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Assessing microbial processes in deep-sea hydrothermal systems by incubation 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 CO_2 into biomass. In addition to producing new organic matter, this process also consumes compounds contained both in vent fluid and entrained seawater (*e.g.* H_2, NO_3^-). 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 examine amendments consisting of different electron donors and acceptors. Microbial activity was observed in all treatments, and time series chemical measurements showed that activity was limited by electron acceptor supply, confirming predictions based on geochemical data. Also consistent with these predictions, the presence of nitrate increased rates of hydrogen consumption and yielded ammonium as a product of nitrate respiration. The stoichiometry of predicted redox reactions was also determined, revealing that the sulfur and nitrogen cycles are incompletely understood at deep-sea vents, and likely involve unknown intermediate redox species. Finally, the measured rates of redox processes were either equal to or far greater than what has been reported in previous studies where *in situ* conditions were not maintained. In addition to providing insights into deep-sea hydrothermal vent biogeochemistry, the methods described herein also offer a practical approach for the incubation of any deep-sea pelagic sample under *in situ* conditions.

1. Introduction

At deep-sea hydrothermal vents, entire ecosystems are supported by primary production in the absence of sunlight. This process, known as chemosynthesis or chemolithoautotrophy, can occur due to chemical disequilibria between reducing hydrothermal vent fluids and oxidizing seawater. Chemosynthetic microbes catalyze thermodynamically-favorable redox reactions and couple this chemical energy to CO_2 fixation, thereby transforming an inorganic energy source into biomass (Jannasch and Mottl, 1985). In addition to supporting productive ecosystems, this process has significant biogeochemical implications. For example, chemosynthetic microbes not only consume reduced inorganic compounds, but they also remove nitrate and oxygen from seawater that mixes with vent fluid.

In the past two decades, the analysis of nucleic acids obtained directly from natural microbial communities as well as the characterization of newly isolated strains of chemolithoautotrophic microbes has revealed insights into the taxonomy, abundance and metabolic potentials of deep-sea vent chemolithoautotrophs (*e.g.* Huber et al., 2007; Nakagawa and Takai, 2008; Sievert and Vetriani, 2012 and references therein). Studies conducted thus far have shown that fluid composition can exert important controls on microbial community structure and function (Amend et al., 2011; Flores et al., 2011, 2012; Dahle et al., 2015; Hentscher and

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Bach, 2012), but the reverse question, *i.e.* how microbes themselves affect fluid composition has received less attention. Although microbial metabolism can be inferred indirectly in low temperature hydrothermal fluids by measuring deviations from the conservative mixing line between high temperature endmember fluids and seawater (Butterfield et al., 2004; Von Damm and Lilley, 2004; Proskurowski et al., 2008; Wankel et al., 2011), this approach cannot provide unequivocal proof that these signatures are microbial nor determine the rates of processes. Therefore, the lack of direct measurements of microbial metabolism hinders our understanding of the biogeochemical role of chemosynthetic processes within vent ecosystems, including their primary productivity (Sievert and Vetriani, 2012).

In order to identify active metabolic pathways and their rates at deep-sea vents, experiments that simulate the natural environment as closely as possible are needed (Sievert and Vetriani, 2012). A conceptually straightforward approach to accomplish this goal is to directly incubate fluids collected from the environment with their resident microbes. This approach is complicated, however, by both inherent challenges of working with deep-sea vent fluids as well as difficulties designing experiments to realistically infer *in situ* processes. Indeed, previous studies have shown that deep-sea microorganisms are sensitive to a decrease in pressure (Bartlett, 2002), suggesting that decompression can affect biological rate measurements. For example, Bianchi et al. (1999) showed that bacterial production rates are underestimated by approximately one half in decompressed pelagic water samples from depths greater than 850 m.

In addition to pressure, the chemical environment is also fundamentally important since the concentration of chemical species directly influences which microbial assimilatory/dissimilatory redox reactions can occur and the rates at which these reactions proceed. The chemical environment in fluid samples collected at deep sea vents may change dramatically if pressure is not maintained during 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 the removal of potential energy sources and alteration of key parameters such as pH, which in turn may affect the composition and activity of the indigenous microbial communities. Artifacts may also be introduced during fluid collection from the natural environment if ambient seawater is entrained. The presence of exogenous seawater not only alters the chemical environment, but also introduces a compositionally distinct microbial community not representative of the vent system.

In addition to technical challenges associated with obtaining representative samples of vent fluid, deriving accurate information on *in situ* processes from incubations of this fluid is complicated by the fact that chemolithoautotrophic microorganisms will have already affected the geochemistry of fluids prior to sampling, depleting some chemicals while producing others (Butterfield et al., 2004; Von Damm and Lilley, 2004; Proskurowski et al., 2008; Wankel et al., 2011). The extent of this effect is poorly known, but must be a function of fluid flow rate, absolute concentrations of chemical species, and the abundance of microorganisms and their metabolic rates. Because diffuse-flow hydrothermal environments are characterized by a continuous replenishment of substrate-bearing fluids, microbial communities can be sustained *in situ* even if relevant metabolic redox couples are present at very low concentrations in sampled fluids. Therefore, in closed-system (batch) growth experiments, low concentrations of reactants in energy-yielding redox reactions is problematic since it will be difficult (if not impossible) to measure some processes, despite their likely occurrence in the natural environment.

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

2. Materials and methods

2.1. Field site

Vent fluids used for incubation experiments were collected from *Crab Spa* (9°50.3981 N, 104°17.4942 W), a well-studied diffuse-flow hydrothermal vent located on the East Pacific Rise at a depth of 2506 m, using the ROV Jason II deployed from the R/V Atlantis during research cruise AT26-10 in January 2014. At this site, warm fluids ($\sim$24 °C) containing microbes emanate from a well-defined orifice, and have maintained a temporally stable chemical and temperature composition since 2007 (Reeves et al., 2014; Sievert, Seewald, Le Bris and Luther, unpublished data). Prior to the first sampling, the site was allowed to stabilize after megafauna (*e.g.*, *Riftia pachyptila, Bathymodiolus* sp.) were cleared to directly access the fluids emanating from the subseafloor.

2.2. Experimental design and methods for incubations

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

Prior to deployment at the seafloor, the IGT samplers were washed with dilute HCl (pH 3) to remove any residual ^{13}C-labeled dissolved inorganic carbonate (DIC) from previous incubations, followed by a 70% ethanol rinse to sterilize the interior, and acetone to dry the ethanol. A Teflon O-ring was added to the IGT sample chamber prior to deployment to allow fluid stirring following chemical additions and prior to time series sampling during the incubations (Fig. 1). The snorkel and sample valve dead volume ($\sim$4 mL) and sample chamber on the back side of the sample piston were filled with filtered bottom seawater while the accumulator chamber on the backside of the accumulator piston was filled with