids by confirming predicted chemosynthetic
108 redox reactions, inferring the stoichiometry of these reactions, and quantifying their rates.

109

110 **2. Materials and Methods:**

111 **2.1 Field Site:**

112 Vent fluids used for incubation experiments were collected from *Crab Spa* ($9^{\circ}50.3981$ N,
113 $104^{\circ}17.4942$ W), a well-constrained diffuse-flow hydrothermal vent located on the East Pacific Rise at
114 a depth of 2,506 m, by the ROV Jason II deployed from the R/V Atlantis during research cruise AT26-
115 10 in January 2014. At this site, warm fluids ($\sim 24^{\circ}\text{C}$) containing microbes emanate from a well-defined

116 orifice, and have maintained a temporally stable chemical and temperature composition since 2008
117 (Reeves et al., 2014). Prior to the first sampling, the site was allowed to stabilize after megafauna (e.g.,
118 *Riftia pachyptila*, *Bathymodiolus* sp.) were cleared to directly access the fluids emanating from the
119 seafloor.

120 **2.2 Experimental Design and Methods for Incubations:**

121 Similar to other low temperature vent fluids, many chemical species at *Crab Spa* are depleted
122 relative to concentrations expected for mixing of the high temperature endmember source fluid with
123 seawater, which may reflect the occurrence of microbial metabolic processes. Therefore, we chose to
124 conduct replicated amendments of these natural vent fluids with electron acceptors and donors (Table
125 1). In particular, dissolved nitrate and oxygen were added to test if their presence would stimulate
126 sulfide oxidation, and dissolved hydrogen was added to test whether microbes were capable of
127 hydrogen oxidation, with or without added nitrate. These conditions were compared to controls (no
128 amendments) to confirm that electron donor oxidation was limited by availability of electron acceptors.
129 Since our goal was to mimic the natural environment, most incubations were carried out at 24°C in the
130 shipboard laboratory, nearly identical to the temperature at which *Crab Spa* fluids exit the seafloor.
131 Two additional incubations were carried out at 50°C to examine biogeochemical processes at higher
132 temperatures that likely characterize deeper seafloor environments.

133 Prior to deployment at the seafloor, the IGT samplers were washed with dilute HCl (pH 3) to
134 remove any residual ¹³C-labelled dissolved inorganic carbonate (DIC) from previous incubations,
135 followed by a 70% ethanol rinse to sterilize the interior, and acetone to dry the ethanol. A Teflon O-ring
136 was added to the IGT sample chamber prior to deployment to allow fluid stirring following chemical
137 additions and prior to time series sampling during the incubations (Fig 1). The snorkel and sample
138 valve dead volume (~4 ml) and sample chamber on the back side of the sample piston were filled with

139 filtered bottom seawater while the accumulator chamber on the backside of the accumulator piston was
140 filled with compressed nitrogen to ~10% of seafloor pressure at the sampling site (Fig 1). During
141 sampling, the sampler inlet valve was opened and fluid entering the sampler forced the sample and
142 accumulator pistons to the opposite ends of their respective chambers. Retraction of the accumulator
143 piston compresses nitrogen in the accumulator chamber allowing it to act as a “spring” that buffers
144 internal pressure fluctuations in the sample chamber caused by external temperature changes during
145 transport from the seafloor to the seafloor. Based on 2°C bottom temperature and 25°C laboratory
146 temperature, internal pressure increases due to sampler warming are <15 %.

147 Vent fluid sampling was performed by positioning the IGT snorkel/thermocouple into the
148 orifice of *Crab Spa* until temperature readings reached a stable maximum of at least 23-24°C, at which
149 point the sampler inlet valve was opened. The sampler was allowed to fill for 2 min, and then the
150 sampler inlet valve was closed to retain pressure during transit back to the surface. After retrieval of the
151 IGT samplers on board the ship, a limited number of fluid aliquots (~10 mL total volume) were
152 removed for analysis of selected chemical species. Dissolved methane was used as a conservative
153 tracer for the amount of endmember hydrothermal fluid in a given sample. Fluids were not used for
154 incubations if the methane concentration was <4 µM, an indication that substantial inadvertent
155 seawater entrainment had occurred during fluid collection. Most shipboard incubations were started
156 within seven hours from sampling at the seafloor, although two IGT samplers used for hydrogen-
157 amended incubations were stored at room temperature for approximately sixteen hours prior to the
158 beginning of the experiment (Table 1).

159 To compensate for pressure loss during fluid withdrawals from the sample chamber, a high
160 performance liquid chromatography (HPLC) pump (Fig 1F) was connected to the pre-charge valve (an
161 on/off high-pressure needle valve; Fig 1B). Distilled water was then pumped into the accumulator

162 chamber until the desired pressure was reached. For fluid additions to the sample chamber, the HPLC
163 pump was connected to the sampler inlet valve to pump in amendments. To relieve excess pressure
164 caused by fluid additions, the pre-charge valve (Fig 1B) was opened briefly as necessary. In both cases,
165 pressure was adjusted to the correct level within seconds by observing a high-pressure gauge connected
166 to the HPLC pump.

167 Incubations were initiated by sequentially injecting solutions of known composition. For all
168 additions, the total volume of fluid injected was ~10 mL, which represents 6-7% of the total volume of
169 the IGT sampler (150 mL). The first fluid to be injected was 2 mL of a $\text{NaH}^{13}\text{CO}_3$ (99% ^{13}C ,
170 Cambridge Isotope laboratories) solution of known concentration made up in N_2 -purged filtered bottom
171 seawater. This was immediately followed by 8 mL of filtered bottom seawater containing chemical
172 species for each of the incubations indicated in Table 1, at a concentration that would yield the desired
173 abundances after mixing with the vent fluid already in the IGT sample chamber. For all treatments, the
174 final concentration of ^{13}C after injection was ~10% of the total DIC (supplemental spreadsheet).
175 Immediately after injection, the IGT samplers were rotated 180° several times to allow the Teflon O-
176 ring to fall through the sample chamber, thereby mixing the fluid contents. Following addition of
177 substrates, fluid chemistry and cell abundance was monitored over an 18 - 24 h period by extracting
178 fluid aliquots from the IGT sample chamber after selected time intervals.

179 Stock solutions for injection of nitrate, oxygen, and hydrogen were prepared just prior to the
180 additions to the IGT sampler. The ^{13}C -bicarbonate solution was used within three days of preparation
181 and refrigerated when not in use. Solutions were prepared anoxically in Hungate tubes or Pyrex bottles,
182 and all glassware was muffled at 480°C for 4 h prior to use to remove organic contaminants.

183 Dissolved oxygen was added as a solution of filtered bottom seawater saturated with ultra-high
184 purity O_2 at atmospheric partial pressure (0.103 MPa) for the lower oxygen concentration incubations

185 and in a solution saturated at 0.206 MPa O₂ partial pressure for the higher concentration incubations.
186 Nitrate solutions were prepared from reagent grade NaNO₃ dissolved in N₂-purged filtered bottom
187 seawater.

188 Due to the low solubility of hydrogen gas in aqueous solution, a modified approach involving
189 the HPLC pump was used to amend the incubations with dissolved hydrogen (Fig. 1c). A length of 1/8"
190 stainless steel tubing with an internal volume of 2 mL was pre-filled with hydrogen gas at atmospheric
191 pressure and connected to the IGT sample inlet valve at one end and the HPLC pump outlet at the
192 other. One mL of the filtered bottom seawater/¹³C label solution was then pumped into the length of
193 hydrogen-filled stainless steel tubing before opening the IGT sample valve, which allowed vent fluid
194 into the sample loop, raising the a pressure to 25 MPa. This solution was allowed to equilibrate for 30
195 min so that the compressed hydrogen gas could dissolve into the filtered bottom seawater/vent fluid
196 mixture. Once dissolved, the contents of the 1/8" stainless tube were injected into the sample chamber
197 using the HPLC pump.

198 Incubations were conducted in the IGT samplers either at *in situ* vent temperature (~24°C) in
199 the ship's laboratory or at 50°C by placing them in an oven. Safety considerations necessitated the
200 addition of a 40 MPa pressure-relief connected to the precharge valve to relieve pressure in the event
201 of runaway temperature within the oven (Figure 1G). Temperature inside the oven was continuously
202 logged at 5 min intervals for all treatments with iButton temperature recorders (Maxim Integrated, San
203 Jose, CA).

204 In addition to the unamended controls described above, killed controls were conducted to
205 examine abiotic processes. Abiotic sulfide oxidation with oxygen was tested at 24°C and 50°C in
206 duplicate experiments by injecting formaldehyde into the IGT sampler to attain a final concentration of
207 2%, followed by the addition of dissolved oxygen (>80 µM) and monitoring the sulfide concentration

208 over time. To test if H₂ diffusion or leakage from the IGT sampler was occurring, H₂ was added to a
209 sampler filled with oxic deionized water and the concentration monitored over time at 24°C. Both
210 controls were conducted on the same timescale as the actual experiments (~24 h).

211

212 **2.3 Analytical Methods:**

213 Fluid Chemistry: Total dissolved sulfide ($\Sigma\text{H}_2\text{S} = \text{H}_2\text{S} + \text{HS}^- + \text{S}^{2-}$) was determined
214 potentiometrically using a sulfide-selective electrode. The electrode was calibrated daily with a serial
215 dilution of a standard sodium sulfide solution. pH (25°C) was measured in fluids withdrawn from the
216 IGT sampler with a Ag/AgCl combination reference electrode calibrated daily. Dissolved methane and
217 hydrogen concentrations were determined shipboard using a gas chromatograph equipped with a 5 Å°
218 molecular sieve packed column and serially connected thermal conductivity and flame ionization
219 detectors following quantitative headspace extraction. DIC concentrations ($\text{DIC} = \text{CO}_3^{2-} + \text{HCO}_3^- +$
220 H_2CO_3^*) were determined after fluid acidification with 25 wt. % phosphoric acid by injecting aliquots
221 of headspace gas directly into a gas chromatograph equipped with a Porapak-Q packed column and a
222 thermal conductivity detector. Dissolved Mg, Cl and SO_4^{2-} concentrations were analysed by ion
223 chromatography with suppressed conductivity detection. Dissolved oxygen concentrations in IGT
224 samplers were determined by passing hydrothermal fluid through a custom designed flow-through cell
225 fitted with a commercially available oxygen optode and associated temperature probe (Pts3, Presens,
226 Germany; see Figure 1D). The oxygen optode spot was calibrated with oxygen-free water (treated with
227 sodium dithionite) and air-saturated water. Prior to measurements, the optode flow-through cell was
228 flushed with N₂-purged filtered bottom seawater or deionized water to remove air bubbles. Oxygen
229 levels were determined in situ by placing an oxygen optode system directly into hydrothermal fluid
230 flow (Model 4330F; Aanderaa Data Instruments, Fall River, MA), which was calibrated by the

231 manufacturer prior to deployment.

232 For determination of dissolved nitrate and ammonium concentrations, fluids were filtered
233 shipboard through a 0.2 μm GTTP membrane (Millipore) and stored frozen at -20°C . The concentration
234 of total nitrate+nitrite was determined on shore by conversion to NO and chemiluminescent detection
235 using the NoxBox instrument (Teledyne, San Diego CA, USA) following the original protocol
236 (Garside, 1982). The concentration of total dissolved ammonia ($\Sigma\text{NH}_3 = \text{NH}_4^+ + \text{NH}_3$) was determined
237 on filtered samples on board the ship by flow injection analysis as previously described (Hall and Aller,
238 1992).

239 Fluid aliquots for total cells counts were removed from the IGT samplers using a separator
240 piston system (Fig 1E), which permitted withdrawal of fluid from the sample chamber with the sample
241 inlet valve completely open. This strategy prevented the shearing and rupture of cells, which would
242 otherwise have occurred by throttling the sample through the small opening in the sample valve stem
243 that would have been required to maintain a high pressure drop across the valve. Fluids are removed by
244 throttling deionized water on the backside of the separator piston into a metering syringe through an
245 additional high pressure valve attached to the separator tube while the IGT sample inlet valve is fully
246 open. Pressure inside the IGT sampler moves the separator piston allowing it to fill with fluid from an
247 incubation, which is then recovered by closing the IGT sample inlet valve, disconnecting the separator
248 and forcing the hydrothermal fluid out with the metering syringe.

249 Cells were preserved by mixing 1.5 mL of fluid with 40 μL of borate-buffered formalin, stained
250 with 200 μL of 0.1% acridine orange solution (Fisher Scientific), filtered under gentle vacuum onto
251 black 0.2 μm polycarbonate filters (GE Healthcare), and enumerated shipboard by fluorescence
252 microscopy (Axioskop 40, Zeiss, Germany). Ten grids were counted per sample and the result
253 extrapolated to the total filtration area to determine the cell concentration. All counts were done in

254 analytical duplicates.

255 Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH): Aliquots of
256 approximately 10 mL of culture were preserved at ~16 and ~24 h after the addition of
257 labels/amendments with paraformaldehyde (1%) and kept for 1 h at room temperature to fix cells. Cells
258 were then filtered under moderate vacuum onto Au/Pd-sputtered 0.2 µm polycarbonate filters
259 (Millipore), washed 2x with 10 mL 1x PBS, air-dried and stored at -20°C prior to further analysis.
260 Filters were subsequently sectioned, embedded in low-melting point agarose, and endogenous
261 peroxidases were inactivated by immersion in 3% H₂O₂ for 10 min. Horseradish-peroxidase labeled
262 probes (Biomers) were hybridized at 46°C for 3 h. Tyramide amplification with Oregon Green 488-X
263 (Molecular Probes, Inc.) was conducted for 20 min at 46°C. Both oligonucleotide probe combinations
264 EPSI549/EPSI914 (Lin et al., 2007; Greuter et al., 2016) and EUBI-III (Daims et al., 1999) were
265 hybridized at 35% formamide.

266

267 **3. Results:**

268 **3.1 Microbial community composition and fluid chemistry at *Crab Spa*:**

269 All the *Crab Spa* fluid samples used for this study had similar chemical concentrations that
270 were consistently different from background seawater (Table 2). CARD-FISH counts demonstrated
271 that the microbial communities in the natural fluids were dominated by *Epsilonproteobacteria* (~80%
272 of total cells, Table 3). Fluids contained elevated levels of methane (~6 µM), sulfide (~190 µM) and
273 ammonium (~11.5 µM), and were slightly acidic (pH=5.6), as well as being depleted in oxygen and
274 nitrate relative to seawater (~22 and 6.8 µM, respectively). Oxygen concentrations measured *in situ*
275 were lower than those measured in the IGT samplers (3.6 µM vs ≥ 15 µM). This excess oxygen can be
276 attributed to air-saturated seawater which was used to fill the sampler dead volume. Small air bubbles

277 entrained in the snorkel tips during vehicle deployment may account for occasional high values
278 observed without oxygen amendments (up to 45 μM O_2).

279 The formation of low temperature vent fluids at oceanic spreading centers occurs as a result of
280 the mixing of high temperature seawater-derived hydrothermal fluids with cold seawater in subseafloor
281 environments (Butterfield et al., 2004; Von Damm and Lilley, 2004). At *Crab Spa*, a likely candidate
282 for the high temperature endmember is the *Tica* vent (located ~14 m NE of *Crab Spa*) which was
283 venting at 194°C during the course of this study. Based on a two-component mixing model for the
284 formation of the *Crab Spa* fluids involving the endmember *Tica* vent fluid and seawater, concentrations
285 of dissolved sulfide, hydrogen, nitrate and oxygen are substantially depleted indicating consumption
286 following mixing in subsurface environments (Table 2).

287

288 **3.2 Incubation results:**

289 In all cases, the natural microbial community remained dominated by *Epsilonproteobacteria*,
290 and in some cases, showed an enrichment compared to background samples (Table 3). Below, we detail
291 specific observations for each condition.

292 *Control treatment* (Fig. 2a): In this case, the only addition to fluids was a ^{13}C bicarbonate label.
293 Although cell numbers did not change consistently during incubations, a clear and replicable pattern in
294 chemical concentrations was observed. Sulfide dropped by ~100 μM in all replicates and stabilized at
295 90-100 μM for the remainder of the experiment, while low initial levels of nitrate and oxygen
296 decreased below the detection limits of ~1 and ~5 μM , respectively. Ammonium concentrations
297 decreased initially by 5-10 μM in two of the replicates, which were elevated at the beginning of the
298 incubation for unknown reasons.

299 *Hydrogen additions* (Fig. 2b): The hydrogen-amended incubations were characterized by a

300 pattern of chemical depletion that was similar to the control treatment. In this case, sulfide
301 concentrations decreased by $\sim 100\ \mu\text{M}$, and neither hydrogen nor sulfide were exhausted during the
302 incubation period. Hydrogen decreased slowly in all three replicates, with a concomitant increase in
303 sulfide. Cell concentrations either did not show a consistent trend or increased gradually. Ammonium
304 increased slightly ($2, 4\ \mu\text{M}$) in two replicates and decreased slightly in one ($2\ \mu\text{M}$), and nitrate and
305 oxygen were exhausted rapidly as in the control treatment to concentrations below their detection
306 limits. Initial sulfide concentrations were lower for the two IGT sampler incubations that were started
307 after a $\sim 10\ \text{h}$ delay.

308 *Nitrate additions* (Fig. 2c): Upon the addition of nitrate, sulfide concentrations decreased to
309 below detection after $\sim 12\text{-}18\ \text{h}$. Oxygen was fully depleted after the second time point, and nitrate
310 concentrations gradually decreased throughout the incubations. Ammonium increased in two out of
311 three replicates and cell concentrations either increased gradually during incubations or showed no
312 consistent pattern.

313 *Oxygen additions* (Fig. 2d): For the oxygen amended incubations conducted at both 80 and 110
314 μM , sulfide and oxygen reached levels below detection after $12\text{-}18\ \text{h}$. Cell counts were consistent
315 between replicates with an initial increase at $\sim 80\ \mu\text{M}$ after $7\ \text{h}$ and a continuous increase at $\sim 110\ \mu\text{M}$
316 throughout the duration of the incubation. Nitrate was eventually depleted across all treatments,
317 although a transient increase was observed in both $\sim 80\ \mu\text{M}$ oxygen replicates. Ammonium decreased in
318 all incubations by $\sim 5\ \mu\text{M}$.

319 *Nitrate/hydrogen additions at 24°C* (Fig. 2e): These incubations were characterized by a
320 complete exhaustion of hydrogen, sulfide, and oxygen and a gradual decrease in nitrate. A consistent
321 and replicable increase in ammonium of around $20\ \mu\text{M}$ was observed for all replicates, and cell
322 concentrations increased from the first time point for all incubations, though later decreased in two

323 cases.

324 *Nitrate/hydrogen additions at 50°C* (Fig. 2f): In these treatments, a continuous increase in cell
325 numbers was observed, accompanied by complete exhaustion of oxygen, hydrogen, sulfide and a large
326 decrease in nitrate. A large increase in ammonium of ~60 μM was observed for both replicates.

327 *Killed controls at 24 and 50°C* (supplemental spreadsheet): For two killed controls at 24°C,
328 sulfide decreased between 6-20 μM over a 17 h period, whereas at 50°C, sulfide either decreased by
329 ~30 μM or increased slightly (18 μM) over a 12 h period. Although oxygen concentrations did decrease
330 in some controls, levels never dropped below 94 μM .

331

332 **4. Discussion:**

333 Our study quantified concentrations of redox reactive species and cellular abundance during
334 short-term laboratory incubations of deep-sea hydrothermal vent fluid, allowing biogeochemical
335 processes to be carefully tracked (Fig. 2). Since these incubations were carried out at deep-sea pressure
336 and temperature with μM concentrations of amendments (Table 1), conditions were only slightly
337 altered from the natural environment. Preservation of natural microbial community structure (Table 3),
338 suggests that our data may accurately reflect processes occurring in the natural environment. Below, we
339 discuss how our incubations can be used to confirm hypotheses derived from observations of *in situ*
340 chemistry, infer the stoichiometry of microbially-mediated processes by quantifying redox reactions
341 catalyzed by the community, and determine the rates of microbial processes (Table 5).

342 **4.1 Chemosynthesis at *Crab Spa***

343 Models for the formation of low temperature ridge-crest hydrothermal fluids involve two-
344 component mixing of a high temperature Mg-free endmember hydrothermal fluid with Mg-rich (53
345 mM Mg) seawater. Within the context of such a model, the Mg content of low temperature fluids

346 provides a direct constraint on the relative contributions of the high-temperature endmember and
347 seawater. The Mg content of the *Crab Spa* fluid (49.3 mM) indicates mixing between seawater and
348 hydrothermal end-member fluid at a ratio of approximately 14:1 (Table 2). This mixing ratio has
349 remained relatively constant over time at *Crab Spa*, since it has been observed that fluids have
350 maintained similar temperature, chemistry and microbial composition since 2007 (Sievert, Seewald, Le
351 Bris and Luther, unpublished). The fluids also carry a strong imprint of subseafloor microbial activity
352 as evidenced by depletions of redox sensitive chemical species such as oxygen, nitrate, sulfide, and
353 hydrogen relative to values predicted for conservative mixing, and enrichment of other species such as
354 ammonium (Table 2). Supporting this presumed microbial activity, both freshly sampled and incubated
355 fluids were dominated by *Epsilonproteobacteria* (Table 3), which are well-known to utilize or produce
356 these compounds during their metabolism (Sievert and Vetriani, 2012; Nakagawa and Takai, 2008).

357 Although sulfate and carbon dioxide are quantitatively the most abundant electron acceptors in
358 these fluids, nitrate, oxygen and intermediate sulfur species (i.e. elemental sulfur, polysulfides) are the
359 only electron acceptors present that are known to be utilized by autotrophic *Epsilonproteobacteria* to
360 generate energy in combination with sulfide or hydrogen as electron donors (Sievert and Vetriani, 2012,
361 Table 2, Figure 2). Redox reactions involving these chemical species likely support autotrophic
362 microbes *in situ*, but evidence is currently lacking for which are preferentially utilized by microbes and
363 which chemicals ultimately limit energy-generating chemosynthetic reactions *in situ*. Important clues
364 about these questions can be found in the chemistry of *Crab Spa* fluids, where both oxygen and nitrate
365 are depleted relative to predictions based on conservative mixing, while sulfide remains high (~190
366 μM). This depletion of electron acceptors is most apparent for oxygen, where *in situ* measurements
367 yielded a value of 3.6 μM , ~103 μM less than predicted for conservative mixing (Table 2).

368 While oxygen and nitrate concentrations measured *in situ* are very low, they would still be more

369 than sufficient to support chemosynthesis in the natural environment if they were available to microbes
370 at these levels. However, since oxygen was measured in exiting fluids just outside the vent orifice,
371 turbulent mixing with ambient seawater could have introduced oxygen not present in the seafloor.
372 In addition, even if 3.6 μM is an accurate value for seafloor fluids, it is likely that there are
373 microenvironments in which these reactants are further depleted, such as in biofilms in the subsurface
374 biosphere. In such an environment, high densities of microbes would draw nitrate and oxygen down to
375 much lower levels and ultimately the diffusion of these electron acceptors would limit energy-
376 generating chemosynthetic reactions despite electron donors being present at high concentrations.

377 Our results support this conjecture by clearly showing that sulfide cannot be completely
378 consumed during experiments (Figures 2a, 2b) without the addition of either nitrate or oxygen (Figures
379 2c-f). The observed depletions in dissolved sulfide are unlikely to be the result of abiotic oxidation with
380 O_2 since killed controls showed no or very low levels of sulfide and oxygen consumption (see
381 supplementary spreadsheet), consistent with other studies assessing the role of biotic and abiotic
382 processes in sulfide oxidation (Luther et al., 2011). In addition, robust growth of *Epsilonproteobacteria*
383 was associated with sulfide consumption in the presence of oxygen (Fig 2d, Table 3), strongly
384 suggesting that microbial sulfide oxidation is tied to oxygen in these incubations (see Table 4 for likely
385 metabolic reactions). In contrast to the oxygen addition experiments, nitrate additions did not result in a
386 consistent pattern of growth, despite rapid and complete consumption of sulfide and enrichment of
387 *Epsilonproteobacteria* compared to background samples (Table 3). This observation, combined with
388 the fact that nitrate was consumed more slowly than oxygen (Table 5), suggests that oxygen serves as
389 the preferred electron acceptor *in situ*, in line with higher growth yields predicted for oxygen relative to
390 nitrate (Chen and Strous, 2013), a phenomenon that has been observed previously in chemostat cultures
391 of *Epsilonproteobacteria* (Timmer-Ten Hoor, 1981).

392 In addition to sulfide, hydrogen oxidation also has the potential to be an important process
393 supporting chemolithoautotrophic production at vents, since it can support higher growth rates and/or
394 yields than sulfide oxidation in autotrophic *Epsilonproteobacteria* (Takai et al., 2006, Han and Perner,
395 2014). Although present in endmember fluids, hydrogen is barely detectable upon sampling at *Crab*
396 *Spa* (Table 2), suggesting that microbes in the seafloor are actively consuming it by coupling its
397 oxidation to either oxygen, nitrate or sulfur reduction. Supporting this idea, hydrogen was rapidly and
398 completely consumed in incubations that also received nitrate (Figures 2e, f), and consumed more
399 slowly in incubations to which only hydrogen was added (Figure 2b). As with sulfide, this oxidation
400 was almost certainly microbially-mediated since abiotic H₂ oxidation by oxygen is kinetically inhibited
401 at the incubation temperatures (Foustoukos et al., 2011). It is noteworthy that drawdown of hydrogen
402 continued after the depletion of nitrate/oxygen and was accompanied by a steady increase in the
403 concentration of sulfide (Figure 2b). This increase in sulfide was most likely due to the oxidation of
404 hydrogen coupled to the respiration of oxidized sulfur species. Although dissimilatory sulfate reduction
405 could potentially result in such sulfide production, it is unlikely to have occurred in these incubations
406 since this process has not been reported in *Epsilonproteobacteria*, which dominated the incubations
407 (Table 3). In contrast, there is ample evidence that *Epsilonproteobacteria* can catalyze the oxidation of
408 hydrogen coupled to the respiration of intermediate sulfur species such as polysulfide, elemental sulfur
409 or thiosulfate (e.g., Hedderich et al., 1998; Sievert and Vetriani, 2012 and references therein; Mino et
410 al., 2014). However, it should be noted that sulfur species with intermediate oxidation states are likely
411 quantitatively less important than oxygen or nitrate as electron acceptors *in situ*, since sulfide
412 production was only observed at hydrogen concentrations 3-4 times the maximum predicted for the
413 natural environment (Table 2), and only after oxygen and nitrate were both depleted.

414 Together, these results provide direct evidence that depletions of redox-active chemicals at *Crab*

415 *Spa* are microbially mediated, and that the supply of electron acceptors limits chemosynthesis *in situ*.
416 Although methane is present at ~6 μM in *Crab Spa* fluids, it was not consumed during incubation
417 experiments even at high oxygen tensions (data not shown). The lack of methane oxidation could be
418 due to the competitive exclusion of methanotrophic communities *in situ* by fast-respiring
419 *Epsilonproteobacteria* drawing oxygen and nitrate down to levels below which aerobic or denitrifying
420 methanotrophs could survive. In this case, the sampled fluids in our study would not contain large
421 populations of methanotrophs. This appears to be the case at the Menez Gwen hydrothermal vent field,
422 where metagenomes indicate the absence of known methane-oxidizing genes (Meier et al., 2016),
423 despite the end-member fluids being rich in methane (Charlou et al., 2000).

424 **4.2 Biogeochemistry of Sulfur and Nitrogen**

425 While the above discussion paints a general picture of processes underlying chemosynthesis at
426 *Crab Spa*, incubation results can also be used to determine the stoichiometry of oxidation and reduction
427 reactions catalyzed by microbes, which in turn constrains possible biochemical mechanisms. For
428 example, in all incubations, the amount of nitrate and oxygen consumed was never sufficient to account
429 for the complete oxidation of sulfide to sulfate (supplemental spreadsheet), suggesting that sulfide was
430 normally oxidized to an intermediate oxidation state sulfur species. Some autotrophic
431 *Epsilonproteobacteria* can catalyze sulfide oxidation to elemental sulfur (S^0) (Wirsen et al., 2002;
432 Sievert et al., 2007), and indeed white flocculent material (likely composed of S^0) continuously
433 emanates from *Crab Spa*.

434 In order to test whether this was occurring in our incubations, the overall redox balance between
435 the total amounts of electron donors and acceptors consumed was determined. This was accomplished
436 by using the reactions summarized in Table 4 to calculate the ratio of electrons transferred from
437 electron donor to acceptor as indicated by changes in the concentrations of relevant chemical species. A

ratio of one indicates a balance between the consumption of electron donors and acceptors, and examination of Figure 3 reveals that the only treatment where electron donors and acceptors were balanced was for the oxygen additions. In contrast, values greater than one were observed for all of the other conditions, indicating that the amount of electrons predicted to be transferred due to oxidation of electron donors exceeds the amount that can be accounted for by the changes in the concentrations of electron acceptors - even assuming that sulfide was normally oxidized to the intermediate S^0 . Although alternative electron acceptors used in microbial respiration could potentially account for this imbalance, we know of no other candidate electron acceptors at *Crab Spa* that could support additional sulfide oxidation.

However, an alternative possibility which can consume sulfide without requiring additional microbial respiration is the abiotic production of polysulfide by reaction of sulfide with elemental sulfur. As mentioned above, elemental sulfur is probably present in fluids upon sampling, which would therefore be available to react with sulfide and contribute to the discrepancy observed in Figure 3. Because the oxidation state of polysulfide varies as a function of chain length, its presence could explain both the high redox balance ratios and inter-sample variability observed in Fig. 3. In addition to this completely abiotic mechanism, this process may be partly biologically-mediated. Polysulfide has been shown to be produced during sulfur oxidation by *Beggiatoa sp.* (Berg et al., 2014) and has been implicated in the reaction mechanism of sulfide:quinone oxidoreductase (Griesbeck et al., 2002), which is thought to be used by some *Epsilonproteobacteria* to oxidize sulfide (Yamamoto and Takai, 2011). In this biologically-mediated process, elemental sulfur produced by microbes during oxidation of sulfide presumably reacts with surrounding sulfide molecules, thereby consuming more sulfide without requiring any extra respiratory electron acceptor to be present.

As discussed above, oxygen is likely the preferred electron acceptor for microbial communities

461 at *Crab Spa*. As a result, oxygen is highly depleted *in situ* (Table 2), and nitrate may serve as an
462 important alternative electron acceptor. This role for nitrate as a secondary electron acceptor is
463 consistent with its consumption during several incubations to which no oxygen was added (Figures 2c,
464 2e, 2f). Because the method used in this study to quantify nitrate consumption actually measures the
465 sum of nitrate + nitrite, the observed nitrate depletions (Fig 2) indicate that nitrate was reduced beyond
466 nitrite to either nitric oxide, nitrous oxide, nitrogen, or ammonium. Since the concentration of
467 ammonium was also determined, our data provide a direct means to assess the relative importance of
468 denitrification versus dissimilatory reduction to ammonium (DNRA), the two major nitrate removal
469 processes expected in this system. Ammonium, which is already present at elevated levels in *Crab Spa*
470 fluids (11.5 μM , Table 2), increased in some incubations when either nitrate or hydrogen was added
471 alone and in one control, suggesting slow rates of DNRA in these cases (Table 5, Fig 2a, 2b, 2c).
472 However, the amount of nitrate converted to ammonium was $\leq 9.4 \mu\text{M}$ and highly variable,
473 representing 0-63% of the initial nitrate (Table 5).

474 In contrast, when both nitrate and hydrogen were added to the incubations (Figures 2e, 2f), the
475 total amount of ammonium produced was not only consistent between replicates, but also of a much
476 greater magnitude than with either amendment alone. While ammonium was produced in both
477 incubations at 24°C and 50°C, the increase in cell counts was more consistent and ammonium
478 production more pronounced at 50°C, supporting previous assertions that DNRA is a more important
479 respiratory pathway at higher temperatures (Sievert and Vetriani, 2012). Despite the more rapid and
480 greater extent of ammonium production at 50°C, the maximum amount of respired nitrate converted to
481 ammonium was never greater than 55% (Table 5), suggesting that denitrification to N_2 or other N
482 intermediates was still occurring at a significant level at elevated temperatures. This could be either due
483 to the simultaneous growth of both denitrifying and DNRA bacteria, or to metabolic pathways within a

484 single organism that can produce both ammonium and intermediate oxidation state nitrogen products
485 from nitrate simultaneously.

486 Although denitrification has been reported for the moderate thermophilic
487 epsilonproteobacterium *Nitratiruptor tergaricus* (Nakagawa et al., 2005; temperature optimum = 55°C),
488 there is also an indication that another organism might be capable of producing both ammonium and
489 other intermediate oxidation state nitrogen compounds from nitrate. Takai *et al.* (2004) report in the
490 description of *Hydrogenimonas thermophila* (*Epsilonproteobacteria*) that during batch cultivation, only
491 ~1/2 of the nitrate consumed is converted to ammonium - nearly identical to the data presented here.
492 Since these authors did not observe nitrite, nitrous oxide, or nitrogen production, the observed
493 imbalanced stoichiometry implies the production of another intermediate oxidation state nitrogen
494 compound during nitrate reduction. Genome sequencing of this strain currently underway as part of
495 phase III of the Genomic Encyclopedia of Bacterial and Archaeal Type Strains (Whitman et al., 2015),
496 will make it possible to look for genetic evidence for such an unusual process.

497 Whether or not the respiration of nitrate to both ammonium and gaseous intermediates is due to
498 one or several different organisms, another important factor that likely constrained the proportion of
499 nitrate converted to ammonium in our incubations is the type of electron donor. DNRA is typically
500 supported by the oxidation of hydrogen, whereas denitrification can occur with either hydrogen or
501 reduced sulfur (Sievert and Vetriani 2012 and references therein). Since we observed both sulfide
502 oxidation and hydrogen oxidation in the 50°C incubation, it may be that the ammonium produced was
503 directly coupled to hydrogen oxidation, whereas the sulfide consumption was coupled to
504 denitrification. Indeed, the proportion of nitrate converted to ammonium closely mirrors the hydrogen
505 consumed on an electron-equivalent level, while the remainder of nitrate loss not coupled to
506 ammonium production can be sufficiently accounted for by sulfide consumption. Overall, these data

507 suggest that the elevated ammonium concentrations observed in *Crab Spa* vent fluids may be the
508 product of bacteria carrying out DNRA in the seafloor and that hydrogen availability is an important
509 factor determining whether or not ammonium is produced as a product of nitrate respiration in natural
510 fluids.

511 **4.3 Rates of Chemosynthetic Processes at *in situ* Pressure and Temperature:**

512 Rate measurements provide an important constraint on biological processes at vents. Previous
513 studies incubating vent fluids at both *in situ* temperature and pressure are, to our knowledge, limited to
514 one experiment conducted by Wirsén et al. (1986). Therefore, this study greatly expands the range of
515 data and can be used for comparison of rates to recent studies that were not carried out at *in situ*
516 pressure (Bourbonnais et al., 2012; Perner et al., 2010; Perner et al., 2011; Perner et al. 2013).

517 In our study, rates were derived for sulfide consumption/production, hydrogen consumption,
518 nitrate consumption, oxygen consumption and ammonia production where at least two time points
519 showed a consistent and measurable change in chemical concentrations. These data are presented as
520 both cell-specific rates (Table 5) and as absolute rates (supplementary spreadsheet). In general, per-cell
521 sulfide consumption rates were consistent across all measured conditions, whereas nitrate consumption
522 rates were more variable and greatly enhanced by the presence of dissolved hydrogen, particularly at
523 50°C. Hydrogen oxidation rates were similarly variable, with the lowest values found where no
524 electron acceptor was added (hydrogen-only amendment) and the highest rates found where nitrate was
525 also added, with incubations at 50°C further enhancing rates. Higher rates of ammonium production
526 were correlated with faster rates of hydrogen oxidation, while sulfide production was only seen in the
527 hydrogen-only treatment. Since oxygen was only measured in four cases, it is difficult to make specific
528 conclusions regarding the rate of its consumption. Below, we discuss how these rates compare to other
529 studies conducted on diffuse-flow vent fluids.

530 Similar to the present study, Perner et al. (2013) performed amendments of deep-sea vent fluids
531 collected at various sites on the Mid-Atlantic Ridge and compared results to unamended samples.
532 There are significant differences in rates between their study and ours, especially for absolute sulfide
533 oxidation rates which were approximately half of those reported here. However, normalization of rates
534 to total cell numbers yielded rates that are comparable. For example, sulfide consumption rates of 239-
535 797 fmol $\text{H}_2\text{S} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ in the present study (Table 5) are consistent with the range of 1.7-432 fmol
536 $\text{H}_2\text{S} \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ reported by Perner et al. (2013) for sulfide addition experiments. Cell count
537 normalized hydrogen oxidation rates in the range of 0.5-2208 fmol $\text{H}_2 \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ determined by
538 Perner et al. (2013) are similar to values of 49.6-1535 fmol $\text{H}_2 \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ determined during this study
539 (Table 5), although their slowest rates were approximately 2 orders of magnitude lower than ours. The
540 reason for this discrepancy is difficult to ascertain, but Perner et al. (2011) note large changes in
541 community composition from background communities, which is an indicator that physicochemical
542 conditions during their incubations changed appreciably from those found *in situ*. Such a change could
543 indicate that not all microbes quantified in their incubations were active, possibly explaining lower per-
544 cell rates. In contrast, our study saw only small changes in community composition, with the already-
545 dominant *Epsilonproteobacteria* becoming more abundant as a result of incubations (Table 3).

546 Rates of nitrate consumption have also been determined in a variety of low temperature vent
547 fluids from the Juan de Fuca Ridge by Bourbonnais et al. (2012) using isotope-labeled nitrogen
548 additions to trace nitrate removal processes. In their study, denitrification rates ranged from ~ 0.002 -
549 77 fmol $\text{NO}_3^- \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$ (Easter Island and Hermosa vents, respectively) which represents a much
550 larger range than in our study (58-655 fmol $\text{NO}_3^- \cdot \text{cell}^{-1} \cdot \text{day}^{-1}$, Table 5). While their highest
551 denitrification rates compare well with our nitrate-only additions, a number of their incubations
552 reported orders of magnitude lower denitrification rates compared to our study. It is unclear why there

553 is such a large difference, since the cell densities in the source fluids used by Bourbonnais et al (2012)
554 are in the same range as the present study and were also mostly dominated by *Epsilonproteobacteria*.
555 One key difference between studies is the concentration of nitrate amendments; in the present study, we
556 used concentrations approximately ten-fold higher. Since Bourbonnais et al. (2012) show that natural
557 nitrate deficits were negatively correlated with denitrification rates, this could imply that samples with
558 large nitrate deficits may have had lower abundances of denitrifiers or that cells present were not
559 expressing denitrification enzymes. Since our study site has similar nitrate deficits (Table 2), yet we
560 observed high per-cell denitrification rates (Table 5), it could be that higher concentrations of
561 amendments permitted the reactivation of these pathways or the growth of denitrifiers. It is also
562 possible that either decompression or experimental manipulation of fluids (or a combination of these
563 factors) accounts for the large range of values observed in their study. Bourbonnais et al (2012) report
564 that fluids were purged with helium in some cases prior to incubation, and were allowed to remain at
565 room temperature for 12 h prior to measurements in other cases - both of which could affect rate
566 measurements. Purging removes volatile redox-active species such as sulfide, hydrogen, and methane,
567 and since our results show that holding samples for 12 h in sealed vessels will deplete all available
568 electron acceptors, not just oxygen (Fig. 2a, b), microbes in either of these situations would have been
569 deprived of energy sources for growth. As a result, they may have died or become less active, possibly
570 accounting for some of the lower rates. Moreover, if most autotrophic energy sources were removed
571 due to purging or consumption, the rates reported by these authors may represent background levels of
572 heterotrophic denitrification instead of autotrophic processes.

573 Bourbonnais et al (2012) also showed that denitrification was the dominant nitrate removal
574 process, with rates exceeding those of DNRA by up to 160-fold. In the presence of hydrogen, the
575 present study demonstrates significantly higher rates of DNRA and that the proportion of NO_3^- respired

576 via DNRA was only 3 to 9-fold lower than denitrification. Since dissolved hydrogen is thought to be
577 the main electron donor for DNRA (discussion above; Sievert and Vetriani 2012 and references
578 therein), experimental approaches that provide hydrogen as an amendment may represent a more
579 accurate constraint on potential DNRA rates in the natural environment.

580 Total nitrogen loss in the subseafloor biosphere was also calculated by Bourbonnais et al.
581 (2012) by multiplying denitrification rates determined in their study with fluid flux and estimated
582 residence time of the fluids. If the rates of denitrification determined in our study are used as input for
583 this calculation, it would make nitrate removal processes by the global deep-sea vent subseafloor
584 microbial community a much more significant sink in the global N-cycle, bringing it into the range of
585 benthic denitrification (Bourbonnais et al., 2012; Gruber, 2004; Codispoti 2007). While this suggests
586 that denitrification at vents may have been underestimated, there are several important caveats. As
587 discussed above, oxygen was more rapidly consumed than nitrate in our incubations, and yielded a
588 higher number of cells (Fig. 2). Therefore, it is likely that in natural environments oxygen is the
589 preferred electron acceptor, and that denitrification will be suppressed where oxygen is non-limiting,
590 although there may be a threshold below which so-called "aerobic denitrification" occurs (Figure 2d
591 and supplemental spreadsheet; Gao et al., 2009). In addition, the residence time of fluids within a
592 subsurface biosphere and the mixing ratio between seawater and vent fluid should ultimately control
593 oxygen concentrations in low temperature hydrothermal vent fluids, and therefore denitrification rates.
594 Higher proportions of seawater will introduce more oxygen and shorter residence times will reduce the
595 opportunity for resident microbes to completely consume oxygen present. Although the mixing ratio
596 can be well-constrained based on fluid composition, residence time and the extent of "aerobic
597 denitrification" are relatively unknown, demonstrating a need for more research to accurately constrain
598 nitrogen loss in the subseafloor biosphere.

599 **5. Summary:**

600 In the present study, novel methods were developed that allowed IGT samplers to be used for
601 incubations of deep-sea hydrothermal vent microbial communities under simulated seafloor conditions.
602 IGT samplers were modified to allow both initial amendments and subsamples during short, ~24 h
603 incubations, permitting time-series measurements of chemical concentrations. Results from eighteen
604 incubations, representing seven separate treatments in biological replicates, provide the basis for future
605 biogeochemical models and reveal important gaps in our understanding of microbially-catalyzed
606 processes at deep-sea hydrothermal vents. Data generated in this study have confirmed predicted
607 microbial processes, quantified their rates, and revealed insights into the stoichiometry of metabolically
608 relevant redox reactions. In addition, because incubations were short (≤ 1 day) and amendment
609 concentrations low (~ 100 μM), the microbial community composition was minimally altered from
610 background communities. Therefore, the results reported here may be representative of rates in the
611 natural environment and can be further combined with vent fluid chemistry to constrain hydrothermal
612 vent microbial communities' contributions to biogeochemical cycling. Finally, since these methods can
613 be applied to any pelagic deep-sea microbial community, incubations conducted in IGT samplers have
614 great potential to increase our understanding of microbial processes and their quantitative importance
615 in a broad range of deep-sea environments.

616

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763
764 **Figure captions:**

765 Figure 1: Isobaric Gas-Tight Sampler (IGT) configurations used to amend *Crab Spa* hydrothermal
 766 fluids with inorganic substrates, monitor chemical consumption and track microbial growth. For each
 767 incubation, the sample draw valve (shown in panel C, D and E) is attached to the sampler inlet valve

768 and is used to regulate fluid flow during both additions of dissolved chemical species and withdrawals
769 for chemical consumption/cell count measurements. Pressure in the IGT is maintained at seafloor
770 values by means of the pre-charge valve (B) which can be opened to bleed off pressure during
771 amendment addition or maintain pressure by adding fluid using an HPLC pump during sample removal
772 (F). In the case of the addition of dissolved hydrogen gas, the configuration in (C) shows how this
773 gaseous substrate was supplied to IGTs. To make instantaneous measurements of dissolved oxygen
774 from IGT fluids, a fiber-optic sensor was held against the optode window in the flow-through cell
775 shown in (D). The separator system (E) used for cell counts allows withdrawals of microbial cells from
776 the IGT without exposure to shear stress. (G) shows the safety setup used for high temperature
777 incubations. The operation of all of these components are explained in greater detail in the text of the
778 methods.

779
780 Figure 2: Incubation results for 6 different treatment types. With the exception of (f), all incubations
781 were carried out at 24°C. a) Control, only $^{13}\text{CO}_2$ label in anaerobic filtered bottom seawater, b)
782 Hydrogen additions, c) Nitrate additions, d) Oxygen additions, e) Nitrate/hydrogen additions, f)
783 Nitrate/hydrogen additions (50°C). For the oxygen additions, triangles indicate the higher oxygen
784 treatment (110 μM) and circles denote the lower oxygen treatment (80 μM).

785
786 Figure 3: Community redox balance for IGT treatments represented as a ratio. A ratio of one indicates
787 balanced use of electron donors and acceptors and a ratio greater than one indicates that more electron
788 donor was consumed than could be accounted for by consumption of electron acceptors (according to
789 the assumptions outlined in the text). Errors are either standard deviations ($n>2$), or ranges ($n=2$).

A - Control, 24°C **B - H_2 addition, 24°C** **C - NO_3^- addition, 24°C** **D - O_2 (80 & 110 μM), 24°C**

E - NO_3^-/H_2 addition, 24°C

F - NO_3^-/H_2 addition, 50°C

Table 4

Important predicted redox reactions in incubations of Crab Spa fluids that are most likely microbially catalyzed. These reactions are used in subsequent calculations of redox stoichiometry (see Methods and Discussion).

Equation #	Predicted microbially catalyzed reaction	Description
1	$2\text{H}_2\text{S} + \text{O}_2 \Rightarrow 2\text{S}^0 + 2\text{H}_2\text{O}$	Sulfide oxidation to sulfur coupled to oxygen reduction
2	$2.5\text{H}_2\text{S} + \text{H}^+ + \text{NO}_3^- \Rightarrow 2.5\text{S}^0 + 3\text{H}_2\text{O} + 0.5\text{N}_2$	Sulfide oxidation to sulfur coupled to denitrification
3	$4\text{H}_2\text{S} + 4\text{H}^+ + \text{NO}_3^- \Rightarrow 4\text{S}^0 + 3\text{H}_2\text{O} + 2\text{H}^+ + \text{NH}_4^+$	Sulfide oxidation to sulfur coupled to DNRA*
4	$2\text{H}_2 + \text{O}_2 \Rightarrow 2\text{H}_2\text{O}$	Hydrogen oxidation coupled to oxygen reduction
5	$2.5\text{H}_2 + \text{H}^+ + \text{NO}_3^- \Rightarrow 3\text{H}_2\text{O} + 0.5\text{N}_2$	Hydrogen oxidation coupled to denitrification
6	$4\text{H}_2 + 2\text{H}^+ + \text{NO}_3^- \Rightarrow 3\text{H}_2\text{O} + \text{NH}_4^+$	Hydrogen oxidation coupled to DNRA*
7	$\text{H}_2 + \text{S}^0 \Rightarrow \text{H}_2\text{S}$	Hydrogen oxidation coupled to sulfur reduction
8	$2\text{NH}_4^+ + 4\text{O}_2 \Rightarrow 2\text{NO}_3^- + 2\text{H}_2\text{O} + 4\text{H}^+$	Aerobic ammonium oxidation (NH ₄ ⁺ /NO ₂ ⁻ ox. steps combined)

* DNRA = Dissimilatory nitrate reduction to ammonium

talyzed.

1).

Evidence for reaction
O ₂ additions
NO ₃ ⁻ additions
H ₂ and NO ₃ ⁻ additions
H ₂ and H ₂ /NO ₃ ⁻ additions
H ₂ and H ₂ /NO ₃ ⁻ additions
H ₂ and H ₂ /NO ₃ ⁻ additions
H ₂ additions
O ₂ additions

1. Sampling

Warm (24°C) microbe-rich fluids obtained from hydrothermal vents at 9°N, East Pacific Rise.

Isobaric gas-tight samplers (IGTs) maintain in situ pressure & prevent loss of volatiles contained in fluids.

2. Shipboard Incubations

Fluids contained in IGT samplers are amended with substrates predicted to support chemoautotrophic growth.

Incubations carried out in IGT samplers for ~ 24 h at vent pressure and temperature.

3. Results

Chemical/biological measurements quantify rates, identify active microbes & constrain redox reactions supporting chemoautotrophy.

Realistic incubation conditions allow extrapolations to natural environment.